Universidade do Minho
Escola de Ciências da Saúde

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Mechanisms utilized by growth factors and cytokines in angiogenesis: role of thrombin in the cross-talk between the FGF1 and Notch signaling pathways

Tese de Doutoramento
Ciências da Saúde – Ciências Biológicas e Biomédicas

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Março de 2006

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Titulo tese:

Mechanisms utilized by growth factors and cytokines in Angiogenesis: role of thrombin in the cross-talk Between FGF1 and Notch signaling pathways

Mecanismos utilizados por factores de crescimento e citoquinas na angiogénese: papel da trombina na ligação dos sistemas de sinalização entre FGF1 e Notch

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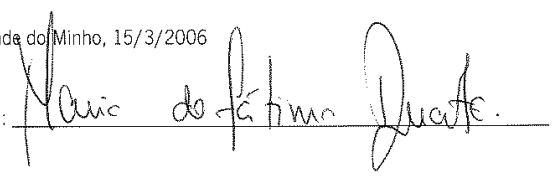
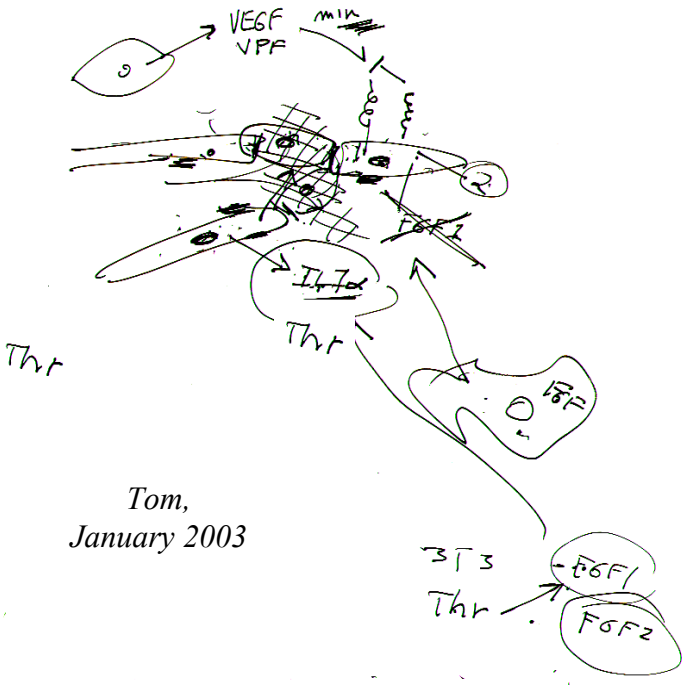
Ano de conclusão: 2006

Designação do Ramo e Área de Conhecimento do Doutoramento:

Ciências da Saúde – Ciências Biológicas e Biomédicas

Universidade do Minho, 15/3/2006

Assinatura: 



Tom,
January 2003

This thesis is dedicated in memory of Tom Maciag, a great mentor in science and a good friend in life.

"…Tom Maciag was a close friend of many of us in vascular biology…For those who do not know this amazing man's accomplishments, Tom purified the first true angiogenic factor, FGF-1, and was a seminal figure in all of vascular biology. The story of his early efforts, in competition with some big names, is a true story of scientific passion. He was to receive the Benditt Award this year, and many of us felt he was a candidate for the Nobel Prize…"

S.M. Schwartz, April 2004

"Somewhere there is something incredible waiting to be known"

Carl Sagan

"Por cada solução encontrada uma panóplia de novas interrogações nasce. Assim evolui a Ciência, assim progride a vida quotidiana do sujeito humano. O que deixa de constituir problema, de inquietar, de preocupar, perde magicamente o fascínio e passa a ficar retido na valência dos temas dominados..."

Pirokas Ricardo

Agradecimentos / Acknowledgements

It is extremely difficult to express all my gratitude, my feelings, my respect and also my deep admiration to all of you that during these four years, directly or indirectly, help me along my way. I just want to say thanks for everything!

É extremamente difícil expressar a minha gratitude, os meus sentimentos, e todo o meu respeito e admiração a todos aqueles que durante estes últimos quatro anos, directa ou indirectamente me ajudaram ao longo deste percurso, apenas vos quero agradecer por tudo.

À Professora Doutora Cecília Leão, orientadora institucional e Directora do Instituto de Ciências da Vida e da Saúde da Escola de Ciências da Saúde da Universidade do Minho, agradeço a oportunidade e a confiança em mim depositada ao aceitar-me como aluna de doutoramento desta instituição e pelo seu incansável apoio, dedicação e constante estímulo à investigação. Muito obrigado, pela prontidão com que sempre se disponibilizou em me ajudar.

Ao Professor Doutor Fernando Schmitt, orientador deste trabalho, quero agradecer todo o apoio, bem como a sua disponibilidade. Ainda que fisicamente muito distante, foi para mim motivo de orgulho ter trabalhado consigo, poder contar com a sua ajuda, o seu rigor, e alguma paciência, que foram preponderantes na concretização deste trabalho, bem como as palavras de incentivo nos momentos mais difíceis, obrigado Dr. Fernando!

To Tom Maciag I want to express all my thankfulness. More than a supervisor he was my "research father" with whom I had learned not only to be a good scientist, but also to be a simple human being always ready to chase my dreams without giving up.

Agradecimentos/Aknoledgments

Thanks Tom for all the wonderful knowledge, the continuous encouragement, for all the dreams, and ideas that you always share; the enjoyable and extremely educative discussions and intensive enthusiasm during the time that we spent together. It was an honor for me, to be your student, and part of your investigation team. Thanks for the great mentor that you were, and above all the good friend. This was a long and sometimes full of curves journey, especially after you were gone. I wish that you would be here with us, sharing this moment.

To Igor Prudovsky, I wish to express my sincere thank for your scientific support, for your supervision, all insightful discussions, and the continuous enthusiasm, without it I don't know if I had finished this thesis. Thanks Igor for taking care of us, after Tom, above all you help me persuading my dreams, thanks!!!

To Robert Friesel, and to Lucy Liaw, the scientific director and the graduate student coordinator, respectively, at Maine Medical Research Institute, I whish to express my sincere and honest thanks for the opportunity to work in your institute, and to make me fell as one of your graduate students.

Thanks to all the PIs from Maine Medical Research Institute, that share their time and expertise in the discussing of this work, specially to Jeong Yoon for all his help in the cloning of FGF1R136K:HA into pTigth and DNA purification for the pronuclear injection, to Lucy Liaw for all her insightful critiques and discussion, which were decisive for the soluble Jagged1 work, and to Carla Mouta-Bellum for her help with the Edman microsequencing. It was a great pleasure to do science will all of you in Maine.

To all the co-authors of the publications, I want to thank their contributions and suggestions.

Also, I thank to the entire lab group, Raffaela Soldi, Irene Graziani, Doreen Kacer, Vihren Kolev, Alek, Peter, for their precious help and friendship. Special thanks to Vihren and Doreen, as part of the Notch work team, sometimes even supervisors of

this work, you were always there, ready to share your time, complicity, knowledge, and skilled technical advices in a such warm and friend way, I could not make it without you guys. Thanks.

A todos os membros do grupo de Braga pela forma acolhedora como sempre me receberam e pela preciosa e paciente ajuda prestada nas várias fazes deste trabalho. À D. Manuela Mendes, muito obrigado por toda a ajuda prestada.

Many thanks to Norma Albrecht for administrative assistance, to Gloria Ledoux for helping with the visa issues, to Barbara Peslee, Karen Korts, Sheryl, Gloria and Joseph Fish that were always there, ready to help.

To P.J. Simpson-Haidaris I would like to thank the dedication and enthusiasm towards my work. I treasure your friendship, and I really appreciate all your scientific support. Thanks PJ!

I thank to Melanie, Irene, Doreen, Laura, Kyoung Sook, and Filipa for their sisterhood, I spent some of the best moments of my life with you girls!!! Thanks for your friendship, for always being there.

Helio, Sonia, Roman and Alexender, my little "Iberian Peninsula" on the other side of the ocean, you were my truly Portuguese Family, all I can say is THANKS. I guess there are no words to express the gratitude that I have for what you always did for me, from the time that I had just move in, until the last day, you were always there, ready to help, to share, to listen… to be my family. All the great times that we spent together laughing, cooking, eating, you guys just made me feel at home. Thanks!!!

A todos os meus "velhos" amigos que sempre estiveram ao meu lado. À Tânia, à Rosinha, ao Sr. António, à D. Fernanda, à Emilinha, ao Sr.P.Zé, a todos os meus colegas e amigos do IPATIMUP, em particular à Joana, à Fernanda, à Sílvia, ao Sérgio, à Ana, ao Gil, ao Rui, ao Paulo, a todos vocês muito obrigado, por todos os momentos que partilharam comigo, as vezes tão longe, mas mesmo assim sempre

prontos para ajudar, cada um à sua maneira. Gosto muito de todos vocês, muito obrigado pela vossa amizade.

A ti Ricardo um obrigado muito especial, pois fizeste com que cada momento fosse vivido muito intensamente. Através de ti, ao longo destes quarenta e dois meses, encontrei a tranquilidade e a paz nos dez minutos mais longos do meu dia. Contigo redimensionei o meu conceito de felicidade, contigo partilhei todas as alegrias, dúvidas e muitas frustrações, de quem sonha ser cientista e ainda assim ser feliz. Nunca me deixaste esmorecer, e mesmo nos momentos mais difíceis, e na contingência da distância física que nos separou tantas vezes, sempre arranjaste nas palavras um modo sublime de me fazeres prosseguir o meu sonho... apenas e só para te dizer obrigado por seres quem és!!!

Finalmente aos meus pais, meus irmãos, Alice e Bárbara um obrigado repleto de muito carinho, pelo amor e confiança com que sempre me souberam presentear todos os dias. Obrigado pela vossa constante dedicação, por sempre estarem presentes, por me fazerem sentir tão perto. Obrigado por todas as bolachinhas e iguarias que no paladar e no carinho me fizeram sentir que Portugal ficava no virar da esquina. Obrigada mamã, papá e Vira, sem vós eu não teria chegado até aqui. OBRIGADO.

Abstract

Angiogenesis, the development of new blood vessels from the existing vasculature, is controlled by different signaling pathways, where diverse cytokines, chemokines and growth factors play important roles. Angiogenesis and blood coagulation are key events of vascular biology. Serine protease, thrombin, which plays a central role in blood coagulation cascade through its ability to cleave fibrinogen and produce fibrin, is also known to be involved in inflammation, wound healing and tissue remodeling, growth factor activation, embryogenesis, and both normal and aberrant cell growth control. The effects of thrombin are associated with the induction of expression of several growth factors including fibroblast growth factor (FGF) 2, platelet derived growth factor, insulin-like growth factor 1, and vascular endothelial growth factor. In this work, the regulation of expression and release of FGF1, a potent pro-angiogenic factor was investigated in the context of thrombin activity.

We found that thrombin has the ability to induce FGF1 transcription and redistribution of FGF1 to the inner leaflet of the plasma membrane, resulting in the non-classical (ER/Golgi independent) export of this growth factor with fast kinetics. FGF1 signaling underlies thrombin mitogenic activity since thrombin does not promote cell proliferation in cells expressing a dominant negative form of FGF receptor 1. In an effort to further define the mechanisms underlying the observed effects of thrombin, we found that both release and expression of FGF1 stimulated by thrombin are dependent on protease activated receptor 1 (PAR1). Additionally thrombin is capable to cleave full-length transmembrane Notch ligand Jagged1 in its extracellular domain and to produce a soluble form of Jagged1 which decreases Notch signaling and induces FGF1 expression and export. Interestingly, we also demonstrated that the long term thrombin treatment can

induce FGF1 release from PAR1 knockout cells, most probably as a result of accumulation of soluble Jagged1.

In conclusion, these studies have identified a novel cross-talk bridging thrombin, FGF1 and Notch signaling pathways, which all play important roles in vascular developing and remodeling.

Resumo

O processo de angiogénese consiste no desenvolvimento de novos vasos sanguíneos a partir de vasos pré-existentes, sendo controlado por diferentes sistemas de sinalização, onde diversas citoquinas, e factores de crescimento têm um papel crucial. Angiogénese e coagulação sanguínea desempenham importantes papéis em biologia vascular. A serina protease trombina, a qual tem um papel principal na cascata de coagulação pela quebra enzimática do fibrinogênio e produção de fibrina, encontra-se também envolvida na inflamação, cicatrização, activação de factores de crescimento, embriogénese e crescimento celular, quer em condições normais quer em condições de crescimento aberrante. A actividade celular da trombina é devida, em grande parte, à indução de expressão de vários factores de crescimento, tais como o "fibroblast growth factor" (FGF) 2, o "platelet derived growth factor", "insulin-like growth factor", e o "vascular endothelial growht factor". Neste trabalho, a regulação da expressão e libertação do FGF1, um conhecido e potente factor pró-angiogénico, foi investigada no contexto da actividade da trombina.

Os resultados obtidos demonstraram que a trombina tem a capacidade de induzir a transcrição e redistribuição do FGF1 na porção celular interna da membrana plasmática, resultando na exportação não clássica (por via independente do retículo endoplásmico\Golgi) deste factor pró-angiogénico com rápida cinética. A libertação do FGF1 evidencia a actividade mitótica da trombina em fibroblastos de ratinho, uma vez que a trombina não é capaz de induzir proliferação celular em células que expressam um mutante do receptor do FGF1 com efeito negativo dominante. Na tentativa de tentar esclarecer este mecanismo, demonstramos que a activação da transcrição e o aparecimento do FGF1 no compartimento extra celular era dependente do receptor da trombina, denominado por "protease activated receptor 1" (PAR1). Adicionalmente, a serina protease trombina foi capaz de clivar o Jagged1 na sua

porção extra celular levando à produção uma forma solúvel deste ligando, a qual é capaz de inibir a activação do mecanismo de sinalização do Notch1 e ao mesmo tempo levar à activação da transcrição e exportação do FGF1. Curiosamente, demonstramos também que a incubação com trombina, por longos períodos de tempo, em células que não expressam PAR1 conduz a libertação do FGF1, mais provavelmente devido à acumulação da forma solúvel do Jagged1.

Em conclusão, estes estudos permitiram no contexto de remodelação vascular identificar uma nova via que interliga as vias de sinalização da trombina, FGF1 e Notch.

Table of Contents

Table of Contents

List of Tables and Figures

Abbreviations

β-gal	***β-gal****actosidase*
AGS	***A****lla****g****ile* ***S****yndrome*
Ang	***Ang****iopoietin*
ANK	***Ank****yrin Repeats*
ATP	***A****dhenosine* ***T****riphos****p****hate*
BCS	***B****ovine* ***C****alf* ***S****erum*
BMP	***B****one* ***M****orphogenic* ***P****rotein*
bp	***b****ase-****p****air*
caN1	***c****onstitutively* ***a****ctive* ***N****otch* ***1***
cDNA	***c****omplementary* ***DNA***
CIR	***C****BF1-****I****nteracting co-****r****epressor*
ColI	*Type* ***I*** ***Col****lagen*
COX	***C****yclo****ox****ygenases*
CR	***C****ysteine* ***R****ich*
CSL	***C****BF-1/Suppressor of Hairless [(****S****u (H)]/* ***L****ag-1*
Dl1ICD	***D****elta* ***1*** ***I****ntra****c****ellular* ***D****omain*
DMEM	***D****ulbecco's* ***M****odified* ***E****agle's* ***M****edium*
dn	***d****ominant* ***n****egative*
dnFGFR1	***d****ominant* ***n****egative* ***F****ibroblast* ***G****rowth* ***F****actor* ***R****eceptor* ***1***
Dox	***Dox****ycycline*
DSL	*[****D****elta,* ***S****errate, (Drosophila homologues),* ***L****ag2 (C. elegans hologue)]*
EC	***E****ndothelial* ***C****ells*
ECM	***E****xtra****c****ellular* ***M****atrix*
EGF	***E****pidermal* ***G****rowth* ***F****actor*
Eph	***Eph****rin*

ER	***E**ndoplasmatic **R**eticulum*
ERK	*phosphorylated-**E**xtracellular signal **R**egulated **K**inase*
FBS	***F**etal **B**ovine **S**erum*
FGF	***F**ibroblast **G**rowth **F**actor*
FGFR1	***F**ibroblast **G**rowth **F**actor **R**eceptor **1***
FITC	***F**luorescein **I**so**th**iocy**c**nate*
FLJ1	***F**ull-**L**ength **J**agged**1***
FLJ1NV5	***F**ull-**L**ength **J**agged1 **N**-terminally **V5** tagged*
FVIIa	***F**actor **FVIIa***
FX	***F**actor **X***
GAPDH	***G**lycer**a**ldehyde-3-**P**hosphate **De**h**h**ydrogenase*
GFP	***G**reen **F**luorescent **P**rotein*
GPCR	***G P**rotein-**C**oupled **R**eceptor*
HEK 293	***H**uman **E**mbryonic **K**idney **293** cell line*
HERP	***HE**S-related **R**epressor **P**rotein*
HES	***H**airy and **E**nhancer of **S**plit*
HIF1	***H**ypoxia-**I**nducible **F**actor 1*
HRP	***H**orse**r**adish **P**eroxidase*
HRT	***H**airy-**R**elated **T**ranscription factor*
HS	***H**eparan **S**ulfate*
HSPG	***H**eparan **S**ulfate **P**roteo**g**lycan*
HUVEC	***H**uman **U**mbilical **V**ein **E**ndothelial **C**ell*
ICAM1	***I**nter**c**ellular **A**dhesion **M**olecule**1***
ICD	***I**ntra**c**ellular **D**omain*
IFN	***I**nterfero**n***
Ig	***I**mmuno**g**lobulin*
IGFR1	***I**nsulin-like **G**rowth **F**actor **R**eceptor 1*
IL	***I**nter**l**eukin*
IP-10	***I**nterferon-gamma-inducible **P**rotein**-10***
kD	***k**ilo-**D**alton*

LDH	***L**actate **D**ehydrogenase*
LDL	***L**ow **D**ensity **L**ipoproteins*
LNR	***L**in-12/**N**otch **R**epeats*
LPS	***L**ipo**p**oly**s**achraride*
MAML	***Ma**ster**m**ind-**L**ike*
MAPK	***M**itogen-**A**ctivated **P**rotein **K**inase*
MBEC	***M**ouse **B**rain **E**ndothelial **C**ells*
MEF	***M**ouse **E**mbryonic **F**ibroblasts*
MG	***M**olten **G**lobule*
MMP	***M**atrix **M**etallo**p**roteinase*
MRC	***M**ultiprotein **R**elease **C**omplex*
mRNA	***m**essenger **RNA***
NCR	***N**otch **C**ytokine **R**esponse Element*
NEP	***N**euro**e**pithelial **P**recursor*
NF_kB	***N**uclear **F**actor **k**appa**B***
NICD	***N**otch **I**ntre**c**ellular **D**omain*
NIH 3T3	*Mouse Fibroblast cell line*
NLS	***N**uclear **L**ocalization **S**ignal*
PAR	***P**rotease **A**ctivated **R**eceptor*
PBS	***P**hosphate-**B**uffered **S**aline*
PCR	***P**olymerase **C**hain **R**eaction*
PDGF	***P**latelet-**D**erived **G**rowth **F**actor*
PECAM	***P**latelet-**E**ndothelial **C**ell **A**dhesion **M**olecule*
PF4	***P**latelet **F**actor **4***
PI3K	***P**hospho**i**nositide **3**-**k**inase*
PKC	***P**rotein **K**inase **C***
PLC	***P**hospho**l**ipase **C***
PNGase	***P**eptide **N**-**G**lycosid**ase***
pS	***p**hosphotidly**s**erine*
pTRE	***T**et-**R**esponsive **E**lement **p**romoter*

PTX	***P**ertussis **T**o**x**in*
Q-RT-PCR	***Q**uantitative **R**everse **T**ranscription **P**olymerase **C**hain **R**eaction*
Rho	***Rho**-family GTPase-activating protein*
RPB-J_K	***R**ecombination Signal-Sequence-**B**inding **P**rotein*
rtTA	***r**everse **t**et **T**rans**a**ctivator*
RT-PCR	***R**everse **T**ranscription **P**olymerase **C**hain **R**eaction*
sD1	***s**oluble **D**elta **1***
SDS-PAGE	***S**odium **D**odecyl**s**ulfate- **P**oly**a**crylamide **G**el **E**lectrophoresis*
sem	***s**tandard **e**rror **d**eviation*
sJ1	***s**oluble **J**agged1*
sJ1 39kDa	***s**oluble **J**agged1 **39kDa***
SK1	***S**phingosine **K**inase **1***
SMC	***S**mooth **M**uscle **C**ells*
sN	***s**oluble Extracellular Domains of **N**otch*
SP	***S**ignal **P**eptide*
Src	*Avian **Sarc**oma Viral proto-oncogene with tyrosine activity*
Syt1	***Syn**ap**t**otagmin **1***
TAD	***T**rans**a**ctivation **D**omain*
TF	***T**issue **F**actor*
TGF	***T**ransforming **G**rowth **F**actor*
TM	***T**rans**m**embrane Domain*
TNF	***T**umor **N**ecrosis **F**actor*
TRAP	***T**hrombin **R**eceptor **A**ctivator **P**eptide*
TTM	***T**etra**t**hio**m**olybdate*
uPA	***u**rokinase **P**lasminogen **A**ctivator*
VCAM	***V**as**c**ular **C**ell **A**dhesion **M**olecule*
VEGF	***V**ascular **E**ndothelial **G**rowth **F**actor*
VEGFR	***V**ascular **E**ndothelial **G**rowth **F**actor **R**eceptor*
VPF	***V**ascular **P**ermeability **F**actor*
VSMC	***V**ascular **S**mooth **M**uscle **C**ells*

Aims

The understanding of the mechanisms involved in blood vessels formation and growth has recently become a principal, yet challenging, objective of the vascular biology. Despite impressive achievements of the last decade, especially in the basic molecular regulatory mechanisms and the consequent discovery of candidates able to stimulate or inhibit ECs, we are still far from satisfactory understanding of complex molecular mechanisms that regulate arteriogenesis, vasculogenesis, and angiogenesis. In this connection, it is especially important to elucidate the crosstalks between different signaling pathways involved in these processes. It is particularly interesting to explore a possible interconnection between three major signaling systems, FGF, Notch and thrombin pathways, that are known to play critical regulatory roles in angiogenesis. Leading in the future to the development of new therapeutic approaches for the regulation of angiogenesis in various clinical contexts.

GENERAL AIM:

The general aim of this thesis was to explore the possible role of thrombin as a bridge between FGF and Notch signaling pathways, in the process of angiogenesis regulation.

SPECIFIC AIMS:

To achieve our goal, the following specific aims were addressed:

1 TO EXPLORE THE ROLE OF THROMBIN IN FGF1 SIGNALING

Since thrombin induces the expression of several growth factors, such as VEGF (76, 149), PDGF (466), and FGF2 (467), we aimed to determine whether thrombin was able to induce the expression of FGF1 in NIH 3T3 cells as well in Swiss 3T3 cells. Additionally, we also explored whether thrombin was able to induce FGF1 release under non-stress conditions. Since thrombin is known to mediate its biological

responses predominantly through the activation of PARs (73), we also characterized the role of PAR1 in thrombin-induced expression and release of FGF1.

2 *TO CHARACTERIZE THE INTERPLAY BETWEEN THROMBIN, NOTCH AND FGF SIGNALING MECHANISMS*

Since thrombin treatment, similarly to expression of sJ1 117kDa (393), induced the expression and release of FGF1 at non-stress conditions, we aimed to determine whether these thrombin effects were due to the production of sJ1 117kDa, as a result of thrombin cleavage of Jagged1 expressed on the cell surface. Additionally, we evaluated the regulation of these effects by Notch signaling.

3 *TO CREATE AN IN VIVO MODEL FOR STUDYING FGF1 RELEASE*

Due to the absence of reliable *in vivo* models, our knowledge about non-classical FGF1 release in the organism is still very limited. Recently, it was demonstrated that tetrathiomolybdate (TTM), which is known to block stress-dependent FGF1 release, represses restenosis provoked by balloon injury of the artery (171). Interestingly, restenosis was also inhibited by adenovirally delivered dnFGFR1. Taken together, these data indicate the participation of FGF1 release *in vivo*. However, there is still no *in vivo* model, which allows direct quantification or study of the regulation of stress-induced FGF1 export. Therefore, we aimed to create transgenic mice with inducible expression of FGF1, which would allow the study of FGF1 release in the bloodstream and peritoneal cavity.

These studies are expected to further our understanding of the molecular mechanisms involved in the regulation of angiogenesis and to yield insight into the complex interactions between blood coagulation and angiogenesis.

Thesis Planning

The present thesis is organized in seven different chapters. In Chapter I a general introduction to the theme thesis is presented, including a literature review focused in the angiogenesis and haemostasis, FGF and Notch signaling mechanisms. Chapter II provides a briefly description of the material and methods used to perform the different experiments of this thesis work. In Chapter III a compendium of the most relevant results, pertaining to the questions raised in the specific aims is presented. The general discussion of the thesis is in Chapter IV, followed by conclusions and future perspectives (Chapter V). The Chapter VI includes the published and under revision papers, supportive of this thesis work. In the last chapter the list of references used is presented (Chapter VII).

CHAPTER I

Introduction

1. Angiogenesis versus Haemostasis

2. Fibroblast Growth Factor Signaling

3. Notch Signaling

1 ANGIOGENESIS VERSUS HAEMOSTASIS

During embryonic vasculogenesis, blood vessels are formed *de novo*, from endothelial cell (EC) precursors (angioblasts) that assemble into a primary capillary plexus. This primitive network then differentiates, and new blood vessels sprout and branch from pre-existing capillaries during the process of angiogenesis (1, 2). Angiogenesis is known to be among the key events in various physiologic processes, such as organ growth and development, wound healing (3), reproduction, development of the *corpus luteum* during ovulation, and placental development (4). Under these circumstances angiogenesis occurs in a highly regulated manner whereby pro-angiogenic factors stimulate a phase of rapid migration, proliferation and differentiation of ECs, and new vessels are formed. Eventually, ECs become quiescent and a local balance of pro- and anti-angiogenic factors tightly regulates the whole process. The moment in which this "normal" balance is disrupted mediates the angiogenic switch towards the pathological angiogenesis, in the course of process such as tumor growth and metastasis (5), rheumatoid arthritis (6), diabetic retinopathy, and psoriasis (7) (4). Although upregulation of angiogenic factors is necessary to stimulate angiogenesis, simultaneous downregulation of angiogenesis inhibitors is also required to sufficiently turn on angiogenesis. The quiescence of the vasculature in a tissue suggests that the tissue either lacks angiogenic stimuli or that endogenous inhibitors suppress angiogenesis.

The haemostatic system (or coagulation cascade), which regulates platelets adherence and fibrin formation to prevent blood loss during vascular damage, is normally inactive in adults due to a balance between pro- and anti-coagulant proteins present in the blood stream. In recent years, it has become more and more evident that the blood coagulation system represents a major regulatory tool in vascular development. Indeed, the EC surface, which is the area of contact between the fluid blood compartment and the vessel wall, is the site where the coagulation cascade conducts its activity, especially when the endothelium is injured or denuded. Under

these circumstances, the permeability of the damaged vessel is increased resulting in the extravasation of fibrinogen and fibronectin and the formation of the fibrin clot which acts as temporary scaffold for migrating ECs. In addition to the release of clotting factors and inhibitors of coagulant enzymes (8) the activation of platelets leads to their degranulation resulting in the release of either positive or negative regulators of angiogenesis (9). These events result in the migration of proliferating ECs into the fibrin mesh in order to repair the vessel wall and in the further adhesion and spreading of ECs.

An increased knowledge of the factors regulating angiogenesis and coagulation has led to the understanding that these two systems are closely interconnected. Indeed, proteins of the haemostasis pathway contribute not only to coagulation, but also to the regulation of angiogenesis.

1.1 ANGIOGENESIS REGULATORS

The complete set of the molecular players involved in the production of each layer of a functional vessel is unknown, even for the most rudimentary of the capillaries. In the course of angiogenesis, the preexisting vessel provides some but not all components and instructions for the formation of its new sprouts and branches. Endothelial and mural cells (pericytes and smooth muscle cells (SMC)) migrate and multiply on extravasated clotted plasma. Other participants of angiogenesis are fibroblasts, which release angiogenic cytokines, as well as chemokines that attract inflammatory cells, and a variety of subtypes of leucocytes. The latter are important sources of chemokines and pro-angiogenic factors [matrix metalloproteinases (MMPs), interleukin (IL) 1 and 8, fibroblast growth factors (FGFs), interferons (IFNs), among others], needed for angiogenesis occurring during wound healing and repair. Imbalance between the expression of pro- and anti-angiogenic factors and their receptors on EC may determine the generation or regression of new blood vessels.

Stimulators of angiogenesis include such mitogens as vascular endothelial growth factor (VEGF) (10, 11), FGF (12, 13), platelet-derived growth factor (PDGF), epidermal growth factor (EGF). Also, hypoxia conditions that frequently occur during

the angiogenic process, are known to result in the stabilization of the hypoxia-inducible factor 1 (HIF1), which up-regulates the expression of certain angiogenic proteins (14). Angiogenesis is also known to be stimulated by transforming growth factors alpha and beta (TGF-α and -β), interleukins, chemokines, angiopoietins (Ang) (15, 16) and small molecules such as sphingosine 1-phosphate (17), that are known to promote cell proliferation, survival and differentiation of ECs (18, 19).

Endogenous inhibitors of angiogenesis include various anti-angiogenic peptides, hormone metabolites, and apoptosis modulators (4). A series of endogenous anti-angiogenic factors have been described, of which many are fragments of naturally occurring extracellular matrix (ECM) and basement membrane proteins (20), while others are non-matrix derived, such as vasostatin, troponin I and angiostatin, among many others (21). Remarkably, many inhibitory molecules, such as "statins", are derived from larger proteins that have no effect on angiogenesis (21). Among these are angiostatin (22), a fragment of plasminogen that binds directly to the adenosine triphosphate (ATP) synthase on the surface of EC, which might play a role in intracellular acidification, thus triggering apoptotic events in EC (23). Other anti-angiogenic proteins resulting from the proteolysis of larger molecules are endostatin (a fragment of collagen XVIII) (24, 25), tumstatin (26), and canstatin (fragments of collagens that bind to integrins) (27). Some full-length extracellular proteins also display anti-angiogenic activity. Among them are thrombospondin-1, 2 and 3 produced by normal fibroblasts (28), IFN-α, produced by leukocytes (29), and platelet factor 4 (PF4) (30).

1.1.1 Coordination of Angiogenesis by Cellular and Molecular Interactions

During angiogenesis, vascular cells break up old and establish new contacts (gap, tight, and adherent junctions), including contacts with the surrounding matrix (31-33). Moreover, angiogenic cells continuously lay down and degrade the ECM to facilitate their migration and proliferation. Thus, angiogenesis likely results from the simultaneous and harmonized occurrence of multiple cellular and molecular

processes. An initial angiogenic stimulus triggers cell activation, which is followed by cell migration, division, and alignment, vessel pruning and maturation, and phenotypic and organotypic differentiation (34) (Figure 1). The onset of hemangiogenesis is the opening of intracellular junctions in the endothelial lining, allowing the leakage of plasma into the subendothelial space. Thus, one of its possibly earliest modulators is vascular permeability factor (VPF) (10, 35). Alternative vasodilators useful in angiogenesis are prostaglandins, lipid products of arachiodonic acid metabolism catalyzed by cyclooxygenases (COX-1 and COX-2).

Resident macrophages, polymorphonuclear leukocytes, and mast cells can be attracted to sites in need of blood vessels by proinflammatory growth factors and by cytokines and chemokines such as interferon-gamma-inducible protein-10 (IP-10) and PF4 (36, 37). Once stimulated under angiogenesis-promoting conditions such as hypoxia, high lactate concentrations, and temperature stress these cells release IL1s, tumor necrosis factor (TNF), FGFs, and proteolytic enzymes that facilitate ECM degradation and activation of MMPs (38). In addition, they also induce ECs, fibroblasts, and keratinocytes to release another set of proteases that further degrade the basal lamina, facilitating EC migration and growth toward the chemotactic source (39). Consequently, there is an overall loosening of the original cell-cell and cell-matrix contacts during the earliest angiogenic events. These processes involve a large group of proteins of the integrin, selectin, and cadherin families, as well as members of the immunoglobulins-like gene family, like intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), platelet endothelial cell adhesion molecule (PECAM) (40). Finally, proinflammatory molecules such as TNF, IL1, and lipopolysachraride (LPS) as well as VEGF induce the expression of certain ephrins (Eph). It is thought that the interaction of Eph receptors (33, 41) with Eph expressed on the surface of adjacent ECs promotes their sprouting, migration, and capillary tube formation (33, 41). Similar events may also be mediated by the Notch ligands, Jagged and Delta (42).

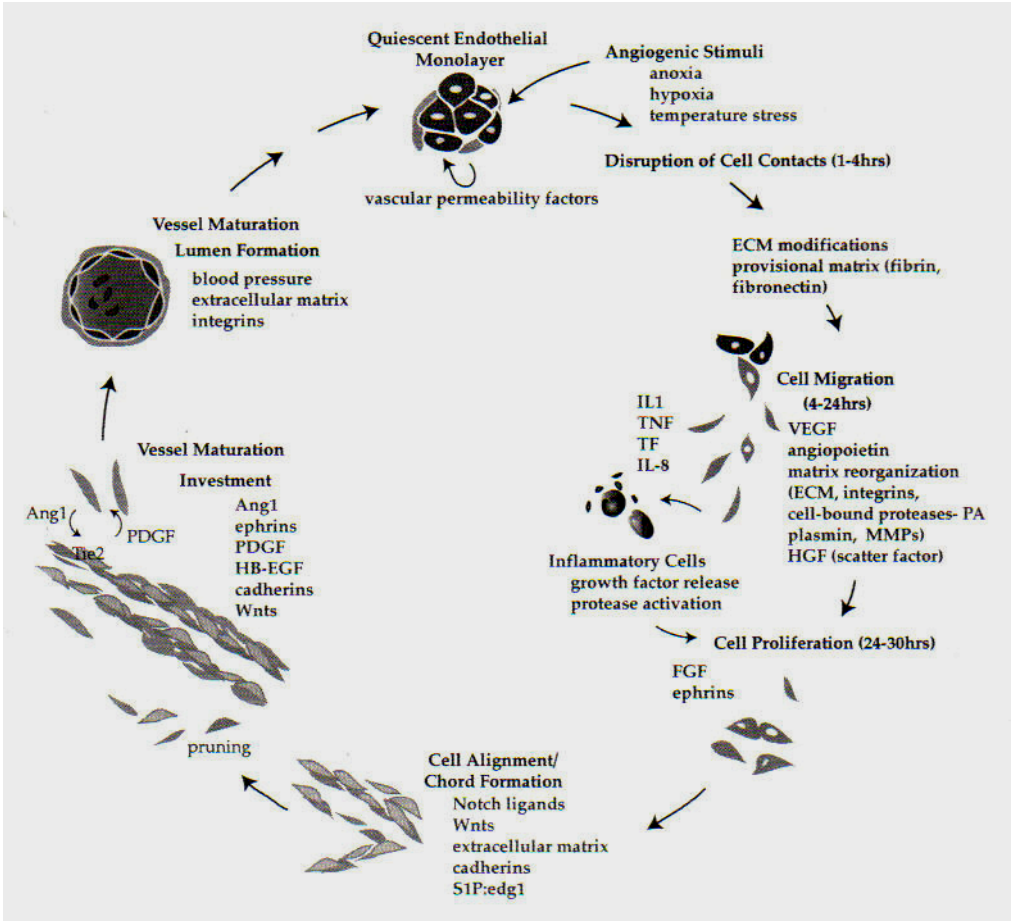


Figure1- Regulation of the angiogenic cycle. The major steps involved in angiogenesis are receipt of an angiogenic stimulus, disruption of endothelial cell contacts, cell migration, cell alignment and tubule formation, and the maturation of vascular structures into vessels by investment with mural cells and formation of a continuous lumen with the circulation. Factors that control the various steps are listed (from Mouta C, Liaw L, and Maciag T. Handbook of Cell Signaling - chapter 334; 2003; 3: 455-462).

A primitive vessel thus assembles along the concentration gradient of angiogenic factors (mitogens, proteases, and others), laying the foundation for a new branch of the vasculature that is still only barely functional. As VEGF, FGFs, and Ang2 levels begin to fall, Ang1 produced by mesenchymal cells activates Tie2 receptor on EC, and this in turn leads to the production and release of a recruitment signal for pericytes (PDGF-BB) and SMCs (PDGF-AA, HB-EGF) (43). Once these cells arrive and contact the endothelium, TGF-β may be activated, inhibiting EC proliferation, altering integrin expression profiles, and stimulating matrix deposition (44). Similarly, it is likely that ECs either secrete or express surface molecules that contribute to pericytes and SMC quiescence (45). As blood vessels mature, they also

establish a different set of interactions with newly deposited ECM that replaces the provisional matrix. These EC-ECM contacts are important in lumen formation, vessel elongation, and acquisition of a vessel-specific EC differentiated phenotype. Also, the EC-mural cell interaction is important for effective function of the new vessel and not just its growth and maintenance (44, 46).

1.2 THE COAGULATION CASCADE

Events that result in vascular damage trigger the blood coagulation cascade in which the participating enzymes are activated by proteolysis, and the final product is fibrin, which forms the basis of blood clots. *In vivo*, the coagulation cascade is initiated when the damaged endothelium interacts with the platelets. This interaction is mediated through von Willebrand factor-GpIbα and collagen-receptor interactions. Further, platelets are recruited to the platelet plug due to their interactions with adhesive proteins such as fibrinogen. Interaction of platelets with adhesive proteins, mediated by integrin receptors results in intracellular signaling and cellular activation. The activated platelets provide a surface enriched in negatively charged phospholipids upon which coagulation factors can assemble. Tissue factor (TF), which is exposed by the vessel injury, is a glycoprotein that forms a complex with Factor VIIa (FVIIa) in the presence of calcium ions and phospholipids to catalyze the activation of the Factor X (FX) (47)- the *extrinsic pathway*. FX can also be activated by a catalytic complex formed by the *intrinsic pathway*. This complex is composed of the serine protease FIXa and its cofactor FVIIIa assembled on appropriate phospholipids in the presence of calcium ions (48). Once FX has been activated, it assembles, together with the nonenzymatic cofactor FVa and calcium, on phospholipids surface into a macromolecular catalytic prothrombinase complex. This complex cleaves prothombin, a serine protease zymogen primarily produced in the liver and secreted into the blood, to thrombin (49) (Figure 2).

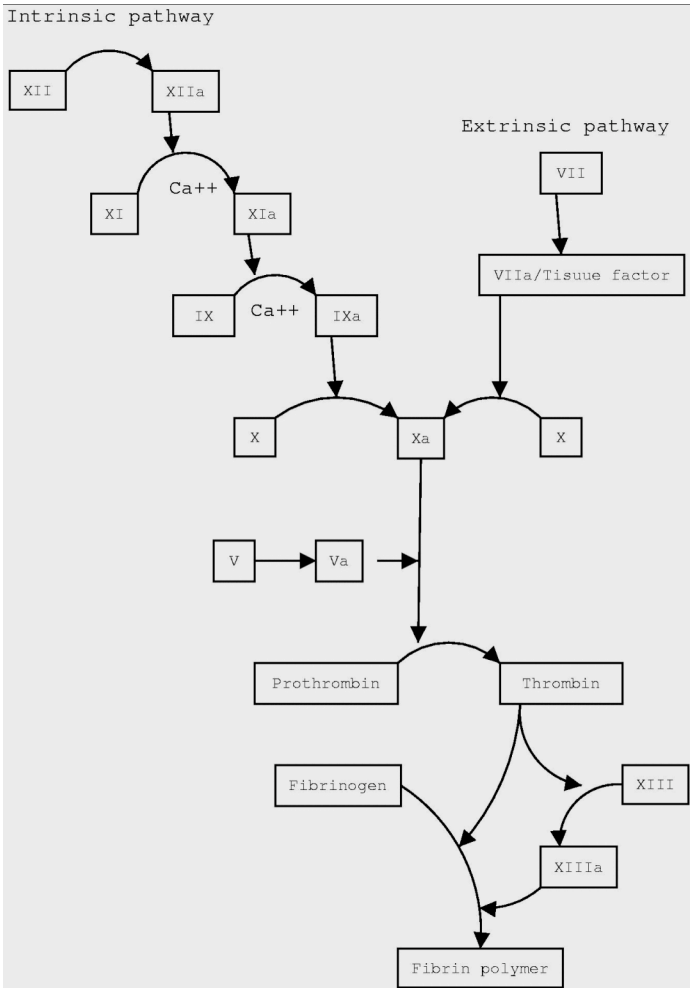


Figure 2- Scheme of coagulation cascade showing the division of its primary stages into the intrinsinc and extrinsinc pathways. The blood coagulation cascade involves proteolytic activation of a series of membrane bound proteases. Activated thrombin cleaves fibrinogen into fibrin, which is crosslinked into clots by the transglutaminase activated factor XIII.

In the dynamic process of thrombin generation in blood, some of the early-generated thrombin feeds back on the cascade system to activate factors V and VII, enabling sustained generation of thrombin (50, 51). Thrombin, as the common final enzyme of the coagulation cascade, can promote clot formation by catalyzing the cleavage of Gly-Arg bonds in circulating fibrinogen converting it to fibrin (52). Thrombin continues to be active until the whole platelet plug is surrounded and stabilized with crosslinked fibrin. However, as the clot continues to expand it reaches areas of intact endothelium, where it encounters thrombomodulin, which has a high affinity for thrombin. Thrombomodulin functions as a sink, draining and redirecting thrombin activity, thereby protecting the intact endothelium from multiple thrombin actions and obstructive thrombus formation (53). All generated thrombin is eventually cleared from the circulation in complex with antithrombin or heparan cofactor II (54).

1.3 INTERACTIONS BETWEEN PHYSIOLOGICAL ANGIOGENESIS AND HAEMOSTASIS

The classical components of the coagulation cascade are well established; however, the mechanisms by which some of these molecules participate in developmental events within the vascular system are not completely understood. The sustained generation of thrombin, for example, at sites of thrombosis has focused attention on the role thrombin may play in vascular remodeling in response to thrombosis and other vascular injuries. Besides of fibrin generation, thrombin is known to stimulate SMCs contraction and proliferation, monocyte chemotaxis, and platelet aggregation as well as many other multiple physiological effects (55-59).

More detailed *in vitro* studies have aimed to investigate the role of thrombin in the individual steps of angiogenesis. Thrombin-induced signaling in the endothelium results in multiple phenotypic changes including: alterations in cell shape, direct and indirect stimulation of proteases, including MMPs and urokinase plasminogen activator (uPA). These enzymes in turn, catalyze the breakdown of the basement membrane and local extracellular matrix (60-62), increasing the endothelial monolayer permeability (63), mobilization of adhesive molecules to the endothelial surface (64), DNA synthesis (65), and cell migration (66). Besides its role in the endothelium, thrombin also stimulates proliferation of rat aortic smooth muscle (67), keratinocytes (68), and fibroblasts (58, 69).

Some of these effects necessary for angiogenesis are independent of thrombin's fibrinogen-cleaving activity and are mediated through thrombin receptors activation and its downstream signaling networks (70, 71).

1.4 THROMBIN RECEPTORS

Thrombin-stimulated cellular events are mediated, at least in part, through the proteolytic activation of seven span transmembrane G protein-coupled receptors (GPCRs) of the protease activated receptor (PAR) family, by a unique proteolytic cleavage of their extracellular domains (72). Thus, the activation of PAR1 occurs when a serine protease binds to a unique site in the amino-terminal extracellular domain of the receptor, resulting in cleavage between Arg^{41} and Ser^{42} leading to the exposure of a new amino-terminus (SFLLRN, in human; SFFLRN, in mouse and rat), referred as the "tethered ligand" (73) which binds to the second extracellular loop of PAR1 to induce transmembrane signaling (67, 74-77). Even without proteolytic cleavage, PAR can be activated by short synthetic peptides (of 5-14 amino acid residues) based on the sequence of the revealed tethered ligand, such as thrombin receptor activator peptides-TRAP (73, 78, 79).

Currently, four members of the PAR family have been identified in mice and human (PAR1, PAR2, PAR3, and PAR4). Of these, PAR1 (73), PAR3 (80), and PAR4 (81, 82) are activated by thrombin (83), whereas PAR2 (84) is activated by trypsin and tryptase but not by thrombin (85, 86). The prototypic member of the PAR family, PAR1 is the predominant thrombin receptor in EC (87) being also detected in a variety of other cell types, including platelets, fibroblasts, monocytes, T lymphocytes, natural killer cells, SMC, epithelial cells, neurons, glial cells, mast cells, cardiomyocytes (88); and in certain tumor cell lines (73, 89-94). PAR1 has been shown to respond to a group of serine proteases that includes thrombin (73), plasmin (95), FVIIa (86), FX (96), and activated protein C (97, 98). Recently, it has been shown that PAR1 could also be activated by matrix metalloprotease-1, a member of the zinc-dependent MMP family (99). It is known that PAR1 plays an important role in angiogenesis. Disruption of PAR1 gene results in 50% embryonic lethality (100, 101). At E8.5, $PAR1^{-/-}$ mice are still indistinguishable from wild-type littermates, but at E9.5, a cohort of PAR1 null embryos are significantly smaller than their siblings, and their hearts do not beat. Hemorrhage, especially in the pericardium, is apparent in

more than a third of the PAR1$^{-/-}$ embryos. Delay in development of the embryo occurs in conjunction with abnormalities in placental development. At this stage about half of the PAR1$^{-/-}$ embryos die, while the other half "catch up" with theirs PAR1$^{+/-}$ littermates. A cause of embryonic death has not been determined definitively, but it is noteworthy that failed hemostasis is not present. Thus activity of the plasma coagulation cascade is not altered, which is consistent with the fact that thrombin ability to cleave fibrinogen is not dependent on the presence or absence of PAR1. These observations indicate that PAR1 is not required for proper platelet responses to thrombin, and PAR3 is now recognized as the receptor responsible for thrombin signaling in mouse platelets (80). Interestingly, transgenic expression of PAR1 under the control of an EC-specific promoter is able to decrease the embryonic lethality to ~ 14% (102). This suggests that lethality in PAR1$^{-/-}$ embryos is related, in large part, to lack of PAR1 in ECs.

1.4.1 Activation and Desensitization of PAR

In common with many GPCRs, PAR1 couples to several different G proteins. The principal pathway of PAR-mediated signaling is through Gαq proteins. It results in activation of phospholipase C (PLC) which catalyses the hydrolysis of inositol trisphosphate to inositol bisphosphate and diacyglycerol, leading to mitogen-activated protein kinase (MAPK) phosphorylation and receptor tyrosine kinase translocation into the nucleus where it phosphorylates or regulates its targets proteins, inducing degranulation and platelets aggregation and integrin activation (103-105). PAR1 is also couples to G13, the activation of this G protein leads to thrombin-stimulated DNA synthesis and cell migration in SMC, and also to the activation of Rho and cytoskeletal changes affecting platelets permeability and cell migration (106, 107). Another set of G proteins are the Gβγ which are involved in the activation of phosphoinositide 3-kinase (PI3K) (108). A complete overview of PAR1 signaling mediated by G-protein signaling is represented in Figure 3.

Activated GPCRs are rapidly desensitized as a result of their fast phosphorylation by G-protein receptor-associated kinases and other kinases (109, 110). In many cases,

phosphorylation enhances receptor affinity to β-arrestin, and β-arrestin binding prevents receptor-G protein interaction, thereby uncoupling the receptor from downstream signaling. However, in the case of PAR1, the binding of arrestins is independent of PAR1 phosphorylation levels (111). Arrestins also interact with components of the endocytotic machinery to facilitate recruitment of GPCRs to

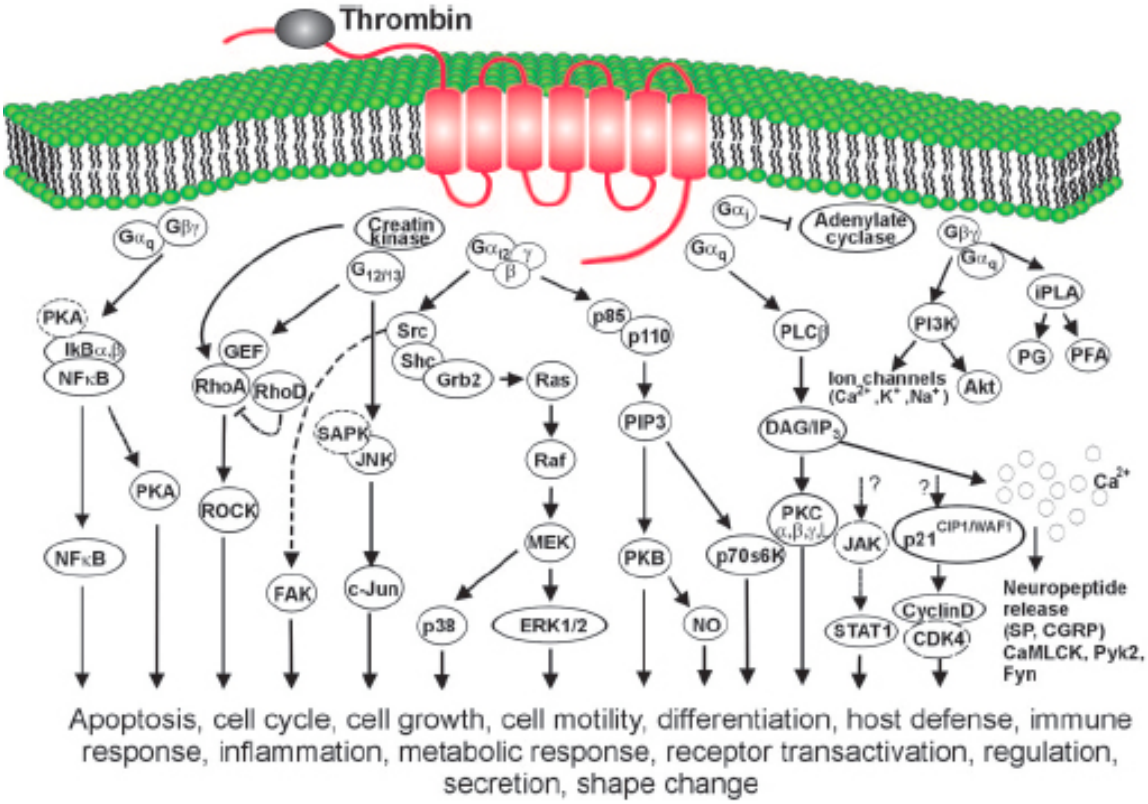


Figure 3- Overview of the major G protein-mediated signaling pathways coupled to PAR1 activated in different tissues and cell types, upon thrombin stimulation. Dashed lines or circles represent signaling pathways or intermediates that are not fully revealed to be activated by PAR1, but are in favor of other G protein-coupled receptors or are typical intermediate-accompanying molecules (from Steinhoff, M. *et al*. Endocrine Reviews 2005; 26(1): 1-43).

clathrin-coated pits and their internalization (112-114). Once internalized into endosomes, GPCRs dissociate from their ligands, become dephosphorylated, and then return to the cell surface where they are capable to mediate the ligand-dependent signaling.

It is possible that proteolytic cleavage of PAR could result in sustained activation of the receptor by the tethered ligand, which does not diffuse away. However,

signaling by PAR1 is rapidly terminated despite the irreversible proteolytic mechanism of receptor activation. It was demonstrated that the cumulative phosphoinositide hydrolysis in response to thrombin precisely correlates with the absolute rate of receptor cleavage during a given time (115). This suggests that each activated PAR1 molecule, generates a defined amount of the second messenger, and then shuts down (at least in terms of Gq activation). Given the irreversible nature of PAR activation, internalization and lysosomal sorting of proteolytically activated PARs may be particularly important for termination of receptor signaling. The process of down-regulation, a decrease in total receptor number, occurs after prolonged agonist exposure for most classic GPCRs. The regulation of the receptor protein levels occurs partially at the level of transcription and RNA stability.

Activated PAR1 and PAR2 are internalized, sorted predominantly to lysosomes and rapidly degraded. Several studies suggest that PAR1 down-regulation by receptor internalization and lysosomal sorting are required to terminate signaling by irreversibly activated receptors (111). It has been recently demonstrated that trafficking of endogenous PAR1 is altered in metastatic breast carcinoma cells, in such way that the activated receptor is not sorted to lysosomes. Consequently, activated PAR1 causes sustained signaling even after thrombin withdrawal. Thus internalization and lysosomal sorting of activated PAR1 is critical for the temporal regulation of thrombin signaling (114). PARs at the cell surface are then replenished from an intracellular pool of uncleaved freshly synthesized PARs.

1.4.2 INHIBITORS OF PAR SIGNALING

PAR activation can be inhibited by strategies that block extracellular domains of the receptor. Thrombostatins, modified bradykinin-derived blocking peptides, appear to directly bind PARs and inhibits their activity (116). Monoclonal antibodies generated against the cleavage site of PAR1 have also been used to block cleavage and activation of PAR1 (117). Small peptide and non-peptide molecule PAR1 antagonists have also been generated, in the last few years, based on the sequence of thrombin receptor activating peptide (118-120). These antagonists function by

blocking interaction of the newly exposed tethered ligand binding sites on the extracellular face of the receptor but do not inhibit thrombin binding or receptor cleavage. However, several limitations such as lack of potency and specificity, low affinity and partial agonist activity, have been reported for the majority of these PAR1 antagonists. Only few PAR1 antagonists have been described in the literature as highly potent and specific, like RWJ-56110 (119); SCH 79797 (121); RWJ-58259 (122) and FR171113 (123).

Other studies have described intracellular inhibitors that disrupt PAR-G-protein interaction. The C-termini of G-protein α subunits are critical for specific binding to their cognate GPCRs. Peptides corresponding to the C-termini of Gα subunits have been used to block PAR1 coupling to specific G protein subtypes in ECs (124). This strategy is useful to dissect out which G protein subtype mediates a particular response but lacks PAR specificity since these peptides would presumably block coupling of G proteins to other GPCRs expressed in the same cell.

In summary, PARs are irreversibly activated, thus the mechanisms that contribute to the termination of signaling are critical determinants of the magnitude and kinetics of PAR1-mediated response in cells. The unusual irreversible proteolytic mechanism of PAR activation is clearly distinct from that involved in activation of other GPCRs. This mechanism appears to have evolved to deal with termination of signaling by these proteolytically activated GPCRs, since all other GPCRs are reversibly activated.

1.4.3 ROLE OF PAR IN VASCULAR BIOLOGY AND TISSUE REMODELING

The activation of PAR triggers a cascade of downstream events, leading to diverse cellular outcomes such as calcium signaling, engagement of integrins, cell adhesion and migration, gene transcription, and mitogenesis. An emerging common theme is that PAR act as high-gain sensors of extracellular protease gradients and allow the cell to react to the proteolytically altered environment. This unique ability to sense proteases can be utilized both for migration toward proteases and for detection of changing microenvironment (125). More recently, PAR have been shown to be critically involved in the tissue remodeling processes necessary for normal

development including angiogenesis and trophoblastic invasion (76, 102, 126-128). Stimulation of PAR1 in atherosclerotic plaques has been implicated in SMC proliferation and restenosis as well as in the repair processes and a variety of acute and chronic inflammatory conditions (129). PAR1 has been proposed to be involved in the invasion and metastasis of cancers such as breast, colon, lung, pancreas, prostate cancers and melanomas (126, 130-134). Even-Ram *et al.* (126) demonstrated that PAR1 expression levels were directly correlated with the degree of invasiveness in both primary breast cancer specimens and established cancer cell lines. High levels of PAR1 were found in infiltrating ductal carcinoma while only undetectable levels were observed in normal and premaligmant lesions (130) (135).

Recent observations support a role of thrombin and PAR1 in the regulation of normal (136-138) and atherosclerotic endothelium (129). In normal human arteries, PAR1 is mostly confined to the endothelium, whereas during atherogenesis, its expression is enhanced in regions of inflammation associated with macrophage influx, SMC proliferation, and an increase in mesenchymal-like intimal cells (129). *In vivo*, a neutralizing antibody to PAR1 has been observed to reduce expression of messenger RNA (mRNA) for the proliferating cell nuclear antigen, and of the index of intimal and neointimal SMC accumulation in rat arteries during balloon angioplastly (139). These data suggest that PAR1 regulates proliferation and accumulation of neointimal SMC during tissue repair.

Several lines of evidence suggest that thrombin, acting through PAR1, contributes to thrombosis and restenosis in patients after angioplasty procedures (57, 129, 140-142). Increased levels of thrombin generation in addition to a high level of thrombin receptor have been detected at the sites of vascular lesions (57, 129, 140). TRAP antagonists and antibodies to PAR1 inhibit thrombin- or TRAP-stimulated platelet aggregation *in vitro* (118, 119, 142-144) as well as experimental arterial thrombosis in primates (57, 142).

The role of PAR1 in vascular biology and tissue remodeling is further stressed by the fact that factors activated upon thrombin induced PAR1 signaling are known to play a crucial role during the process of vascular remodeling. Malik and coauthors (104, 145) studied the involvement of PAR1 in the activation of nuclear factor

kappaB (NF_kB) in ECs, demonstrating that Gαq and Gβγ dimers are responsible for NF_kB activation and ICAM1 transcription in ECs which are induced by activation of PAR1 by thrombin or by a synthetic peptide. Overall, the expression and/or release of several growth factors, including FGF2 (146), PDGF (147, 148), VEGF (76, 149), the upregulation of the insulin-growth factor receptor 1 (IGFR1) (150), and the activation of fibroblast growth factor receptor 1 (FGFR1) (151) were demonstrated to be induced in response to thrombin.

A number of different ligands have been implicated in the development and maintenance of the vasculature including: VEGF family, angiopoietins and their receptor-Tie2, PDGF, ephrins, and its receptors, FGF family as well as many other cytokines and chemokines. The Notch family receptors and ligands also play a significant and non-redundant role in angiogenesis and arterial specification.

In this literature review, we will discuss more in detail the effects of FGF and Notch signaling upon vasculature.

2 FIBROBLAST GROWTH FACTORS SIGNALING

2.1 FGF AND FGFR

The FGF signaling system has been identified in multicellular organisms ranging from *C.elegans* to vertebrates but not in unicellular organisms (152). FGF1 was first isolated as mitogen from bovine and sheep brains tissue in the 1970s (153), and few years later Abraham *et al.* isolated FGF2 (154). By now, 24 structurally-related members of the FGF family, with a broad range of biological activities, have been identified (152, 155-158). Despite their status of "prototype" FGFs, FGF1 and FGF2 differ from most other FGFs in several important aspects. Most FGFs (FGF 3-8, 10, 17-19, 21, and 23) have N-terminal signal peptides (SP) and are secreted from cells. By contrast, FGFs 9, 16, and 20 lack a conventional SP sequence, but are nevertheless secreted through endoplasmatic reticulum (ER)/Golgi apparatus. FGF1 and FGF2 also lack SP, however, unlike FGF9, 16, and 20, the prototypes FGFs are secreted by novel secretion mechanisms, independent of the ER-Golgi traffic (159, 160), FGF22 with a putative N-terminal SP remains attached to the cell surface rather than being secreted. FGFs 11-14 lack SP, remain in the intracellular compartment and function within cells in a receptor-independent manner. These FGFs might be intracellular components of a tissue-specific protein-kinase signaling module and seem to share structural, but not functional, homology with other FGFs (161).

FGFs are widely expressed in developing and adult tissues and have various biological activities both *in vitro* and *in vivo*, including roles in neurulation, mesoderm formation, somite segmentation (162, 163), angiogenesis (164-166), nail and teeth growth, tissue injury repair during postnatal life, neurogenesis (167, 168), inflammation (169), liver fibrosis (170), restenosis (171), vascular remodeling (172, 173) and in pathogenesis of some tumors (160, 174-176).

An important feature of FGF biology involves the interaction between FGF and heparin or heparan sulfate (HS) proteoglycan (HSPG) (177). These interactions

stabilize FGFs to thermal denaturation and proteolysis and may severely limit their diffusion and release into interstitial spaces (178-180). It has been established that heparin or HS are required for FGF in order to more efficiently activate its receptors (155, 181).

The FGF receptors, FGFR1-4, are tryrosine kinases that belong to the immunoglobulin (Ig) superfammily, with an extracellular ligand-binding domain, a transmembrane domain (TM) and a split intracellular tyrosine kinase domain (182). The extracellular domain contains two or three Ig loops that arise as a result of alternative splicing, and a heparin-binding domain (177, 183). The two membrane proximal Ig loops bind the FGF ligand, resulting in the formation of a complex containing at least two FGFs, two FGFRs and the glycosaminoglycan moiety (184). Upon ligand binding, receptor dimers are formed and their intrinsic tryrosine kinase is activated causing phosphorylation of multiple tryrosine residues on the receptors (182). These then serve as docking sites for the recruitment of src homology 2 or phosphotyrosine binding domains of adaptors, docking proteins or signaling enzymes (185). Signaling complexes are assembled and recruited to the active receptors resulting in a cascade of phosphorylation events (186). Genetic and biochemical experiments have helped to elucidate the signal transduction pathways concomitantly activated by FGFs in most cell types. The best understood of these are the RAS-MAPK pathways which include ERK 1/2, p38 and JNK kinases; the PI3K-AKT pathway, and the PLCγ pathway (185). The activation of ERK1/2 and p38 in response to FGF has been observed in all cell types, while the activities of other signal transduction pathways varies depending on the cell type (187). Receptor signaling can be negatively modulated by down-regulation of FGFR through internalization, or by the induction of proteins such as Sprouty (188, 189) and Sef (190, 191) that inhibit the downstream signaling pathways.

A central issue in FGF biology is to understand how diverse cellular responses are determined and how similar signaling inputs can generate distinct patterns of gene expression that govern the specificity of the cellular responses.

2.2 FGF TARGET GENES

FGF signaling results in changes in the steady-state levels of many gene transcript levels. The mechanism of these FGF-induced alterations has only been studied for a few target genes. While in many cases the effects are indirect, FGFs were shown to affect the transcription of several genes directly, independently of protein synthesis. Exogenous FGFs can be translocated into the nucleus and are found in the nuclear matrix, the nucleolus, and in association with chromatin (192). The effect of FGFs on gene expression is cell-type dependent. For example, while FGFs increase collagenase 1 mRNA levels in fibroblasts, the same FGFs, inhibit the expression of this metalloprotease in keratinocytes (193). Among the FGF-regulated genes (including direct and indirect targets) are immediate early response genes (c-*fos*, c-*jun*, c-*myc*, *egr-1*, *thrombospondin-1, fnk, and AP-2*), delayed early response genes (*proliferin, ornithine decarboxylase, glyceraldehydes 3-phosphate dehydrogenase, and an aldose reductase-related protein*), homeobox genes (*msx-1*, *evx-1*, *xnot*, *xcad*, *xhox*, *eve1*, *hoxb9*, *hox-d11-d13*, and *lim-1*), patterning genes (*Xbra/ntl*, *en-2*, *cad-1*, and *Shh*), growth factors and their receptors (nerve growth factor, human chorionic gonadotropin, PDGFA, IGFII, IGF-binding protein 6, FGFs, and FGFR2), skeletal muscle regulatory factors (myoD1, and myogenin), matrix proteins (integrins; collagens I, III, and IV; tenascinC; actin, and neural cell adhesion molecule), proteases, protease activators and inhibitors (plasminogen activators, nexin-1, collagenases, and metalloproteinase inhibitors), and genes coding for low density lipoproteins (LDL) receptor, fatty acid synthase, and ER Ca^{2+} ATPase, osteocalcin and proenkephalin (193-197). While some of the FGF-induced genes encode transcription factors (EGR-1, HOX proteins, the zinc finger proteins SLUG, STAT1) leading to late responses, others such as proteases and matrix proteins, effect cell behavior more directly and rapidly (198, 199).

FGFs can work either synergistically or antagonistically with other growth factors. Some of the growth factors that often colocalize and act in cooperations with

FGFs are members of the TGF-β, IGF1, and vertebrate homolog of Drosophila wingless families (200).

2.3 THE BIOLOGICAL ACTIVITIES OF FGFS, DIVERSITY OF CELLULAR RESPONSES TO FGF SIGNALING

As the name implies, FGFs were originally identified as proteins capable of promoting fibroblast proliferation. This proliferative response to FGF is in fact shared by a broad range of cell types, including keratinocytes, immature osteoblasts, oligodendrocyte progenitors, and ECs (201-203). However, as FGF field developed, it has become clear that FGF signaling leads to very different responses in other cell types, being implicated in a variety of physiological and pathological processes. FGFs can induce *in vitro* a complex pro-angiogenic phenotype in ECs that recapitulates several aspects of the *in vivo* angiogenesis process, including modulation of EC proliferation, migration, protease production, integrin and cadherin receptor expression, and intercellular gap-junction communication (Figure 4) (204). However, different cell types, or even the same cell, may display alternate, sometimes opposite responses to FGFs, depending on the state of differentiation, biochemical status, and the cellular, physical and chemical environment of the cell.

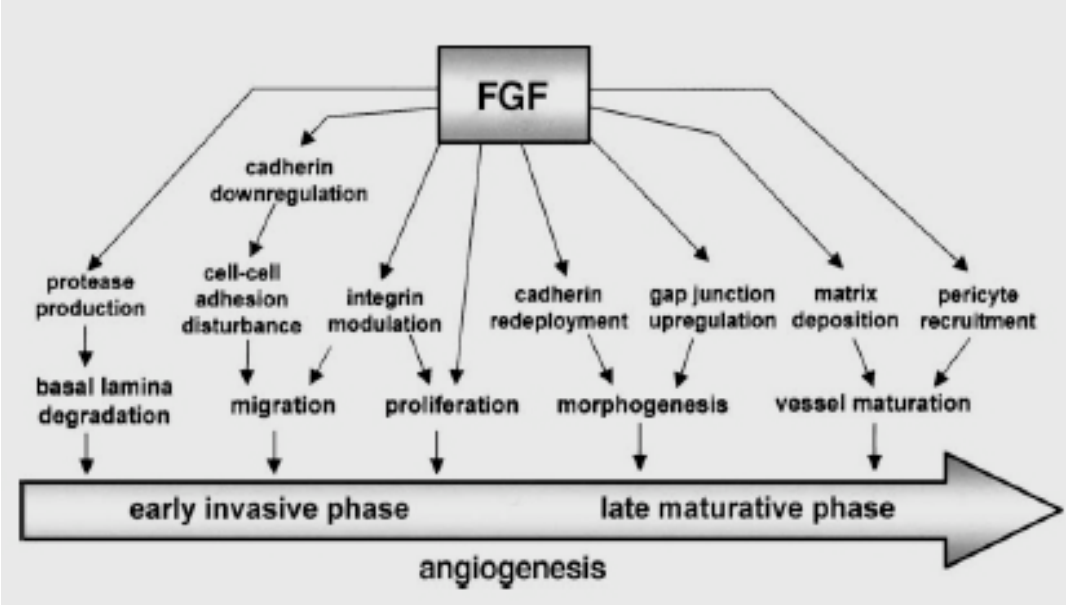


Figure 4- Schematic representation of the events triggered by FGFs in endothelial cells that contribute to the acquisition of the angiogenic phenotype *in vitro* and to neovascularization *in vivo* (from Presta *et al*. Cytokine and Growth Factor Reviews 2005; 16:159-178).

The importance of the proper spatial and temporal regulation of FGF signals is evident from human and mouse genetic studies, which show that mutations leading to the dysregulation of FGF signals cause a variety of developmental disorders including dominant skeletal diseases and tumor growth. The analysis of expression patterns and activities of FGFs during embryonic development has provided further insight into their normal biological functions. In a vertebrate embryo, the activities of FGFs are required from the earliest stages of development through the detailed patterning of organs. A number of gene knockout experiments in the mouse have shown a complementary role of FGFs in several development processes. Many members of the FGF family have been disrupted by homologous recombination in mice. The phenotypes range from very early lethality to subtle phenotypes in adult mice. The major phenotypes observed in FGF knockout mice are shown on Table 1.

Table 1 - FGF Knockout mice (adapted from Ornitz, *et al.* Genome Biology 2001; 2(1): 1-12)

Gene	Survival of null mutant*	Phenotype	References
Fgf1	Viable	None identified	(205)
Fgf2	Viable	Mild cardiovascular, skeletal, and neuronal defects	(205-209)
Fgf3	Viable	Mild inner ear, skeletal (tail), and CNS defects	(210)
Fgf4	Lethal, E4-5	Inner cell mass proliferation	(211)
Fgf5	Viable	Long hair, angora mutation	(212)
Fgf6	Viable	Subtle, muscle regeneration	(213-215)
Fgf7	Viable	Hair follicle growth, ureteric bud growth	(216, 217)
Fgf8	Lethal, E7	Gastrulation defect, CNS, and limb development	(218-221)
*Fgf*9	Lethal, P0	Lung mesenchyme, heart, XY sex reversal, gastrointestinal tract, and skeleton	(155, 222)
*Fgf*10	Lethal, P0	Development of multiple organs, including limb, lung, thymus, pituitary	(223-225)
Fgf12	Viable	Neuromuscular phenotype	(155)
Fgf14	Viable	Neurological phenotype	(155)
Fgf15$^{\phi}$	Lethal, E9.5	Not clear	(155)
*Fgf*17	Viable	Cerebellar development	(226)
Fgf18	Lethal, P0	Skeletal and lung development	(155)

*E, embryonic day and P, postnatal day; CNS, central nervous system. $^{\phi}$ Human *FGF19* and mouse *FGF15* are orthologous genes

2.3.1 FGF1 BIOLOGICAL ACTIVITY

The best known function of FGF1 is the stimulation of DNA synthesis followed by cell proliferation (227). But this growth factor is also responsible for many other biological effects induced in different cell types. FGF1 is crucial at numerous stages of embryonic development, due to its ability to regulate morphogenesis and differentiation (228, 229). In adults, FGF1 is involved in the regulation of such important physiological processes as angiogenesis, osteogenesis, tissue injury repair (172), cell migration, and chemotaxis (227, 230, 231). In animal models of brain ischemia, FGF1 has been documented to prevent cell death resulting from ischemic damage (232), being effective as a neuro-protective agent (233). It was also shown that FGF1 contributed to brain protection after an acute stroke (234), and also stimulated spinal injury repair (235). In addition, FGF1 appears to be helpful in the healing of non-union fractures, due to its competence to induce osteogenesis *in vivo* (236). However, the widest application of FGF1 can be expected in cardiovascular diseases. In particular, patients with coronary artery disease, having occluded vessels to be bypassed or with other limitations of surgery are potential candidates for angiogenesis-based therapy (228, 237). Nevertheless, the first attempts to use FGF1 as an angiogenic factor in ischemic dog myocardium were ineffective (238), probably due to its rapid inactivation *in vivo* (239). A subsequent study on an animal model demonstrated improved myocardial perfusion and function after administration of modified FGF1 with increased half-life (240). FGF1 is known to be susceptive to thrombin cleavage that can explain low efficiency of its application *in vivo*. Interestingly, the development of FGF1 thrombin resistant mutant (R136K) demonstrated that even in the absence of heparin, FGF1R136K is able to induce more pronounced migration of EC than wild type FGF1. Moreover, FGF1R136K maintained this heparin-independent pro-migratory activity even after inhibition of EC growth with mitomycin D, additionally FGF1R136K does not induce intimal hyperplasia suggesting a potential clinical efficacy of this mutant, if delivered to the site of vascular intervention (241). Recently, Zakrzewska *et al.* (242) engineered

highly stable FGF1 mutants that show prolonged half-life, strong resistance to proteolysis and enhanced mitogenic activity, suggesting their potential role for therapeutic applications.

2.4 THE NON-CLASSICAL RELEASE

The majority of FGF family members have been characterized as oncogenes as the result of the presence of a classical NH_2-terminal SP sequence (159, 160). Indeed, early studies from different laboratories, have demonstrated that if one adds a SP sequence to either FGF1 or FGF2, it also becomes a transforming gene (243), and if one deletes the SP sequence from an oncogenic FGF gene family member, it looses its oncogenic potential (244).

A number of secretory proteins with defined extracellular functions have been shown not to contain a functional SP, and they do not represent substrates for the ER membrane translocation machinery (245-248). Furthermore, the extracellular appearance of such molecules is not sensitive to brefeldin A, a drug known to block ER/Golgi-dependent secretory transport. These observations led to the postulation of alternative secretory mechanisms that are fully functional in the absence of an intact ER/Golgi system and therefore have been collectively termed unconventional or non-classical secretion (245-248). Intriguingly, unconventional secretory proteins comprise growth factors and cytokines including IL1β (249), IL1α (250), FGF2 (251-254), as well as secretory transglutaminase (255), thioredoxin (256-258), Annexin 1 and 2 (259, 260), all of these proteins having significant relevance to physiological processes such as cell growth and differentiation, inflammation, and angiogenesis (Table 2).

Table 2 - Proteins exported through non-classical pathways (from Prudovsky *et al.* Journal Cell Science 2003; 116: 4871-4881)

Protein	Release Characteristics	References
Secretory transglutaminase	Constitutive, through membrane blebbing	(255)
Thioredoxin	Induced by antigen-specific T cells, intracellular vesicles not involved	(256-258)
Galectins	Constitutive, through membrane blebbing	(261-264)
IL1α	Stress-induced, Cu^{2+}-dependent, in complex with S100A13	(250, 265)
IL1β	Stress-induced, ABC-transporter-dependent, through the endolysosomal pathway	(249, 266)
FGF1	Stress-induced, Cu^{2+}-dependent, in complex with S100A13 and p40 Syt1	(267-272)
FGF2	Constitutive, Na^+/K^+ ATPase-dependent	(252-254)
Sphingosine Kinase	Constitutive, inhibited by cytochalasin	(273)
Annexin 1	Glucocorticoid-induced, ABC-transporter-dependent	(259)
Annexin 2	Thrombin-induced, in complex with p11	(260)
p40 Synaptotagmin 1	Constitutive	(268, 269)
S100A13	Constitutive	(271, 272)
HIV Tat	Constitutive	(274)
Herpes VP 22 protein	Constitutive	(275)
Foamy virus Bet protein	Constitutive	(276)
Engrailed 2	Attenuated by the CK2-dependent phosphrylation	(277-279)
HMGB1	Stress-induced, through an endolysosomal pathway	(280-284)
Leishmania HASPB protein	Constitutive, acylation-dependent	(285)

2.4.1 THE NON-CLASSICAL FGF1 RELEASE

Since 1990's our group has been studying the non-classical release of FGF1, demonstrating that FGF1 could be released in ER/Golgi-independent away in response to different cellular stresses, or in the presence of soluble Notch ligands.

2.4.1.1 CELL STRESS-DEPENDENT FGF1 RELEASE

Under normal conditions the cells expressing FGF1 do not release this protein. However, several types of stress, such as heat shock (267), hypoxia (286), cultivation under low serum conditions (287), and cell treatment with oxidized LDL (288) induce release of FGF1 from NIH 3T3 cells. Similar stresses can occur *in vivo*, in the course of inflammation, angiogenesis, and tumor development. The inhibitory analysis of stress-induced FGF1 release demonstrated that it is sensitive to methylamine, verapamil, and brefeldin A, a drug known to block protein transport from the ER to the Golgi apparatus (252, 289). At the same time, FGF1 release requires ATP synthesis, translation and transcription (267, 290).

FGF1 release from stressed NIH 3T3 cells is exported as a copper (Cu^{2+}) -dependent multiprotein release complex (MRC) which includes a cysteine-mediated FGF1 dimer, a p40 form of the membrane docking protein, Synaptotagmin 1 (Syt1) (268, 269), a small calcium-binding protein, S100A13 (270, 271), and sphingosine kinase 1(SK1) (Soldi R, Prudovsky I, and Maciag T. unpublished results) (Figure 5).

p40 Syt1 encompasses the extravesicular domain of p65 Syt1 and contains two calcium (Ca^{2+}) -binding C2 domains which are responsible for the binding of Syt1 to the plasma membrane. Interestingly, p40 Syt1 is also exported from cells independently of FGF1, both at 37^0C and 42^0C. p40 Syt1 was considered to be a product of proteolytic cleavage of p65 Syt1 in its extravesicular portion close to transmembrane domain. However, it has been found that p40 Syt1 is actually a product of the alternative translation of p65 Syt1 complementary DNA (cDNA) due to translational initiation from one of two closely located internal start codons.

Interestingly, the expression of p65 Syt1 cDNA with mutated alternative start codons attenuates stress-induced FGF1 release (291). Apparently, the transmembrane p65 Syt1 binds FGF1 and diverts it from p40 Syt1-mediated non-classical release pathway (Figure 5).

The small Ca^{2+}-binding protein, S100A13, a member of the large S100 protein family, is another component of FGF1 MRC. The molecular mechanism of activation of S100 proteins in the presence of calcium involves the exposure of two hydrophobic patches, which provide the interaction surface for the target proteins (292). In marked contrast, in the presence of calcium a drastic decrease in the solvent-exposed non-polar surface is observed in S100A13 (293). Therefore, it is believed that the mechanism of activation of S100A13 is distinctly different from those of the other S100 members. Although all S100 proteins lack a SP in their primary structure, several members of the family are released into the extracellular compartment (294). Our laboratory demonstrated that S100A13 is released from NIH 3T3 cells at 37^0C and 42^0C and it is involved in the non-classical export of other signal-peptide-less proteins such as FGF1 and IL1α (246). The co-expression of S100A13 and FGF1 results in the inhibition of S100A13 export at 37^0C. However at heat-shock, S100A13 is released in complex with FGF1 and p40 Syt1 (271). Amlexanox, an anti-inflammatory drug known to bind S100A13 with high affinity (295, 296), efficiently blocks FGF1 release indicating the importance of S100A13 for this process. Recently, Sivaraja *et al*. demonstrated that S100A13 binds to FGF1 with a moderate binding affinity (K_d ~ 80 μM), forming a complex with two molecules of FGF1 and one molecule of the S100A13 dimer (297) (Figure 5).

Timothy Hla and colleagues (273) had reported that SK1 is released through a non-classical pathway. SK1 is responsible for the biosynthesis of shingosine-1-phosphate, a lipid mediator involved in the regulation of a variety of cellular events, including apoptosis, growth and motility (298-300). While SK1 lacks a classical SP sequence, it is constitutively released at 37^0C from cells through a brefeldin A-insensitive pathway (273). Interestingly, when co-expressed with FGF1, SK1 is released in response to temperature stress. Moreover, the co-expression of SK1 in FGF1 background inhibits the constitutive release of SK1; the immunoprecipitation of

FGF1 from cell lysates and media conditioned by temperature stress reveals the presence of SK1 as a component of FGF1 MRC, since it is able to interact with S100A13 and the presence of SK1 enhances the copper-dependent formation of FGF1 complex in a cell-free system, suggesting that SK1 may act as a copper donor which facilitates the formation of FGF1 MRC in response to temperature stress (Soldi R. 2005 submitted paper) (Figure 5).

Annexin 2 is another protein participating in FGF1 release. Interestingly, when FGF1 was purified from bovine brain as a non-covalent high molecular weight complex containing p40 Syt1 and S100A13, Annexin 2 was also found in this complex ((270) and Soldi R, Prudovsky I, and Maciag T. unpublished results). Annexin 2 is known to flip from the inner to the outer leaflet of the cell membrane (260), where it functions as a receptor for plasminogen activator (301, 302). The N-terminus domain of Annexin 2 is known to associate with p11, an 11 kDa member of the S100 protein family forming a heterotetramer by association of two molecules of Annexin 2 and two molecules of p11 (303). Interestingly, studies with affinity chromatography were also able to resolve Annexin 2 in a non-covalent complex with S100A13 (295). Furthermore, since Annexin 2 associates with the inner surface of the plasma membrane (304) and the assembly of the FGF1 MRC also occurs in this locale (305), it is possible that Annexin 2 interacts with MRC and participates in its stress-induced translocation through the cell membrane (Figure 5). Indeed, preliminary results from our group demonstrated that the mouse embryonic fibroblasts (MEFs), derived from Annexin 2 knockout mice are unable to exhibit FGF1 release, when exposed to heat shock conditions.

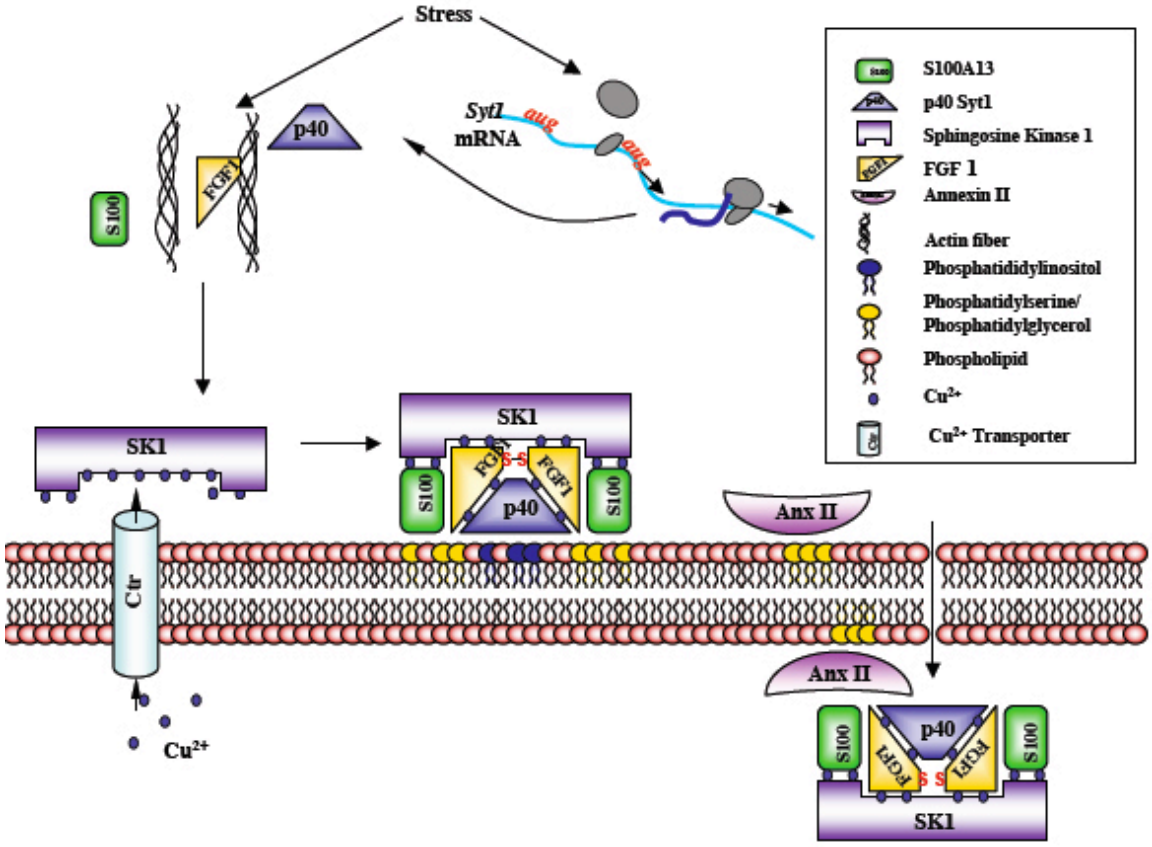


Figure 5 – Overview of the current knowledge regarding stress-induced FGF1 release. Stress induces the actin cytoskeleton-mediated transport of FGF1, S100A13, and p40 Syt1 to the cell membrane. SK1 serves as a donor of copper ions needed to dimerize FGF1 and to form the MRC associated with specific pL-mediated flipping of the FGF1 release complex through the cell membrane (with permission from Raffaella Soldi).

2.4.1.1.1 The role of the cytoskeleton in stress-mediated intracellular transport of FGF1

Previous data demonstrating that latrunculin (306) and amlexanox (307), reagents known to induce the disassembly of F-actin cytoskeleton, are able to attenuate the release of FGF1 in response to stress (270, 307), argue that the cytoskeleton plays a role in stress-induced FGF export. Further, the expression of a dominant-negative (dn) mutant of Src, an established regulator of F-actin stress fiber assembly (308) was also able to repress FGF1 release in response to heat shock, suggesting that the F-actin cytoskeleton participates in the stress-mediated release of FGF1. Indeed, real-time confocal studies of cells transfected with FGF1:GFP chimera have demonstrated

stress-induced migration of the cytosolic FGF1 to the vicinity of cell membrane, and a similar pathway is followed by S100A13 and p40 Syt1 (305). Moreover, the stress-induced redistribution of FGF1 to the cell periphery is inhibited by amlexanox (305). Taken together, these data indicate that the stress-induced translocation of FGF1 is mediated by the actin cytoskeleton.

2.4.1.2 CELL STRESS-INDEPENDENT FGF1 RELEASE

It has been reported that the suppression of Notch-mediated signaling by the ectopic expression of the soluble(s) non-transmembrane form of Notch ligands, Jagged1 (sJ1) in NIH 3T3 cells induces a phenotype reminiscent of angiogenic ECs, inducing chord formation *in vitro* and formation of highly vascularized tumors *in vivo* (42, 309). Recently, it has also been shown that NIH 3T3 cells with Notch signaling downregulated due to expression of sJ1 117kDa or soluble Delta1 (sD1), released FGF1 under non-stress conditions (310, 311). Interestingly, when these cells were co-transfected with constitutively active Notch1 (caN1), FGF1 secretion was blocked under non-temperature stress conditions, however the caN1 is unable to block the stress-induced FGF1 release (310). In addition, sJ1 117kDa expression leads to an increase in FGF1 transcription, and the development of FGFR1-dependent transformed cell phenotype (310). Although, little is known about the mechanism that regulates FGF1 release under the conditions of downregulation of Notch signaling, it appears clear that these two signaling mechanism are interacting, and this interaction plays a role in the regulation of many common physiological process.

2.4.2 POTENTIAL ROLE OF THE MOLTEN GLOBULE STATE OF PROTEINS IN FACILITATING NON-CLASSICAL PROTEIN EXPORT

The most enigmatic and intriguing aspect of FGF1 MRC release is the penetration of the MRC through the cell membrane. Translocation of a protein across the lipid bilayer might require conformational changes that increase its hydrophobicity. Proteins can achieve this by adopting a “molten globule” (MG) conformation (312)

defined by unfolded states or transitions in which the structure of the protein attains multiple conformational states with high secondary but low tertiary structure, and contains a loosely packed hydrophobic core that increases the hydrophobic surface accessible to solvent (313). As a result, these partially unfolfed protein conformations achieve low solubility in aqueous environments resulting in their association with lipid bilayers which they are able to traverse (314). A model proposed by Mach and Middaugh (315) suggests that FGF1 has a MG character. These MG states are usually formed at low pH and physiological temperatures (312) although in the case of FGF1 this process begin to be exaggerated at temperatures between 37^0C and 42^0C reaching a maximum at 50^0C to 55^0C (315). Further, in the case of FGF1, MG transition is also facilitated by the presence of acidic phospholipids including phosphatidlyserine (pS) (315). FGF1, p40 Syt1 and S100A13 exhibit pS-binding, an acidic phospholipids (316-318), are known to flip from the inner to the outer leaflet of the lipid bilayer in response to stress (319) suggesting that transmembrane translocation of MRC FGF1 may be due to the interaction with acidic phospholipids and further acquisition of the MG conformation.

3 NOTCH SIGNALING

3.1 NOTCH RECEPTORS AND LIGANDS

The Notch gene family encodes evolutionarily conserved transmembrane cell surface receptors that initiate signaling between neighboring cells in multicellular organisms (320). There is only one Notch in *Drosophila melanogaster* (321), but mammalian genomes encode four Notch receptors (322-325) (Table 3). The cloning of Notch gene showed that it encodes a single transmembrane receptor (324), with a N-terminal extracellular domain containing large number of extracellular tandemly positioned EGF-like repeats, which mediate direct contact between the ligands and the receptor (326). Glycosylation of some of the EGF repeat motifs regulates Notch-ligand interactions and downstream signaling (327). The extracellular domain contains also three cysteine-rich (CR) Lin-12/Notch repeats (LNR) that have negative regulatory activities. The intracellular domain containing two nuclear localization sequences (NLS) flanking a RAM signaling domain (328) juxtaposed to six ankyrin repeat (ANK) domain and a COOH-terminal PEST (proline, glutamate, serine, threonine) sequence (329) (Figure 6).

The ligands for Notch are also transmembranes proteins. There are six ligands, including three Delta genes (330), two Jagged genes (320, 331) and F3/contactin (332) that have been currently identified (Table 3). The ligands for the Notch receptors have traditionally been divided into two subclasses, Delta/Delta-like and Jagged/Serrate-like, defined by the absence and presence, respectively, of an additional CR domain in the extracellular portion of the polypeptide (333). The Jagged and the Delta genes encode an evolutionarily conserved domain structures consisting of extracellular, transmembrane and intracellular domain. The N-terminal extracellular domain of these ligands contains the SP plus a DSL domain [Delta, Serrate, (*Drosophila* homologues), Lag2 (*C. elegans* hologue)], a variable number of EGF repeats, and in case of Jagged1 a CR domain. The DSL domain is important in

the receptor recognizition (334). The intracellular domain contains one or two NLS, and a C-terminal PDZ-binding domain (335) (Figure 6).

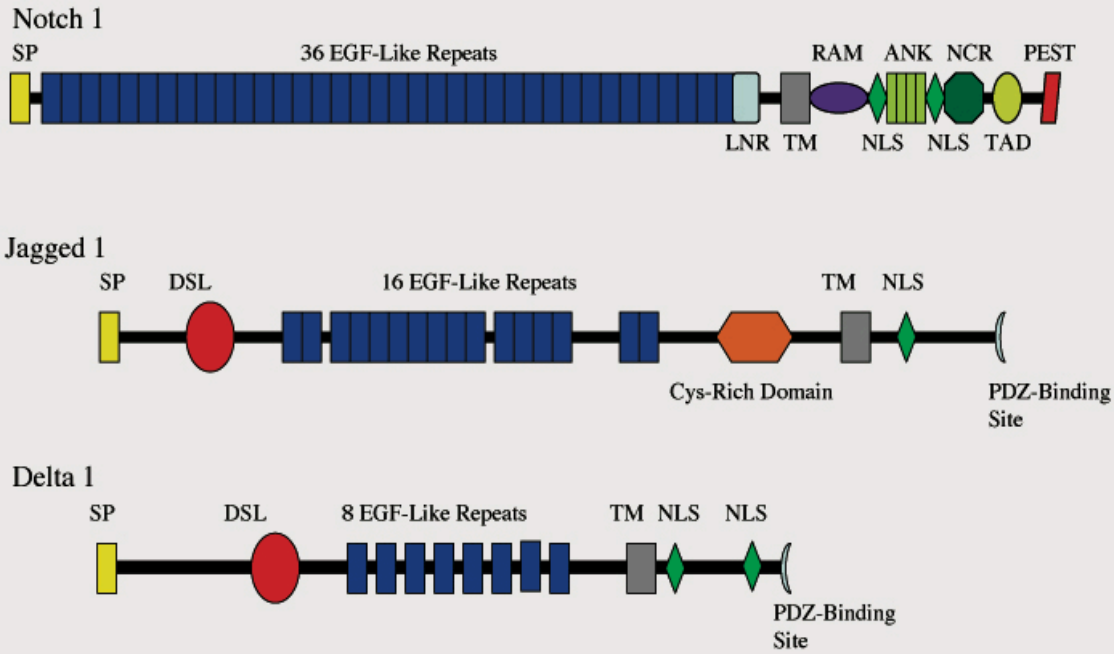


Figure 6 – Domain structure of representative Notch receptor and ligands Jagged1 and Delta 1.SP-signal peptide (yellow), DSL-Delta-Serrate-Lag-2 domain (red), EGF-epidermal growth factor (dark-blue), LNR- Lin 2/Notch repeats (sky blue), TM- transmembrane domain (gray), NLS-nuclear localization sequences (light green), ANK-ankyrin repeats (mustard green), NCR-Notch cytokine response element (dark green), TAD-transactivation domain (dark yellow) (with permission from Vihren Kolev).

Table 3- Notch pathway components in different species (from Haines *et al.* Nature Reviews Molecular Cell Biology 2003; 4: 786-797)

Protein type	*Drosophila melanogaster*	Mammals	*Caenorhabditis elegans*
Receptors	Notch	Notch1	Lin-12
		Notch2	Glp-1
		Notch3	
		Notch4	
		F3/Contactin	
Ligands	Delta	Delta1	Lag-2
		Delta3	Apx-1
		Delta4	Arg-2
	Serrate	Jagged1	F16B12.2
		Jagged2	
CSL protein	Suppressor of the Hairless [Su(H)]	CBF-1/RBF-J_K	Lag-1
O-FucT-1	OFUT1	POFUT1	A15C7.1
Fringe	Fringe	Lunatic Fringe	
		Manic Fringe	
		Radical Fringe	

CSL (CBF-1/Suppressor of Hairless/Lag1); RPB-J_K(recombination signal-sequence-binding protein)

3.2 NOTCH ACTIVATION AND DOWNSTREAM SIGNALING MECHANISMS

The Notch signaling is initiated by the interaction of Notch receptors with their ligands, through the DSL domain, on the surface of neighboring cells. This leads to two proteolytic cleavages, one outside and one within the transmembrane domain, which releases the Notch intracellular domain (NICD) that migrates into the nucleus (333, 336). The extracellular cleavage event is catalysed by an ADAM17 protease (a disintegrin-metalloprotease) (337), and the intracellular cleavage by a γ-secretase/Presenilin complex (338, 339, 340).

The generation and stability of NICD is regulated by several E3 ubiquitin ligases, which influence the intensity and duration of Notch signals (341). In the nucleus, NICD forms a ternary complex with a highly conserved transcription factor CSL (CBF1/Su(H)/Lag-1) (320, 333, 336, 342) and transcriptional coactivators of the mastermind-like (MAML) family (343-345). Within the nucleus CSL binds DNA in a complex containing histone deacetylase complex (346) and associated corepressors [silencing mediator for retinoic acid (347), CBF1-interacting co-repressor (CIR) (348) and KyoT2 (349)]. When NICD enters the nucleus and binds the CSL, the corepressors are displaced, and coactivators (MAML1, MAML2 (350); p300 (351)) and acetlytransferases such as PCAF and GNC5 are recruited (352). Activation of CSL-binding promoters upregulates the transcription of target genes (353). The primary target genes of Notch signaling are hairy and enhancer of split (HES)- 1, -5, -7 and HES-related repressor protein (HERP)- 1 to -3 in mammals (354-356). The members of HES and HERP families are basic helix-loop-helix-type transcriptional repressors that bind E-box motifs of target promoters, acting as Notch effectors by negatively regulating expression of downstream target genes (357-359). Other identified genes with CSL-binding promoters are: MAPK phosphatase LIP1 (360), and the cell cycle regulators $p21^{WAF1/Cip1}$ (361) and CDK2 (362). Several studies support the idea that Notch activation can stimulate or inhibit proliferation by modulating target gene expression in a cell type-specific manner (363, 364). While the mechanism of Notch activation signaling via CSL factors has been extensively

documented in a variety of biological settings, recent genetic and biochemical evidence indicates that Notch proteins can also signal via an alternative intracellular CSL-independent pathway (365, 366). This alternative pathway, which requires the cytoplasmic protein Deltex, known to associate with NICD upon ligand binding (367, 368), appears to prevent cell differentiation (369). Interestingly, the domains of Notch required for this pathway are not the same as those needed for Notch signaling via CSL family members (370, 371). Some experiments also suggest that Notch signaling via a Deltex-independent and CSL-independent pathway suppresses the expression of Wnt target genes (372, 373).

Notch signaling has also been demonstrated to interact with various other pathways that are important in the vascular cell phenotype. In several cases, the interactions comprise feedback loops between Notch and the interacting pathway. Bone morphogenic proteins (BMPs) in the presence of activated Notch1 have been reported to synergistically induce HERP1 in murine embryonic ECs, this synergy is thought to be regulated by enhanced association of NICD with Smads, the downstream effectors of BMP signaling (374, 375). Several signaling pathways (VEGF/ephrin/Ang/PDGF; FGFs) are essential for the complex process of vascular remodeling. Both *in vivo* and *in vitro* data suggest that Notch signaling interfaces with several of these angiogenic signaling pathways.

VEGF is one of the most critical factors in various aspects of physiological and pathological neovascularization including arteriogenesis. VEGF lies upstream of the Notch signaling pathway and upregulates Notch1 and Delta4 expression in arterial ECs (376, 377). The combination of VEGF and FGF2 upregulates Notch1 and Notch4 mRNA in human umbilical vein endothelial cells (HUVECs) (377). Notch activation in turn downregulates VEGF receptor (VEGFR) 2, suggesting that through this negative feedback loop, Notch may stabilize the vasculature by inhibiting vascular permeability and uncontrolled proliferation (378). In human microvascular ECs, constitutively active Notch4 inhibits both FGF2 and VEGF-induced *in vitro* tube formation and VEGF-induced angiogenesis on the chick chorioallantoic membrane (379). Interestingly, it has been shown that upon FGF-stimulated angiogenesis *in vitro*, HUVECs grown on fibrin exhibit dramatic upregulation of Jagged1 expression

(380). At the same time, inhibition of Jagged1 using antisense oligonucleotides promotes a marked increase in invasion and tube formation by bovine microvasculature ECs grown on collagen gel in response to FGF1, but not VEGF (42). All these different signaling pathways are tightly regulated and the final balance leads to different Notch biological activities.

3.2.1 THE ROLE OF SOLUBLE LIGANDS IN NOTCH SIGNALING

While several studies have suggested that the soluble forms of the Notch ligands are able to activate Notch receptors (381-386), there are numerous reports showing that the soluble forms of the Notch ligands act as antagonists of Notch signaling by impeding the interaction between Notch receptors and their full-length ligands (311, 387-394). Interestingly, Notch-dependent proteolytical cleavage was observed for the Delta and Jagged ligands (395-397). Upon interaction with Notch, the ectodomains of Jagged and Delta are cleaved respectively by ADAM17 and Kuzbanian metaloproteases, yielding membrane tethered C-terminal fragments. Presenilin/γ-secretase mediates a second cleavage that releases Delta and Jagged intracellular domains (ICDs). Delta1 ICD (Dl1ICD) is able to enter the nucleus (397), and the intracellular domain of Serrate1 is known to suppress primary neurogenesis (398, 399). On the other hand, soluble N-terminal fragments of Notch ligands can induce dramatic phenotypic changes. An alternatively spliced transcript encoding Jagged1, devoid of transmembrane and intracellular domain, has been isolated from cDNA library screening (42), the expression of this soluble non-transmembrane Jagged1 (sJ1 117kDa) antagonizes Notch signaling in NIH 3T3 cells and induces significant changes in their phenotype including FGFR1-dependent transformation (310, 393). Intradermal injection of such cells into the flank of nude mice results in formation of tissue masses (non-metastatic tumors) with prominent vasculogenesis (309), suggesting a positive role of sJ1 in angiogenesis. sJ1 117kDa cells exhibit the loss of growth contact inhibition, which is in accordance with their tumor forming ability (393). The sJ1 117kDa transfectants also display the attenuation of cell-matrix interaction, focal adhesion formation, and migration while exhibit exaggerated

expression of N-cadherin on cell-cell contacts (400). The type I collagen (ColI) α1 and α2 chain synthesis (309) is repressed in these cells. When exposed to ColI as an extracellular matrix *in vitro*, the sJ1 117kDa NIH 3T3 cell transfectants rapidly form the chord-like structures similar to those normally formed by EC *in vitro* (393, 401). Interestingly, sJ1 117kDa enhances Src Kinase activity and phosphorylation of a major Src substract, the filament binding protein, cortactin. When the sJ1 117kDa cells were stably cotransfected with a dn form of Src, the expression of ColIα1 chain was rescued and the formation of the ColI-dependent chords repressed (393).

The transfection of NIH 3T3 cells with the soluble extracellular domains of Notch1 (sN1) and Notch2 (sN2) results in a phenotype similar to sJ1 117kDa, although not as strongly pronounced. sJ1 117kDa, sN1 and sN2 transfectant cells display a significantly attenuated actin cytoskeleton, presenting a low level of actin stress fibers as well as a decrease of CSL-dependent transcription activity (393). Thus, soluble extracellular portions of Jagged1, Notch1 and Notch2 act as inhibitors of Notch signaling probably by interfering with the binding of transmembrane Notch receptors and ligands. Recently, Aho *et al*. isolated an alternatively spliced transcript encoding sJ1, by yeast two hybrid screening through interaction with thrombospondin-1 (402). This transcript devoid of sequences encoding the transmembrane and intracellular domains of Jagged1, is specific for keratinocytes, and carries an ability to induce keratinocyte differentiation (402). sJ1 has also been reported, *in vitro*, to play a role in hematopoietic stem cell self-renewal (394). According to the authors, Jagged1 immobilization on stromal cell layer or on Sepharose-4B beads is required for the induction of self-renewing divisions of days 28-35 cobblestone area-forming cell. However, sJ1 has a dominant-negative effect on self-renewal in the stem cell compartment (394). In contrast, soluble as well as immobilized Jagged-1 promoted growth factor-induced colony formation of committed hematopoietic progenitor cells (394). Recently, Trifonova *et al*. reported that the extracellular domain of Delta1 induces phenotypic changes similar to sJ1 117kDa (311). Secreted forms of Delta perturb association between full-length Delta and Notch (403) and inhibit the Notch-dependent repression of myoblast (404) and hematopoietic progenitor cell (385) differentiation *in vitro*. Taken together these

findings suggest that endogenous Delta1/Jagged1/Notch may act to maintain cell interaction with matrix and to activate the migratory ability of cells, possibly by decreasing cell-cell contacts. However, the expression of soluble Notch ligands may interfere with Notch signaling and attenuate its effects.

3.3 THE CROSS-TALK BETWEEN FGF AND NOTCH SIGNALING

The literature on this subject is scarce, limiting the overview of this important signaling cross-talk. The interaction of FGF and Notch signaling pathways has been described in the development of the teeth. FGF10 stimulates the expression of Lunatic Fringe, and the activity of Hes1 in the epithelium of developing teeth (405, 406). FGF10 also maintains Notch activation in pancreatic progenitors (407) cells by inducing the expression of Notch1, Notch2, Jagged1 and Jagged2 (408). Ikea and Hayashi established that in the process of tracheal branching in Drosophila, Notch signaling is activated by signaling of the FGF homolog, Branchless, through the FGFR homolog, Breathless (409). Observations by Faux *et al*. (410) described the stimulation of Notch signaling and treatment with FGF1 and FGF2 inhibits differentiation of mouse neuroepithelial precursor (NEP) cells *in vitro*. The response of NEP to FGFs can be overcome by downregulation of Notch1, and by the blockage of Notch cleavage (ablation of Presenilin1 gene) (410). Notch1 signaling in the embryonic telencephalon has been demonstrated to promote a proliferative response due to FGF2 stimulation *in vitro* (411). Moreover, the same authors described FGFR2 expression in telencephalic radial glia, and they reported that activation of this receptor induces the morphological differentiation of cells (411). Thus, regulation of Notch signaling by FGF is demonstrated in several development situations. As described in the FGF section of this literature review, there are *in vitro* data supporting the interconnection of Notch and FGF1 signaling pathways, particularly the downregulation of Notch signaling correlates with the non-classical release of FGF1 (310). These results suggest an important role of Notch/FGF cross-talk in vascular biology.

3.4 ROLE OF NOTCH SIGNALING IN VASCULAR BIOLOGY

Notch signaling is critical for determination of cell fates in invertebrates, and vertebrates (412, 413), in multiple tissues it contributes to self-renewal and survival of undifferentiated, multipotent cells throughout development and adulthood (320). The Notch signaling pathway is involved in strikingly diverse biological processes including neurogenesis (414, 415), retinal development (416-418), somitogenesis (419-421), adipogenesis (422), limb development (423), myogenesis (369, 414, 424), hematopoiesis (425, 426), vascular development, skin differentiation and immune response (427-432). The role of Notch signaling during vascular development as well as maintenance of vessel homeostasis is being extensively reported (430, 433). Indeed, mice with defects in genes enconding Notch receptors, Notch ligands, and components of the Notch signaling cascade invariably display vascular defects (Table 4) (430). Null mutations of several components of the Notch pathway, including Notch1, and Jagged1 resulted in embryonic lethality in mice with vascular remodeling defects (434, 435). Vasculogenesis proceed normally in these mutants whereas the next step, angiogenesis, was disrupted, suggesting that Notch signaling plays a more important role in angiogenesis. Although Notch4 deficient mice were viable and fertile (434), embryos with Notch1 and Notch4 knockouts displayed a more severe phenotype in angiogenesis than single Notch1 knockout embryos (434). These findings suggest a more important role of Notch1 than Notch4, as well as their redundant function in angiogenesis. Mouse embryos that are rendered null for Jagged1 exhibit defects in vascular remodeling (435). Interestingly, constitutive activation of Notch4, specifically in EC, also causes defects in vascular remodeling (379, 436). Mutation of the Presenilin1 gene, which is involved in processing of Notch, produced a complex phenotype, including abnormal blood vessel development and intracranial hemorrage, additionally supporting the idea that Notch pathway regulates vascular development (437-439). Hairy-related transcription factor (HRT)2 knockout mice displayed significant cardiac development defects, but no vascular phenotype (440-442). In contrast, the HRT1/HRT2 double knockout mouse embryos

Table 4- Phenotype of mice deficient for components of Notch pathway (from Iso *et al.* Arterioscler Thromb Vasc Biol 2003; 23:543-553)

Gene Disrupted	Lethality	Vascular Phenotype	References
Delta like1	E12	Hemorrhage	(452)
Delta like3	Perinatal	ND	(453, 454)
	Viable	ND	
Jagged1	E11.5	A large hemorrhage adjacent to the optic vesicle, lack of obvious large vessels in the yolk sac, failure to remodel the primary plexus in the yolk sac, less intricate network and a reduced diameter of vessels in the head	(435)
Jagged2	Perinatal	ND	(423, 455)
	Viable	ND	
Notch1	E11.5	Lack of large vitelline blood vessels in the yolk sac, disorganized, confluent vascular plexus in the yolk sac, defect of the main trunk of the arterior cardinal vein, lack of intersomitic vessels, the collapsed aortae	(434)
Notch2	E11.5	ND	(456, 457)
	Perinatal	Widespread hemorrhage near the surface of the skin, no capillary tuft of mature glomeruli (majority), capillary aneurysm-like structure of glomeruli (minority), numerous capillaries emanating from an aberrant bulbous structure, at the terminus of the hyaloid artery	
Notch4	Viable	Normal development	(434)
Notch1/ Notch4	E9.5	More severe than Notch1 null mutant	(434)
HES1	Perinatal	ND	(358)
HES5	Viable	ND	(458)
HES7	Perinatal	ND	(459)
HERP1	Perinatal	No vascular abnormality	(440, 441)

exhibit a global lack of vascular remodeling and massive hemorrhage after embryonic day 9.5 (443). Strongly reduced staining of arterial markers was reported in these embryos, and the paired dorsal aorta was poorly developed or absent (443). The similar vascular phenotypes that are induced by either constitutive activation, constitutive loss and further downstream signaling effects of Notch suggest a requirement for specific and finely tuned activation of Notch within the context of the developing vasculature.

3.4.1 INVOLVEMENT OF NOTCH IN VASCULAR DISEASES

There are at least two examples of congenital diseases that affect the vasculature in which Notch signaling is impaired. Allagile Syndrome (AGS) is an autosomal dominant disorder associated with abnormalities of the liver, heart, eye, and skeleton with variable expressivity (444). Between 60 to 75% of the patients affected by AGS are haploinsufficient for the Jagged1 gene (381, 445). There are also evidences showing that AGS patients carry mutant Jagged1 transcripts that are highly stable (446). Even if some mutant transcripts are present at low levels, it is believed that these residual mRNA can be translated and lead to functionally important expression of truncated soluble proteins (447) acting in a dominant negative manner. The most frequent cardiovascular anomaly in the patients is peripheral pulmonic stenosis, and anomalies of peripheral arteries comprising aneurysms and stenoses have recently been recognized (448, 449).

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is an autosomal dominant syndrome (450, 451) associated with Notch3 mutations commonly clustered in the first five EGF repeats. The disease is histologically characterized by deposition in the media of a nonatheromatous, nonamyloidotic substance, normally found in the brain as well as in the peripheral vascular tree (460). Clinically, the arteriophaty develops slowly, resulting in destruction of smooth muscle cells and thickening and fibrosis of the walls of small and medium-sized arteries with consequent narrowing of the lumen (461).

Furthermore, Notch signaling also seems to be involved in arterosclerosis and restenosis. Lindner *et al*. have studied expression of Jagged1, Jagged2, and Notch1-4 before and after balloon catheter denudation of rat carotid artery (400). Although expression of only Jagged1, Jagged2 and Notch1 was observed in intact endothelium, all the other Notch components were strongly induced in injured EC. Importantly, all the six Notch components were greatly increased in injured SMC after denudation of ECs (400), which is in contrast with normal SMCs, which expressed only Jagged1 and Notch3 (462).

The Notch signaling is also known to play an important role in tumor angiogenesis. It has been reported that Delta4 mRNA is expressed in some but not all microvessels of tumors (463). Moreover, examination of human cirrhotic livers demonstrated strong Jagged1, as well as Notch2 and Notch3 expression in many of the small neovessels, implying a role for Notch signaling in the process of neovascularization (464, 465).

CHAPTER II

Material and Methods

1. Material

2. Methods

1 MATERIAL

GENERATION OF EXPRESSION CONSTRUCTS: The descriptions of all mutants and expression constructs except FGFR136K:HA in pcDNA3 vector and FGF1R136K:HA in pTRE-Tight vector are detailed in Paper 3.

A thrombin-resistant human FGF1 mutant was constructed by polymerase chain reaction (PCR)-based site-directed mutagenesis of the FGF1pMEXneo expression vector (267). The codon encoding arginine 136 (AGA) was changed to a lysine (AAA) (FGF1R136K). FGF1R136K N-terminally tagged with a FLAG epitope (DYKDDDD) was constructed by PCR-based site-direct mutagenesis of the FGF1:FLAG (Soldi R. unpublished results). FGF1R136K was cloned into *SalI* and *EcoRI* restriction sites of pcDNA3/HA vector (generous gift from Jeong Yoon, Maine Medical Research Center, Maine, US) originating FGF1R136K:HA-pcDNA3. Further we inserted FGF1R136K:HA into the *HindIII* and *XhoI* restriction sites of pTRE-Tight expression vector (Biosciences Clontech, Worcester, MA, US) to obtain the FGF1R136K:HA-pTRE construct. Full-length Jagged1 (FLJ1) (393) was transferred to the expression vector pcDNA3.1/Hygro(+). The V5-His tag was excised from the pcDNA^{TM}4/V5-His vector, and inserted into the FLJ1-pcDNA3.1/Hygro(+) between the SP and the DSL domain of FLJ1, originating FLJ1NV5 construct. Soluble Jagged1 39kDa (sJ1 39kDa) was obtained from FLJ1NV5 by insertion of a stop codon at position 349, followed by a *PmeI* restriction site, which was then used to clone the fragment back into the pcDNA3.1/Hygro(+) vector. Thrombin-uncleavable Jagged1 mutant (FLJ1NV5R348K) was obtained through site-direct mutagenesis of FLJ1NV5, by changing the codon at position 348 from arginine to lysine.

Further, FGF1R136K, FLJ1NV5, and sJ1 39kDa were also cloned in the adenoviral shuttle vector, pAdlox (generous gift from Lisa Phipps, Somatix Therapy Corporation, California, US). All the mutagenesis reactions were performed using the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA, US), following the manufacturer's instructions. Generated sequences were confirmed by DNA

sequencing. A list of construct, including those already described in the literature, is presented below (Table 5).

Table 5- Expression constructs used for transfection and transduction experiments

Gene	Vector	References
FGF1	pMEXneo	(267)
FGF1:HA	pCR3.1	gift from Andrew Baird (Kings College, London, UK)
FGF1R136K	pMEXneo	Paper 3
FGFR136K	pAdlox	Paper 3
FGF1R136K:FLAG	pExchange-3	u.d.
FGF1R136K:HA	pcDNA3	u.d.
FGFR136K:HA	pTRE-Tight	u.d.
dnFGFR1	pcDNA3.1	(466)
IL1α	pMEXneo	(250)
FLJ1	pcDNA 3.1/Hygro(+)	(393)
FLJ1NV5	pcDNA 3.1/Hygro(+)	Paper 3
FLJ1NV5	pAdlox	Paper 3
FLJ1NV5R348K	pcDNA3.1/Hygro(+)	u.d.
FLJ1NV5R348K	pAdlox	u.d.
sJ1 39kDa	pcDNA3.1/Hygro(+)	Paper 3
sJ1 39kDa	pAdlox	Paper 3
sJ1 117kDa	pcDNA3.1^{+}/Neomycin	(393)
caN1	pAdlox	(310)
β-galactosidase	pAdlox	(310)
S100A13:Myc	pAdlox	(467)
p40 Syt1	pAdlox	Bagala, C. unpublished results
Luciferase under CBF1 response elements	GL2pro	(468)

pMEXneo - expression vector; pAdlox – adenoviral shuttle vector; u.d - unpublished data; dn – dominant negative; ca – constitutive active

STABLE NIH3T3 TRANSFECTANTS AND OTHER CELL LINES: NIH 3T3 cell transfectants stably expressing FGF1R136K, FGF1R136K:FLAG, sJ1 39kDa, and insert-less control vector pcDNA3.1/Hygro(+) were generated by utilizing the FuGENE 6 reagent (Roche Molecular Biochemicals, Indianapolis, US), and further selected by using 400 μg/L Geneticin (GIBCO, Life Technologies, Piscataway, NJ, US), 2.5 mg/L Puromycin (Roche Molecular Biochemicals) or 200 μg/ml Hygromycin (Roche Molecular Biochemicals), respectively. Selected clones were screened for protein expression by utilizing an anti-FGF1 rabbit antibody for FGF1R136K, M2 anti-FLAG monoclonal antibody (Sigma, St. Louis, MO, US) for the FGF1R136K:FLAG, and an anti-V5 antibody (Invitrogen, Foster City, CA, US) for sJ1 39kDa. The genome incorporation of insert-less control vector pcDNA3.1/Hygro(+) was screened by PCR. NIH 3T3 cells (ATCC, Manassas, VA, US), Swiss 3T3 cells (ATCC), IL1α (250), FLJ1 (393) and sJ1 117kDa NIH 3T3 transfectants (393) were maintained at 37°C, 5% CO_2, humidified atmosphere in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO, Life Technologies) containing 10% Bovine Calf Serum (BCS; Hyclone, San Diego, CA, US) and 1x antibiotic-antimycotic mixture (GIBCO, Life Technologies). Human embryonic kidney 293 cell line (HEK 293) (ATCC), PAR1 null MEFs and PAR1 null fibroblasts transfected with PAR1, a generous gifts from S. Coughlin (University of California, San Francisco, California, US) were grown in DMEM supplemented with 10% Fetal Bovine Serum (FBS; Hyclone).

2 METHODS

Most methods and techniques utilized in the experiments are described in papers 2, 3, 4, 5, and 6. Methods, not described in papers, are presented here more in detail.

PRODUCTION OF ADENOVIRUS: Recombinant adenoviruses were produced, purified and titrated as described (469). Briefly, CRE8 cells were transfected with *SfiI*-digested FGF1R136K, FLJ1NV5, or sJ1 39kDa pAdlox DNA, and infected with the ψ5 virus. Lysates were prepared 2 days after infection. Viruses were passed twice through CRE8 cells, and purified from the second passage using a Cesium density gradient. The viruses were quantified by optical density at 260 nm readings, and the bioactivity was determined by the plaque forming unit assay (more detailed information can be found on Papers 2, 3, and 5).

ADENOVIRAL TRANSDUCTION: The adenoviral transduction was performed in serum-free DMEM with approximately 10^3 viral particles/cell in the presence of poly-D-Lysine hydrobromide (Sigma) ($5x10^3$ molecules/viral particle) for 2 hours at 37^0C. Then the adenovirus-containing media was removed and replaced with serum-containing medium. Cells were plated for experiments 24-48 hours after transduction. The efficiency of transduction for FLJ1NV5, caN1, and S100A13 was assessed by immunofluorescence using an anti-V5 monoclonal antibody (Invitrogen), and anti-Myc monoclonal antibody for S100A13 (Oncogene™, West Grove, PA, US).

HEAT SHOCK AND THROMBIN/TRAP STIMULATION ASSAYS: The heat shock-induced FGF1 release assay was performed by incubation of cells at 42^0C for 110 minutes in serum-free DMEM containing 5 U/ml of heparin (Sigma), as previously described (267). Control cultures were incubated at 37^0C for the same time period. Thrombin or TRAP stimulation experiments were performed by incubation of cells at 37^0C, for different time periods, in the presence of 1 U/ml (equivalent to 10 nM) thrombin, (gift

from John W. Fenton, New York State Department of Health, Albany, New York, US) or 5.7 μM TRAP (Sigma). Control cells were incubated in the absence of thrombin or TRAP for the same time periods. Further, conditioned media were collected, filtered, and FGF1 was isolated for immunoblot analysis by using heparin-sepharose chromatography (for details see Paper 3), the IL1α was purified from the medium using Cu^{2+} affinity chromatography, as previously described (250). In both heat shock or thrombin/TRAP stimulation experiments cell viability was assessed by measuring lactate dehydrogenase (LDH) activity in conditioned medium after filtration (269, 470). In pertussis toxin (PTX; Sigma) experiments, FGF1R136K NIH 3T3 transfectants were pre-incubated with 100 ng/ml of PTX for 60 minutes at 37^0C.

IMMUNOBLOTTING AND IMMUNOPRECIPITATION: Immunoblot analysis was performed using rabbit antibodies against FGF1 (267) and Syt (269), a monoclonal anti-Myc antibody (Oncogene) to detect S100A13:Myc, and a goat anti-IL1α polyclonal antibody (Roche Molecular Biochemicals). In all the experiments, conditioned media were obtained from one 150 mm cell culture dish for each time point. Also 1/10 of the cell lysate derived from one 150 mm plate was loaded to each gel for FGF1 expression control, as described in Paper 3.

Immunoprecitation was used to assess the thrombin-induced production of sJ1 39kDa upon treatment of HEK 293 cells transduced with FLJ1NV5. Control cells were transfected with the adenovirus expressing β-galactosidase (β-gal). Conditioned media were collected, after thrombin, thrombin plus hirudin or thrombin plus protease inhibitor cocktail treatments. sJ1 39kDa was immunoprecipitated from the conditioned media using 1 μg of anti-V5 antibody (Sigma), resolved on 12% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted using the anti-V5 antibody.

NUCLEAR RUN-ON, RT-PCR, AND REAL TIME RT-PCR ANALYSIS: Nuclear run-on analysis of FGF1 expression, reverse transcription polymerase chain reaction (RT-PCR) and Real-time PCR (Q-RT-PCR) was performed as described in papers 2 and 3. In RT-PCR experiments, *jagged1* and *fgf1* cDNA were amplified using specific primers,

generating a 620 base-pair (bp) and 578bp products, respectively. Amplification of the *gapdh* cDNA was used as the endogenous normalization standard. Each sample was amplified in triplicate.

IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY: Cells were plated on fibronectin-coated glass coverslips in 6-well TC plates at 10^5 cells/well. The next day, the cells were transfected with FGF1:HA. The following day, the cells were stimulated with 1U/ml thrombin or 5.7 μM TRAP, fixed and immunofluorescently stained with anti-HA antibodies (Covance, Berkeley, CA, US) followed by a fluoresceine-conjugated anti-mouse IgG antibody (Sigma). For the pharmacology experiments, 24 hours after FGF1:HA transfection cells were incubated overnight with 250 μM TTM (Sigma) or 0.375 mM amlexanox (a generous gift of Takeda Chemical Industries, Osaka, Japan). Next day the cells were washed and further incubated in the presence of fresh TTM, or amlexanox, plus thrombin or TRAP. Control cells were kept all the time in the absence of TTM, or amlexanox. Actin stress fibers were visualized by fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma) in stable FLJ1 NIH 3T3 transfectants ($5x10^4$ cell/well) fixed with formaldehyde after thrombin or TRAP stimulation. Untransfected NIH 3T3 served as control. Fluorescently stained cells were analyzed and studied using the LTCS-SP confocal microscope (Leica) as described in papers 2, 3, and 6.

PREPARATION OF CELL MEMBRANES: Cell fractionation experiments were performed as described in paper 3. Briefly, cells were washed with serum-free DMEM containing 5 U/ml of heparin, and stimulated with 1U/ml thrombin. Control cells were incubated in the absence of thrombin. Next, the cells were washed in phosphate-buffered saline (PBS), scraped, and quickly spun down. The cell pellet was then resuspended in hypotonic buffer and incubated on ice. The pellets were homogenized in a Dounce homogenizer and centrifuged. The supernatants were collected and further ultracentrifuged to precipitate the membranes.

BIOTINYLATION OF CELL SURFACE PROTEINS: After thrombin and heat shock stimulation, stable FGF1R136K:FLAG NIH 3T3 transfectants were washed with cold PBS (pH 8.0). The biotinylation reaction was performed at 4^0C for 30 minutes, using biotin solution (PBS with 200 mg/L $CaCl_2$, 97.67 mg/L $MgCl_2$, 2.5 mM biotin (EZ-Link sulphosuccinimidyl-6-(biotin-amido) hexanoate (sulfo-NHS-LC-Biotin)) as recommended by the manufacturer (Pierce, Rockford, IL, US). Biotinylated cells were lysed in 1ml of buffer containing 0.1% TritonX100, and the lysates were immunoprecipitated with M2 anti-FLAG monoclonal antibody (Sigma) overnight before being washed three times in ice-cold 0.1% TritonX100, and resolved using 12% SDS-PAGE followed by Western blotting using horseradish peroxidase (HRP) conjugated straptavidin antibody (Pierce).

DNA SYNTHESIS ASSAY: A combination of [^{3}H]-thymidine autoradiography and immunohistochemistry was used to evaluate DNA synthesis levels in Swiss 3T3 cells expressing a dn FGFR1 (dnFGFR1) mutant as described in paper 3.

CELL-FREE TRANSLATION AND THROMBIN CLEAVAGE: A plasmid containing human FLJ1 was transcribed and translated *in vitro* in the presence of a [^{35}S]-Met/Cys protein-labeling mixture, using the T7-coupled reticulocyte lysate system according to manufacturer's instruction (Promega, Madison, WI, US). Thrombin cleavage was performed as described in paper 3. The samples were resolved by 12% SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane, and analyzed by autoradiography.

AUTOMATED EDMAN MICROSEQUENCING: The bands corresponding to the thrombin cleavage products were excised and subjected to automated Edman microsequencing. The products of each cycle were collected prior to resolution by high pressure liquid chromatography (HPLC) and quantified by liquid scintillation spectroscopy as described in paper 3.

DUAL LUCIFERASE REPORTER ASSAY OF CSL-DEPENDENT TRANSCRIPTION: Insert-less vector control, FLJ1, sJ1 117kDa and sJ1 39kDa NIH 3T3 cell transfectants were plated on fibronectin-coated cell culture dishes at approximately 50% confluency, and transiently cotransfected using FuGENE 6 (Roche Molecular Biochemicals) with 500 ng of a luciferase construct driven by four tandem copies of the CBF1 response element, and 100 ng of the TK *Renilla* (Promega) construct used as internal control for transfection efficiency. Forty-eight hours after transfection, the cells were harvested and the luciferase/renilla activity was measured by utilizing the Dual Luciferase Reporter Assay System (Promega). Each experiment was performed in triplicate.

GLYCOSILATION ASSAY: Stable sJ1 39kDa NIH 3T3 cell transfectants were plated at equal density. Twenty-four hours after plating the cells were washed in PBS, scraped, and quickly spun down. The cell pellet was then ressuspended in 1x glycoprotein denaturating buffer (0.5% SDS plus 1% β-mercaptoethanol) and incubated for 10 minutes at 100^0C. The pellets were then incubated in 1xG7 reaction buffer (50mM sodium phosphate pH 7.5) in the presence of 1%NP-40 and 1U of peptide N-Glycasidase (PNGase; New England BioLabs, Beverly, MA, US) for 1 hour at 37^0C. The enzymatic reaction was then stopped with loading buffer.

SOFT AGAR COLONY GROWTH: Approximately a total of 1800 sJ1 117kDa NIH 3T3 transfectants cells were plated in DMEM containing 10% BCS and 0.5% agar. Every 2 days, the soft agar culture was overlaid with fresh medium containing 1U/ml thrombin or 5.7 μM TRAP. Control plates were kept in DMEM containing 10% BCS. Three weeks after plating, colonies were visualized by staining with p-iodonitrotetrazolium violet (Sigma).

ISOLATION OF JAGGED1 NULL MOUSE EMBRYONIC FIBROBLAST: Pregnant mice heterozygous for the Jagged1^{DSL} mutant allele (435) were obtain from Thomas Gridley (Jackson Laboratory, Bar Harbor, ME, US). Females were sacrificed at embryonic

day (E) 9.5, uteri removed, placed in PBS and embryos extracted. Then embryos were dissociated by passing them through an 18-gauge needle. Embryo cells were plated in DMEM supplemented with 10% FBS, 100 U/ml penicillin/streptomycin and 2 mM of glutamine (GIBCO, Life Technologies). Four days later, a portion of cells was frozen down and the rest used to expand cell cultures. We obtained three strains out of twelve original embryos. The resultant cultures were checked for the presence of Jagged1 by PCR using the following set of primers: forward 5'-GGCGGCTGGGAA GGAACAAC-3' and reverse 5'-TCACCGGCTGGAGACTGGAAGA-3'. One of strains was demonstrated to be Jagged1$^{-/-}$. This strain was propagated and further transfected with simian virus 40 (SV40) T-antigen (gift of James deCaprio, Harvard University, MA, US) using FuGENE 6 (Roche Molecular Biochemicals) in order to obtain a Jagged1 null MEFs immortalized cell line.

DEVELOPMENT OF TRANSGENIC MICE WITH THE INDUCIBLE EXPRESSION OF FGF1: To produce transgenic mice with inducible FGF1 expression, we used a Tet-on system. Since FGF1 is cleaved by thrombin (241), we utilized a FGF1R136K mutant C-terminally tagged with the HA epitope, which was clone in the pTRE-Tigth vector. The final construct (FGF1R136K:HA-pTRE) was then purified and ressuspended in injection buffer (10 mM PIPES, 5 mM NaCL and 150 mM KCl) for the pronuclear injection into fertilized oocyte from the Balb/c strain. The microinjection was performed by Anne Harrington in the Transgenic Animal Facility of Maine Medical Center Research Institute (Scarborough, ME, US).

CHAPTER III

Results

1. *Study of Thrombin Role in FGF1 Signaling*
2. *Interplay Between Thrombin, Notch and FGF1 Signaling Pathways*
3. *Creation of an in Vivo Model for Studying FGF1 Release*

In this chapter, a compendium of the most relevant results is presented. A more detailed presentation of results is in papers 2, 3, 4, 5, and 6. The paper 1, a review focused on the non-classical export of two pro-angiogenic polypeptides (FGF1 and IL1α) gives the general picture of the current knowledge about the routes that FGF1 and IL1α utilize to be exported. This review also discusses the important questions related to non-classical protein release that remain to be answered. Some of these questions are further studied and discussed in papers 5 and 6. The papers 2 and 4 address different aspects of Notch and PAR1 signaling mechanisms, respectively. The results of these papers will not be presented here, however they will be discussed later, since they provide important information for the understanding of the FGF, Notch and thrombin role in angiogenesis and vascular remodeling.

Following are the results, including some unpublished data, pertaining to the questions raised in the specific aims.

1 Study of Thrombin Role in FGF1 Signaling

1.1- Study of thrombin role in the induction of FGF1 expression and release

1.1.1- Thrombin Induces FGF1 Expression

We analyzed by run-on assay the expression of the FGF1 transcript in NIH 3T3 cells in response to thrombin. The eight-fold induction of FGF1 mRNA was initially detected 15 minutes after the addition of thrombin to NIH 3T3 cells. The level of FGF1 mRNA increased over time, and reached a plateau after 2 hours of thrombin treatment (Figure 7A). Similar kinetics were obtained with Swiss 3T3 cells, although for these cells the induction of FGF1 transcript as slower (Figure 7B).

1.1.2- THROMBIN RAPIDLY INDUCES THE NON-CLASSICAL RELEASE OF FGF1

Based on the result that thrombin induced FGF1 expression, we sought to determine whether it also induces FGF1 release. Since FGF1 is susceptible to thrombin cleavage at arginine 136 (241), we utilized a thrombin-resistant FGF1 mutant (FGF1R136K). The heparin binding affinity of FGF1R136K was evaluated, and this mutant carries a binding to sulfate glycosaminoglycans similar to the wild type FGF1 (12) (data not shown). NIH 3T3 cells stably transfected with FGF1R136K were stimulated for 5, 15, 30, and 60 minutes with 1 U/ml thrombin. The addition of thrombin to FGF1R136K NIH 3T3 cell transfectants at 37^0C resulted in the rapid, sustainable appearance of the FGF1R136K mutant in the extracellular compartment (Figure 8A). We also evaluated the effect of thrombin upon the release of IL1α, a proinflammatory cytokine known to exhibit stress-induced non-classical release similarly to FGF1 (250, 265). As demonstrated on Figure 8B, similarly to FGF1, thrombin induced the IL1α release after 5 minutes of incubation.

The release of FGF1R136K mutant was dependent on the concentration of thrombin with a maximal response at 1 U/ml (10 nM) (Figure 8C). It is worthwhile to mention, that thrombin induced FGF1 release pertains to experiments in which the concentration of LDH in the conditioned media did not exceed LDH levels in media conditioned by non-stimulated cells (466) showing the absence of cell damage.

1.1.3- THROMBIN INDUCES THE REDISTRIBUTION OF FGF1 TO THE CELL MEMBRANE

1.1.3.1- IMMUNOFLUORESCENCE CONFOCAL MICROSCOPY

It has been demonstrated that heat-shock conditions stimulating FGF1 release induced the translocation of FGF1 to the cell membrane (305). We performed similar experiments applying immunofluorescence confocal microscopy to evaluate the effect of thrombin on the intracellular localization of C-terminally HA tagged FGF1 (FGF1:HA). We observed that short term thrombin treatment of NIH 3T3 cells transiently transfected with FGF1:HA resulted in the translocation of FGF1 to cell

periphery, near the cell membrane (Figure 9A).

1.1.3.1.1- PHARMACOLOGY OF THROMBIN-INDUCED FGF1 REDISTRIBUTION

The similarity between the kinetics of thrombin-induced FGF1R136K release and the peripheral redistribution of intracellular FGF1 prompted us to examine the pharmacology of FGF1 redistribution using reagents known to inhibit FGF1 export including amlexanox (270) a known attenuator of actin stress fiber formation (307). We demonstrated that amlexanox was able to completely repress thrombin induced redistribution of the cytosolic FGF1:HA (Figure 9B upper panel). We have also studied the effect of TTM a specific copper chelator, known to repress the heat shock induced export of FGF1 from NIH 3T3 cells (272). Interestingly, the incubation of FGF1:HA NIH 3T3 cell transfectants with TTM did not attenuate the peripheral redistribution of cytosolic FGF1:HA upon thrombin treatment (Figure 9B lower panel), strengthening previous results from our laboratory, that contrary to amelexanox, which was able to completely block stress-induced FGF1 translocation to the cell periphery, TTM did not interfere with FGF1 redistribution (305).

1.1.3.2- CELL FRACTIONATION

In order to confirm the confocal microscopy data, we additionally performed subcellular fractionation of FGF1R136K NIH 3T3 cell transfectants, treated and untreated with thrombin for 30 minutes at 37^0C. We found that thrombin induced the appearance of FGF1R136K in the membrane fraction (Figure 9C). After thrombin treatment, 29% of FGF1R136K was translocated to the membrane fraction, as determined by densitometric gel analysis (note that all of the cell lysate from one 150 mm cell culture dish was used for each treatment analysis).

1.1.3.3- BIOTINYLATION OF CELL SURFACE PROTEINS

Based on the cell fractionation and immunofluorescence results, we went further to evaluate whether FGF1 released upon thrombin treatment is associated with the

cell surface. Stable FGF1R136K:FLAG NIH 3T3 cell transfectants stimulated with thrombin or heat shock, were subjected to surface protein biotinylation and specific immunoprecipitation of FGF1, using anti-FLAG monoclonal antibody. Next the precipitated complexes were analyzed by Western blotting using streptavidin-HRP. We detected the appearance of biotinylated FGF1 on the cell surface, upon both thrombin stimulation and heat shock, and its absence on the surface of non-stimulated cells (Figure 9D).

1.1.4- Thrombin Treatment Induces the Export of S100A13 and p40 Syt1

FGF1 is known to be released under stress conditions as a part of a copper (Cu^{2+})-dependent multiprotein complex, which includes S100A13 (270, 271) and p40 form of Syt 1 (268, 269) proteins. We assessed whether thrombin was able to induce the export of S100A13 and p40 Syt1. As shown in Figure 10 (upper panel), anti-Myc immunoblotting analysis of medium conditioned at 37^0C by NIH 3T3 cells co-expressing S100A13:Myc and FGF1R136K revealed that upon thrombin stimulation S100A13 was released into the extracellular compartment with kinetics identical to that observed for FGF1R136K. Similar results were obtained for p40 Syt1 release when thrombin was applied to cells cotransduced with FGF1R136K and p40 Syt1 (Figure 10, lower panel).

1.1.5- The Mitogenic Activity of Thrombin is FGFR1 Dependent

Since thrombin induces both the expression of FGF1 and its release into the extracellular compartment, it is possible that these two effects contribute to the mitogenic activity of thrombin. To assess this hypothesis, we analyzed the ability of a dominant-negative mutant of FGFR1 to attenuate cell proliferation stimulated by thrombin. We utilized Swiss 3T3 cells since, unlike the NIH 3T3 cells, they exhibit a low level of apoptosis and endogenous DNA synthesis in response to serum deprivation. Swiss 3T3 cells express significant levels of FGFR1 (467). A dnFGFR1 construct was transfected into Swiss 3T3 cells, and their proliferative index was measured in the presence and absence of exogenous thrombin. The expression of

dnFGFR1 was verified by immunohistochemistry, and DNA synthesis was revealed by [^{3}H]-thymidine radioautography. As shown in Figure 11, the expression of dnFGFR1 not only reduced the ability of FGF1 to induce the appearance of replicating nuclei by approximately 70%, but it also decreased the DNA synthesis frequency in the presence of thrombin to a level consistent with quiescence.

Thus, thrombin efficiently and rapidly induced FGF1 expression and FGF1 release under non-stress conditions and the latter effect appears to be mediated by the translocation of FGF1 to the cell membrane. Thrombin-induced FGF1 translocation to cell membrane seems to be dependent on actin stress fibers. Moreover, similarly to the stress-induced FGF1 export (267, 286-288), the release of FGF1 in response to thrombin is associated with the export of S100A13 and p40 Syt 1. Interestingly, the mitogenic effect of thrombin depends on FGFR1 activity, bringing together thrombin and FGF signaling.

1.2- INVESTIGATION OF THE ROLE OF PAR1-MEDIATED SIGNALING IN THE THROMBIN-INDUCED FGF1 EXPRESSION AND RELEASE

Since thrombin is known to mediate its biological responses predominantly through the activation of PARs (73), we sought to evaluate the role of PAR1 in mediating thrombin-induced FGF1 expression and release.

1.2.1- ACTIVATION OF PAR1 BY TRAP RAPIDLY INDUCES FGF1 EXPRESSION

We assessed whether the PAR1-activating peptide, TRAP, which is devoid of proteolytic activity but is well known to induce the activation of PAR1 (78), was able to mimic the ability of thrombin to induce FGF1 expression. As shown on Figure 12A, similarly to thrombin, TRAP was able to induce the expression of FGF1 transcript in NIH 3T3.

1.2.2- PAR1 MEDIATES RAPID THROMBIN-INDUCED FGF1 RELEASE

To evaluate the role of PAR1 in FGF1 release, we utilized embryonic fibroblasts obtained from PAR1 null mice, as well as control PAR1 null fibroblasts transfected with PAR1 (100) that were transduced with the FGF1R136K adenovirus. Thrombin was unable to rapidly induce the export of FGF1R136K from PAR1 null cells (Figure 12B, upper panel). However, PAR1 null cells exported FGF1R136K in response to temperature stress (42^0C), suggesting that they were not defective in mediating stress-induced non-classical FGF1 export. At the same time, thrombin induced rapid FGF1R136K release from control PAR1 null fibroblasts transfected with PAR1 (Figure 12B, lower panel). Additionally, we assessed whether TRAP, which was able to induce FGF1 expression, would also mimic the ability of thrombin to induce FGF1R136K release. TRAP rapidly induced the export of FGF1R136K (Figure 12C). To further characterize PAR1 role in FGF1 induced release and since PAR1 signaling is coupled to PTX-sensitive inhibition of adenylate cyclase, we assessed the ability of PTX to block FGF1 release. As expected, PTX was able to repress the release of FGF1R136K in response to thrombin (Figure 12D).

1.2.3- TRAP INDUCES FGF1:HA TRANSLOCATION TO CELL PERIPHERY

Based on the finding that TRAP was able to mimic thrombin-induced FGF1 expression and release, we performed the immunofluorescence confocal microscopy studies in order to evaluate the effect of TRAP on the intracellular localization of FGF1:HA, as well as the effects of amlexanox and TTM upon this process . We observed that TRAP treatment of FGF1:HA NIH 3T3 cell transfectants resulted in the translocation of FGF1 to cell periphery, near the cell membrane (Figure 12E), and similarly to thrombin, TTM was not interfering with this translocation process. Conversely, amlexanox blocked TRAP-induced peripheral translocation of FGF1:HA suggesting the importance of actin stress fibers in this process (Figure 12F).

Thus we demonstrated that thrombin-induced FGF1 expression and rapid release were mediated by PAR1 activation. Particularly, TRAP, a synthetic peptide known to activate PAR1, induces FGF1 expression, rapid FGF1 release and amlexanox sensitive peripheral translocation of FGF1.

2 *INTERPLAY BETWEEN THROMBIN, NOTCH AND FGF1 SIGNALING PATHWAYS*

In the past we have demonstrated that the expression of sJ1 117kDa in NIH 3T3 cells represses Notch-mediated CSL-dependent transcription, and induces both FGF1 expression and constitutive non-classical FGF1 release at 37^0C (310). Since thrombin was able to induce also the expression and FGF1 release under non-stress conditions, and based on thrombin proteolytic activity, we hypothesized that additionally to PAR1, thrombin could also act through cleavage of Jagged1 and production of soluble ligand Jagged1.

2.1- THROMBIN-MEDIATED CLEAVAGE PRODUCES SOLUBLE JAGGED 1

2.1.1- IN VITRO TRANSLATED JAGGED1 IS CLEAVED BY THROMBIN

Computer analysis[1] of human Jagged1 amino acid sequence revealed two putative thrombin cleavage sites within the extracellular domain of Jagged1 (R113 and R348) (Figure 13A). In order to assess Jagged1 as a thrombin substrate, *Jagged1* was transcribed and translated *in vitro* in the presence of a [^{35}S]-Cys/Met mixture; and the 134kDa Jagged1 translation product was incubated with or without thrombin. Autoradiographic analysis of the reaction products revealed cleavage of the Jagged1

[1] www.us.expasy.org/peptidecutter

protein into 39 kDa and 95 kDa fragments (Figure 13B), the same sizes as theoretically expected according to one of Jagged1 thrombin cleavage site (R348↓G349) revealed by computer analysis.

2.1.2- Characterization of the Thrombin Cleavage Site by Edman Sequencing

Next we sought to confirm the identity of thrombin cleavage site in Jagged1. In the human Jagged1 sequence there are three cysteine residues (351, 360 and 362) close to the position R348. Based on this fact, we performed the *in vitro* transcription/translation of Jagged1 in the presence of [^{35}S]-Cys/Met mixture, following thrombin treatment and electrophoresis. Further, the band corresponding to the 95 kDa fragment was excised, and subjected it to automated Edman degradation, and the products of each cycle were monitored by liquid scintillation counting. We observed [^{35}S]-Cys radioactivity in cycles 2, 11, and 13 (Figure 13C), which agrees with the presence of Cys at positions 351, 360, and 362 (Figure 13A). These results suggest that thrombin cleaves Jagged1 between residues R348 and G349, which are located between EGF repeats 3 and 4 (468). This cleavage yields an amino terminal fragment with a molecular mass of approximately 39 kDa. To further confirm this cleavage site, we utilized a Jagged1 thrombin non-cleavable mutant (FLJ1R348K) N-terminally with V5 (Figure 13A), to perform the thrombin cleavage experiments. As expected, this mutant was not cleaved by thrombin, confirming the identity of Jagged1 thrombin cleavage site at position 348 of the amino acid sequence. Moreover, we tried another protease involved in blood coagulation process - plasmin, however, it was not able to cleave Jagged1 (data not shown).

2.1.3- Thrombin Cleaves Jagged1 Expressed in Culture Cells

To verify that thrombin cleaves Jagged1 expressed in living cells, we transduced HEK 293 cells with a FLJ1NV5 adenoviral construct (Figure 13A) for 48 hours, and used transduced cells for thrombin treatment. The percentage of HEK 293 FLJ1NV5 positive cells was around 90%, as determined by immunofluorescence using anti-V5

antibody. After 1 hour of treatment at 37^0C with 1 U/ml of thrombin, the serum-free medium was collected, immunoprecipitated with the anti-V5 antibody, resolved by SDS-PAGE, and immunoblotted with the anti-V5 antibody. As shown in Figure 13D, thrombin induced the cleavage and release of a N-terminal fragment of Jagged1 with the molecular weight of approximately 39 kDa into the medium. Jagged1 cleavage was completely blocked by a protease inhibitor cocktail (Figure 13D). Moreover, we found that hirudin, a highly specific thrombin inhibitor, was able to block the appearance of sJ1 39kDa (Figure 13D).

2.2- CHARACTERIZATION OF THE BIOLOGICAL ACTIVITY OF JAGGED1 THROMBIN CLEAVAGE PRODUCT (SJ1 39KDA)

The extracellular domain of Jagged1 is involved in receptor binding, and consists mainly of 16 EGF-like repeats. Since thrombin cleaves Jagged1 between the third and fourth EGF repeat, and because the glycosylation of some EGF repeat motifs is essential for the regulation of Notch-ligand interactions and downstream signaling (327) we sought to evaluate the biological activity of the resulting soluble Notch ligand, particularly its glycosylation pattern, and the ability to regulate Notch and FGF1 signaling.

2.2.1- SJ1 39KDA IS NORMALLY GLYCOSYLATED

We assessed sJ139kDa glycosylation, by incubating stable sJ1 39kDa NIH 3T3 cell transfectant lysates in the presence of PNGase, an N-endoglycosidase, known to cleave polysaccharide chains from glycoproteins. As shown in Figure 14A, PNGase treatment results in the electrophoretic shift of sJ1 39kDa to approximately 37kDa, revealing the presence of polysaccharide chains in sJ1 39kDa.

2.2.2- sJ1 39kDa Expression Decreases the CSL-Mediated Transcription

To determine whether sJ1 39kDa carries the same capacity to decrease Notch signaling as sJ1 117kDa, which represents the whole extracellular domain of Jagged1 (393), we assayed vector control, FLJ1, sJ1 117kDa, and sJ1 39kDa NIH 3T3 stable transfectants for CSL-dependent transcription by utilizing a luciferase reporter assay (344, 469). While FLJ1 transfectants exhibited an increase in CSL-mediated transcription, NIH 3T3 sJ1 39kDa transfectants displayed a decrease of the CSL-dependent transcription (Figure 14B), which was similar to that in sJ1 117kDa transfectants (393).

2.2.3- sJ1 39kDa Induces FGF1 Expression

We next assessed by RT-PCR untransfected NIH 3T3 cells, vector-transfected control, sJ1 117kDa, and sJ1 39kDa transfectant NIH 3T3 cells for the expression of *fgf1*. sJ1 117kDa and sJ1 39kDa transfectants expressed *fgf1*, while both untransfected and vector control transfected cells did not (Figure 14C, upper panel). Further (Q)-RT-PCR analysis results demonstrated that sJ1 39kDa induced significantly higher *fgf1* mRNA levels than sJ1 117kDa (Figure 14C, lower panel).

2.2.4- sJ1 39kDa Induces FGF1 Release Under Normal Growth Conditions

Since sJ1 39kDa induced the expression of FGF1, we next assessed whether it induced FGF1 release. Vector control and sJ1 39kDa NIH 3T3 transfectants were transduced with FGF1R136K adenovirus, and analyzed for FGF1 release under normal or heat shock conditions. Whereas both vector control and sJ1 39kDa transfectants exported FGF1 in response to temperature stress (42^0C), FGF1 release under non-stress temperature conditions (37^0C) was only observed in the sJ1 39kDa NIH 3T3 transfectants (Figure 14D).

Thus sJ1 39kDa resulting from thrombin cleavage was not inferior to sJ1 117kDa in its ability to repress Notch signaling, and to induce FGF1 expression and release.

2.3- Study of Notch Signaling Role in Thrombin-Induced FGF1 Expression and Release

To further understand the role of thrombin in bridging FGF1 and Notch signaling and based on the results obtained with the sJ1 39kDa transfectant cells, where the CSL-dependent transcription was downregulated, we aimed to determine whether thrombin treatment, would be able to attenuate Notch signaling, and whether the activation of Notch signaling would interfere with thrombin-induced FGF1 release.

2.3.1- Thrombin Attenuates CSL-Dependent Transcription in Jagged1 NIH 3T3 Cell Transfectants

We used the luciferase reporter assay to evaluate the ability of thrombin to attenuate the activity of the CSL-dependent promoter in NIH 3T3 cells. While treatment of the FLJ1 NIH 3T3 stable cell transfectants with thrombin reduced the level of CSL-dependent transcription, TRAP, an agonist peptide of PAR1 devoid of proteolyical activity, did not affect it (Figure 15A).

2.3.2- Thrombin-Induced FGF1 Expression and Release Is Repressed by the Expression of caN1

To explore the role of Notch signaling in thrombin effects, we utilized FGF1R136K NIH 3T3 cell transfectants adenovirally transduced with constitutively active Notch1 (caN1), and stimulated with thrombin for short time periods. About 90% of the cells expressed caN1 48 hours after transfection (data not shown). Interestingly, the expression of caN1 abolished the expression as well the release of

FGF1 induced by thrombin (Figure 15B). As shown in Figure 15C, thrombin stimulated the release of FGF1R136K from control cells; however, it was unable to initiate the release from cells expressing caN1 (Figure 15C). At the same time, the expression of caN1 in FGF1R136K NIH 3T3 cell transfectants did not affect the heat shock-induced FGF1 release (Figure 15C).

2.3.3- JAGGED1 NULL EMBRYONIC FIBROBLAST CONSTITUTIVELY RELEASE FGF1

Small *et al.* data (310) and the results of the present work demonstrate that sJ1, either genetically expressed or produced as a result of thrombin-dependent cleavage, induces FGF1 release, apparently due to the downregulation of Notch signaling. To further verify this working hypothesis, we explored FGF1 release from immortalized Jagged 1 knockout fibroblasts. We found that, unlike wild type fibroblasts, these cells constitutively released FGF1 at non-stress conditions (Figure 16A). Most probably, this effect is the result of downregulation of Notch signaling, by knock out of Jagged1, a major Notch ligand.

2.3.4- THROMBIN INDUCES CELL GROWTH IN SOFT AGAR IN THE ABSENCE OF EXOGENOUS FGF1

sJ1 117kDa NIH 3T3 cells transfectants are able to grow in an anchorage-independent manner and to form colonies in soft agar. The size of these colonies is significantly increased when exogenous FGF1 is added to the medium, however the number of colonies does not change (310). Based on these results, we sought to determine whether thrombin or TRAP, which both are able to induce the release of FGF1, would enhance the anchorage-independent growth. When sJ1 117kDa NIH 3T3 cell transfectants were treated with thrombin, not only the size but also the number of colonies significantly increased (Figure 16B). However, TRAP did not have any effect upon colony growth (Figure 16B).

2.3.5- FLJ1 NIH 3T3 Transfectants Exhibit an Attenuation of Actins Stress Fibers Upon Thrombin Treatment

Our laboratory in the past had demonstrated that sJ1 117kDa displayed a decrease of focal adhesion sites number, exaggerated expression of N-cadherin on the intercellular borders and a decrease in actin stress fibers (400). Based on these data, we hypothesized that stable FLJ1 NIH 3T3 cell transfectants treated with thrombin, should recapitulate the same effects. Indeed, the FLJ1 NIH 3T3 cell transfectants exhibited significantly reduced actin stress fibers upon thrombin treatment when compared either to control or TRAP treated cells. Thrombin untreated FLJ1 and NIH 3T3 cell transfectants served as control (Figure 16C).

Based on the disparate effects of thrombin and TRAP upon the anchorage-independent cell growth, it appears that the long-term mitogenic effect of thrombin is mediated by Jagged 1 cleavage followed by downregulation of Notch signaling and FGF1 expression and release. Moreover, the difference in actin stress fibers abundance between thrombin treated- and TRAP-treated cells also supports this premise. The results presented strengthen our hypothesis about the existence of Notch-FGF-thrombin signaling triangle.

2.4- Study of the kinetics of thrombin-induced FGF1 release in PAR1 null cells

Since thrombin failed to stimulate rapid FGF1 release from PAR1 null mouse embryonic fibroblasts, we explored the dynamics of thrombin-induced FGF1 export in order to determine whether the long-term thrombin treatment would produce enough sJ1 39kDa to induce FGF1 release, in absence of PAR1.

2.4.1- Jagged1 Expression in PAR1 Null Mouse Embryonic Fibroblasts

At the beginning of these studies, we assessed Jagged1 expression in PAR1 null cells and found, using RT-PCR, that they expressed Jagged1 transcripts at levels similar to those found in NIH 3T3 cells (Figure 17A).

2.4.2- Long-Term Thrombin Incubation Induces FGF1 Release from PAR1 Null Cells

To study the dynamics of thrombin-induced FGF1 release, PAR1 null cells were incubated with thrombin or TRAP in complete cell culture medium for 2 or 48 hours. In parallel, other PAR1 null cells were transduced with FGF1R136K adenovirus. Forty-eight hours after transduction, the cells were carefully washed in serum-free medium containing heparin, and incubated for an additional 2 hours in the medium conditioned by untransduced PAR1 null cells treated with thrombin or TRAP. As shown in Figure 17B, medium conditioned for 2 hours by thrombin-treated cells failed to induce FGF1 release. At the same time, the medium conditioned for 48 hours by cells treated with thrombin, but not with TRAP, was able to induce FGF1 release from PAR1 null cells (Figure 17B).

2.4.3- Short-Term Thrombin Stimulation Induces FGF1 Release from PAR1 Null Cells Overexpressing Jagged1

We hypothesized that the continuous presence of thrombin during 48 hours resulted in accumulation of sJ1 39kDa in the extracellular compartment, which induced FGF1 export. In order to further assess this hypothesis, we overexpressed Jagged1 in PAR1 null cells by adenoviral transduction. Cells transduced with FLJ1NV5 were stimulated with thrombin for 2 hours. Conditioned media were collected and added for 2 hours to PAR1 null cells transduced with FGF1R136K adenovirus. Conditioned medium from thrombin-treated PAR1 null cells

overexpressing FLJ1 induced FGF1 release (Figure 17C); however, conditioned medium from thrombin-treated control β–gal-transduced cells did not exhibit such an effect. These data demonstrate that the accumulation of sJ1 39kDa in the medium conditioned by thrombin-treated cells can result in PAR1-independent FGF1 release.

Thus long-term stimulation of cells with thrombin induced FGF1 release even in the absence of PAR, apparently due to the accumulation of sJ1 39kDa in the extracellular compartment. At the late stages of thrombin stimulation when PAR1 receptors are desensitized, high levels of extracellular FGF1 may be maintained in a PAR1-independent manner, due to continuous sJ1 39kDa production.

3 CREATION OF AN IN VIVO MODEL FOR STUDYING FGF1 RELEASE

Because of the absence of reliable *in vivo* models, our knowledge about non-classical FGF1 release in the organism is very limited, and all the studies have been performed on cell culture models. The establishment of an *in vivo* model would allow the study of the regulation of FGF1 release in the organism under normal or stress conditions.

3.1- DEVELOPMENT OF TRANSGENIC MICE WITH INDUCIBLE EXPRESSING OF FGF1

We used a Tet-based system in order to produce transgenic mice with inducible FGF1R136K C-terminally HA tagged expression in all tissues. This system represents an easy and reliable approach to reversibly induction of gene expression. Upon administration of the tetracycline analog doxycycline (Dox), the reverse tet transactivator (rtTA), interacts with tetO element located upstream of the gene of

interest and induces activation of gene transcription ("tet-on"). Dox removal switches off the transcription.

3.1.1- Doxycycline Treatment Induces Expression of FGF1R136K:HA Cloned in the pTRE-Tight Vector

In order to check the inducibility of transgene expression and expression leakage in the absence of Dox, FGF1R136K:HA-p*TRE*-Tight was transiently transfected into NIH 3T3 stably transfected with rtTA. Cells were incubated with or without 10ng/ml Dox for 24 hours. The expression of FGF1R136K:HA transgenes was checked by FGF1 immunoblotting (Figure 18A) and by anti-HA immunofluorescence (data not shown). FGF1R136K:HA expression was potently induced in response to Dox, and no expression leakage in the absence of Dox was observed.

3.1.2- Production of Transgenic Mice

The production of transgenic mice was performed in the Molecular Genetics Core of Maine Medical Research Institute. Transgenic mice were generated by pronuclear injection of the K136RFGF1:HA-p*TRE*-Tight DNA into fertilized oocytes of the Balb/c mouse strain. One month old mice originating from microinjected oocytes were genotyped by PCR, using genomic DNA extracted from the tails. Of a total of 20 animals, 5 of them turned out to be transgenic Figure 18B. At the present moment, transgenic mice are being bred with FVB mice to expand the transgenic population. In parallel, we are also breeding FGF1R136K:HA transgenic mice with the rtTA transgenic mice, received from Jackson Laboratory (Bar Harbor, ME, US) in order to produce double transgenic mice with inducible FGF1 expression.

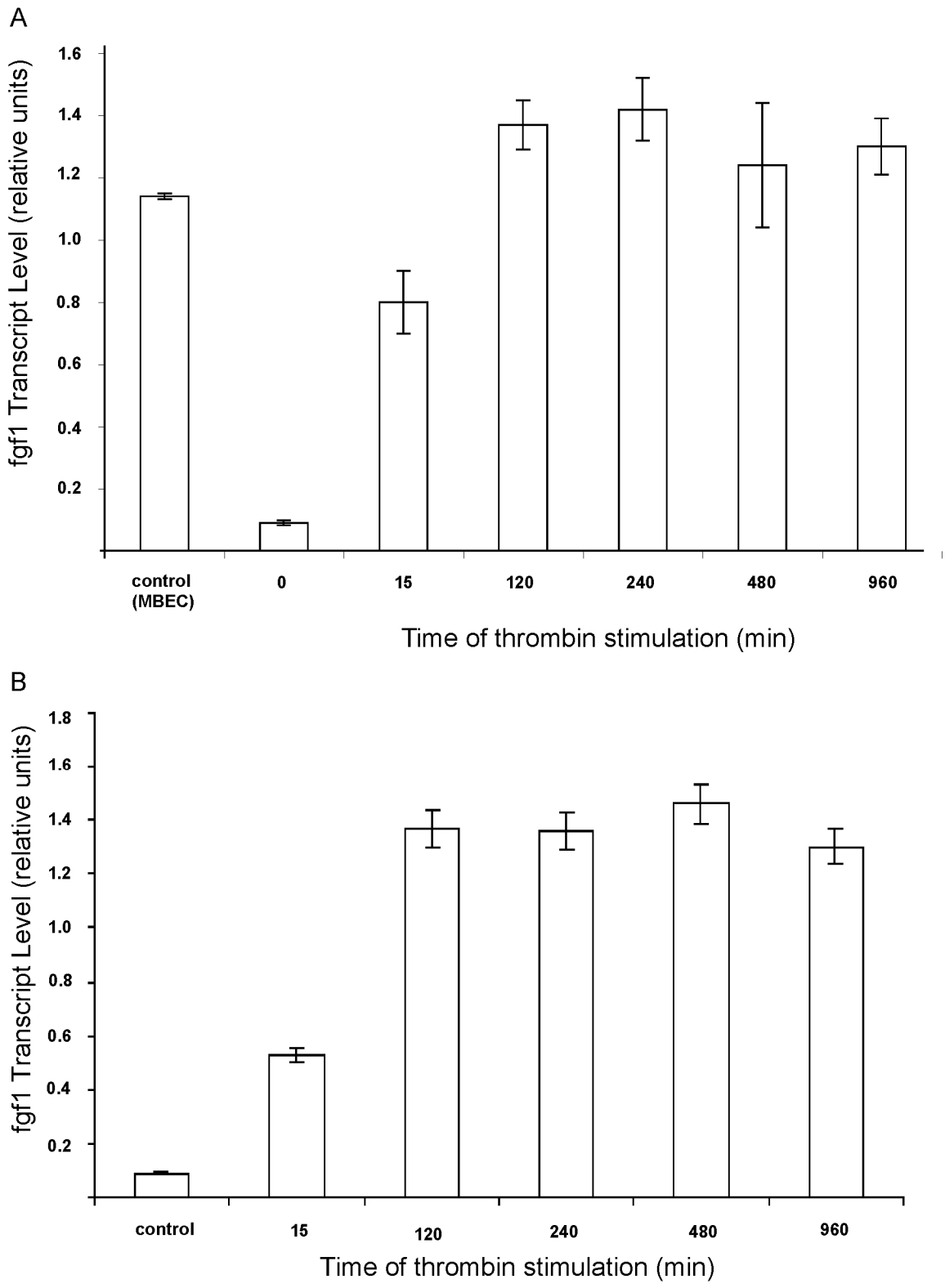


Figure 7- Thrombin induces FGF1 expression. The expression of *fgf1* in thrombin-treated NIH3T3 cells **(A)** or Swiss 3T3 **(B)** was evaluated by nuclear run-on analysis. Cells were harvested at various intervals after thrombin addition, the nuclei isolated and the transcription rate for *fgf1* gene was determined as described in Material and Methods. The bar graphs represent the normalized ratio of [^{32}P]-labeled *fgf1* to the *gapdh* transcript ±s.e.m. Mouse brain endothelial cells (MBEC) that expressed endogenous FGF1 served as positive control (A). Unstimulated Swiss 3T3 served as negative control (B).

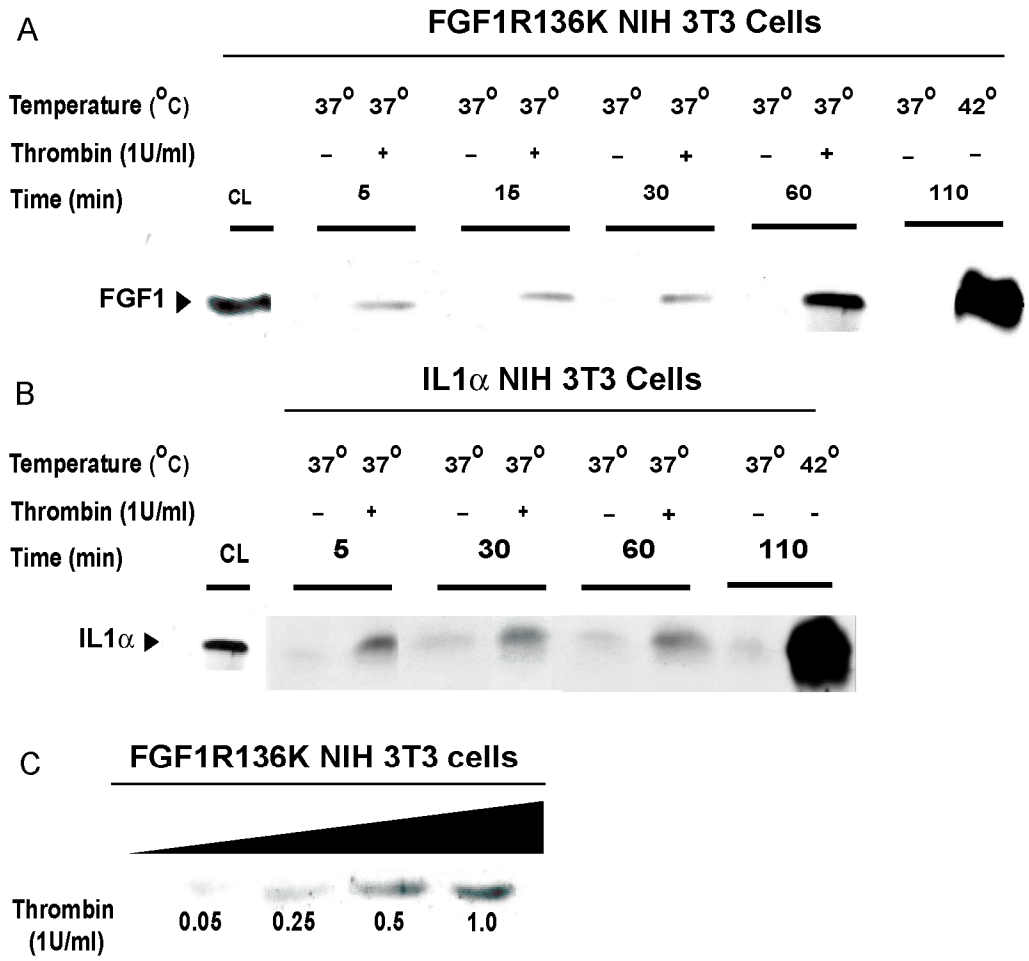


Figure 8- Thrombin induces rapid release of FGF1R136K and IL1α at 37°C. **(A)** FGF1 immunoblot analysis was used to identify the presence of FGF1R136K in media conditioned by FGF1R136K NIH 3T3 cell transfectants when stimulated with thrombin for 5, 15, 30 and 60 minutes at 37°C. **(B)** The IL1α NIH 3T3 cell transfectants were stimulated with thrombin for 5, 30 and 60 minutes at 37°C, and conditioned media analyzed by immunoblot with a goat polyclonal antibody. For both FGF1 and IL1α immunoblots, media conditioned in the absence of thrombin at 37°C or by heat shocked cells (42°C, 110 minutes) served as control. Cell lysates (CL) are shown in the left panel (1/10 of the total cell lysate was loaded). **(C)** Thrombin-induced FGF1 release is dose-dependent. FGF1 immunoblot analysis of media conditioned by FGF1R136K NIH 3T3 cells transfectants in response to 15 minutes treatment with 0.05, 0.25, 0.5 and 1 U/ml thrombin is shown.

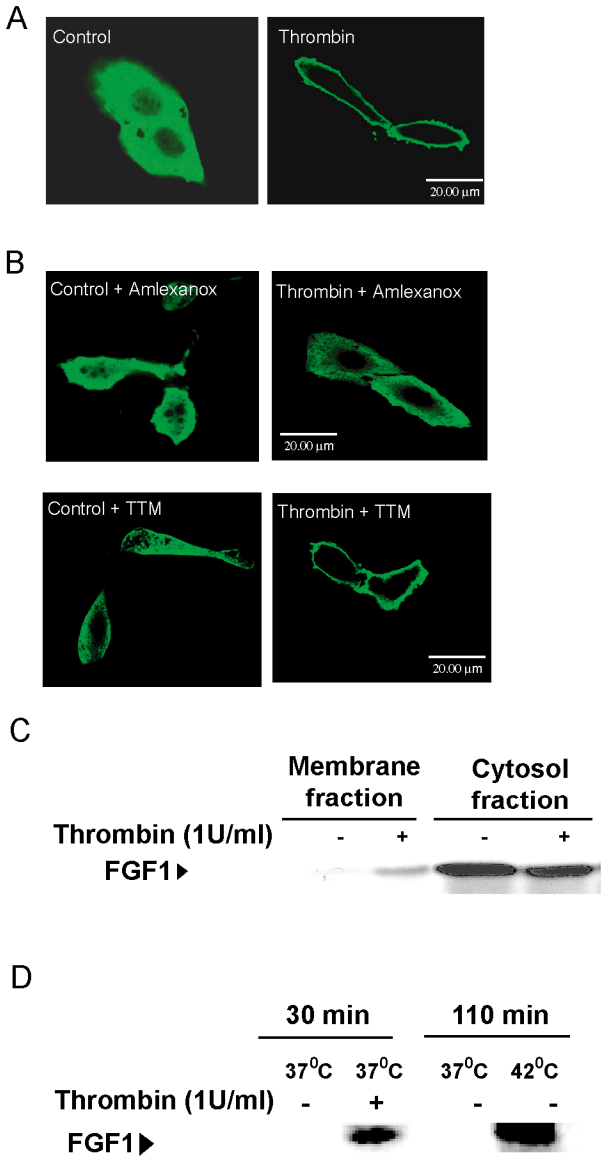


Figure 9- Thrombin induces the redistribution of FGF1 to the cell membrane. **(A , B)** FGF1:HA NIH 3T3 cell transfectants, unstimulated (control), stimulated with 1 U/ml thrombin for 30 minutes at 37^{0}C in the presence or absence of either amlexanox or TTM were fixed and processed for immunofluorescence microscopy as described in Material and Methods. Confocal images of median horizontal cell sections were taken using the100X objective. Bar, 20 µm. **(C)** FGF1R136K NIH 3T3 cell transfectants were incubated with or without thrombin for 30 minutes at 37^{0}C. Further cytosol and membrane fractions were prepared as described in Material and Methods. **(D)** Biotinylation of cell surface proteins. After thrombin and heat shock stimulation in the presence of heparin, FGF1:FLAG NIH 3T3 cell transfectants were biotinylated for 30 minutes at 4^{0}C, cell lysates were prepared, and FGF1 was immunoprecipitated with anti-FLAG antibodies and detected by streptavidin-HRP immunoblotting.

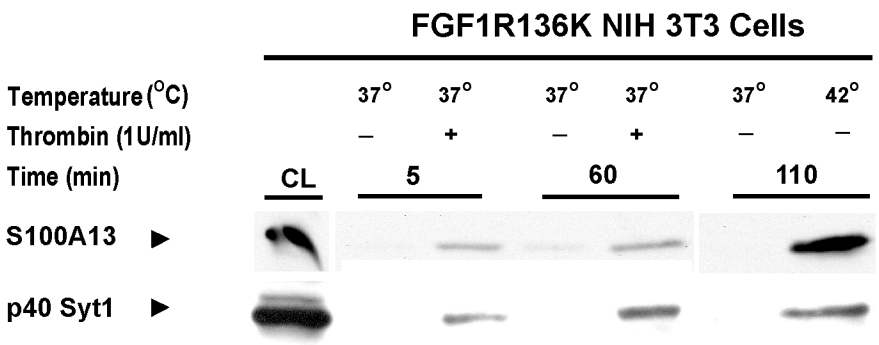


Figure 10- Thrombin induces the rapid release S100A13 and p40 Syt1 at 37^{0}C. Myc and Syt1 immunoblot analysis was used to identify the presence of respectively S100A13:Myc and p40 Syt1 in media conditioned by FGF1R136K NIH 3T3 cell transfectants adenovirally transduced with either S100A13:Myc or p40 Syt1 at 37^{0}C following the addition of 1 U/ml thrombin for 5 and 60 minutes. Media conditioned in the absence of thrombin or by heat shock (42^{0}C, 110 minutes) served as controls. Cell lysates (CL) from these cells are shown in the left panel.

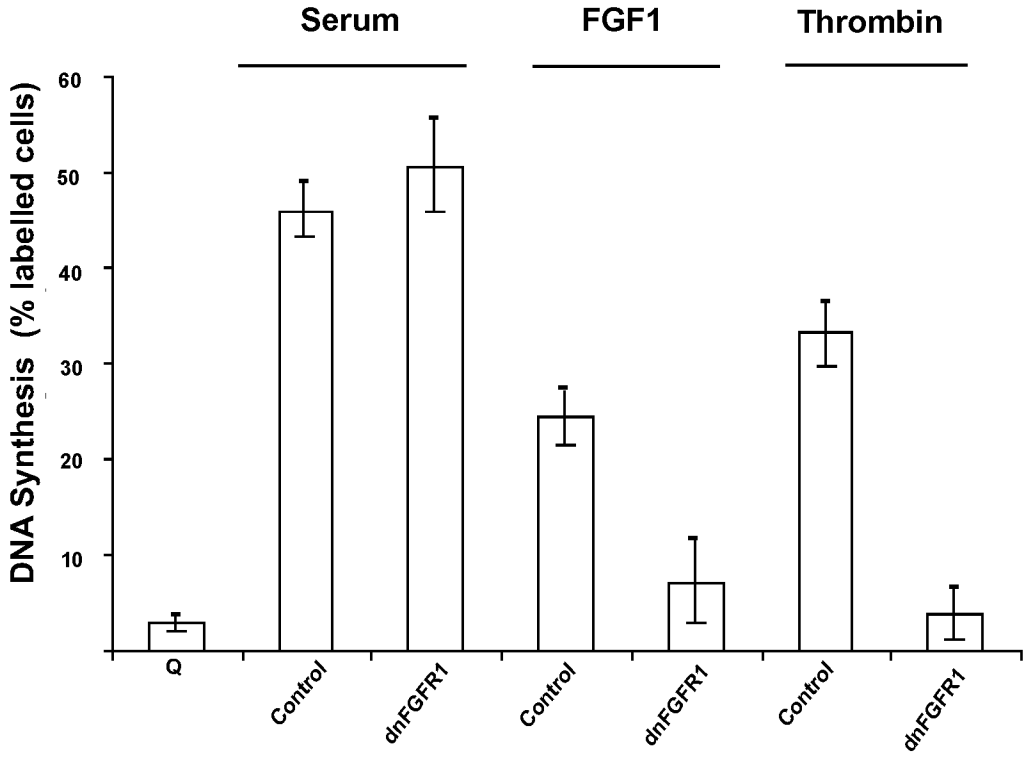


Figure 11- The mitogenic activity of thrombin is FGFR-mediated. A dnFGFR1 mutant inhibits thrombin-induced DNA synthesis in Swiss 3T3 cells. Quiescent Swiss 3T3 cells transiently transfected with a FGFR1 deletion mutant were stimulated with either 10% BCS, 10 ng/ml FGF1 plus 10 U/ml of heparin or thrombin for 24 hours. DNA synthesis in control and dnFGFR1$^+$ cells was determined using [^{3}H]-thymidine incorporation assay combined with anti-FGFR1 immunoperoxidasestaining. "Q" refers to quiescent cells.

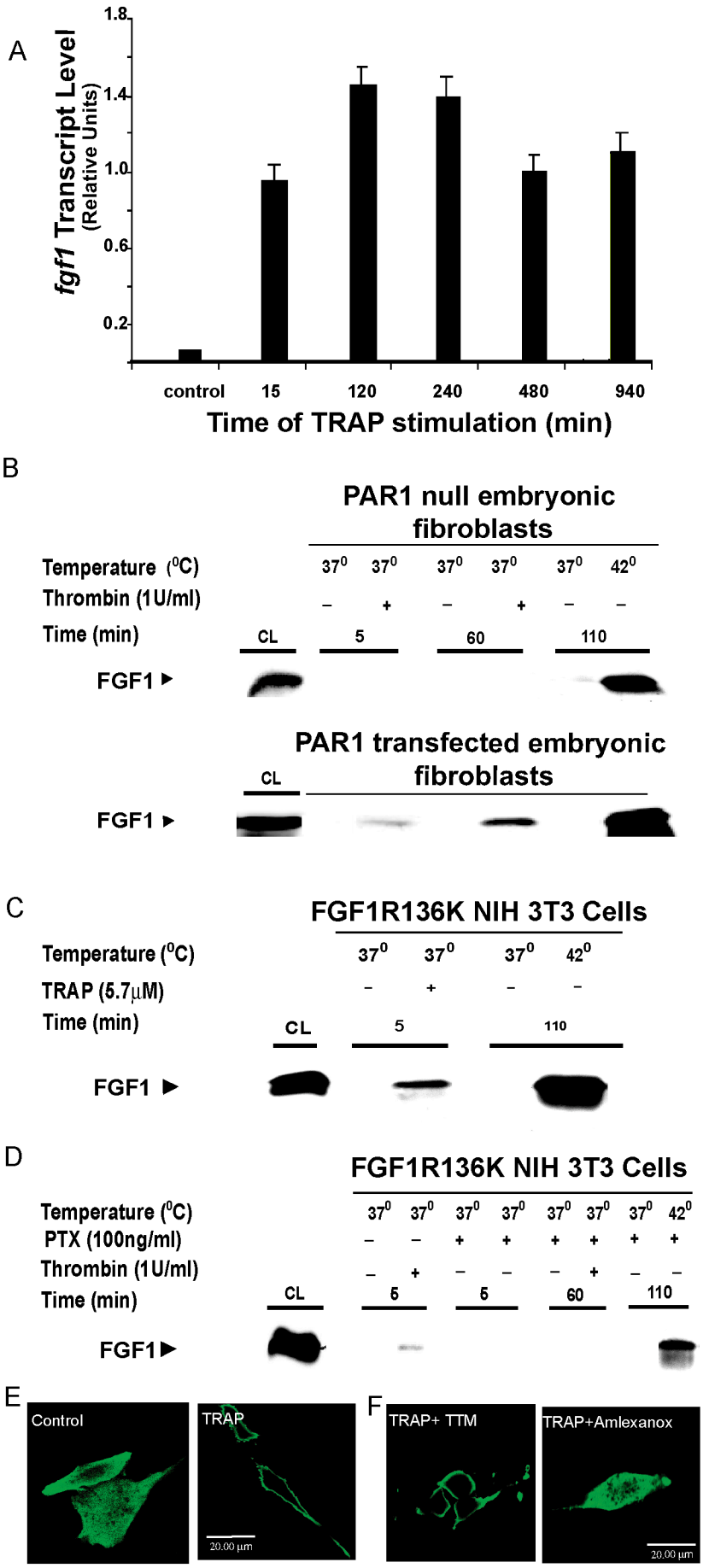


Figure 12- PAR1 mediates rapid thrombin-induced FGF1 release. **(A)** TRAP rapidly induces FGF1 expression. The expression of *fgf1* in TRAP-treated NIH 3T3 cells was evaluated by nuclear run-on analysis, as described for thrombin treatment (figure 1). Unstimulated NIH 3T3 cells served as a negative control for FGF1 expression. The bar graphs represent the normalized ratio of [^{32}P]-labeled *fgf1* to *gapdh* ± s.e.m. **(B)** PAR1 mediates rapid thrombin induced release of FGF1. FGF1 immunoblot analysis of media conditioned by PAR1 null or PAR1-transfected PAR1 MEF adenovirally transduced with FGF1R136K and stimulated with thrombin for 5 and 60 minutes. Media conditioned by heat shocked of PAR1 null cells served as positive control. The cell lysates (CL) from PAR1 null and control PAR1-transfected MEF are shown. **(C)** TRAP rapidly induces FGF1 release. FGF1 immunoblot analysis of media conditioned by FGF1R136K NIH 3T3 cell transfectants in response to the addition of TRAP is presented. **(D)** PTX blocks the thrombin-induced release of FGF1. FGF1 immunoblot analysis of media conditioned by FGF1R136K NIH 3T3 cell transfectants after 1 hour of PTX treatment in the presence and absence of thrombin. PTX and/or thrombin treatment did not affect the cytosolic levels of FGF1 and a representative cell lysate (CL) is shown. **(E, F)** FGF1:HA NIH 3T3 cell transfectants unstimulated (control), stimulated with TRAP for 30 minutes at 37^{0}C, in the presence or absence of either amlexanox or TTM were fixed and processed for immunofluorescence microscopy as described in Material and Methods. Confocal images of median horizontal cell sections were taken using the 100X objective. Bar, 20 μm.

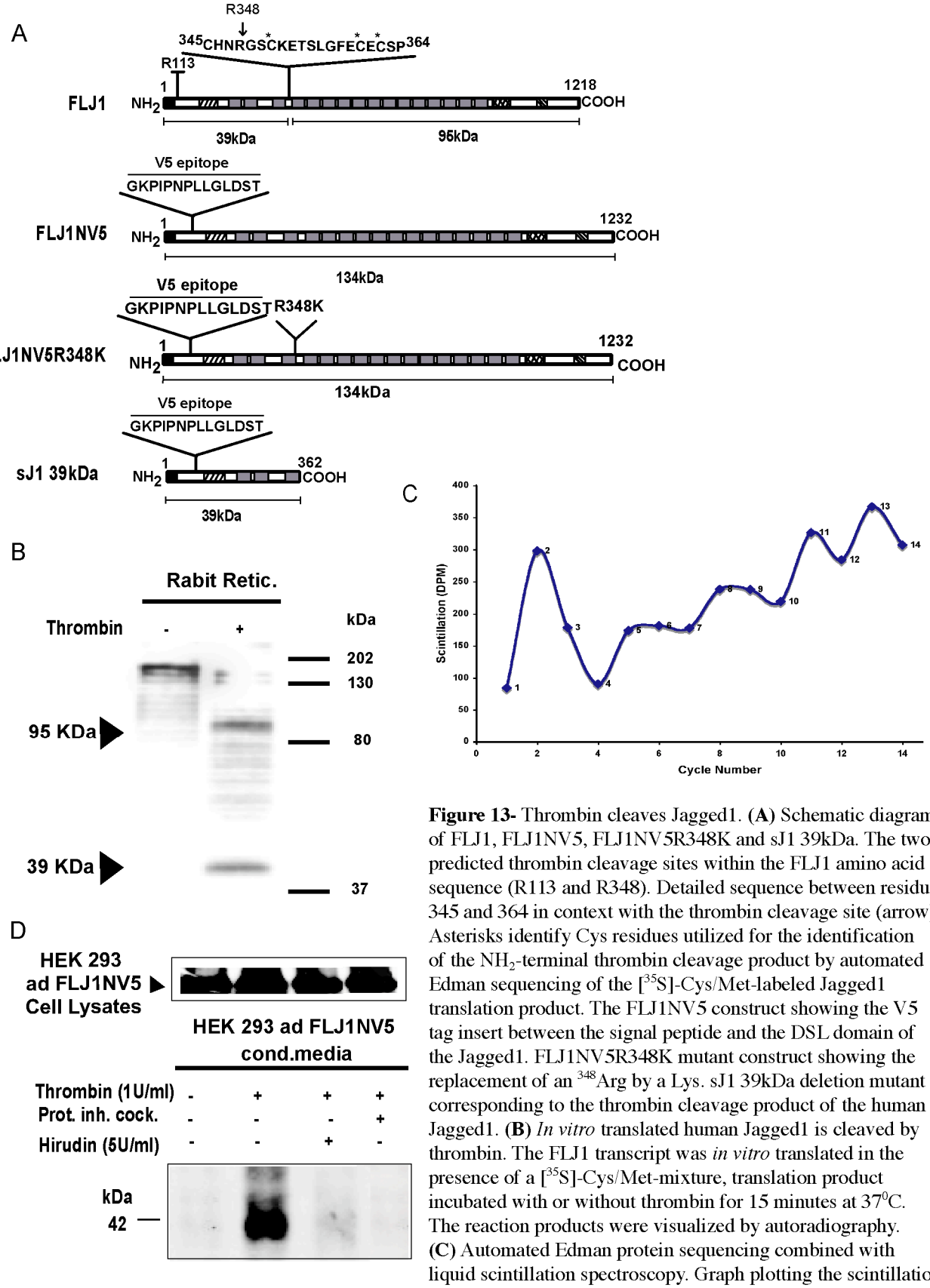


Figure 13- Thrombin cleaves Jagged1. **(A)** Schematic diagram of FLJ1, FLJ1NV5, FLJ1NV5R348K and sJ1 39kDa. The two predicted thrombin cleavage sites within the FLJ1 amino acid sequence (R113 and R348). Detailed sequence between residues 345 and 364 in context with the thrombin cleavage site (arrow). Asterisks identify Cys residues utilized for the identification of the NH_2-terminal thrombin cleavage product by automated Edman sequencing of the [^{35}S]-Cys/Met-labeled Jagged1 translation product. The FLJ1NV5 construct showing the V5 tag insert between the signal peptide and the DSL domain of the Jagged1. FLJ1NV5R348K mutant construct showing the replacement of an ^{348}Arg by a Lys. sJ1 39kDa deletion mutant corresponding to the thrombin cleavage product of the human Jagged1. **(B)** *In vitro* translated human Jagged1 is cleaved by thrombin. The FLJ1 transcript was *in vitro* translated in the presence of a [^{35}S]-Cys/Met-mixture, translation product incubated with or without thrombin for 15 minutes at 37^{0}C. The reaction products were visualized by autoradiography. **(C)** Automated Edman protein sequencing combined with liquid scintillation spectroscopy. Graph plotting the scintillation counting per each cycle. **(D)** Thrombin cleaves Jagged1 expressed in HEK 293 cells. Forty-eight hours after FLJ1NV5 transfection, cells were treated either with thrombin, thrombin plus hirudin or thrombin plus protease inhibitor cocktail for 1hour at 37°C. Control FLJ1NV5-transduced cells were incubated in serum-free media without thrombin. Cleaved Jagged1 was immunoprecipitated from the conditioned medium by anti-V5 antibodies. Immunoprecipitated sJ1 39kDa was detected by V5 immunoblotting. The corresponding cell lysates from the FLJ1NV5 HEK 293 transduced cells are shown on the top panel.

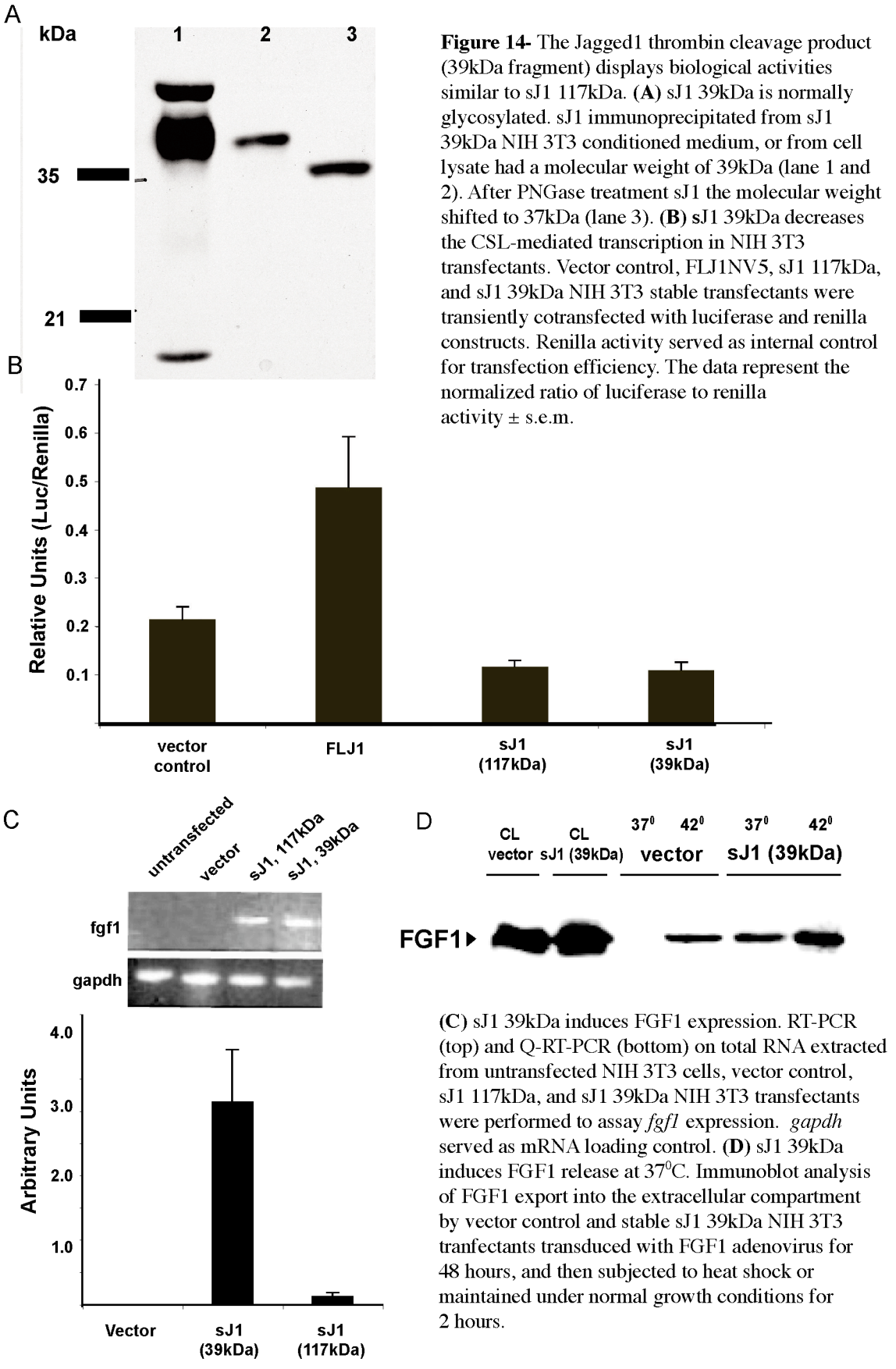


Figure 14- The Jagged1 thrombin cleavage product (39kDa fragment) displays biological activities similar to sJ1 117kDa. **(A)** sJ1 39kDa is normally glycosylated. sJ1 immunoprecipitated from sJ1 39kDa NIH 3T3 conditioned medium, or from cell lysate had a molecular weight of 39kDa (lane 1 and 2). After PNGase treatment sJ1 the molecular weight shifted to 37kDa (lane 3). **(B)** sJ1 39kDa decreases the CSL-mediated transcription in NIH 3T3 transfectants. Vector control, FLJ1NV5, sJ1 117kDa, and sJ1 39kDa NIH 3T3 stable transfectants were transiently cotransfected with luciferase and renilla constructs. Renilla activity served as internal control for transfection efficiency. The data represent the normalized ratio of luciferase to renilla activity ± s.e.m.

(C) sJ1 39kDa induces FGF1 expression. RT-PCR (top) and Q-RT-PCR (bottom) on total RNA extracted from untransfected NIH 3T3 cells, vector control, sJ1 117kDa, and sJ1 39kDa NIH 3T3 transfectants were performed to assay *fgf1* expression. *gapdh* served as mRNA loading control. **(D)** sJ1 39kDa induces FGF1 release at 37^{0}C. Immunoblot analysis of FGF1 export into the extracellular compartment by vector control and stable sJ1 39kDa NIH 3T3 tranfectants transduced with FGF1 adenovirus for 48 hours, and then subjected to heat shock or maintained under normal growth conditions for 2 hours.

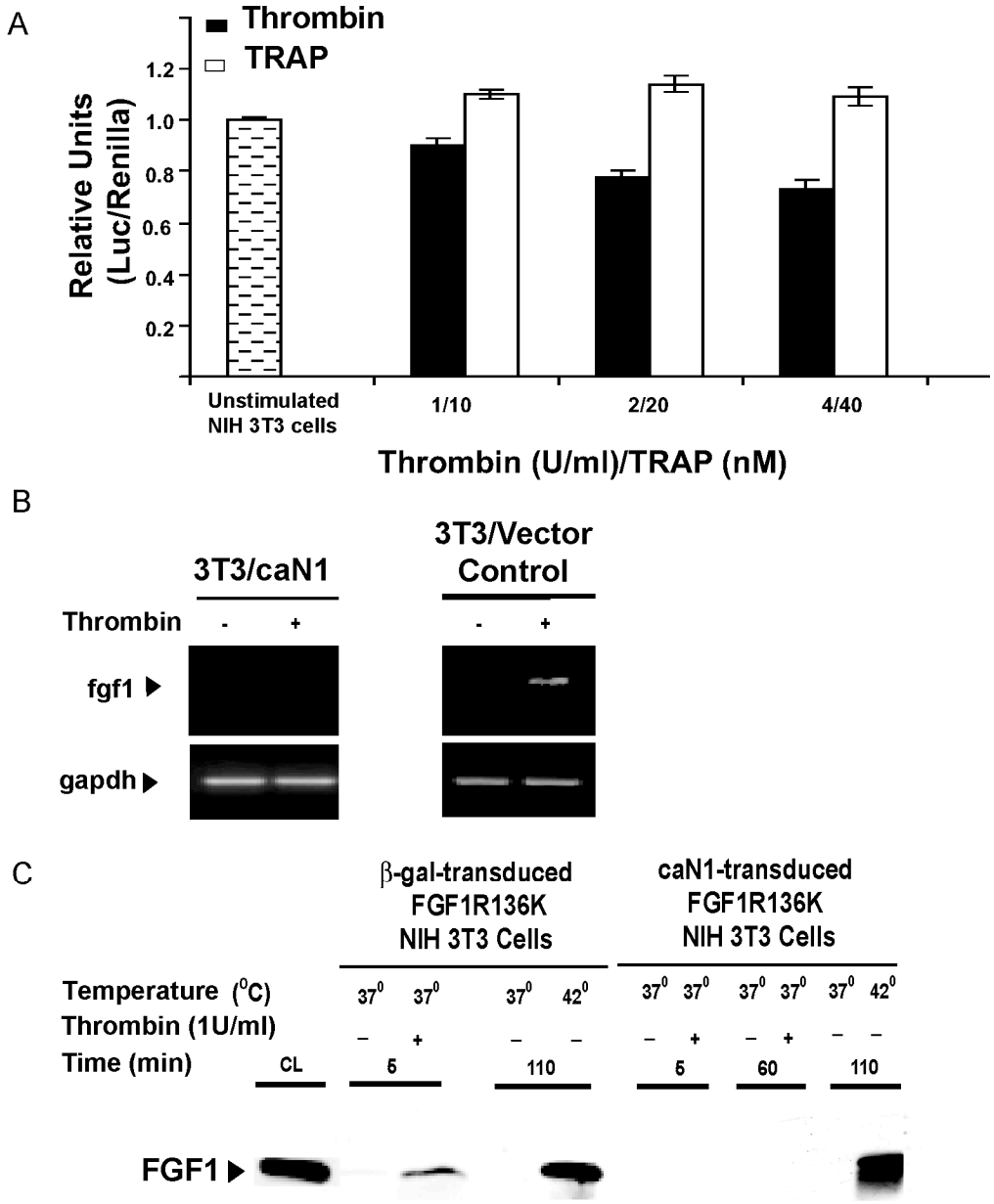


Figure 15- Role of Notch signaling suppression in thrombin-induced FGF1 release and expression. **(A)** Thrombin attenuates CSL-dependent transcription in Jagged1 NIH 3T3 cell transfectants. FLJ1 NIH 3T3 transfectants were treated with thrombin or TRAP for 12 hours before and 48 hours after luciferase and renilla cotransfection. CSL1-mediated transcription was assayed, as described in the Materials and Methods. The bar graphs represent the normalized ratio of luciferase to renilla activity ± s.e.m. as a function of the concentration of thrombin or TRAP. The CSL-dependent transcription activity from unstimulated NIH 3T3 cells served as a control. **(B)** Thrombin-induced expression of FGF1 is repressed by caN1. NIH 3T3 cells transduced with caN1 adenovirus and vector control were treated for 30 minutes with thrombin. RT-PCR experiments were performed as described in the Materials and Methods. gapdh served as mRNA loading control **(C) T**hrombin-induced release of FGF1 is repressed by the expression of caN1. FGF1R136K NIH 3T3 cell transfectants were adenovirally transduced with caN1; the levels of the FGF1 in media conditioned by the addition of thrombin were assessed using immunoblot analysis. β-gal-transduced cells were used as a control. The representative cell lysate (CL) from FGF1R136K-transduced cells is shown in the left panel.

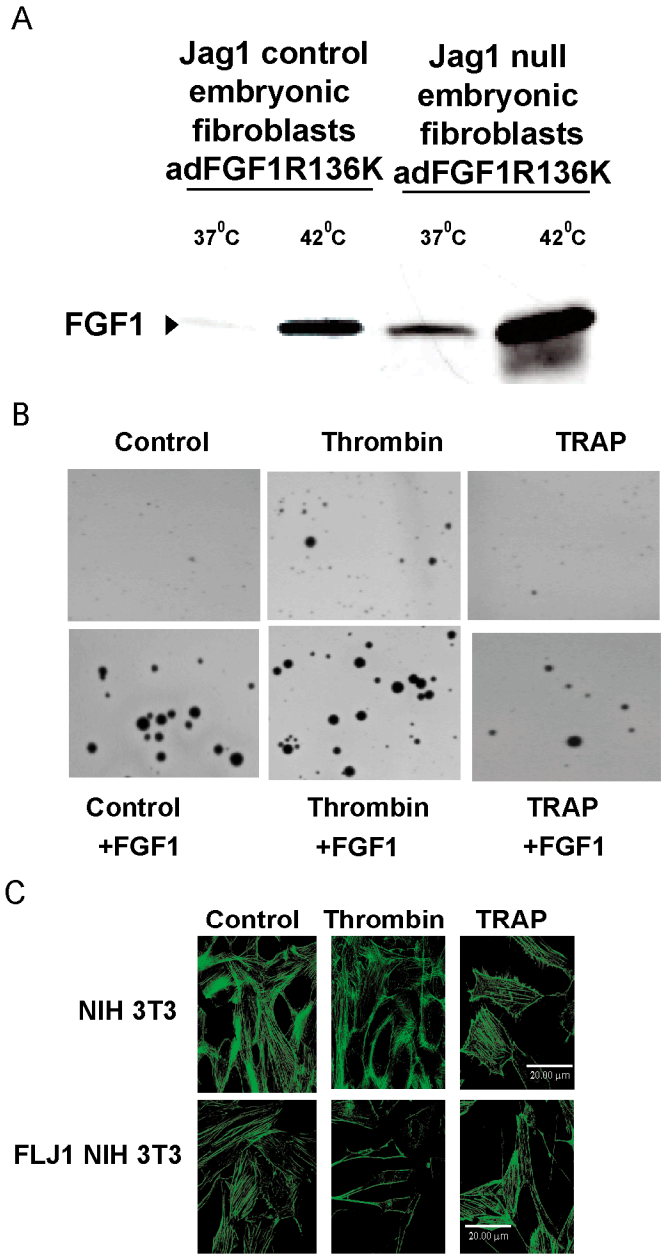


Figure 16- Role of thrombin inducing the Jagged1 cleavage, in the anchorage-independent growth and in the actin stress fibers.
(A) Jagged1 null MEFs constitutively release FGF1. Jagged1 null MEFs were established as described in Material and Methods. FGF1 immunoblot analysis was used to identify the presence of FGF1R136K in media conditioned by Jagged1 null MEFs transduced with FGF1R136K adenovirus and kept at 37^{0}C or 42^{0}C for 2 hours, Jagged1 positive MEFs served as control. **(B)** Thrombin treated cells are able to growth in anchorage independent manner. Cells were grown in soft agar as described in Material and Methods, treated with thrombin, or thrombin plus FGF1, TRAP or TRAP plus FGF1. Untreated cells or untreated cells plus FGF1 served as negative controls. Representative fields for each growth condition are shown (original magnification, x4). **(C)** Thrombin treatment decreases the expression of actin stress fibers. FLJ1 NIH 3T3 cell transfectants treated with thrombin, TRAP or unstimulated (control) were stained with rhodamine-phalloidin for the presence of actin stress fibers and analyzed by fluorescent confocal microscopy. Confocal images of median horizontal cell sections were taken using the 100X objective. Bar, 20 µm. NIH 3T3 cells served as control.

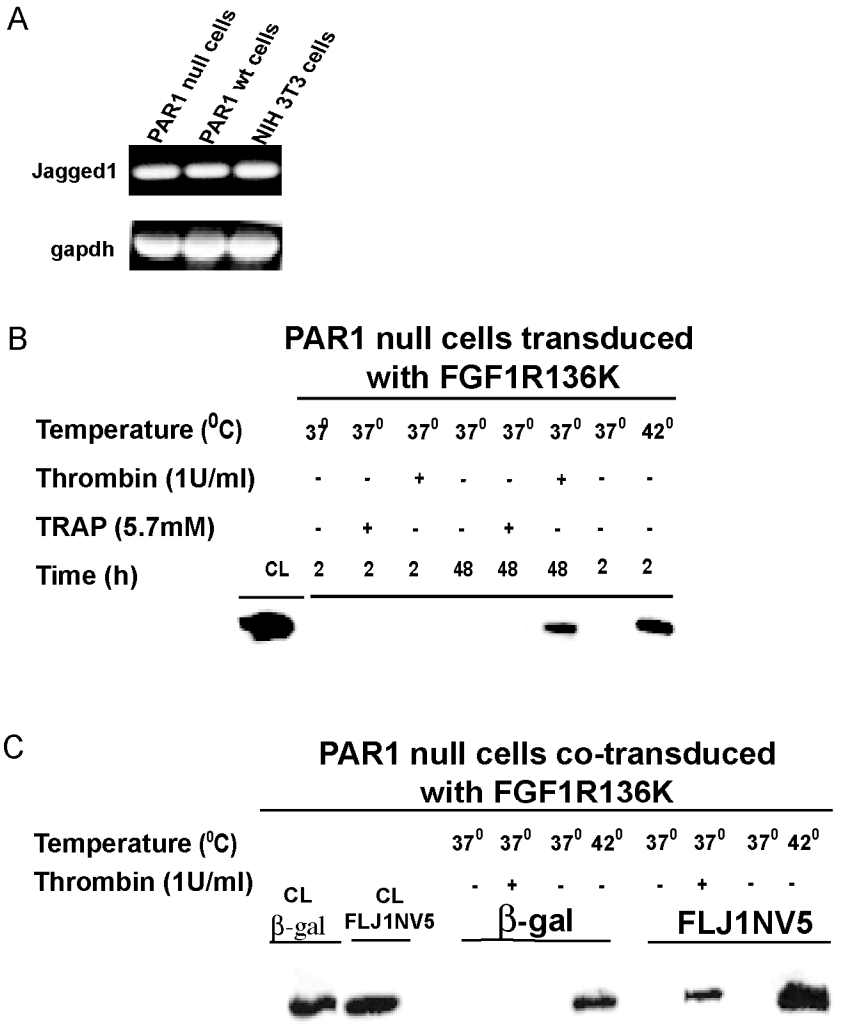


Figure 17- Long-term thrombin incubation induces FGF1 release from PAR1 null MEFs, and overexpression of Jagged1 accelerates FGF1 release from PAR1 null cells in response to thrombin. **(A)** Jagged1 expression in PAR1 null MEFs. The expression of Jagged1 in PAR1 null, PAR1 wt control MEFs and NIH 3T3 cells was determined by RT-PCR. gapdh served as control for mRNA loading. **(B)** Long-term thrombin incubation induces FGF1 release from PAR1 null cells. PAR1 null cells were stimulated either with thrombin or TRAP for 2 or 48 hours. Conditioned media were collected and added for 2 hours to PAR1 null cells transduced with FGF1R136K adenovirus. Detection of FGF1 in the conditioned media was performed as described in the Materials and Methods. The cell lysate (CL) from these cells is shown in the left panel. **(C)** Short-term thrombin stimulation induces FGF1 release from PAR1 null cells overexpressing Jagged1. PAR1 null cells transduced with FLJ1NV5 or β-gal adenoviruses for 48 hours were stimulated with thrombin for 2 hours. Conditioned media were collected and added for 2 hours to PAR1 null MEFs transduced with FGF1R136K. Detection of FGF1 in the conditioned medium was performed, as described in the Materials and Methods. The representative lysate (CL) from these cells is shown in the left panel.

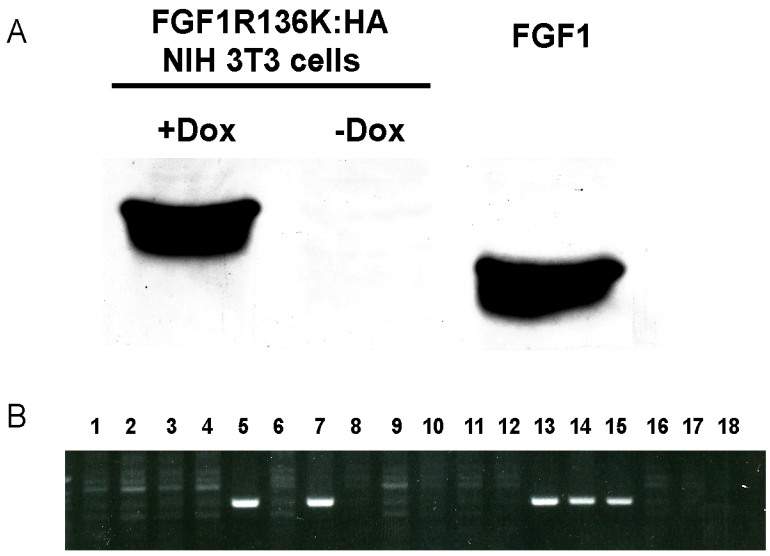


Figure 18- Development of transgenic mice with inducible expression of FGF1R136K:HA **(A)** Doxycycline-inducible expression of FGF1R136K:HA. NIH 3T3 cell stably transfected with rtTA were transiently transfected with pTRE-Tight plasmid containing FGF1R136K:HA. Cells were incubated for 12 hours with 10 ng/ml Dox. The expression of FGF1R136K:HA was confirmed by immunoblotting using anti-FGF1 antibodies. Recombinant FGF1 protein was loaded as positive control. **(B)** Genotyping by PCR analysis of genomic DNA extracted from the tails of mice derived from oocytes microinjected with FGF1R136K:HA.

CHAPTER IV

General Discussion

1. *Thrombin is Able to Induce FGF1 Expression and Release Under Non-Temperature Stress Conditions*

2. *Thrombin Cleaves Jagged1 Producing a Novel Soluble Ligand that Displays Important Biological Activities in Vitro*

3. *Notch Activation Downregulates Thrombin Induced FGF1 Signaling*

4. *The Interplay Between Thrombin, FGF1 and Notch Signaling Has a Biphasic Character*

Thrombin, FGF and Notch signaling pathways play important roles in angiogenesis, vascular repair and remodeling (1, 433, 466, 467). In the past, it has been demonstrated that the presence of soluble Jagged1, a Notch 1 ligand, could interfere with the regulation of angiogenesis *in vitro* and *in vivo*, through cooperation with FGF1 signaling (42, 309, 310). Since thrombin has a potential to produce soluble Notch ligands, it was interesting to evaluate its role in the interplay between FGF and Notch signaling mechanisms.

The results of this study demonstrate the existence of a novel and unanticipated cross-talk between three major signaling systems, bringing new insights about FGF, Notch and thrombin in the context of angiogenesis. Indeed, we found that: 1) thrombin is able to induce FGF1 expression and release under non-temperature stress conditions; 2) thrombin cleaves Jagged1 producing a novel ligand that displays important biological activities; 3) Notch activation downregulates thrombin induced FGF1 signaling; 4) the interplay between thrombin, FGF1 and Notch signaling has a biphasic character.

1 THROMBIN IS ABLE TO INDUCE FGF1 EXPRESSION AND RELEASE UNDER NON-TEMPERATURE STRESS CONDITIONS

Tissue injury induced by stress as a result of physical trauma, infection, metabolic stresses (acidosis, hypoxia, etc) or inflammation is often accompanied by a thrombotic event (468), induced by the conversion of prothrombin to thrombin. The role of the latter serine protease in the response to an injury is complex, evolving in a dynamic way, as the microenvironment of the wound changes over the course of healing (469, 470). Initially, thrombin is pivotal in formation of the fibrin clot (52), aggregation of platelets, and stimulation of proinflammatory responses. At the intermediate stage, thrombin induces a cascade of signaling events, stimulating cell proliferation and migration, needed to restore the integrity of vascular wall. Later,

thrombin triggers events that limit its own formation, lead to dissolution of the clot, and contribute to cessation of inflammatory cell activity.

According to several authors, in the vascular injury context, thrombin plays a critical role not only by forming the extracellular matrix, but also by regulating neovascularization through the induction of expression and/or release of certain growth factors in the course of wound healing (76, 146-149). It is well established that thrombin mediates its biological responses predominantly through the activation of PARs (73, 80, 82, 471-475).

PAR1 is an important player in promoting inflammation and abnormal remodeling during restenosis (neointima formation after vascular injury) and fibrosis in the injured lung (476-478). Due to the presence of several PAR1-coupled G-proteins, each of which activates different pathways, the signaling network induced upon PAR1 activation is complex. In most cell types, thrombin modulates the activity of adenylyl cyclase (67, 74), activates phospholipase C and A2, protein kinase C (PKC), Ras/MAPK signaling pathway, and also regulates the expression of a broad range of transcription factors. Specific inhibitors of PKC, Src, and PI3K suppress PAR1-induced VEGF expression (76), demonstrating the need of proper PAR1 downstream signaling.

VEGF (76, 149), FGF2 (479), PDGFA (480), TGF-β (481) have been shown to be some of the factors, whose expression is induced in vascular endothelial and smooth muscle cells (VSMCs), upon thrombin stimulation. A common and interesting denominator among these growth factors is the time that is required for thrombin to increase their transcript level, none of them seem to be upregulated with particularly rapid kinetics.

In our study, the expression of FGF1 transcript in fibroblasts increases already 15 minutes after thrombin stimulation, suggesting that hemostatic and thrombotic events occurring during the earliest stages of vascular response to injury might involve FGF1 signaling. Identical results were obtained, when PAR1 was activated by a synthetic peptide - TRAP (73, 78, 79). The nuclear run-on studies demonstrated that either thrombin or TRAP induced FGF1 mRNA expression within 15 minutes. We further demonstrated that besides the induction of FGF1 expression, thrombin as well as

TRAP were able to induce its release, under non-stress conditions, with rapid kinetics. The role of PAR1 in mediating the rapid FGF1 release, is crucial since thrombin was unable to stimulate rapid FGF1 export from PAR1 null MEFs also suggesting that other members of this protease activated receptor family (482), might not be involved in the process of thrombin induced FGF1 release.

It has been extensively demonstrated that proinflammatory cytokines are induced very early after vascular injury and may play a crucial role in the attraction of mononuclear cells during the early stages of restenosis and experimental arteriosclerosis (483). Indeed, the inhibition of mononuclear cells recruitment to mechanically injured arteries that are devoid of endothelium may also limit the delivery of essential cytokines and growth factors responsible for the initiation of migration of the VSMC, a prerequisite for the development of a neointima (484, 485). Therefore, it was important to determine whether besides of FGF1, thrombin had the capability to stimulate the release of proinflammatory cytokines with rapid kinetics. According to our results the release of IL1α was induced after a short period of thrombin stimulation. These results indicate that at the early stages of vascular remodeling thrombin not only induces the release of a proangiogenic factor, FGF1, but it is also involved in the export of proinflammatory cytokines.

One could argue, whether thrombin really stimulates FGF1 secretion or it simply causes a rise in the FGF1 expression level, which, in turn, results in an increased secretion rate. However, the pMEXneo mammalian expression construct (486), where FGF1R136K was cloned, is a pUC18-based vector in which the polylinker sequences are flanked by Moloney murine sarcoma virus long-term repeats. We should not expect major fluctuations of transcription driven by this powerful and ubiquitously active promoter. When NIH 3T3 cells stably transfected with FGF1R136K were stimulated by thrombin, the FGF1 intracellular protein levels were unchanged over the incubation period (data not shown). Speaking about the novel cross-talk between FGF1 and thrombin, is important to note that thrombin concentrations, for all the experiments, were in the physiological range. Thrombin circulates in the plasma in micromolar concentrations; indeed the levels of prothrombin in circulation are 1-2

μM (487), although the sequestration of thrombin within the clot decreases its local concentration to the nanomolar range (10-30 nM) (487).

Thrombin and TRAP stimulation also lead to the redistribution of FGF1 from a cytoplasmatic pool to the inner leaflet of the plasma membrane. The fractionation and biotinylation data further demonstrated that upon thrombin stimulation FGF1 translocates across the cell membrane, getting access to the extracellular compartment, where it apparently binds to heparan sulfate proteoglycan sites (12). As already discussed, FGF1 contains a thrombin cleavage site (241), but its proteolysis is inhibited by heparin (488). Thus, we suggest that the saturation of heparan sulfate proteoglycan sites by extracellular FGF1 may assure that excess of FGF1 is proteolytically inactivated by thrombin (241, 489). The release of active FGF1 is a critical outcome in this new FGF1/thrombin interplay since thrombin has little mitogenic activity when NIH 3T3 or Swiss 3T3 cells express a dominant-negative form of FGFR1.

The ability of thrombin to stimulate the rapid non-classical release of FGF1 is noteworthy since it appears to utilize proteins involved in the stress-induced FGF1 release, such as S100A13 (271) and the alternative p40 kDa translation product (291) of the Syt1 transcript. Few years ago, the dependency of FGF1 multiprotein release complex formation upon copper ions has been demonstrated (272). Interestingly, copper depletion of cells by a specific copper chelator did not interfere with the peripheral redistribution of cytosolic FGF1, upon thrombin or TRAP stimulation. However, the inhibition of the FGF1 intracellular redistribution was observed after co-treatment of cells with thrombin or TRAP and amlexanox suggesting that F-actin stress fibers may be used by thrombin to direct FGF1 to the inner surface of plasma membrane. These observations were similar to what has already been reported previously for FGF1 redistribution under stress conditions (305).

As reviewed by Walter Nickel (248), recent studies have established a subgroup of unconventional secretory proteins capable to translocate from the cytoplasm directly across the plasma membrane, in order to get access to the extracellular compartment. FGF1 is one of these proteins that do not have a SP in their primary structure (490). While it was first assumed that angiogenic growth factors might be

released from mechanically injured tissue to promote wound healing (491), several evidences demonstrated that FGF1 is exported from cultured cells in the absence of appreciable cell death (265, 267, 286). We made similar observations in case of thrombin or TRAP treatment, based on low levels of LDH activity in conditioned medium.

The mechanism involved in FGF1 translocation from the cytosol to the extracellular compartment remains to be determined. Previously, it was demonstrated that at stress conditions, FGF1 is released into the extracellular compartment as a multiprotein complex (246). However, the machinery that mediates transmembrane translocation of these proteins remains to be elucidated. Translocation of proteins across the lipid bilayer might require conformational changes that increase their hydrophobicity. It was demonstrated that FGF1 is able to permeabilize membranes containing an acidic phospholipid, phosphatidylglycerol (315). Moreover, FGF1, Synaptotagmins, and S100 proteins bind to phosphatidylserine (316, 318) known to flip from the inner to outer leaflet of the lipid bilayer in response to stress (319), suggesting that FGF1 translocation across the membrane may be due to the interaction with acidic phospholipids. Another hypothesis explaining FGF1 translocation involves Annexin 2, a protein known to exhibit a stress-induced flip-flop through the cell membrane (304). Annexin 2 has been identified as a member of the brain-derived FGF1-containing multiprotein complex and seems to participate in the export of the FGF1 release complex (Soldi, R., Prudovsky, I. and Maciag, T. unpublished results). Recently, Peterson *et al.* demonstrated the capability of thrombin to enhance the presence of Annexin 2 and p11 (a member of the S100 family forming heterotetramers with Annexin 2 (303)) on the EC surface, through their translocation from the inner to outer leaflet of plasma membrane (260). These findings lead us to the hypothesis that thrombin induces the Annexin 2-mediated flip-flop of the FGF1 release complex across the cell membrane. It still remains to be elucidated whether thrombin induces FGF1 translocation through a mechanism similar to FGF1 stress-induced release or through an alternative mechanism.

Since the stress-induced pathway of FGF1 export exhibits slower kinetics (267), we suggest that the thrombin-dependent release of FGF1 may be utilized to rapidly

establish low levels of FGF1 in the extracellular compartment to function primarily as a cell survival factor *in vivo*. This event would be the consequence of a tissue damage involving the activation of either the intrinsic or extrinsic coagulation pathways, which would provide for fibrin deposition as well as for presence of thrombin at the damage site. However, should the time period of the initial stress be extended beyond this immediate-early phase by additional physiological and/or pathophysiologic stress (hypoxia, temperature, etc), it is likely that the non-classical export of FGF1 will be further maintained by the function of the stress-induced pathway (267, 286-288) or by the accumulation of soluble Jagged1 ligands, as we will suggest in the next part of this discussion.

Within the vascular system, FGF1 has been reported to participate in vascular remodeling (172, 492) and to promote angiogenesis after injury (173, 492, 493). Indeed, as shown by different groups, the delivery or *in vivo* expression of FGF1 enhances the vascularization of the myocardium, and stimulates repair of infarctic lesions (166, 466, 494-496). Thus, thrombin-induced FGF1 release mediated by PAR1 activation may have a crucial role in initiating the earlier stages of wound healing, by providing a potent mitogen and regulator of cell survival (497) in the process of neovascularization.

Although the induction of FGF1 expression and release is promoted by thrombin stimulation, that leads to PAR1 activation, with rapid kinetics, it can be assumed that over a certain period of time this local PAR1 population would get desensitized. Indeed, PAR1 is activated by an unusual irreversible proteolytic mechanism in which thrombin binds and cleaves the amino-terminal exodomain of the receptor (73). Thus, the mechanisms that contribute to the termination of signaling are critical determinants of the magnitude and kinetics of the thrombin response in cells (109, 110, 114).

Interestingly, we observed that FGF1 amount released into the conditioned medium, upon thrombin treatment increases over the time, suggesting that thrombin

should be able to induce FGF1 release, through some other molecular mechanism(s), when the PAR1 population gets desensitized.

2 THROMBIN CLEAVES JAGGED1 PRODUCING A NOVEL SOLUBLE LIGAND THAT DISPLAYS IMPORTANT BIOLOGICAL ACTIVITIES IN VITRO

We demonstrated that the serine protease thrombin is able to induce FGF1 release under non-temperature stress conditions. Since it was described that Jagged1 is a FGF response gene in human ECs undergoing differentiation on fibrin clots (42, 380), and since more recently the constitutive release of FGF1 in the presence of a soluble ligand Jagged1 – sJ1 117kDa was demonstrated (310), it was reasonable to hypothesize that thrombin, as a protease, could be responsible for the proteolytic cleavage of Jagged1, inducing the appearance of FGF1 in the extracellular compartment after the desensitization of PAR1 population.

Based on this hypothesis, we studied the amino acid sequence of human Jagged1. Surprisingly, the analysis revealed two putative thrombin cleavage sites within the extracellular domain of Jagged1, none of them corresponding to Jagged1 soluble forms previously described in the literature (42, 309, 393, 394, 402, 498). After thrombin treatment, followed by Edman sequencing, we found that thrombin was able to cleave Jagged1 between EGF repeats 3 and 4 (499). This cleavage, that yields an amino terminal fragment with a molecular mass of approximately 39kDa (sJ1 39kDa), could be completely blocked in the presence of a potent and specific thrombin inhibitor – hirudin.

We further demonstrated that sJ1 39kDa is an active ligand, normally glycosylated, that induces downregulation of Notch signaling as indicated by the decrease of CSL-mediated transcription *in vitro*. Our results suggest that the presence of sJ1 39kDa may also regulate FGF1 signaling, since FGF1 mRNA is upregulated in the presence of this soluble ligand, but not in vector control stable NIH 3T3 cell transfectants, indicating that interference with endogenous Jagged1/Notch signaling alters the level of FGF1 expression. In addition, the presence of sJ1 39kDa induces

FGF1 release under normal growth conditions, as it could be anticipated since Small *et al*. (310) had described similar effects of sJ1 117kDa upon FGF1 transcription.

Numerous studies have demonstrated that the activity of Notch signaling is highly dependent on cell type and environmental context, and this is particularly true for the activity of soluble ligands and for Notch regulation of cell growth (402). Thus, in the context of NIH 3T3 cells, Jagged1 thrombin cleavage product, sJ1 39kDa, acts as a dominant negative regulator of Notch signaling, and induces FGF1 expression and release. Interestingly, sJ1 39kDa, roughly three times smaller than the soluble Jagged1 previously described by Wong and Small *et al*. (309, 310, 393), exhibits similar biological effects upon Notch signaling, FGF1 expression and FGF1 release. Thus, we narrowed down the extracellular Jagged1 region involved in the downregulation of Notch signaling, to the DSL and the first three EGF repeats, since those were the common domains between these two soluble ligands. These results do not support the findings by Li *et al.* where the addition of a peptide carrying only the DSL domain of Jagged1 was shown to activate Notch in hematopoietic precursor cells (500). Indeed, the role of soluble ligands in Notch signaling is still poorly understood. Naturally occurring soluble forms of Notch ligands arising as a result of proteolytic cleavage (383) or differential mRNA processing (42, 381, 402) have been identified; yet the functional activities of these modified ligands are not clear, and highly controversial. A total of 71.5% of the human Jagged1 mutations found in Alagille Syndrome (501, 502) patients (381, 446, 503-505) lead to the appearance of a premature termination codon giving rise to sJ1 ligands. Both agonist (381, 384, 386, 500) and antagonist (311, 387, 388, 391-394, 506) effects of soluble ligands upon Notch signaling have been reported. The molecular mechanisms underlying these opposite effects are not known. Some explanations involve oligomerization or immobilization of the ligands (391, 392), which are needed for efficient stimulation of Notch signaling. If these explanations are correct, soluble ligands, unlike transmembrane ones, should be unable to activate Notch receptors. It has been suggested by Small *et al*. (393) that the negative effects of soluble ligands upon Notch signaling may be explained by competition with functional transmembrane ligands for binding to Notch receptors.

Over the last three years, there have been a number of publications demonstrating that the Presenilin/γ-secretase-mediated cleavage of Notch ligands results in the translocation of their ICDs into the nucleus (395-397, 507), suggesting that Notch pathway, in addition to signaling through NICDs, includes the activity of soluble ligand extracellular domains, as well as the biological effects of ligand ICD. The presence of a PDZ binding site (335) within the ICD of Jagged1, was revealed to be essential for cellular transformation (508). Also the potential nuclear localization sequence in J1ICD, allows to anticipate a relevant role for this domain, similarly with what has been, recently, determined for Dl1ICD (399) [1].

We did not specifically address, in our studies, the role of the J1ICD after thrombin cleavage, neither the role of the leftover extracellular portion (from the fourth EGF-like repeat to the transmembrane domain) of Jagged1. However, there are at least three different situations to consider: i) after thrombin cleavage, both the leftover extracellular domain and the ICD are proteolytically inactivated; ii) the leftover extracellular domain interacts with Notch receptor, since it still carries a reasonable number of EGF-like repeats, inducing the activation of Notch signaling, and simultaneously, the formation of J1ICD; and iii) the leftover extracellular domain interacts with Notch receptor, but instead of activation it induces downregulation of Notch signaling.

The ICD of Jagged1 might carry an important role in providing the bidirectionality of Notch signaling after cell-cell contact establishment. We recently demonstrated that the expression of the Dl1ICD resulted in a non-proliferating senescent-like phenotype of HUVECs (paper 2) while J1ICD induced a severe apoptotic phenotype (Kacer D, Kolev V, Duarte M, and Prudovsky I. unpublished results).

Proteolysis of extracellular matrix components has long been known to play an important role in both *in vitro* and *in vivo* angiogenesis (401). The capability of thrombin to cleave Jagged1 might represent an example of it, since this proteolytic

[1] A more complete discussion of biological activity of the intracellular domain of Delta can be found in paper 2.

modification yields a soluble ligand able to induce the expression and release of FGF1, a well-known angiogenic factor.

Thrombin has been reported to regulate a broad range of biological processes as a result of its proteolytic activity. Here we reported a novel and unanticipated link between the enzymatic activity of thrombin and Notch signaling. We further characterized this new thrombin proteolytic role in Jagged1/Notch signaling, demonstrating that thrombin, but not TRAP, was able to induce a prominent transformed phenotype on sJ1 117kDa NIH 3T3 cell transfectants enhancing their growth in anchorage-independent manner and formation of large multicellular, spheroid-like colonies. The colonies formed upon thrombin treatment were bigger than ones formed in the absence of thrombin, and even bigger when exogenous FGF1 along with thrombin was added to the cells, most likely due to a synergy effect between FGF1 and thrombin, unmasking the role of sJ1 39kDa in FGF1 and Notch signaling.

Previous reports have described a decreased expression of stress fibers in NIH 3T3 cells, when Notch signaling was downregulated (393, 400). We obtained similar results upon thrombin treatment of NIH 3T3 cells whereas TRAP did not interfere with stress fiber organization. It has been reported that the induction of Src activity (393) may be responsible for the manifestation of many of the phenotypic characteristics in the sJ1 117kDa NIH 3T3 cell transfectants, including the decrease in actin stress fibers (400), loss of pro-α 1 (I) collagen expression and decreased sensitivity to contact inhibition (309). Moreover, the constitutively active v-*src* induces in a dramatic down-regulation of actin stress fibers (509), and loss of contact inhibition is documented as a consequence of v-*src* expression in a variety of cell types (510). Therefore, it is possible that thrombin treatment-dependent alteration of stress fibers might be mediated by Src, promoting the stabilization of the chord-like phenotype in sJ1 39kDa transfectant cultures, similar to what has been described for sJ1 117kDa NIH 3T3 cell transfectancts (393).

Several reports indicate that sJ1 may play crucial role in hematopoietic stem cell self-renewal (394), angiogenesis (42, 309), and vascular repair (400). Jagged1 knockout (435) resulted in embryonic lethality with major vascular defects in mice. *In*

vitro studies demonstrated that NIH 3T3 cell stably expressing sJ1 117kDa form chord-like structures, similar to those formed by EC in the course of angiogenesis (309). In addition, intradermal injection of these cells into the flank of nude mice resulted in formation of tissue masses with prominent vascularization (309), underlying a crucial role of sJ1 in the process of angiogenesis.

We hypothesized that the export of FGF1 at the early stage of thrombin-induced response may contribute to the increase of Jagged1 expression level. Indeed, the induction of Jagged1 transcription by FGF1 stimulation has been described in EC and NIH 3T3 cells (42, 310). The continuous presence of thrombin in the extracellular compartment may result in the accumulation of sJ1 39kDa, and consequent release of FGF1 after the desensitization of PAR1 population. Based on this premise, the role of PAR1 signaling may be limited to the immediate-early events of thrombin-mediated response upon vascular injury, whereas the activity of sJ1 39kDa may be important later, in the process of vascular repair.

According to our hypothesis, thrombin through its proteolytic activity is able to induce FGF1 release through PAR1 signaling at the early stages of vascular injury, when cell-cell contact have not yet been reestablished. The presence of thrombin leads to the formation and accumulation of sJ1 39kDa into the medium. This soluble ligand induces FGF1 expression and release, through downregulation of Notch signaling. As wound healing proceeds and the cell proliferation is augmented by the sustained presence of FGF, cell-cell contacts start to be reestablished, and Notch signaling is activated as a result of receptor-ligand interaction between neighboring cells. The upregulation of Notch signaling blocks FGF1 expression and release, leading to the termination of the proliferative phase of wound healing.

3 NOTCH ACTIVATION DOWNREGULATES THROMBIN INDUCED FGF1 SIGNALING

Notch signaling plays a key role in normal development through diverse effects on differentiation, survival, and proliferation and these events are highly dependent on signal strength and cellular context (412, 511). Ligand binding to Notch receptors triggers a cascade of proteolytic events, resulting in the production of a NICD which translocates to the nucleus (338-340, 344, 512-515), where it binds to CSL, displacing co-repressors in order to form a ternary complex with coactivating factors, inducing transcription activation of Notch target genes (347, 348, 352, 356, 516). According to our results, while thrombin antagonizes CSL-dependent signaling in FLJ1 NIH 3T3 cell transfectants, TRAP, a PAR1 activating peptide devoid of any proteolytic activity, does not affect it. These results underlie the proteolytic activity of thrombin, which results in cleavage of Jagged1 and production of sJ1 39kDa, a soluble ligand able to induce FGF1 expression and export. Interestingly, the interrelations between FGF1 and Notch signaling are reciprocal. Indeed, Small *et al*. (310) demonstrated that exogenous FGF1 was able to repress CSL-dependent transcription in NIH 3T3 cells.

Since we demonstrated that PAR1 activation induced FGF1 expression and release, and TRAP was not able to decrease either the CSL-dependent transcription or the anchorage-independent cell growth or the actin-stress fibers expression (all decreased when Notch1 signaling is downregulated) we suggest that PAR1 induced FGF1 transcription and export are not mediated through Notch signaling. Instead, the role of sJ1 39kDa in downregulation of Notch signaling seems to be crucial for FGF1 release, since thrombin was unable to induce both the expression and the export of FGF1, when Notch signaling was maximized by adenoviral transduction of caN1. Thus, thrombin induced FGF1 expression and release are mediated by PAR1 and soluble Jagged1 39kDa, most probably at two different temporal stages. Based on the premise that downregulation of Notch signaling is a key step in FGF1 signaling activation, we anticipated that in the absence of Jagged1, FGF1 should be

constitutively released. Indeed, FGF1 was constitutively released from Jagged1 null embryonic fibroblasts, most likely due to the downregulation of Notch signaling. Since PAR1 expression levels in Jagged1 null cells were unchanged comparatively to control embryonic fibroblasts (data not shown), we expect that those cells would also release FGF1 under thrombin stimulation, through PAR1 activation. However, due to the constitutive FGF1 release we were unable to verify this premise.

Although aberrant activation of Notch signaling pathways has been reported to be associated with neoplastic growth in mammals (322, 334, 517, 518), it is clear that Notch pathway regulates cell growth depending upon cell type and environmental context (363, 386, 426, 519). Indeed, Notch has been reported by several groups to be a suppressor of cell growth. For example, the activation of Notch1 causes the arrest of cell cycle progression in the chicken B-cell line DT40 (520) as well as in small cell lung cancer cells (364), and prevents myeloid but not erythroid cell proliferation in the absence of polypeptide mitogens (521). Also the proliferation of ECs has been shown to be inhibited by Notch activation (361, 377), through inhibition of retinoblastoma protein phosphorylation (361). Furthermore, down-regulation, not up-regulation, of Notch1 signaling is required for progression into the late stages of human papillomavirus-induced cervical carcinogenesis (522). The observation that thrombin suppresses CSL-mediated transcription suggests that Notch may protect the NIH 3T3 cell from abnormal growth through the transcriptional regulation of Notch/CSL-responsive genes. FGF1 export, under thrombin stimulation, would then reinforce the inhibition of Notch/CSL. This sort of regulatory mechanism is consistent with the requirement for Notch/CSL-dependent induction of $p21^{waf/cip}$ for the stimulation of keratinocyte differentiation, which involves growth arrest (keratinocytes require exogenous FGF for cell division) (363). However, this regulatory mechanism may also contain a cell- and tissue-specific as well as an age-dependent components because in the developing tooth bud, FGF10 is able to induce the Notch/CSL-dependent transcription of *hes1* (406). It also may be complicated by specificity for only some of 24 members of the *fgf* gene family.

To our knowledge, the link between Notch and FGF signaling and thrombin activity represents a novel facet of the thrombotic cascade in response to vascular

injury. Notch signaling is involved in multiple aspects of vascular biology and angiogenesis (430, 433). Several studies have found that Notch signaling plays a critical role in vascular formation during early embryonic development (376, 434, 435, 523-526). As recently demonstrated by Limbourg *et al*. (526) mutant embryos lacking endothelial Notch1 died with profound vascular defects in placenta, yolk sac, recapitulating the vascular defects and embryonic lethality of global Notch1-deficient mice (434), underlying the essential role of Notch1 signaling in physiological angiogenesis and vascular development.

The interaction of Notch signaling with various molecular mechanisms important in the vascular cell phenotype, like VEGF (376, 377), BMP/TGF-β (527), hepatocyte growth factor (528), HIF1α (529), and FGF1 (42, 310) have been described.

As a result of our studies, we found a new link between Notch signaling and haemostasis in the context of vascular remodeling and repair, where thrombin appears to be the common denominator between the non-classical export of FGF1 and the antagonism of Notch signaling.

4 THE INTERPLAY BETWEEN THROMBIN, FGF1 AND NOTCH SIGNALING HAS A BIPHASIC CHARACTER

The long-term thrombin stimulation induced FGF1 release from PAR1 null cells, which express endogenous Jagged1 at the level comparable to NIH 3T3 cells. This long-term thrombin stimulation rescued the FGF1 export from PAR1 null cells, most probably due to the accumulation of sJ1 39kDa into the medium, as a result of thrombin proteolytic activity. Additionally, overexpression of Jagged1 in PAR1 null cells enabled them to accelerate the process of FGF1 release, indicating that thrombin may induce FGF1 export through two pathways: one PAR1-dependent, and the other mediated by the cleavage of Jagged1.

We hypothesize that tissue damage-induced FGF1 release proceeds through at least two stages. The early stage is caused by tissue damage and it involves the

activation of coagulation pathway, which provides fibrin deposition, as well as increase of thrombin level at the damage site. Thrombin induces FGF1 expression and release through PAR1 activation. FGF1 serves as a survival and mitogenic factor for the cells of damaged tissue. Additionally, released FGF1 enhances Jagged1 expression as it has been previously demonstrated (42). Interestingly, a marked increase of Jagged1 expression has been detected in the regenerating endothelium following balloon injury of the rat carotid artery (400). In agreement with these observations, MAPK activation upon cell stimulation with growth factors has recently been shown to induce Jagged1 expression (530). Since PAR1 signaling is known to induce MAPK activation (531-533), we suggest that PAR1 may stimulate Jagged1 expression not only through FGF1 expression and release, but also directly. The persistence of thrombin in damaged tissue results in accumulation of sJ1 39kDa (combined effect of induced expression and continuous cleavage of Jagged1).

While PAR1 population gets desensitized over time, the release of FGF1 at the later stage of cell response to tissue damage becomes dependent upon the sJ1 39kDa-induced downregulation of Notch signaling. As cell-cell interactions disturbed by initial tissue damage start to be re-established, inductive signaling between Notch ligands and their receptors on neighboring cells ensure the proper development of vascular structures. This signaling may have a bi-directional character. Indeed, interactions of transmembrane Notch ligands with their receptors results in the proteolytic cleavage not only of receptors, but also of ligands and in the production of ligand ICDs, untethered to cell membrane (396, 397, 507, 508). We demonstrated that Dl1ICD blocked cell proliferation where cell synchronization, tissue sculpting and repair might take place (399), while J1ICD induces an apoptotic phenotype (Kacer D, Kolev V, Duarte M, and Prudovsky I. unpublished results). In addition to acting as a "gatekeeper" and restricting the number of cells responding to various inductive cues, bi-directional Notch signaling might also provide stabilization and maintenance of a quiescent and mature endothelium. FGF1 and Notch1 signaling pathways apparently differentially regulate each other at different stages of tissue stress response. At the early stage of vascular repair process, FGF1 plays a pivotal role in providing cell

survival and proliferations, and later the activation of Notch signaling drives cellular differentiation, and tissue sculpting throughout the vascular tree.

The interplay between thrombin, Notch and FGF1 signaling pathways may also be important for stem cell renewal and differentiaion. Although there is no direct study linking the enzymatic activity of thrombin to any aspect of developmental biology, Vas *et al*. (394) described the ability of a soluble form of Jagged1 to promote clonal expansion of hematopoietic stem cells *in vitro*. Combined with our results about thrombin proteolytic activity towards Jagged1 protein, these data may link thrombin to stem cell biology. Indeed, the observation that PAR1 in one of eight genes upregulated in embryonic, neurosphere and hematopoietic stem cell populations (534) supports this premise.

In the process of vascular development, there are two fundamentally different processes that establish blood vessels. Initial vasculogenesis generates a primitive network of vessels through aggregation and tube formation of angioblast precursor cells. Then angiogenesis leads to further growth, branching, and remodeling of the vascular tree (1). The formation of capillary sprouts from the existing microvasculature occurs secondary to an inciting stimulus that results in increased vascular permeability, accumulation of extravascular fibrin, and local proteolytic degradation of the basement membrane (535). The ECs overlaying the disrupted region become "activated", change shape, and extend elongated processes into the surrounding tissue. Direct migration toward the angiogenic stimulus results in the formation of a column of ECs. Just proximal to the migrating tip of the column there is a region of proliferating ECs. According to our hypothesis, the edge of the tip might represent the cell that has partially lost contact with the neighboring cells, and where FGF1, along with other growth factors would induce the active proliferation, but where Notch signaling does not play a pivotal role. Proximally to the proliferative zone, the ECs undergo another shape change, adhere tightly to each other, and begin to form a tube, where cell-to-cell contacts activate Notch signaling. Notch signaling is frequently modulated by other proteins expressed on interacting cells, and also by intrinsic Notch regulators such as Presenilins, Numbs (536-538), Fringes (539-541) and Deltex (367, 368, 542). These regulatory mechanisms ensure that Notch

activation is restricted to discrete cells at specific times of the vascular development. Thrombin also may regulate Notch signaling through the production of sJ1 39kDa and by stimulation of the expression and export of FGF1. Indeed, mutual regulatory relationship between Notch and FGF pathways has been suggested, to play role in different biological processes, like oncogenic transformation (543), *in vitro* angiogenesis (42, 380), during development of the teeth (405, 406), in the development of *Drosophila* tracheal system (409) and differentiation of neuroepithelial precursor cells *in vitro* (410).

After the stimulation of endothelial cell proliferation with growth factors, they usually return to the state of quiescence (544, 545). Interestingly, it has been recently demonstrated that non-transformed cell cultures stimulated with FGF1 transit through only one cell cycle, and then are blocked in the G1 phase of the second cycle (546). Various studies raise the notion that Notch activation may be required for the establishment of a mature, quiescent endothelial phenotype, in part by downregulating VEGFR2 (377, 378), and maybe also through FGF1 signaling. Our model of interplay between FGF and Notch signaling pathways is indirectly supported by the phenotype of Notch- and Notch ligand-deficient mice that display inappropriate apoptosis and (or) proliferation in the vascular system (434, 435, 457). The primary vascular plexus is laid down, but remodeling of this initial endothelial network does not take place. Thus it is possible that whereas FGF1, most probably along with other growth factors, plays an important role in the induction of cell proliferation at the early stages of vasculogenesis, Notch activation is required to maintain endothelial viability during the reorganization or mature vasculature.

The crosstalk between thrombin, Notch and FGF1 described here may have ramifications for some pathological states, such as restenosis and tumor development. The role of PAR1 in restenosis is well recognized, since preclinical studies demonstrated that perivascular administration of a selective PAR1 antagonist significantly reduced neointimal thickness after balloon angioplasty in a rat restenosis model (120) and intravenous administration of a PAR1 antagonist prevented thrombotic occlusion of carotid arteries in a nonhuman primate model of vascular

injury, (116)[2]. FGFs are known to contribute to vessel wall pathology in response to injury, especially when an excess of FGF family proteins is present (172, 547-550), suggesting that these secreted proangiogenic factors function in a dose-dependent manner. Moreover, PAR1, FGF1 and Notch-mediated signaling events have been implicated in tumor growth (126, 130, 131, 133, 551-554). Therefore, exaggerated induction of FGF1 transcription and export through PAR1 activation, and through soluble Jagged1 production may play an important role in the context of carcinogenesis, where vascular remodeling is a constant requisite, and either Notch, FGF1 or PAR1 have been extensively described to play pivotal roles and even proposed as strong therapeutic targets.

Given that thrombin, FGF and Notch as individual signaling systems have a significant role in the vascular system, the crosstalk between these three pathways might open new routes for the development of new therapeutics and diagnostic strategies for vascular related diseases and for the plethora of other pathological scenarios.

[2] Integrating in the role of PAR1 in the vascular system we recently characterized the multiple biological effects of SCH 79797 in different cells lines (Paper 4).

CHAPTER V

Conclusions and Future Perspectives

CONCLUSIONS

Following are the main conclusions of the present study:

- The serine protease thrombin induces the upregulation of FGF1 transcription and redistribution of FGF1 to the inner leaflet of the plasma membrane, resulting in the of export this proangiogenic growth factor with fast kinetics.

- PAR1 activation is essential for the rapid induction of FGF1 release.

- Mitogenic activity of thrombin is dependent upon FGFR signaling.

- Thrombin is able to cleave the extracellular domain of Notch ligand Jagged1 and to generate the soluble N-terminal fragment sJ1 39kDa.

- sJ1 39 kDa exhibits a normal glycosylation pattern, it is capable to decrease CSL-mediated transcription and to induce FGF1 expression and export under non-temperature stress conditions.

- The activation of Notch signaling abrogates thrombin-induced FGF1 expression and release.

- Long-term thrombin stimulation of PAR1 null cells induces FGF1 release, most probably due to sJ1 39kDa accumulation.

The data presented here demonstrate the existence of a link between thrombin/PAR1 signaling and two other major signaling pathways: FGF1 and Notch1. They indicate that thrombin can regulate FGF1 export, by using both PAR1 and Notch pathways.

We feel tempted to provide a hypothetical scheme for this cross-talk between thrombin, FGF, and Notch signaling (Figure 19).

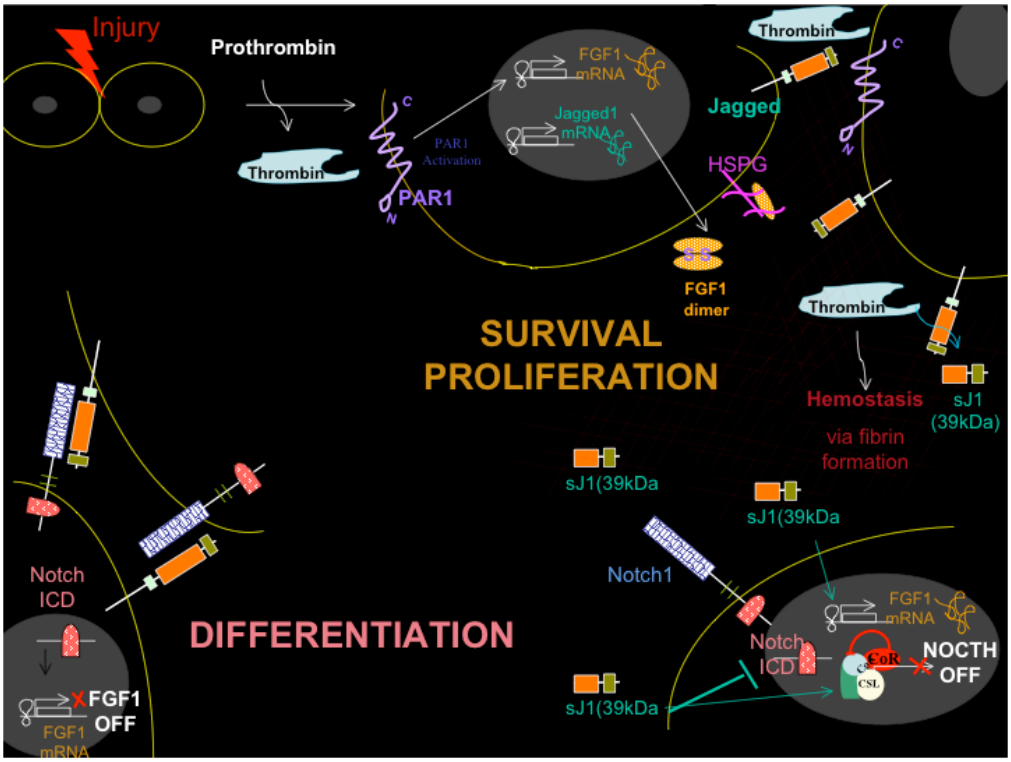


Figure 19- Schematic representation of the crosstalk between thrombin, FGF1 and Notch signaling pathways in the context of vascular injury.
Following initiation of coagulation as part of the haemostatic response to injury, thrombin is generated from its inactive precursor prothrombin by factor Xa (a part of the prothrombinase complex). One of the prime functions of thrombin is conversion of fibrinogen to fibrin, which forms the fibrin mesh. Thrombin also activates PARs, inducing the transcription and release of several growth factors, among them FGF1, a potent cell survival and pro-angiogenic factor, which is release with rapid kinetics after PAR1 activation. The interaction of FGF1 with FGFR induces a broad range of biological responses resulting in activation of transcription of FGF1 response genes including Jagged1. Thrombin present in the damaged tissue promotes Jagged1 cleavage, resulting in the accumulation of soluble Jagged1 - sJ1 39kDa. While PAR1 population gets desensitized, the release of FGF1 at the later stages may be dependent upon sJ1 39kDa induced downregulation of Notch signaling, which results in FGF1 expression and release. When cell-cell interactions start to be re-established, binding of transmembrane Notch ligands and receptors activates Notch signaling which blocks FGF1 transcription and release.

FUTURE PERSPECTIVES

A number of problems concerning the understanding of thrombin role in angiogenesis, and translation of information about Notch/thrombin/FGF cross-talk to other biological contexts remain to be solved. Our long-term goal has been to understand the molecular mechanisms responsible for the regulation of angiogenesis in an attempt to apply this information for the repair of tissue and organ damage or for the inhibition of angiogenesis during restenosis and solid tumor growth. The results present in this thesis add new insights to our current knowledge regarding the roles of FGF1, Notch, PAR1 and thrombin in the vascular biology, however, they also raise additional questions that should be addressed in future studies:

- Giving the importance of PAR1 for inducing the rapid FGF1 expression and release, it is important to elucidate the molecular mechanism underlying this induction.

- Since PAR1 upregulation seems to be associated to several pathological situations, it would be highly interesting to study the role of FGF1 signaling in the processes of PAR1-dependent cancer cell invasion and vascular restenosis.

- It remains to be explored the mechanisms of induction of FGF1 expression and release induced by sJ1 39kDa.

- Based on our results sJ1 39kDa in cell culture play an important role upon both FGF1 and Notch signaling mechanism. It remains to be determined the role of this soluble ligand *in vivo*.

CHAPTER VI

Papers 1- 6

Paper 1

"The non-classical export routes: FGF1 and IL-1α point the way"

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Journal of Cell Science 116 (24): 4871-4881, 2003

The non-classical export routes: FGF1 and IL-1α point the way

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Journal of Cell Science 116, 4871-4881 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00872

Summary

Non-classical protein release independent of the ER-Golgi pathway has been reported for an increasing number of proteins lacking an N-terminal signal sequence. The export of FGF1 and IL-1α, two pro-angiogenic polypeptides, provides two such examples. In both cases, export is based on the Cu^{2+}-dependent formation of multiprotein complexes containing the S100A13 protein and might involve translocation of the protein across the membrane as a 'molten globule'. FGF1 and IL-1α are involved in pathological processes such as restenosis and tumor formation. Inhibition of their export by Cu^{2+} chelators is thus an effective strategy for treatment of several diseases.

Key words: Fibroblast growth factor 1, FGF1, Interleukin 1α, IL-1α, Release, Non-classical, Copper, Synaptotagmin 1, S100A13

Introduction

Many biological processes involve polypeptide translocation across phospholipid membranes. Among them are export to the extracellular milieu (Blobel, 1995), transport in and out of the nucleus (Weis, 2003), and import into mitochondria (Endo et al., 2003; Gordon et al., 2000) and peroxisomes (Holroyd and Erdmann, 2001). These processes use specific transporters and frequently involve transmembrane pores and channels. The proteins translocated usually possess appropriate signal sequences and, in the case of classical protein secretion, this is a hydrophobic N-terminal sequence that allows the protein to enter the ER-Golgi pathway (Blobel, 2000).

However, several extracellular proteins lack signal sequences, and their export proceeds through endoplasmic reticulum (ER)-Golgi-independent non-classical routes (Table 1) (reviewed by Nickel, 2003). Currently, we know little about the export mechanisms of most of these proteins, the only two common features being the absence of a signal sequence in the protein and the insensitivity of the pathway to brefeldin A [a drug that specifically inhibits ER-to-Golgi transport (Misumi et al., 1986)]. Nevertheless, what is apparent is that non-classical export is not a single pathway but instead comprises several transport mechanisms. Here, we concentrate on two biologically important and functionally related proteins, fibroblast growth factor 1 (FGF1) and interleukin (IL)-1α, and their non-classical export pathways.

Structure and function of FGF1 and IL-1α

FGF1 and FGF2 are prototypical members of the FGF family (Szebenyi and Fallon, 1999). FGFs have a wide variety of biological activities. During embryogenesis, these growth factors regulate mesodermal induction, neurulation, and the formation of the circulatory and skeletal systems (Friesel and Maciag, 1999). Subsequently, they play a crucial role in angiogenesis, tissue regeneration, inflammation and the formation of some tumors (Friesel and Maciag, 1999). Their biological effects are mediated through activation of four transmembrane phosphotyrosine kinase receptors (FGFR1-4), with the participation of cell-surface heparan sulfate proteoglycans (HSPGs), and consequently require release of the polypeptide (Friesel and Maciag, 1999). Most members of the family therefore possess classical signal sequences but FGF1 and FGF2 are devoid of such sequences and thus are released by novel secretion mechanisms (Coulier et al., 1997; Friesel and Maciag, 1999).

The existence of FGF1- and FGF2-specific secretion pathways might represent a protective mechanism developed in the course of evolution and might be related to their high mitogenic potential and widespread expression. Indeed, a recombinant derivative of FGF1 that has an attached N-terminal signal sequence is a potent oncoprotein (Forough et al., 1993). Significantly, the FGFs of *Caenorhabditis elegans* and *Drosophila* have signal sequences (Coulier et al., 1997). Apparently, strictly programmed mosaic development of these organisms can rely on the regulation of FGF availability solely at the level of its expression. The more complicated and less hierarchical developmental strategies of chordates probably required the evolution of signal-peptide-less FGFs, whose accessibility might be more flexibly regulated post-translationally.

The IL-1 family (Dinarello, 1996; Stylianou and Saklatvala, 1998), of which IL-1α and IL-1β are prototypical members, numbers at least ten proteins. Nine of these, including IL-1α and IL-1β, do not have signal sequences despite acting through transmembrane receptors and thus requiring export (Dinarello, 1998; Stylianou and Saklatvala, 1998). These proteins are potent pro-inflammatory cytokines (Dinarello, 1996), inducing

biosynthesis of a variety of inflammation-related molecules, such as tumor necrosis factor (TNF), transforming growth factor (TGF)-β, granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), cyclooxygenase 2, endothelin-1, phospholipase A2, and inducible nitric oxide synthase (Dinarello, 1996). Unlike the FGFs, IL-1s are synthesized as higher molecular weight precursor (p) proteins. pIL-1α is cleaved by calpain or calpain-like proteases to form mature (m) IL-1α, and pIL-1β is cleaved by the IL-1-converting enzyme to form mIL-1β (Dinarello, 1996; Stylianou and Saklatvala, 1998).

Comparison of the crystal structures of FGF1, FGF2, IL-1α and IL-1β (Graves et al., 1990; Venkataraman et al., 1999; Zhu et al., 1991) reveals that they have very similar folds in spite of their very low sequence similarity (Fig. 1). These proteins contain β-barrel structures that are often found in transmembrane proteins, including bacterial pore-forming proteins (Chen and Funk, 2001; Heuck et al., 2000; Montoya and Gouaux, 2003) and are crucial for membrane insertion of some proteins (Heuck et al., 2000). This provided the first indication that similarities might exist between the release mechanisms of the IL-1 and FGF prototypes. It was especially interesting to compare the release of IL-1α and FGF1 since these two proteins have antagonistic effects upon the proliferation and migration of endothelial cells (Maier et al., 1990; Friesel and Maciag, 1999).

Cell stress induces FGF1 and IL-1α release

Under normal conditions, cells expressing FGF1 and IL-1α do not release these proteins. However, several stresses, such as heat shock (Jackson et al., 1992), hypoxia (Mouta Carreira et al., 2001), cultivation under low serum conditions (Shin et al., 1996) and cell treatment with low-density lipoproteins (LDLs) (Ananyeva et al., 1997), induce release of FGF1 from NIH 3T3 cells. Heat shock induces the export of mIL-1α from human promonocytic leukemia cells and activated peripheral mononuclear cells (Tarantini et al., 2001; Mandinova et al., 2003). The two latter cell types also exhibit heat-shock-induced export of pIL-1α (Mandinova et al., 2003). Interestingly, similarly to FGF2 (Shi et al., 1997), pIL-1α is not secreted from stressed NIH 3T3 cells (Tarantini et al., 2001). The retention of pIL-1α is most likely because of the nuclear localization sequence (Wessendorf et al., 1993) in its cleavable N-terminal precursor domain. Although pIL-1α is cleaved in monocytes/macrophages (Dinarello, 1992;

Table 1. Proteins exported through non-classical pathways

Protein	Reference	Signal peptide	Export sensitivity to brefeldin A	Release characteristics
Secretory transglutaminase	Aumuller et al., 1999	–	Insensitive	Constitutive, through membrane blebbing
Thioredoxin	Rubartelli et al.,1992; Rubartelli et al., 1995; Angelini et al., 2002	–	Insensitive	Induced by antigen-specific T cells, intracellular vesicles not involved
Galectins	Hughes, 1999; Sato et al., 1993; Lindstedt et al., 1993	–	Insensitive	Constitutive, through membrane blebbing
Il-1α	Tarantini et al., 2001; Mandinova et al., 2003	–	Insensitive	Stress-induced, Cu^{2+}-dependent, in complex with S100A13
Il-1β	Rubartelli et al., 1990; Andrei et al., 1999	–	Insensitive	Stress-induced, ABC-transporter-dependent, through the endolysosomal pathway
FGF1	Jackson et al., 1992; Tarantini et al., 1998; LaVallee et al., 1998; Mouta Carreira et al., 1998; Landriscina et al., 2001a; Landriscina et al., 2001b	–	Insensitive	Stress-induced, Cu^{2+}-dependent, in complex with S100A13 and p40 Syt1
FGF2	Florkiewicz et al., 1995; Mignatti et al., 1992; Engling et al., 2002	–	Insensitive	Constitutive, Na^+/K^+ ATPase-dependent
Sphingosine kinase	Ancellin et al., 2002	–	Insensitive	Constitutive, inhibited by cytochalasin
Annexin I	Chapman et al., 2003	–	Not tested	Glucocorticoid-induced, ABC-transporter-dependent
Annexin II	Peterson et al., 2003	–	Not tested	Thrombin-induced, in complex with p11
p40 Synaptotagmin 1	LaVallee et al., 1998; Tarantini et al., 1998	–	Insensitive	Constitutive
S100A13	Landriscina et al., 2001a; Landriscina et al., 2001b	–	Insensitive	Constitutive
HIV Tat	Chang et al., 1997	–	Insensitive	Constitutive
Herpes VP 22 protein	Elliott and O'Hare, 1997	–	Insensitive	Constitutive
Foamy virus Bet protein	Lecellier et al., 2002	–	Insensitive	Constitutive
Engrailed 2	Joliot et al., 1998; Maizel et al., 1999; Maizel et al., 2002	–	Insensitive	Attenuated by the CK2-dependent phosphorylation
HMGB1	Gardella et al., 2002; Passalacqua et al., 1997; Passalacqua et al., 1998; Sparatore et al., 1996	–	Insensitive	Stress-induced, through an endolysosomal pathway
Leishmania HASPB protein	Denny et al., 2000	–	Insensitive	Constitutive, acylation-dependent

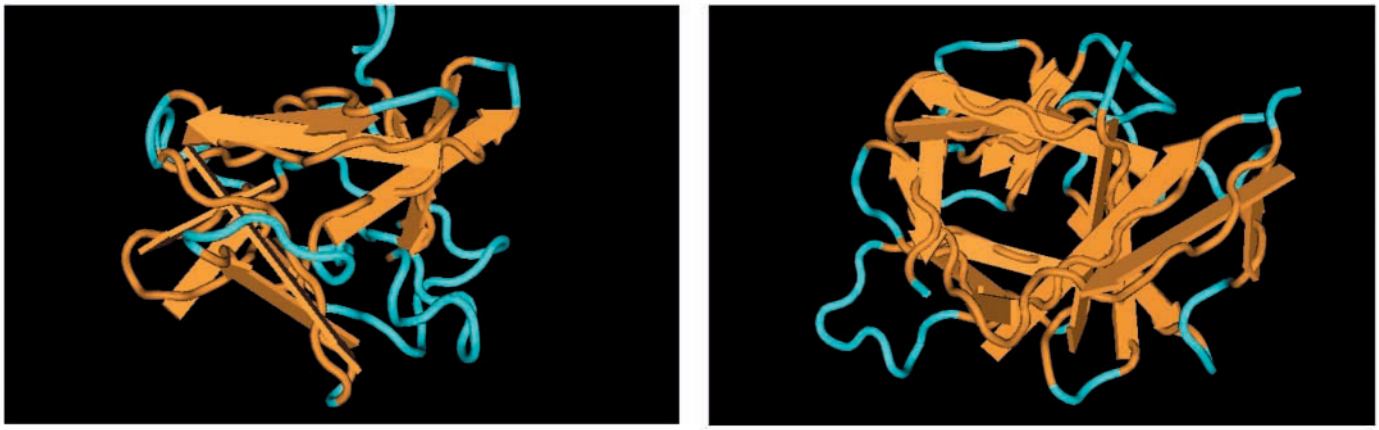

Fig. 1. Three-dimensional representation of the β-barrel structures of human mIL-1α (Graves et al., 1990) and human FGF1 (Lozano et al., 2000). β-sheet domains are indicated in yellow and are depicted as rotating counter clockwise around the open centers of the structures. The structures were downloaded from the Protein Data Bank of the NCBI (http://www.rcsb.org/pdb/).

Dinarello, 1996), the cleavage does not occur in NIH 3T3 cells (Tarantini et al., 2001), perhaps because of high levels of the calpain inhibitor calpastatin (Goll et al., 2003) in the cytosol of these cells. In macrophage-like cells, the control of pIL-1α nuclear localization is less stringent since these cells display pIL-1α both in the nucleus and in the cytoplasm (Beuscher et al., 1988; Kobayashi et al., 1990).

Significantly, co-expression of pIL-1α and FGF1 in NIH 3T3 cells inhibits the stress-induced release of FGF1 (Tarantini et al., 2001). It appears that the release pathways used by FGF1 and IL-1α interact. Thus, pIL-1α could bind some important protein(s) shared by these pathways and sequester it in the nucleus. Indeed, FGF1 and IL-1α release pathways share several similarities, including the delayed character of export, which becomes detectable only after 90 minutes of stress (Jackson et al., 1992; Tarantini et al., 2001). This delay presumably reflects the need for stress-induced synthesis of proteins that participate in the export of IL-1α and FGF1 because both IL-1α release and FGF1 release are sensitive to inhibition of transcription and translation (Jackson et al., 1992; Tarantini et al., 2001).

FGF2 and IL-1β also exhibit non-classical release. However, their export mechanisms appear to be quite different. Unlike FGF1 and IL-1α, FGF2 is exported constitutively (Florkiewicz et al., 1995; Mignatti et al., 1992). The release of FGF2 is highly sensitive to the inhibitors of Na^+/K^+ ATPase (Dahl et al., 2000; Florkiewicz et al., 1998), whereas the export of FGF1 is refractory to these compounds (F.T., I.P. and T.M., unpublished).

The release of IL-1β is induced by lipopolysaccharides (Andrei et al., 1999; Rubartelli et al., 1990) but not by heat shock (A.M. and T.M., unpublished). In addition, unlike the export of FGF1 and IL-1α (Jackson et al., 1992) (F.T., I.P. and T.M., unpublished), IL-1β release is sensitive to methylamine (Rubartelli et al., 1990), an inhibitor of exocytosis, and to sulfonylurea glybenclamide, an inhibitor of the mammalian ATP-binding cassette (ABC) translocator ABC1 (Andrei et al., 1999). Another notable difference is that, unlike FGF1 and IL-1α, which are distributed homogeneously in the cytoplasm (Prudovsky et al., 2002), IL-1β is contained within cytoplasmic vesicles expressing lysosomal but not ER-Golgi markers (Andrei et al., 1999). Export of IL-1β thus appears to be based on its intracellular translocation into lysosome-like vesicles and the subsequent exocytotic fusion of these vesicles with the cell membrane (Andrei et al., 1999). It will be interesting to determine whether the constitutive release of FGF2 is also sensitive to these pharmacological agents. Thus, in spite of the very similar 3D structures of FGF1 and IL-1 prototypes, FGF2 and IL-1β appear to be secreted through pathways different from IL-1α and FGF1.

The stress-mediated intracellular transport of FGF1 and IL-1α

The inhibition of FGF1 and IL-1α release by 2-deoxyglucose (Jackson et al., 1992; Tarantini et al., 2001) demonstrates that these pathways are dependent on ATP. In addition, an intact actin cytoskeleton is important, since release of FGF1 and IL-1α is sensitive to agents that attenuate actin stress fibers, such as latrunculin and amlexanox (Landriscina et al., 2000; Mouta Carreira et al., 1998; Tarantini et al., 2001). By contrast, microtubule inhibitors, such as nocodazole, fail to inhibit FGF1 release (F.T., I.P. and T.M., unpublished). Real-time confocal studies of cells transfected with an FGF1-GFP chimera have demonstrated stress-induced migration of cytosolic FGF1 to the cell membrane 60 minutes after heat shock and this translocation can be completely inhibited by amlexanox (Prudovsky et al., 2002). Likewise, heat shock also induces translocation of an IL-1α-RFP chimera from the cytosol to the cell membrane (Mandinova et al., 2003). Although the actin cytoskeleton transports different types of cytoplasmic membrane vesicle (Rogers and Gelfand, 2000), at least at the level of fluorescence microscopy, neither FGF1 nor IL-1α appears to be present in vesicular structures under normal conditions or during heat shock (Prudovsky et al., 2002).

The stress-induced formation of multiprotein FGF1 and IL-1α release complexes

FGF1 is released during stress as a covalent cysteine-linked

homodimer (Jackson et al., 1992). The evolutionarily conserved Cys30 residue is crucial for its stress-mediated release (Tarantini et al., 1995). Interestingly, the FGF1 homodimer exhibits a low heparin affinity (compared with the monomer), as well as low mitogenic activity in vitro (Engleka and Maciag, 1992). Dimer formation might therefore be a way of storing and possibly transporting FGF1 in an inactive form. However, low heparin affinity makes dimeric FGF1 potentially more susceptible to proteolysis, since heparin and HSPGs protect FGF family members from proteases (Friesel and Maciag, 1999; Rosengart et al., 1988). The balance between the monomeric and dimeric forms of FGF1 in the extracellular compartment might be regulated by stress-induced extracellular reducing and oxidizing agents. For example, the reducing agent thioredoxin is a signal-peptide-less protein released through a non-classical pathway (Rubartelli et al., 1992; Rubartelli et al., 1995), and Cu^{2+} ions are potential extracellular oxidizing agents that could convert reduced monomeric FGF1 to the dimeric form. Indeed, in a cell-free system, Cu^{2+} efficiently induces FGF1 dimerization (Engleka and Maciag, 1992). However, the role of intracellular Cu^{2+} in the release of FGF1 and IL-1α is even more significant (see below).

IL-1α release does not appear to depend on covalent dimerization. mIL-1α is exported as a monomeric, biologically active cytokine (Mandinova et al., 2003; Tarantini et al., 2001). Moreover, it has no evolutionarily conserved equivalent of Cys30 in FGF1 (Furutani et al., 1986; Lomedico et al., 1984), and a cysteine-free IL-1α mutant is released normally in response to cellular stress (A.M., I.P. and T.M., unpublished).

Both mIL-1α and FGF1 are exported as components of multiprotein release complexes that, at least in the case of FGF1, assemble near the inner surface of the plasma membrane (Prudovsky et al., 2002). The first evidence for such complexes resulted from HPLC analysis of high-molecular-weight FGF1-containing fractions from bovine and ovine brains (Maciag et al., 1982; Mouta Carreira et al., 1998). Brain-derived FGF1 is associated with at least four other polypeptides, which include S100A13 and the p40 form of synaptotagmin 1 (Syt1) (Burgess et al., 1985; Mouta Carreira et al., 1998). S100A13 belongs to the S100 family of polypeptides, which are small acidic proteins that have two Ca^{2+}-binding EF-hand domains (Heizmann et al., 2002). The biological functions of most S100s are not defined but, significantly, the intracellular distributions and/or expression levels of some family members are modified in response to cellular stress (Breen et al., 1999; Du et al., 2002; Duarte et al., 1999; Hoyaux et al., 2000; Hsieh et al., 2002; Kucharczak et al., 2001; Lam et al., 2001; Mandinova et al., 1998; Migheli et al., 1999; Zhang et al., 2002). A specific structural characteristic of S100A13 is the presence of a C-terminal domain rich in basic residues (Wicki et al., 1996). The expression of S100A13 in NIH 3T3 cells is detectable by RT-PCR analysis (Landriscina et al., 2001a).

All S100 proteins lack classical signal sequences, but at least some of them are released into the extracellular compartment (Heizmann and Cox, 1998). S100A13 transfected into NIH 3T3 cells is constitutively released (Landriscina et al., 2001a); however, when it is co-expressed with either FGF1 or mIL-1α, its release becomes stress-dependent (Landriscina et al., 2001a; Mandinova et al., 2003). This observation in conjunction with experiments using a dominant-negative S100A13 deletion mutant lacking the basic C-terminal domain demonstrated that S100A13 is an indispensable part of the multiprotein FGF1 release complex (Landriscina et al., 2001a). Similar experiments provided evidence that S100A13 expression is also critical for IL-1α release (Mandinova et al., 2003). Interestingly, although a cysteine-free FGF1 mutant is not released in response to stress (Tarantini et al., 1995), its co-expression with S100A13 results in the stress-induced export of both proteins (Landriscina et al., 2001a). It appears that overexpression of S100A13 induces the non-covalent dimerization of cysteine-free FGF1.

The p40 Syt1 component of the brain-derived FGF1-containing multiprotein complex represents the extravesicular portion of the transmembrane p65 Syt1 protein. Syt1 participates in the docking of a variety of secretory vesicles, including synaptic vesicles, at the cell membrane prior to their subsequent exocytosis (Sudhoff and Rizo, 1996). Similarly to other members of the synaptotagmin protein family, Syt1 displays two Ca^{2+}-binding C2 domains in its extravesicular portion (Marqueze et al., 2000). p40 Syt1 is believed to be produced by proteolytic cleavage of p65 near its transmembrane domain (Marqueze et al., 2000; Sudhoff and Rizo, 1996). In contrast to p65 Syt1, which displays a classical N-terminal signal peptide in its primary structure and localizes primarily to the ER-Golgi apparatus, cytoplasmic vesicles and cell membrane, signal-peptide-less p40 Syt1 displays a diffuse cytosolic distribution (C.B., I.P. and T.M., unpublished). Interestingly, like S100A13, p40 Syt1 is also constitutively released from cells under normal cell culture conditions (LaVallee et al., 1998). Experiments using either an antisense strategy or the expression of a dominant-negative p65 Syt1 mutant, as well as immunoblot analysis of the exported FGF1 complex at non-reducing low denaturation conditions for electrophoresis, demonstrated that, similarly to S100A13, p40 Syt1 is a crucial component of the FGF1 release complex (LaVallee et al., 1998; Tarantini et al., 1998).

Surprisingly, unlike S100A13, p40 Syt1 is dispensable for IL-1α release (Tarantini et al., 2001). However, it is conceivable that IL-1α uses another member of the synaptotagmin family or other C2-domain-containing polypeptides, for example calpain, the intracellular protease responsible for pIL-1α cleavage, which contains a C2 domain (Goll et al., 2003) and associates with annexin II (Barnes and Gomes, 2002). Interestingly, although expression of FGF1 in the presence of S100A13 inhibits the constitutive release of S100A13, it does not affect release of p40 Syt1 (LaVallee et al., 1998).

Annexin II might also be a part of the FGF1 and IL-1α release complexes. This protein exhibits inducible flipping from the inner to the outer surface of the cell membrane (Peterson, 2003), where it functions as a receptor for plasminogen and plasminogen activators (Hajjar et al., 1994; Hajjar et al., 1998). Studies using amlexanox affinity chromatography were able to resolve annexin II in a non-covalent complex with S100A13 (Oyama et al., 1997), and we have recently demonstrated the presence of annexin II in the brain-derived FGF1-containing multiprotein complex (R.S., I.P. and T.M., unpublished). Since annexin II forms heterotetramers with S100A10 (p11) (Kim and Hajjar, 2002), its participation in the multiprotein complexes might rely upon interactions with S100A13. Furthermore, since annexin II

associates with the inner surface of the plasma membrane (Goll et al., 2003) and the assembly of the FGF1 multiprotein complex also occurs near the inner surface of the plasma membrane (Prudovsky et al., 2002), it is possible that annexin II serves as the site of assembly for the non-classical export of these multiprotein complexes. However, more experiments are needed to verify its role in FGF1 and IL-1α release.

The role of Cu^{2+} in FGF1 and IL-1α export

How do the FGF1 and IL-1α release complexes assemble? Association of the members of these multiprotein aggregates might involve Cu^{2+}. FGF1, IL-1α, S100A13 and p40 Syt1 are Cu^{2+}-binding proteins (Engleka and Maciag, 1992; Landriscina et al., 2001b; Mandinova et al., 2003). Also, Cu^{2+} specifically induces formation of FGF1 but not FGF2 homodimers even though two of the three Cys residues present in FGF1 are conserved in FGF2 (Engleka and Maciag, 1992). In addition, several studies have demonstrated angiogenic and pro-inflammatory effects of Cu^{2+} (Brewer, 2001; Gullino, 1983; Hannan and McAuslan, 1982; Raju et al., 1982; Zoli et al., 1998), indicating that Cu^{2+} might participate in the non-classical release of angiogenic and pro-inflammatory polypeptides. The role of Cu^{2+} in mediating the release of FGF1 and IL-1α export has been examined in some detail, and indeed we and others have demonstrated in a cell-free system that Cu^{2+} is able to induce the formation of a complex containing p40Syt1, FGF1 and S100A13 at a molar ratio of 1:2:2, respectively, as well as the formation of a heterotetrameric (2:2) IL-1α-S100A13 complex (Landriscina et al., 2001b; Mandinova et al., 1998). The depletion of intracellular free Cu^{2+} through continuous application of a specific chelator, tetrathiomolybdate (TTM), can attenuate the stress-induced release of IL-1α and FGF1, as well as of S100A13 when co-expressed with IL-1α or FGF1 (Landriscina et al., 2001b; Mandinova et al., 2003). These data indicate that the stress-induced Cu^{2+}-dependent assembly of IL-1α and FGF1 multiprotein release complexes is indeed a prerequisite for the non-classical export of these proteins in vitro (Fig. 2).

The plasma membrane as a platform for the assembly of release complexes

Considerable experimental evidence indicates that the Cu^{2+}-dependent formation of IL-1α and FGF1 multiprotein release complexes occurs at the inner leaflet of the cell membrane. Indeed, TTM treatment does not prevent the stress-induced migration of FGF1 to the cell membrane (Prudovsky et al., 2002), and thus complex formation (including formation of the FGF1 homodimer) does not appear to be important for the intracellular transport of FGF1 to the cell periphery. Moreover, dominant-negative mutants of S100A13 and p40 Syt1 that are known to inhibit FGF1 release are transported to the cell membrane in response to heat shock, and they do not prevent the stress-induced translocation of FGF1 to the periphery (Prudovsky et al., 2002). Apparently, the members of the FGF1 multiprotein complex follow independent stress-induced pathways to the cell periphery. Interestingly, FGF1, IL-1α, p40 Syt1 and members of the S100 family can all bind acidic phospholipids in a cell-free system (Heizmann et al., 1998; Marqueze et al., 2000; Mandinova et al., 2003; Tarantini et al., 1995). Furthermore, mutational analyses have revealed specific acidic phospholipid-binding domains in FGF1 (Tarantini et al., 1995) and Syt1 (Fernandez et al., 2001). Interestingly, a few of these acidic phospholipids are asymmetrically distributed between the leaflets of the plasma membrane (Pomorski et al., 2001) and thus, under normal conditions, acidic phospholipids such as phosphatidylserine localize preferentially to the inner leaflet. However, in response to a variety of different stresses, including heat shock, phosphatidylserine flips to the outer leaflet (Sims and Wiedmer, 2001).

Phosphatidylserine could drive the transmembrane translocation of the IL-1α and FGF1 release complexes since immunofluorescence data suggest that the inner side of the cell membrane is a platform for the assembly of IL-1α and FGF1 release complexes after the participant proteins reach the membrane through heat-shock-induced, actin-dependent transport. Cu^{2+} ions needed for the assembly of release complexes might be provided by transmembrane Cu^{2+} transporters (Finney and O'Halloran, 2003), and it is noteworthy that the recently characterized human Cu^{2+} transporter 1 (hCtr1) is activated by cellular stress (Lee et al., 2002). Since free Cu^{2+} is virtually absent from the cytosol (Rae et al., 1999), the inner leaflet of the cell membrane is the most likely locale for the function of transient Cu^{2+} ions in the assembly of the FGF1 and IL-1α multiprotein complexes. However, it is unclear whether the Cu^{2+} ions involved in the formation of these complexes are released into the extracellular compartment with the exported polypeptides or whether they are recycled back to their intracellular transporters.

Potential role of detergent-like properties and the molten globule state of proteins in facilitating non-classical protein export

The key moment in non-classical export is translocation across the cell membrane. The mechanism might involve local destabilization of the phospholipid bilayer at the inner surface of the plasma membrane, which would allow the protein to insert into the membrane and eventually exit the cell. Several proteins including bactericidal peptides (Wiese et al., 2003) and viral fusion proteins (Dutch et al., 2000) have detergent-like properties that destabilize and permeabilize phospholipid bilayers. It has been observed that FGF1 has similar properties, demonstrating that it can induce temperature-dependent permeabilization of phosphatidylserine/phosphatidylglycerol liposomes (Mach and Middaugh, 1995). IL-1α also behaves similarly (Oku et al., 1995; Mandinova et al., 2003). The phospholipid-binding and detergent-like activities of these proteins indicate that the inner leaflet of the cell membrane could contain sites that recognize the multiprotein complexes destined for release. These sites could contain specific acidic phospholipid 'signatures' that determine both the composition of assembled protein aggregates and the export mechanism.

Translocation of a protein across the bilayer might require conformational changes that increase its hydrophobicity. Proteins can achieve this by adopting a 'molten globule' conformation (Ptitsyn, 1995). This is a partially unfolded intermediate conformation assumed during denaturation and renaturation (Arai and Kuwajima, 2000; Ptitsyn, 1995). It is characterized by (1) the presence of secondary structure, (2)

the absence of most of the tertiary structure normally produced by tight packing of side chains, (3) a relative compactness (a radius of gyration only 10-30% larger than that of the native state), and (4) the presence of a loosely packed hydrophobic core that increases the hydrophobic surface accessible to solvent (Arai and Kuwajima, 2000). The fourth characteristic could allow proteins to traverse lipid bilayers (Bychkova et al., 1988), and it has been reported that FGF1 exhibits a temperature-dependent molten globule conformation (Sanz et al., 2002). Additional studies using two-dimensional nuclear magnetic resonance have confirmed this and underlined the importance of an all-β-barrel structure for formation of the molten globule (Srisailam et al., 2002). This structural feature might therefore enable FGF1, IL-1α and the other polypeptide components of the release complex to lose their solubility in an aqueous environment and simultaneously become soluble in a non-aqueous lipophilic environment, which is a prerequisite for their transport through the plasma membrane. It is interesting to note that the β-barrel structure may be responsible for the ability of FGF1 to form amyloid-like fibrils (Srisailam et al., 2003). However, whether this feature contributes to the Cu^{2+}-induced assembly of the FGF1 (Landriscina et al., 2001b) and IL-1α (Mandinova et al., 2003) high-molecular-weight complexes formed prior to export is not known. Interaction with acidic phospholipids might also significantly contribute to the transition of FGF1 and possibly IL-1α to a molten globule conformation. The importance of unfolded or partially unfolded protein conformations is

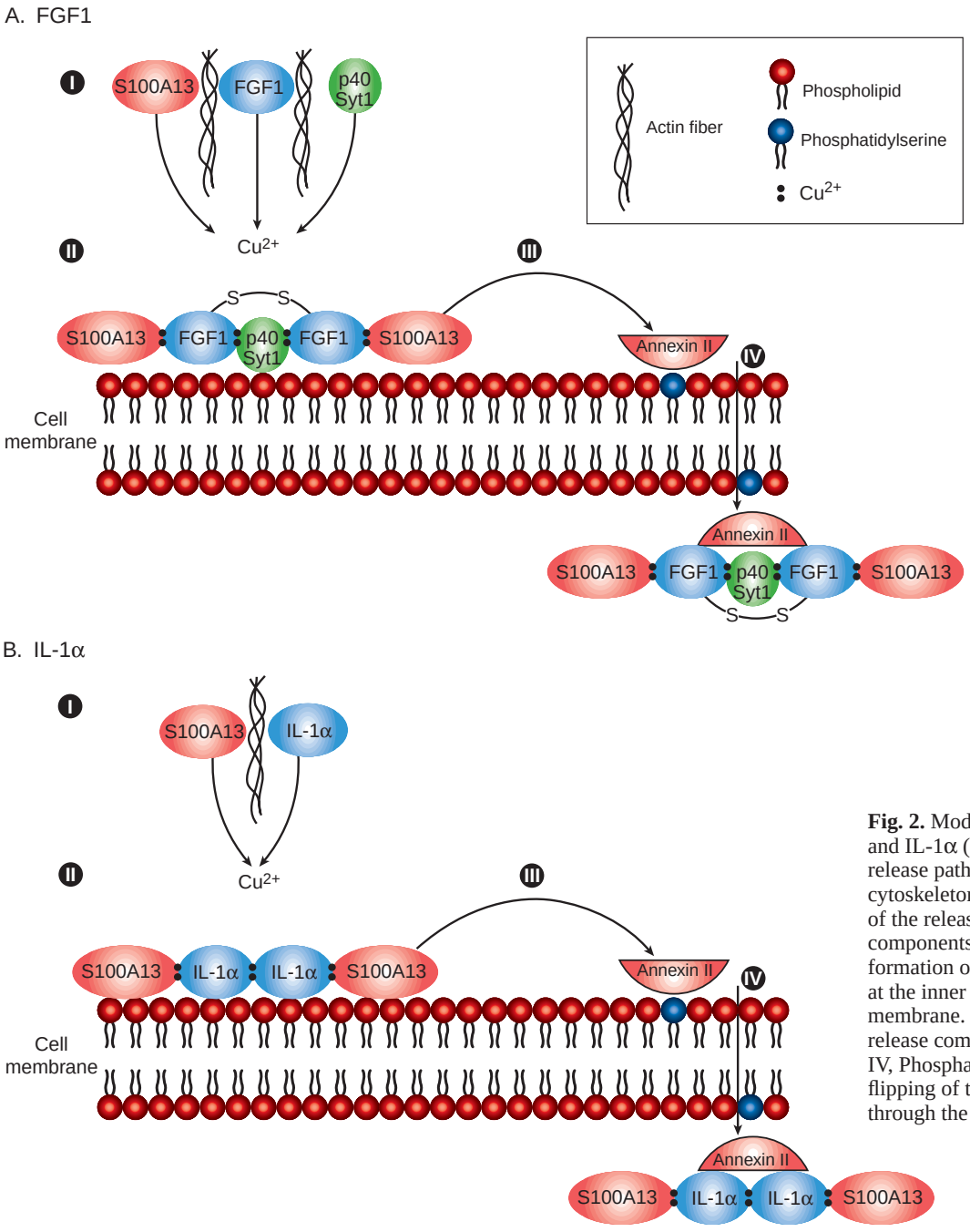

Fig. 2. Models of FGF1 (above) and IL-1α (below) stress-induced release pathways. I, Actin cytoskeleton-dependent transport of the release complex components. II, Cu^{2+}-dependent formation of the release complex at the inner leaflet of the cell membrane. III, Association of the release complex with annexin II. IV, Phosphatidylserine-dependent flipping of the release complex through the cell membrane.

stressed by results demonstrating that, upon overexpression of rhodanese and GFP, excess unfolded proteins are evacuated from the cells through non-classical export pathways (Sloan et al., 1994; Tanudji et al., 2002).

Whereas there is no direct biophysical evidence for the ability of either S100A13 or p40 Syt1 to assume the molten globule conformation, both proteins are known to be constitutively released independently of FGF1 or IL-1α (Landriscina et al., 2001a; LaVallee et al., 1998) and interact with acidic phospholipids. They could therefore play a role as chaperones that stabilize FGF1 and possibly IL-1α in a molten globule conformation. Indeed, observing the interaction between a bacterial pilin and its chaperone, Knight and co-authors (Zavialov et al., 2003) have recently demonstrated that chaperones can maintain polypeptides in a partially folded, high-energy state. Previously, cytosolic chaperones, such as members of the Hsp70 family, had been shown to maintain the mitochondrial pre-proteins in a translocation-competent conformation, which is crucial for their post-translational import into mitochondria (Gordon et al., 2000). It is possible that S100A13 performs a chaperone-like service needed for membrane translocation since its overexpression alleviates the requirements of IL-1α and FGF1 export for new transcription and translation (Landriscina et al., 2001b; Mandinova et al., 2003). Indeed, some other members of the S100 family have chaperone activity (Heizmann et al., 2002), and S100A10, also known as p11, serves as a chaperone for the hepatitis virus B polymerase and is needed for its nuclear translocation (Choi et al., 2003). Also, acting as a chaperone, it appears to be crucial for insertion of annexin II into the plasma membrane, as well as its thrombin-induced flipping to the outer surface of the plasma membrane (Peterson et al., 2003).

The pathological significance of non-classical FGF1 and IL-1α export

The elucidation of the mechanisms responsible for the non-classical export of FGF1 and IL-1α has required the use of in vitro methods of analysis and, as a result, it has been difficult to determine the role of these mechanisms in vivo and/or in pathological processes. However, clinical studies pioneered by G. Brewer and S. Merajver (Brewer et al., 2000; Cox et al., 2001) in which the Cu^{2+} chelator TTM was used to manage the progress of stage IV tumors in humans have provided insight into the potential role of TTM as an angiogenic inhibitor capable of attenuating mammary gland tumor formation in the Her transgenic mouse (Pan et al., 2002). Since these studies suggested that TTM can repress the transcriptional activation of NF-κB, and NF-κB lies downstream of IL-1 receptor signaling (Baldwin, 1996), it appeared possible that TTM functions as a repressor of non-classical IL-1α export. Both FGF1 and IL-1 play a pro-angiogenic role in vivo (Friesel and Maciag, 1999; Voronov et al., 2003) although, in vitro, FGF1 stimulates proliferation and migration of endothelial cells (Maciag et al., 1979; McMahon et al., 1997), whereas IL-1α inhibits both of these activities (Maier et al., 1990). It appears that the regulation of angiogenesis and inflammation involves a coordination of non-classical FGF1 and IL-1α release. Indeed, IL-1α stimulates the infiltration of tissues with macrophages (Dinarello, 1996), which present an abundant source of the FGF prototypes (Sano et. al., 1990; Brogi et al., 1993). The absence of extracellular IL-1α in a tumor setting would limit the recruitment of FGF1-laden mononuclear cells (Sano et al., 1990; Sano et al., 1992) to tumor sites exhibiting an anoxic and/or hypoxic microenvironment. Thus, in the absence of mononuclear cell infiltration, FGF1 would not be delivered to the tumor environment and, even if FGF1 was available within the tumor microvasculature itself, TTM would also repress its export.

Interestingly, a similar mechanism has been proposed to explain the response to injury in large vessels as a result of catheter-mediated clinical management of atherosclerotic arteries (Mandinov et al., 2003). Since the infiltration of mononuclear cells into the injured area in response to the release of IL-1α could result in the generation of an FGF1-rich microenvironment, and FGF1 is a potent mitogen for the vascular smooth muscle cells (Winkles et al., 1987), its export into the extracellular compartment could be responsible for the onset of restenosis. Indeed, the long-term administration of TTM significantly suppresses restenosis induced by catheter injury in the rat carotid artery (Mandinov et al., 2003). The arterial walls of TTM-treated rats display a strong attenuation of neointimal growth, impaired vasa vasorum formation, little, if any, macrophage/monocyte infiltration and, most importantly, very low levels of FGF1 and IL-1α expression when compared with injured arteries from control animals. Thus, the inhibition of restenosis by TTM could be due to the ability of the Cu^{2+} chelator to repress the stress-induced release of pro-inflammatory IL-1α, which would prevent infiltration of mononuclear cells known to be a source of pro-angiogenic and pro-restenotic FGF1 in the wall of the damaged vessel. These data also suggest that the repression of non-classical FGF1 and IL-1α export by Cu^{2+} chelation might ultimately be useful for the clinical management of pro-inflammatory angiogenesis in humans.

These data corroborate the preclinical and clinical reports on the ability of TTM to inhibit solid tumor growth (Brewer et al., 2000; Cox et al., 2001), which depends on the availability of pro-angiogenic polypeptides (Folkman, 2002). Thus, the potential significance of the role of Cu^{2+} as a mediator of the non-classical export of FGF1 and IL-1α could provide an alternative approach for the clinical management of other pathological conditions dependent on pro-inflammatory angiogenesis, such as rheumatoid arthritis (Maini and Taylor, 2000). Indeed, studies have demonstrated that Zn^{2+}/Cu^{2+} chelation can repress the onset of Alzheimer's disease in the β-amyloid transgenic mouse (Cherny et al., 2001). Because the β-amyloid gene is known to be regulated by IL-1α in human endothelial cells (Goldgaber et al., 1989), it is likely that the repression of Alzheimer's disease is due, at least in part, to the absence of extracellular IL-1α. The recent report (Voronov et al., 2003) that IL-1α- and IL-1β-null mice cannot sustain an active angiogenic environment to support tumor growth is consistent with the premise that the function of these signal-peptide-less polypeptides is crucial for the regulation of pro-inflammatory angiogenic responses in vivo.

Future directions

Several important questions related to non-classical polypeptide release remain to be answered. Are there other alternative pathways for non-classical export of FGF1 and IL-

1α and, if so, how are they regulated? Is the molten globule a common feature of such mechanisms? What are the transporter molecules responsible for actin-dependent translocation of the proteins from the cytosol to the cell membranes? How is this translocation induced by cellular stress? Which phospholipids or which groups of phospholipids interact with specific protein members of the release complexes? Do phospholipid signatures permanently exist in the inner leaflet of the cell membrane or are they arranged in response to cellular stress? How does the Cu^{2+}- and phospholipid-dependent formation of multiprotein release complexes induce their subsequent translocation across the cell membrane? What is the source of energy used for stress-induced transmembrane translocation? Solving these problems will result in a better understanding of the non-classical protein release and eventually in an improved ability to regulate both inflammation and angiogenesis.

We thank Norma Albrecht for expert administrative assistance. This work was supported in part by NIH grants RR15555, HL35627 and HL32348 to T.M.

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Paper 2

"The intracellular domain of Notch ligand Delta 1 induces cell growth arrest"

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FEBS Letters 579: 5798-5802, 2005

FEBS Letters 579 (2005) 5798–5802 FEBS 30035

The intracellular domain of Notch ligand Delta1 induces cell growth arrest

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Received 31 August 2005; accepted 17 September 2005

Available online 4 October 2005

Edited by Ned Mantei

The article is dedicated to the memory of Tom Maciag, scientist, friend, and mentor.

Abstract Notch signaling involves proteolytic cleavage of the transmembrane Notch receptor after binding to its transmembrane ligands, Delta or Jagged; and the resultant soluble intracellular domain of Notch stimulates a cascade of transcriptional events. The Delta1 ligand also undergoes proteolytic cleavage upon Notch binding, resulting in the production of a free intracellular domain. We demonstrate that the expression of the intracellular domain of Delta1 results in a non-proliferating senescent-like cell phenotype which is dependent on the expression of the cell cycle inhibitor, p21, and is abolished by co-expression of constitutively active Notch1. These data suggest a new intracellular role for Delta1.

Keywords: Notch; Delta intracellular domain; p21; DNA synthesis; Senescence

1. Introduction

The Notch signaling pathway plays a critical role in cell fate determination at all stages of organism development [1]. The current model of the Notch signaling pathway suggests that the Notch transmembrane receptor molecule is activated via direct interaction with transmembrane ligands expressed on the surface of neighboring cells. This interaction results in consecutive cleavages of Notch by an ADAM metalloprotease and by a presenilin-dependent γ-secretase. The generated Notch intracellular domain (icd) translocates into the nucleus where it interacts with the transcription factors of the CSL family or activates CSL-independent signaling pathway(s) (for review see [2]).

We have demonstrated that the expression of the soluble extracellular domain of Delta1 (sDl1) enhances cell proliferation, and induces the non-classical release of FGF1 [3]. Recently Notch-dependent proteolytic cleavage was reported for *Drosophila* and mammalian Notch ligands of the Delta family [4–6]. Upon interaction with Notch, Delta is cleaved by an ADAM metalloprotease and presenilin/γ-secretase that release Delta icd from the plasma membrane. Immunohistochemistry experiments demonstrate that *Drosophila* Delta icd is able to enter the nucleus [5]. Interestingly, the icd of mammalian Delta1 (Dl1icd) contains a PDZ-binding site [7,8].

The ability of Delta to undergo proteolytic cleavage of its icd suggests a bidirectional character of Notch signaling. In the present study, we investigated the biological effects of Dl1icd expression in cell cultures. We demonstrate that Dl1icd induced p21-dependent blockage of DNA synthesis and cell proliferation arrest. Interestingly, constitutively active Notch1 (N1icd) was able to reverse Dl1icd1- induced phenotype.

2. Materials and methods

2.1. Cell cultures

Human umbilical vein endothelial cells (HUVEC) (ATCC) at passages 7–12 were grown in EBM medium supplemented with EGM-2 growth factor cocktail (Cambrex). NIH 3T3 murine fibroblasts (ATCC), HEK293 cells (ATCC), p21−/−, p27−/− and wt mouse embryo fibroblasts (MEF) (gift of Dr. C. Sherr, St. Jude Children's Research Hospital in Memphis, Tennessee) were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (Hyclone).

2.2. DNA constructs, transfection, preparation of adenoviruses and adenoviral transduction

To study the biological role of human Dl1icd, nucleotide sequence coding for amino acids 569–723 was cloned in pcDNA 3.1-Zeo vector (Invitrogen) in restriction sites *Xba*I and *Hin*dIII. Additionally,

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Abbreviations: CSL family, CBF, SuH, Lag-1; cdk, cyclin-dependent kinase; flDl1, full length Delta 1; HUVEC, human umbilical vein endothelial cells; Dl1icd, intracellular domain of Delta1; MEF, mouse embryo fibroblast; N1icd, Notch1 intracellular domain; NLS, nuclear localization sequence; sDl1, soluble Delta 1

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doi:10.1016/j.febslet.2005.09.042

the V5 tag was introduced in the N-terminus of Dl1icd. NIH 3T3 cells were transfected using FuGene (Roche) transfection reagent according to the manufacturer's instructions. Selection of stably transfected NIH 3T3 cells was described earlier [9]. Dl1icd was also cloned in the multiple cloning site of the pAdlox shuttle vector (Invitrogen). The corresponding adenoviruses were prepared as described [10], and used to transduce HUVEC and MEF. In a series of experiments, an adenoviral construct expressing human N1icd [10] was used to transduce HUVEC 16–24 h before Dl1icd transduction. The control LacZ adenoviral construct was described earlier [10]. Alternatively, the control pcDNA3.1(−) Myc-His/LacZ construct (Invitrogen) was used for transient transfection. Full length human Delta 1 (flDl1) used for transient transfection was cloned into the EcoR1 and Bam H1 sites of the plasmid pcDNA3.1A (−) Myc-His (Invitrogen) [11]. The efficiency of Dl1icd and N1icd transduction was controlled using immunofluorescence anti-V5 staining, and it was always above 90%.

2.3. Site-directed mutagenesis

To mutate the nuclear localization sequences (NLS) of Dl1icd, we used a PCR-based strategy. Mutations were introduced with following primers: Dl1icd-nls1 – (s) cagaagcacgccccagccgacccctg and ggacggctggggcgtgcttctgcagcc (as); Dl1icd-nls2 – (s) gaagcatctgaacaaaggccggactcgggctgttc and (as) cagccgagtccggcctttgttcagatgcttctccaccc, using a Stratagene site-directed mutagenesis kit following the manufacturer's instructions. PDZ-binding site deletion mutant was generated by introducing a stop codon at amino acid 720 by using the following primers: (s) gatgagtgcgtctgagcaactgaggtftaa and (as) cacctcagttgctcagacgcactcatccttctc.

2.4. Immunoblot analysis

Lysates of LacZ- and Dllicd-transduced HUVEC were prepared, resolved by 12% or 15% SDS–PAGE and immunoblotted as described previously [12] using either a mouse anti-p21 (BD Biosciences), mouse anti-p27 (BD Biosciences), rabbit anti-cyclin A (Santa Cruz), rabbit anti-cyclin E (BD Biosciences), rabbit anti-cyclin D1 (Santa Cruz), rabbit anti-β-actin (Sigma) or rabbit anti-pErk1/2 antibodies (Cell Signaling).

2.5. Immunofluorescence confocal microscopy

Cells growing on glass coverslips were fixed 24 h after Dl1icd of flDl1 transfection with 4% (w/v) paraformaldehyde. Anti-V5 (Invitrogen) or anti-Myc (Covance) antibodies followed by FITC-conjugated secondary antibody were used to visualize, respectively, Dl1icd or flDl1. TO-PRO3 (Molecular Probes) was used to stain DNA as described previously [13]. Immunofluorescently stained cells were analyzed using a TC-SP confocal microscope (Leica).

2.6. DNA synthesis assay

[^{3}H]-Thymidine autoradiography was used to evaluate the levels of DNA synthesis as described previously [13]. The percentage of ^{3}H-labeled nuclei was calculated using an inverted Olympus microscope. In experiments with transient transfection, transfected cells were identified by immunoperoxidase staining as described [14] using antibodies against V5 or against β-galactosidase.

2.7. Acidic β-galactosidase staining

Cells transduced with Dl1icd were washed in PBS, fixed for 5 min in 2% formaldehyde/0.2% glutaraldehyde, washed, and stained for acidic β-galactosidase as described [15].

2.8. Real time RT-PCR

Total RNA from LacZ- and Dl1icd-transduced HUVEC was isolated using RNAeasy (Qiagen) according to the manufacturer's protocol. cDNA was obtained from 5 μg of total RNA with SuperScript™ (Invitrogen) reverse transcriptase by using an oligo(dT) primer (Invitrogen). Real-time PCR was performed using the Icycler IQ Real-Time PCR (Bio-Rad) according to the manufacturer's recommendations. Amplification of the *gapdh* cDNA was used as the endogenous normalization standard. Each sample was amplified in triplicate. The following specific primers were used for RT-PCR analysis of p21: (s) gattagcagcggaacaagga, (as) caactactcccagccccata.

3. Results

To study the biological effects of Dl1icd in cell culture, we transfected NIH 3T3 cells for further selection of cells stably expressing Dl1icd. Surprisingly, unlike sDl1 transfectants [3], cells transfected with Dl1icd failed to form colonies. Instead, Dl1icd transfectants surviving selection assumed morphology reminiscent of senescent fibroblasts: large, well-spread cells with hypertrophic cytoplasm (Fig. 1A). Since clones of stable Dl1icd transfectants did not arise, we prepared an adenoviral construct for Dl1icd expression, which allowed us to efficiently express Dl1icd in non-immortalized cells, such as HUVEC. To assess the ability of Dl1icd to inhibit DNA synthesis in HUVEC, Dl1icd- and control LacZ-transduced HUVEC were labeled with [^{3}H]-thymidine 48 h after transduction for a period of 16 h. The expression of Dl1icd resulted in the dramatic inhibition of DNA synthesis (Fig. 1B). Similar results were obtained with NIH 3T3 (data not shown) or HEK293 cell transduced with Dl1icd (Fig. 1F).

To further evaluate the status of Dl1icd-transduced HUVEC, we assessed the expression of β-galactosidase active at pH 6, a common biomarker of senescent non-immortalized cells [15]. Dl1icd transduction induced the activity of acidic β-galactosidase in HUVEC after 2 days, and most of the cells were acidic β-galactosidase positive after 4 days (Fig. 1C). The growth of Dl1icd-transduced HUVEC stopped, and cells remained viable and non-proliferating for at least 2 months (data not shown). Since cell senescence in vitro is normally accompanied by the reduction of telomere length [16], we assessed this parameter by using the Telomere Length Assay kit (Roche). Interestingly, no significant difference in telomere length was observed between Dl1icd- and LacZ-transduced cells 4 days after transduction (data not shown).

Recent studies demonstrated nuclear localization of *Drosophila* Delta icd [5]. To evaluate the ability of mammalian Dl1icd to localize into the nucleus, we transiently transfected HEK293 cells with C-terminally Myc-tagged human flDl1 and N-terminally V5-tagged Dl1icd. Confocal microscopy analysis, using the anti-Myc antibody, demonstrated cytoplasmic distribution of flDl1 (Fig. 1E). Conversely, Dl1icd was found both in the nuclei and cytoplasm of transfected cells (Fig. 1E). Analysis of the amino acid sequence of Dl1icd reveals two potential NLS domains – ^{575}KHRPP^{579} and ^{689}RKRPP^{692} (Fig. 1D). To investigate the functionality of Dl1icd NLS and their importance for Dl1icd biological effect, we prepared a series of mutants: in Dl1icd-nls1, amino acids ^{575}KHRPP^{579} were mutated to KHAP; and in Dl1icd-nls2, amino acids ^{689}RKRPP^{692} were mutated to RQP. In Dl1icd-nlsDM (double mutant), both hypothetical NLS in Delta1 were mutated as described above. While both Dl1icd-nls1 and Dl1icd-nls2 exhibited nuclear and cytoplasmic localization similarly to wild type Dl1icd, Dl1icd-nlsDM was detected exclusively in the cytoplasm of transfected cells (Fig. 1E). The autoradiographic studies of DNA synthesis in transiently transfected HEK293 cells demonstrated that when one or both of the Delta's NLS were mutated, the percentage of labeled nuclei was similar to that in the cells transduced with wild type Dl1icd, i.e., 3 times lower than in cells transfected with LacZ (Fig. 1F). In a similar series of experiments, we assessed the role of the C-terminal PDZ-binding domain of Dl1icd in its antiproliferative effect. We produced a deletion mutant of

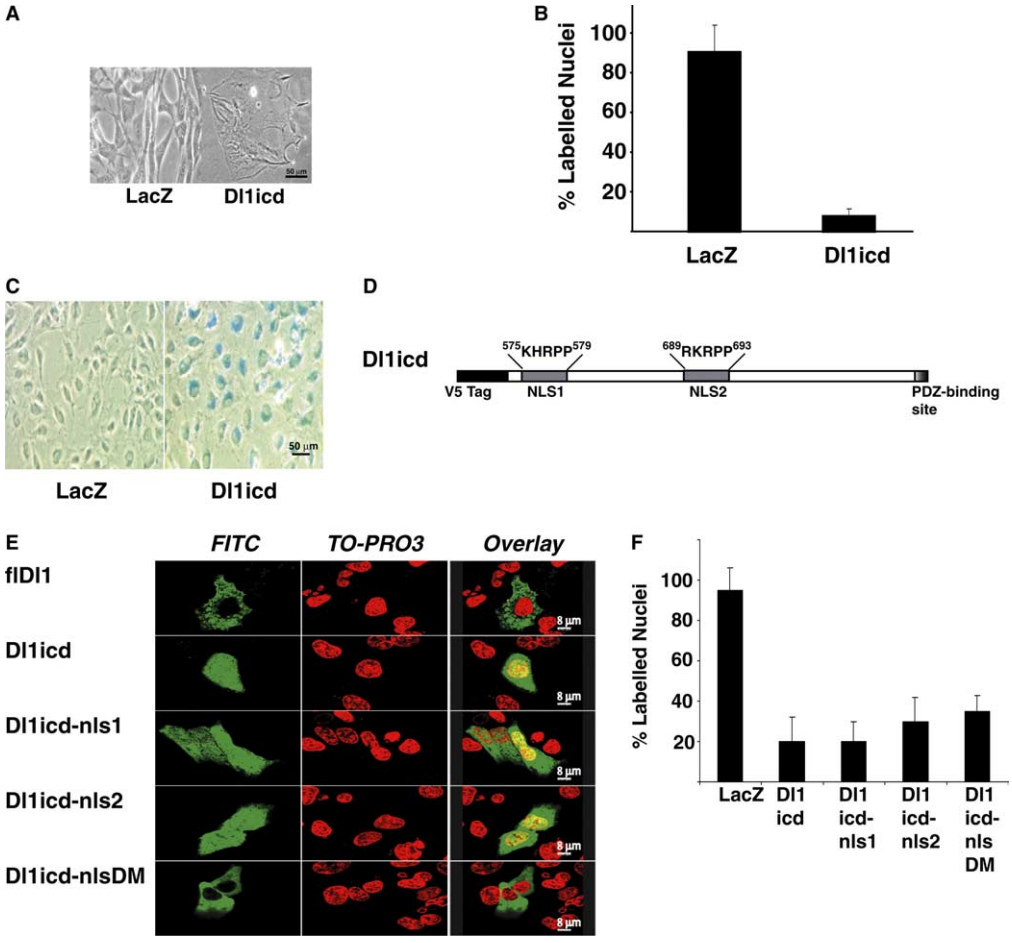

Fig. 1. Cells expressing Dl1icd adopt a senescent-like phenotype. (A) Cell morphology. Dl1icd-transfected NIH 3T3 cells 4 days after transfection and zeocin selection (phase contrast). (B) DNA synthesis. HUVEC were labeled for 16 h with 1 μCi/ml [^{3}H]-thymidine starting at 48 h after transduction with Dl1icd or LacZ. Bars represent average percentage of ^{3}H-labeled nuclei in LacZ- and Dl1icd-transduced HUVEC ± standard deviation (S.D.). (C) Acidic β-galactosidase expression. HUVEC adenovirally transduced with Dl1icd stained for acidic β-galactosidase 4 days after transduction. (D) Scheme of Dl1icd structure showing NLS and the PDZ-binding domain. (E) Nuclear localization of Dl1icd and its NLS mutants. HEK293 cells were transiently transfected either with flDl1, Dl1icd, or its corresponding NLS mutants as indicated. Cells were fixed, immunostained with anti-V5 antibody (Dl1icd and derived NLS mutants), or anti-Myc antibody (flDl1), and co-stained with TO-PRO3 48 h after transfection, and studied using confocal microscopy as described earlier [13]. (F) DNA synthesis. HEK293 were transiently transfected with LacZ, Dl1icd or Dl1icd-NLS mutants. DNA synthesis after transfection was determined using [^{3}H]-thymidine incorporation as described in Section 2. The average percentage of labeled nuclei ± S.D. is represented.

Dl1icd lacking the C-terminal PDZ-binding domain (^{721}TEV^{723}), and found that it induces the inhibition of DNA synthesis similarly to the wild type Dl1icd (data not shown). Thus, nuclear localization is not required for the anti-proliferative activity of Dl1icd, and PDZ-binding domain is dispensable for this effect. It can be hypothesized that PDZ-binding site is instead relevant to the interaction of transmembrane flDl1 with its cytoplasmic partners. The particular region(s) of Dl1icd required for the inhibition of cell proliferation remains to be elucidated.

Progression through the cell cycle is controlled by a group of cyclin-dependent kinases (cdks) and their inhibitory proteins [17]. Therefore, we assayed the expression of the cdk inhibitors, p21 and p27, in Dl1icd expressing cells. Western blot analysis revealed significant induction of p21 and p27 expression in HUVEC transduced with Dl1icd (Fig. 2A). At the same time, the expression of cyclins D1, A, and E as well as the levels of phosphorylated Erk 1 and Erk 2 in Dl1icd-transduced cells were not significantly changed (data not shown). We hypothesized that Dl1icd may induce growth arrest through upregulation of p21 or p27 expression or both. To assess this hypothesis, we utilized p21−/− and p27−/− MEF. Similarly to wild type (wt) MEF, Dl1icd transduction resulted in DNA replication blockage in p27 knockout MEF but the p21 knockout MEF were refractory to the inhibitory effect of Dl1icd (Fig. 2B). To further elucidate the stage of expression at which Dl1icd regulates p21 levels, we performed quantitative RT-PCR analysis and p21 promoter assay. We demonstrated that Dl1icd expression resulted in a strong increase of both p21 promoter activity in HEK293 cells (data not shown) and p21 mRNA levels in HUVEC (Fig. 2C).

Notch signaling determines the fate of many cell types through regulation of cell proliferation, differentiation, and apoptosis [1]. Since the expression of Notch1 overlaps the expression patterns of its ligands, Delta1 and Jagged1 [18–20], and since ligand-activated Notch cleavage results in the production of the soluble intracellular fragment of Notch, we sought to determine whether N1icd interferes with the biological effects of Dl1icd. To this end, we transduced HUVEC with N1icd-adenovirus 16 h prior to Dl1icd adenoviral transduction. Expression

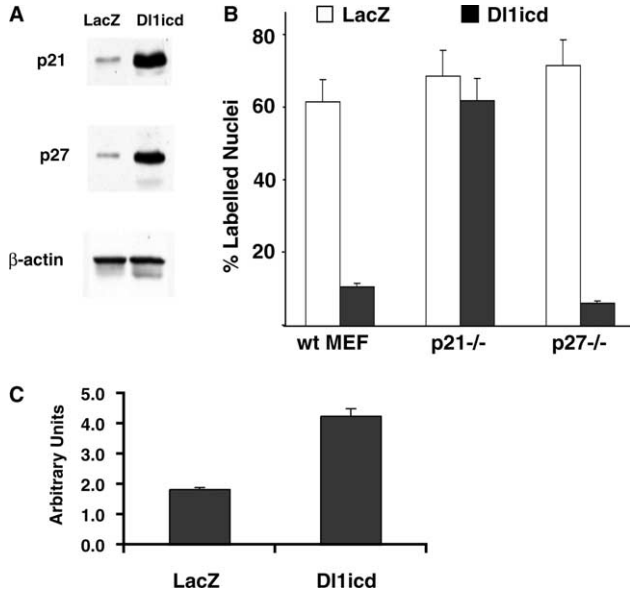

Fig. 2. Dl1icd-induced proliferation blockage is p21-dependent. (A) Dl1icd- and LacZ-transduced HUVEC were harvested 48 h after adenoviral transduction. Cell lysates were resolved by 15% SDS–PAGE and immunoblotted for cdk inhibitors, p21 and p27. Immunoblot for β-actin served as control of equal protein loading. (B) DNA synthesis in p21 and p27 knockout MEF expressing Dl1icd. p21−/−, p27−/−, and control wt MEF were labeled for 16 h with 1 μCi/ml [^{3}H]-thymidine starting at 48 h after transduction with Dl1icd. Bars represent average percentage of ^{3}H-labeled nuclei ± S.D. (C) Dl1icd induced expression of p21 mRNA in HUVEC. The expression of p21 was assessed by real time RT-PCR using the primers and conditions described in Section 2. The bars represent p21 mRNA levels normalized to *gapdh* mRNA levels ± S.D.

of N1icd abrogated the Dl1icd-induced senescence-like phenotype, as it was manifested by prevention of the expression of acidic β-galactosidase and of DNA synthesis blockage (Fig. 3B and A). Also, N1icd expression abrogated the induction of p21 expression by Dl1icd (Fig. 3C).

4. Discussion

We found that Dl1icd induced a p21-dependent inhibition of cell proliferation. Under the same experimental conditions the artificial expression of both LacZ and N1icd failed to inhibit DNA synthesis and, moreover, N1icd specifically abrogated the effect of Dl1icd, demonstrating that the antiproliferative activity of Dl1icd was not due to its overexpression. The irrelevance of nuclear localization of Dl1icd for its anti-proliferative effect indicated that Dl1icd does not participate directly in the activation of p21 transcription. This effect is most probably mediated through a cytoplasmic signaling pathway. Interestingly, HUVEC express Notch1, Delta1, and glycosyltransferase Lunatic Fringe (LFng) (data not shown). LFng potentiates the interaction between Notch1 and Delta1 [21]. The ability of HUVEC to proliferate may be maintained due to the simultaneous production of N1icd and Dl1icd, which may be a result of the efficient interaction of Notch 1 and Delta 1 promoted by LFng activity.

In the developing *Drosophila* wing, activation of Notch results in direct upregulation of cell proliferation without affecting cell fate determination [22]. In hematopoietic cells, the expression of N1icd results in delays of cell differentiation and diminishes the number of cells in the G_0/G_1 phase of the cell cycle, which also suggests induction of cell proliferation [23]. In order for proper Notch signaling to occur, there must be a distinction between a signaling cell versus a receiving cell. Based on the observations that Dl1icd induced non-proliferating phenotype, we suggest that its role in developing organisms is related to cell synchronization, tissue sculpting, and repair. In this scenario, at least three hypothetical situations may exist: (i) when a cell expressing Delta and Notch is surrounded by

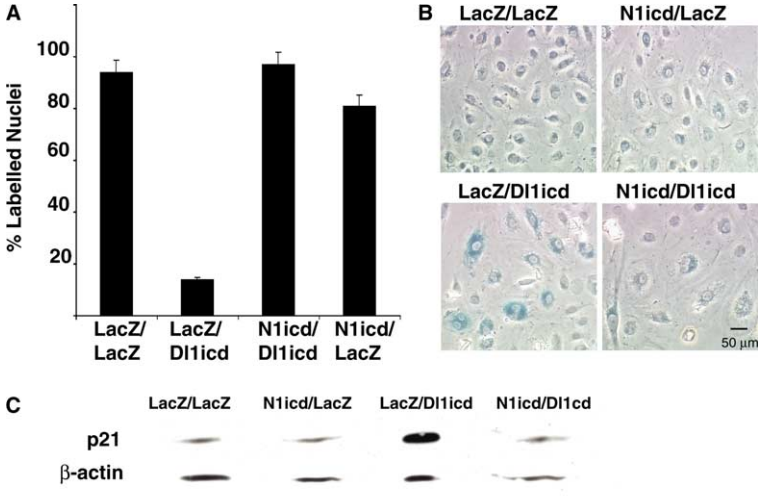

Fig. 3. N1icd expression prevents the effects of Dl1icd. (A) Dl1icd-induced inhibition of DNA synthesis. HUVEC were transduced with N1icd or LacZ, and 16 h later, the second transduction with Dl1icd or LacZ was performed. Cells were labeled for 16 h with [^{3}H]-thymidine 36 h after the second transduction [13]. The average percentages of [^{3}H]-thymidine-labeled nuclei ± S.D. are presented. (B) Dl1icd-induced acidic β-galactosidase activity. HUVEC were transduced with N1icd or LacZ; and 16 h later, cells were additionally transduced with either Dl1icd or LacZ. Cells were stained for acidic β-galactosidase 4 days after the second transduction. (C) p21 expression. HUVEC were transduced with N1icd or LacZ; and 16 h later, cells were additionally transduced with either Dl1icd or LacZ. 48 h later cell lysates were prepared, resolved by 15% SDS–PAGE, and immunoblotted for p21. Immunoblot for β-actin served as control of equal protein loading.

similar cells, the signals conducted through ligand and receptor are balanced and normal tissue homeostasis is maintained; (ii) when signaling through Notch is downregulated, e.g., by Numb [24], Delta signaling dominates over Notch signaling and cells stop proliferating as a result of Delta icd production; (iii) when Notch activation by Delta from a neighboring cell is potentiated by Fringe, the balance of signaling through Notch and Delta is skewed, and cell proliferation is upregulated.

Acknowledgments: Dr. C. Sherr, St. Jude Children's Research Hospital in Memphis, Tennessee for p21−/−, p27−/− and wt MEF; Dr. G.P. Dotto, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA for p21 promoter reporter construct; Drs. Lucy Liaw, Robert Friesel, Jeong Kyo Yoon and Douglas Spicer for helpful discussions, and Norma Albrecht for editorial assistance. This work was supported in part by NIH Grants HL 35627, HL 32348, and RR15555 (Project 1) to I.P.

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Paper 3

"Thrombin mediates FGF1 release through PAR1-dependent, and Jagged1-dependent pathways"

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TRAFFIC

(Manuscript under revision)

To: TRAFFIC

Thrombin mediates FGF1 release through PAR1-dependent, and Jagged1-dependent pathways

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Running title: Thrombin enables FGF1 release

Key words: Thrombin, PAR1, FGF1, Jagged1, Non-classical release

Word count: 7986

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This work was supported in part by NIH grants, HL 32348, HL 35627 to IP, HL 70865 to LL, HL 65301, DE 13248 to RF and RR 15555 to RF and IP.
This work was performed by MD from the Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, Braga, Portugal in partial fulfillment of the requirements for Ph.D. degree.

The article is dedicated to the memory of Tom Maciag, friend, scientist, and mentor.

The authors wish to thank J. Fenton (N.Y. State Dept. of Health) for human α-thrombin, S. Coughlin (University of California, San Francisco, CA) for the PAR1 null and control mouse embryonic fibroblasts, and A. Baird (Human BioMolecular Research Institute, San Diego, CA) for FGF1:HA.

Summary

Thrombin, a mediator of blood coagulation, induces cell proliferation and migration upon vascular injury. The mitogenic effect of thrombin is not well understood. We report that thrombin induced expression and non-classical release of fibroblast growth factor 1 (FGF1). FGF signaling underlies thrombin mitogenic activity, since this protease did not stimulate proliferation in cells expressing a dominant-negative form of FGF receptor 1. Thrombin failed to induce the rapid release of FGF1 in fibroblasts with the knockout for protease activated receptor 1 (PAR1), suggesting that this rapid effect is dependent on PAR1. Thrombin cleaved the Notch ligand, Jagged1, in its extracellular domain, and produced a soluble form of Jagged1, an inhibitor of Notch signaling and inducer of FGF1 expression and release. Long-term thrombin stimulation induced FGF1 release from PAR1 null cells, which express endogenous Jagged1. Overexpression of Jagged1 in PAR1 null cells enabled them to accelerate the release of FGF1 in response to thrombin. Thus, in addition to its role as an effector of the blood coagulation, thrombin may induce FGF1 release through two pathways: one is PAR1-dependent, and the other is mediated by the cleavage of Jagged1. These data demonstrate the existence of novel crosstalk between the thrombin, FGF, and Notch signaling.

Introduction

Thrombin, a multifunctional serine protease released at sites of vascular injury elicits blood coagulation (Fenton, 1986). Thrombin is also a well known mitogen (Van Obberghen-Schilling et al., 1985) and regulator of angiogenesis (Richard et al., 2001). Thrombin-induced signaling in the endothelium results in multiple phenotypic changes including: alterations in cell shape; endothelial monolayer permeability (Rabiet, 1996); mobilization of adhesive molecules to the endothelial surface (Kaplanski, 1998); DNA synthesis (Herbert et al., 1994); cell migration (Pankonin, 1991); and angiogenesis in vivo (Hirano and Kanaide, 2003; Minami et al., 2004; Vu et al., 1991). Thrombin signaling mediates proliferation of vascular smooth muscle cells in vivo (Hirano and Kanaide, 2003). Thrombin induces its biological responses predominantly through protease-activated receptors (PAR) (Vu et al., 1991) expressed in various cell types (Algermissen et al., 2000). Activation of these G protein-coupled receptors occurs through a

proteolytic modification in their amino-terminal domains, leading to a cascade of downstream effects (Vu et al., 1991). The expression and/or release of several growth factors are induced in response to thrombin including fibroblast growth factor 2 (FGF2) (Benezra et al., 1993), platelet-derived growth factor (Daniel, 1986; Harlan et al., 1986), vascular endothelium growth factor (VEGF) (Bassus et al., 2001; Tsopanoglou and Maragoudakis, 1999). Thrombin also upregulates the insulin-like growth factor receptor 1 (Delafontaine et al., 1996), and induces the activation of fibroblast growth factor receptor 1 (FGFR1) (Rauch et al., 2004). However, mechanisms underlying the mitogenic activity of thrombin remain obscure.

Similarly to thrombin, fibroblast growth factor 1 (FGF1) is involved in the process of tissue and vascular repair (Bjornsson et al., 1991), and the mechanism of its mitogenic activity is more clearly defined (Grieb and Burgess, 2000). FGF1 lacks a classical signal peptide sequence and is exported through a non-classical ER-Golgi-independent pathway (Prudovsky et al., 2003). Other growth factors and cytokines including interleukin 1β (Rubartelli et al., 1990), interleukin 1α (Tarantini et al., 2001), and FGF2 (Engling et al., 2002; Florkiewicz et al., 1995; Florkiewicz et al., 1998; Mignatti et al., 1992) are also released through non-classical pathways, and non-classical protein release is being studied intensively (Nickel, 2005; Prudovsky et al., 2003). We demonstrated that FGF1 is released from NIH 3T3 cells into the extracellular compartment in response to cellular stress as a non-covalent complex with the small calcium-binding protein, S100A13, and the p40 form of the membrane-docking protein, Synaptotagmin 1 (Prudovsky et al., 2003). These results are consistent with our previous findings that FGF1 is purified from bovine brain as a non-covalent complex containing FGF1, p40 Synaptotagmin 1, S100A13, and Annexin 2 ((Mouta Carreira et al., 1998) and Soldi, R., Prudovsky, I. and Maciag, T. unpublished results). The assembly of FGF1 release complex occurs at the inner leaflet of the cell membrane (Prudovsky et al., 2002), which is the Annexin 2 localization site (Kim and Hajjar, 2002). The N-terminus of Annexin 2 associates with p11, a member of the S100 family (Glenney, 1986). Peterson et al. demonstrated the capability of thrombin to enhance the presence of Annexin 2 and p11 on the endothelial cell surface, through their translocation from the inner to outer leaflet of plasma membrane (Peterson et al., 2003), leads to the hypothesis that thrombin

induces the Annexin 2-mediated flip-flop of the FGF1 release complex across the cell membrane.

We reported that the suppression of Notch-mediated signaling by the soluble non-transmembrane form of the Notch ligand, Jagged1, a 117 kDa soluble Jagged1 (sJ), induces non-classical release of FGF1 under non-stress conditions (Small et al., 2003). In addition, sJ1 expression induces FGF1 transcription, and development of a FGFR1-dependent transformed cell phenotype (Small et al., 2003). Premature truncations, leading to the production of non-transmembrane forms of human Jagged1, result in Alagille Syndrome, a disease characterized by spontaneous bleeding, congenital heart defects, and pulmonary stenosis (Joutel and Tournier-Lasserve, 1998). Since our preliminary amino acid sequence analysis revealed two potential thrombin cleavage sites in the extracellular domain of the Jagged1 sequence, we hypothesized that thrombin could be a protease involved in the production of soluble non-transmembrane forms of Jagged1.

We questioned whether thrombin can induce the expression and release of FGF1 into the extracellular compartment and, if so, whether this effect is mediated by the cleavage of Jagged1. We demonstrated that thrombin stimulated the expression and release of FGF1 under non-stress conditions. We also found that thrombin enabled the production of a short extracellular form of Jagged1. The analysis of FGF1 release dynamics from thrombin-treated $PAR1^{+/+}$ and $PAR1^{-/-}$ cells demonstrated that FGF1 export could be stimulated by at least two mechanisms, the early one PAR1-dependent and the later mediated by Jagged1 cleavage. Our results confirm earlier observations of the interplay between FGF and Notch pathways, and add thrombin and PAR1 as new participants in this signaling network.

Materials and Methods

Generation of expression constructs and stable NIH 3T3 transfectants

A thrombin-resistant FGF1 mutant was constructed by site-directed mutagenesis of the FGF1pMEXneo vector (Jackson et al., 1992). The codon encoding arginine 136 (AGA) was changed to a lysine (AAA) (FGF1R136K) by using the primers: 5'-CTGCAAACGCGGTCCTAAAACTCACTATGGAG-3' forward and 5'-CTGGCCATAGTGAGTTTTAGGACCGCGTTTGCAG-3' reverse. The N-terminally V5-tagged full-length Jagged1 (FLJ1NV5) construct was obtained by cloning the complete human Jagged1 ORF into

BamHI and *XhoI* restriction sites of pcDNA3.1/Hygro(+) (Invitrogen). The V5-His tag was excised from the pcDNA^{TM}4/V5-His vector, and inserted into the FLJ1-pcDNA3.1/Hygro(+) between the signal peptide and the Delta, Serrate, Lag-2 (DSL) domain of full-length Jagged1 (FLJ1). For this purpose, the two new restriction sites, *NotI* and *EcoRI*, were introduced in FLJ1-pcDNA3.1/Hygro(+) construct by PCR mutagenesis. sJ1 (39kDa) was obtained from the N-terminally V5-His-tagged FLJ1-pcDNA3.1/Hygro(+) construct by insertion of a stop codon at position 349, followed by a *PmeI* restriction site, which was then used to clone the fragment back into the pcDNA3.1/Hygro(+) vector. The mutagenesis reactions were performed using the Quickchange site-directed mutagenesis kit (Stratagene), and the generated sequences were confirmed by DNA sequencing. NIH 3T3 cell transfectants expressing FGF1R136K, sJ1 (39kDa), and insert-less control vector pcDNA3.1/Hygro(+) were generated by utilizing the Fugene 6 reagent (Roche), and selected by using geneticin (GIBCO) or hygromycin (Roche). Transfectants were screened for gene expression by utilizing an anti-FGF1 rabbit antibody for FGF1R136K or an anti-V5 antibody (Invitrogen) for the sJ1 (39kDa) clones. The genome incorporation of insert-less control vector pcDNA3.1/Hygro(+) was screened by PCR. The FGF1R136K, FLJ1NV5, and sJ1 (39kDa) constructs were also cloned in the the adenoviral shuttle vector, pAdlox, and recombinant adenoviruses were produced and purified as described (Hardy et al., 1997).

Cell culture

NIH 3T3 cells (ATCC), Swiss 3T3 cells (ATCC), FGF1R136K NIH 3T3 transfectants, and FLJ1 NIH 3T3 transfectants (Small et al., 2001) were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies) containing 10% Bovine Calf Serum (BCS; Hyclone) and 1x antibiotic-antimycotic mixture (Life Techonologies, Inc.). Stable NIH 3T3 cell transfectants cultures were supplemented with 400 μg/L Geneticin (GIBCO) or 200 μg/ml Hygromycin (Roche). PAR1 null mouse embryonic fibroblasts and PAR1 null fibroblasts transfected with PAR1 (gifts from S. Coughlin, University of California, San Francisco, CA), and HEK 293 cells (ATCC) were grown in DMEM supplemented with 10% Fetal Bovine

Serum (Hyclone). The cells used in the FGF1 release experiments were grown on human fibronectin-coated (10 μg/cm^2) dishes, as described (Jackson et al., 1992).

Adenoviral transduction

PAR1 null mouse embryonic fibroblasts, NIH 3T3 or HEK 293 cells were transduced with different adenoviruses by incubation in serum-free medium with approximately 10^3 PFU of adenovirus, as described (Mandinova et al., 2003). In addition to FGF1R136K, FLJ1NV5, and sJ1 (39kDa) adenoviruses, we used the previously reported adenoviruses expressing β-galactosidase and the constitutively active form of Notch1 (caN1) (Small et al., 2003). Adenoviral transductions were performed 24 or 48 hours before plating the cells. The efficiency of transduction for FLJ1NV5 and caN1 was assessed by immunofluorescence by using an anti-V5 monoclonal antibody.

Thrombin stimulation and immunoblot analysis of FGF1 release

Stable FGF1R136K NIH 3T3 cell transfectants, adenovirally transduced NIH 3T3 cells or adenovirally transduced PAR1 null cells were washed with serum-free DMEM containing 5 U/ml of heparin (Sigma), and stimulated with either 1 U/ml thrombin (a gift from J. Fenton, New York State Department of Health, Albany, NY) or 5.7 μM Thrombin Receptor-Activator Peptide (TRAP, Sigma) at 37°C for different time periods. Control cells were incubated in the absence of thrombin or TRAP for the same time periods. The heat shock-induced release of FGF1 was stimulated by incubation of cells at 42°C for 110 minutes. Conditioned media and cell lysates were collected and tested for FGF1 content by heparin chromatography and immunoblot analysis (Jackson et al., 1992). Processed conditioned medium from one 150 mm cell culture dish was used at each time point. Also, 1/10 of the cell lysate derived from one 150 mm plate was loaded to each gel for FGF1 expression control. Cell viability was assessed by measuring lactate dehydrogenase (LDH) activity in conditioned medium after filtration (Mandinova et al., 2003). In the long-term thrombin stimulation experiments, PAR1 null cells were adenovirally transduced with FGFR136K for

48 hours. Further, the cells were washed in serum-free DMEM containing 5 U/ml of heparin, and incubated for additional 2 hours in the complete medium that was preconditioned for 2 or 48 hours with untransduced PAR1 null cells in the presence of either thrombin (1 U/ml) or TRAP (5.7 μM). To evaluate the effect of Jagged1 overexpression upon the ability of PAR1 null cells to release FGF1 in response to short-term thrombin stimulation, PAR1 null cells were transduced for 48 hours with FLJ1NV5 or control β-galactosidase adenoviruses. Then the cells were stimulated for 2 hours with thrombin, and conditioned media were collected and added for 2 hours to PAR1 null cells transduced with FGF1R136K. After 2 hours of incubation, conditioned media were tested for FGF1 release, as described above.

Preparation of cell membranes

FGF1R136K NIH 3T3 cells were washed with serum-free DMEM containing 5 U/ml of heparin, and stimulated with 1 U/ml thrombin at 37°C for 30 minutes. Control cells were incubated in the absence of thrombin for 30 minutes. Next, the cells were washed in PBS, scraped, and quickly spun down. The cell pellet was then resuspended in 1 ml of hypotonic solution (2.5 mM HEPES pH 7.0, 25 mM Sucrose and protease inhibitor cocktail (Roche)), and incubated on ice for 20 minutes. The pellets were homogenized in a Dounce homogenizer and centrifuged for 10 minutes at 10,000 g 4°C. Then the supernatants were collected and centrifuged at 40,000 rpm for 18 hours to precipitate the membranes.

The cell-free translation of Jagged1, thrombin cleavage, and automated Edman microsequencing

A plasmid containing FLJ1 (Zimrin et al., 1996) was transcribed and translated in vitro in the presence of a [^{35}S]-Met/Cys protein-labeling mixture (Amersham), using the T7-coupled reticulocyte lysate system according to the manufacturer's instructions (Promega). After 60 minutes of incubation at 30°C, the reaction was stopped by the addition of 0.05% DTT. Half of the reaction mixture was incubated with 1 U of thrombin for 15 minutes at 37°C, and the reaction was stopped by boiling in the presence of SDS-PAGE sample buffer. The samples were resolved by 12% SDS-PAGE, transferred to a PVDF membrane, and analyzed by autoradiography. The bands corresponding to the thrombin cleavage products were excised

and subjected to automated Edman microsequencing (Applied Bio Sciences, Maine Medical Center Research Institute, Protein, Nucleic Acid, and Cell Imaging Core). The products of each cycle were collected prior to resolution by HPLC and quantified by liquid scintillation spectroscopy (Beckman).

Cleavage of Jagged1 expressed on the cell surface, and immunopreciptation of soluble Jagged1 from conditioned medium

HEK 293 cells were transduced with the FLJ1NV5 adenovirus. Control cells were transfected with the adenovirus expressing β-galactosidase. Forty-eight hours after transduction, the cells were washed with DMEM and incubated with 1 U/ml thrombin for 1 hour in serum-free medium at 37˚C. In control dishes, the cells were incubated with or without 1 U/ml thrombin in the presence of hirudin (Sigma) at a final concentration of 5 U/ml or in the presence of protease inhibitor cocktail. Conditioned media were collected and concentrated by Centricon devices (Millipore). Then, 1 ml of conditioned medium was immunoprecipitated with 1 μg of anti-V5 antibody, resolved on 12% SDS-PAGE, and immunoblotted using the anti-V5 antibody.

Nuclear run-on, RT-PCR, and real time RT-PCR analysis of FGF1 expression

Nuclear run-on analysis of FGF1 expression in Swiss 3T3 and NIH 3T3 cells was performed as previously described (LaVallee et al., 1998). Linearized and alkali denaturated plasmid constructs (0.4 μg/dot) containing either the *fgf1* or *gapdh* cDNA were used as probes. Membranes were analyzed utilizing a phosphoimager (Molecular Dynamics). RT-PCR was performed with total RNA isolated, using the RNeasy kit (Qiagen) from PAR1 null mouse embryonic fibroblasts, insert-less vector control, sJ1 (117kDa) (Small et al., 2001), and sJ1 (39kDa) NIH 3T3 cell transfectants, as well as from NIH 3T3 cells adenovirally transduced with β-galactosidase or caN1. The following PCR primers were utilized: *Jagged1* 5'-GGCGGCTGGGAAGGAACAAC-3' forward and 5'-TCACCGGCTGGAGACTGGAAGA-3' reverse; *fgf1*: 5'-ATGGCTGAAGGGGAGATCACAACC-3' forward and 5'-CGCGCTTACAGCTCCCGTTC-3' reverse. RT-PCR was performed with 1 μg RNA, using the Platinum Taq One Step RT-PCR kit

(Invitrogen). *gapdh* expression served as a control for RNA loading. Real-time PCR was performed using the Icycler IQ Real-time PCR (Bio-Rad), according to the manufacturer's recommendations. Amplification of the *gapdh* cDNA was used as the endogenous normalization standard. Each sample was amplified in triplicate.

DNA synthesis assay

A combination of [^{3}H]-thymidine autoradiography and immunohistochemistry was used to evaluate DNA synthesis levels in Swiss 3T3 cells expressing a dominant-negative (dn) FGFR1 mutant. Cells were plated on coverslips for 24 hours. The cells were transfected with an *X. laevis* dnFGFR1 (Neilson and Friesel, 1995) deletion mutant lacking the FGFR1 intracellular domain, using the Fugene 6 reagent. After 24 hours, the medium was changed to DMEM containing 0.25% BCS; following a 48-hour incubation in low BCS, the cells were stimulated for 24 hours with either 1 U/ml of thrombin, 10% BCS or 10 ng/ml of recombinant FGF1 plus 10 U/ml of heparin prior to [^{3}H]-thymidine (1 μCi/ml, NEN) addition for 12 hours. The cells were fixed and immunostained using a polyclonal antibody against the extracellular domain of *X. laevis* FGFR1, followed by an immunoperoxidase-conjugated goat anti-rabbit IgG secondary antibody. Immunostained cells were processed for autoradiography, as described (Prudovsky and Tsong, 1991).

Dual luciferase reporter assay of CSL-dependent transcription

Insert-less vector control, sJ1 (117kDa) (Small et al., 2001) and sJ1 (39kDa) NIH 3T3 cell transfectants were plated on fibronectin-coated cell culture dishes, and transiently transfected using Fugene 6 at approximately 50% confluency with 500 ng of a luciferase construct driven by four tandem copies of the CBF1 response element (Small et al., 2001). Cotransfection with 100 ng of the TK *Renilla* (Promega) construct was used as an internal control for transfection efficiency. In additional experiments, stable insert-less vector control and Jagged1 NIH 3T3 cells transfectants were treated with or without 1, 2 or 4 U/ml thrombin or 10, 20 or 40 nM TRAP for 12 hours before and 48 hours after transfection. Forty-eight hours after transfection, the cells were harvested and the luciferase/renilla activity was measured by utilizing the

Dual Luciferase Reporter Assay System (Promega). Each experiment was performed in triplicate.

Immunofluorescence and confocal microscopy

NIH 3T3 cells were plated on fibronectin-coated glass coverslips in 6-well TC plates at 10^5 cells/well. The next day, the cells were transfected with FGF1:HA cloned in the pCR3.1 vector (1μg of DNA per well) (a gift from A. Baird, Human BioMolecular Research Institute, San Diego, CA) using the Fugene 6 reagent. The following day, the cells were stimulated for 30 minutes with 1 U/ml thrombin. The cells were fixed, immunofluorescently stained for the HA tag, and studied using the LTCS-SP confocal microscope (Leica) as described (Prudovsky et al., 2002).

Densitometric analysis

After scanning the immunoblots, the optical densities of individual bands were analyzed by ImageQuant software (Molecular Dynamics). The area of each band analyzed was kept constant for each blot. All samples were normalized against FGF1 level in cell lysates.

Results

Thrombin induces the expression of FGF1

Since thrombin induces the expression of several growth factors (Bassus et al., 2001), we questioned whether FGF1 was among them. The expression of the FGF1 transcript in NIH 3T3 and Swiss 3T3 cells, in response to thrombin, was analyzed by nuclear run-on assay. Induction of FGF1 mRNA was initially detected 15 minutes after the addition of thrombin to NIH 3T3 cells, increased over time, and reached a plateau after 2 hours (Fig. 1). Similar results were obtained with Swiss 3T3 cells (data not shown).

Thrombin rapidly induces the non-classical release of FGF1

The majority of FGF family members contain a N-terminal hydrophobic signal peptide that facilitates the direct import into the endoplasmic reticulum for transport via the Golgi apparatus to the extracellular compartment (Blobel, 1995). However, FGF1 and FGF2 do not have a signal peptide; they are released

through non-classical export mechanisms (Prudovsky et al., 2003; Nickel, 2005). Since thrombin induced FGF1 expression (Fig. 1), we sought to determine whether it induces FGF1 release. Because FGF1 is susceptible to thrombin cleavage at arginine 136 (Erzurum et al., 2003), we utilized a thrombin-resistant FGF1 mutant (FGF1R136K). NIH 3T3 cells stably transfected with FGF1R136K were stimulated for 5, 15, 30, and 60 minutes with 1 U/ml thrombin. The addition of thrombin to FGF1R136K NIH 3T3 cell transfectants at 37°C resulted in the rapid, sustainable appearance of the FGF1R136K mutant in the extracellular compartment (Fig. 2A). At the same time, similarly to wild type FGF1 (Jackson et al., 1992), the release of the FGF1R136K mutant required 90 minutes of temperature stress at 42°C in order to be detected by immunoblotting (data not shown). Similarly to heat shock, thrombin treatment did not induce the release of lactate dehydrogenase from the cells (data not shown); thus neither treatment resulted in cell damage. Furthermore, release of the FGF1R136K mutant was dependent on the concentration of thrombin with a maximal response at 1 U/ml (10 nM) (Fig. 2B). This concentration is physiologically relevant because the level of prothrombin in circulation is 1-2 μM, and fibrin clot formation occurs at 10-30 nM thrombin concentration (Mann, 2003). Heat shock conditions stimulating FGF1 release induce the translocation of FGF1 to the cell membrane (Prudovsky et al., 2002). We applied immunofluorescence confocal microscopy to evaluate the effect of thrombin on the intracellular localization of FGF1:HA. We observed that short thrombin treatment of NIH 3T3 cells transiently transfected with FGF1:HA resulted in the translocation of FGF1 to cell periphery, near the cell membrane (Fig. 2C, top panel). We additionally performed subcellular fractionation of FGF1R136K NIH 3T3 cell transfectants, treated and untreated with thrombin for 30 minutes at 37°C. Thrombin induced the appearance of FGF1R136K in the membrane fraction (Fig. 2C, lower panel). After thrombin treatment, 28.7% of FGF1R136K was translocated to the membrane fraction, as determined by densiometric gel analysis (note that all of the cell lysate from one 150 mm cell culture dish was used for each treatment). Thus, thrombin efficiently induced the release of FGF1 under non-stress conditions with rapid kinetics, and apparently this effect is mediated by the association of FGF1 with cell membrane.

The mitogenic activity of thrombin is FGFR-dependent

Since thrombin induced both the expression of FGF1 and its release into the extracellular compartment, it is possible that these two effects contribute to the mitogenic activity of thrombin. To assess this hypothesis, we analyzed the ability of a dominant-negative mutant of FGFR1 to attenuate cell proliferation stimulated by thrombin. We utilized Swiss 3T3 cells since, unlike the NIH 3T3 cells, they exhibit a low level of apoptosis and endogenous DNA synthesis in response to serum deprivation. Swiss 3T3 cells express significant levels of FGFR1 (Andreeva, 2004). A dnFGFR1 construct was transfected into Swiss 3T3 cells, and their proliferative index was measured in the presence and absence of exogenous thrombin. The expression of dnFGFR1 was verified by immunohistochemistry, and DNA synthesis was revealed by [^{3}H]-thymidine radioautography. As shown in Fig. 3, the expression of dnFGFR1 not only reduced the ability of FGF1 to induce the appearance of replicating nuclei by approximately 70%, but it also decreased the DNA synthesis frequency in the presence of thrombin to a level consistent with quiescence. Thus, the mitogenic effect of thrombin depended on FGFR signaling.

Thrombin cleaves Jagged1

Previously, we demonstrated that the expression of sJ1 in NIH 3T3 cells represses Notch-mediated CSL (CBF1/Su(H)/Lag1)-dependent transcription, and induces both FGF1 expression and constitutive non-classical FGF1 release at 37°C (Small et al., 2003). We hypothesized that the ability of thrombin to induce the expression and release of FGF1 may be due to the production of sJ1, as a result of cleavage of the Jagged1 expressed on the surface of NIH 3T3 cells (Small et al., 2003). To evaluate this hypothesis, we determined whether the Jagged1 translation product was susceptible to proteolytic cleavage by thrombin. Examination of the human Jagged1 amino acid sequence revealed two putative thrombin cleavage sites within the extracellular domain of Jagged1 (R113 and R348). In order to assess Jagged1 as a thrombin substrate, Jagged1 was transcribed and translated in vitro in the presence of a [^{35}S]-Cys/Met mixture; and the 134 kDa Jagged1 translation product was incubated with or without thrombin. Autoradiographic

analysis of the reaction products revealed cleavage of the Jagged1 protein into 39 kDa and 95 kDa fragments (Fig. 4A). Because the fragment sizes were consistent with a Jagged1 cleavage site between residues R348 and G349 (Fig. 4B, upper panel), we sought to confirm the identity of this putative site. The 95 kDa fragment was excised, subjected to automated Edman degradation, and the products of each cycle were monitored by liquid scintillation spectroscopy. We observed [^{35}S]-Cys radioactivity in cycles 2, 3, 12, and 14, which agrees with the position of Cys at residues 351, 360, and 362 (Fig. 4B, upper panel). These analyses suggest that thrombin is able to cleave Jagged1 between residues R348 and G349, which are located between epidermal growth factor (EGF) repeats 3 and 4 (Shimizu et al., 1999). This cleavage yields an amino terminal fragment with a molecular mass of approximately 39 kDa. To verify that thrombin cleaves Jagged1 expressed in living cells, we transduced HEK 293 cells with a FLJ1NV5 adenoviral construct (Fig. 4B, middle panel) for 48 hours, and used transduced cells for thrombin treatment. After 1 hour of treatment at 37°C with 1 U/ml of thrombin, the serum-free medium was collected, immunoprecipitated with the anti-V5 antibody, resolved by SDS-PAGE, and immunoblotted with the anti-V5 antibody. As shown in Fig. 4C, lane 2, thrombin induced the cleavage and release of a N-terminal fragment of Jagged1 with the molecular weight of approximately 39 kDa into the medium. Jagged1 cleavage was completely blocked by a protease inhibitor cocktail (data not shown). Moreover, we found that hirudin, a highly specific thrombin inhibitor, was able to block the appearance of sJ1 (39kDa) (Fig. 4C, lane 3).

sJ1 (39kDa) corresponding to the Jagged1 thrombin cleavage product induces the expression and release of FGF1

The extracellular domain of Jagged1 is involved in receptor binding, and consists mainly of 16 tandem epidermal growth factor-like (EGF-like) repeats. Since thrombin cleaves Jagged1 between the third and fourth EGF repeat, we sought to evaluate the biological activity of the resulting soluble Notch ligand, particularly its ability to downregulate Notch signaling, and to induce FGF1 expression and release. We prepared a construct coding for the product of thrombin-mediated cleavage of Jagged1, sJ1 (39kDa) (Fig.

4B, lower panel). To determine whether sJ1 (39kDa) carries the same capacity to decrease Notch signaling as sJ1 (117kDa), which represents the whole extracellular domain of Jagged1 (Small et al., 2001), we assayed vector control, FLJ1, sJ1 (117kDa), and sJ1 (39kDa) NIH 3T3 stable transfectants for CSL-dependent transcription by utilizing a luciferase reporter assay (Hsieh et al., 1996; Jarriault et al., 1995). While FLJ1 transfectants exhibited an increase in CSL-mediated transcription, NIH 3T3 sJ1 (39kDa) transfectants displayed a decrease of the CSL-dependent transcription (Fig. 5A), which is similar to sJ1 (117kDa) transfectants (Small et al., 2001). We next assessed by RT-PCR untransfected NIH 3T3 cells, vector-transfected control, sJ1 (117kDa), and sJ1 (39kDa) transfectant NIH 3T3 cells for the expression of *fgf1*. sJ1 (117kDa) and sJ1 (39kDa) transfectants expressed *fgf1*, while both untransfected and vector control transfected cells did not (Fig. 5B, top panel). Further (Q)-RT-PCR analysis results demonstrated that sJ1 (39kDa) induced significantly higher *fgf1* mRNA levels than sJ1 (117kDa). Since sJ1 (39kDa) induced the expression of FGF1, we next asked whether it induced FGF1 release. Vector control and sJ1 (39kDa) NIH 3T3 transfectants were transduced with FGF1R136K adenovirus, and analyzed for FGF1 release under normal or heat shock conditions. Whereas both vector control and sJ1 (39kDa) transfectants exported FGF1 in response to temperature stress (42°C), FGF1 release under non-stress conditions (37°C) was only observed in the sJ1 (39kDa) NIH 3T3 transfectants (Fig. 5C). Thus sJ1 (39kDa) resulting from thrombin cleavage was not inferior to sJ1 (117kDa) in its ability to repress Notch signaling, and to induce FGF1 expression and release.

Thrombin antagonizes CSL-dependent signaling, and constitutively active Notch1 inhibits thrombin-induced expression and release of FGF1

Because CSL-dependent transcription in NIH 3T3 cells is significantly repressed by the expression of sJ1 (39kDa), we questioned whether thrombin treatment attenuates Notch signaling. We used the luciferase reporter assay to evaluate the ability of thrombin (1-4 U/ml) to attenuate the activity of the CSL-dependent promoter in NIH 3T3 cells. While treatment of the FLJ1 NIH 3T3 stable cell transfectants with thrombin reduced the level of CSL-dependent transcription, TRAP, an agonist peptide of PAR1 devoid of

proteolyical activity, did not affect it (Fig. 6A). To further determine whether Notch signaling is involved in thrombin-induced FGF1 release, we utilized FGF1R136K NIH 3T3 cell transfectants adenovirally transduced with constitutively active Notch1 (caN1), and stimulated with thrombin. About 90% of the cells expressed caN1 48 hours after transfection (data not shown). As shown in Fig. 6B, thrombin stimulated the release of FGF1R136K from control cells; however, it was unable to initiate the release from cells expressing caN1 (Fig. 6B). At the same time, the expression of caN1 in FGF1R136K NIH 3T3 cell transfectants did not affect heat shock-induced FGF1 release (Fig. 6B). Interestingly, the expression of caN1 also abolished the expression of FGF1 induced by thrombin (Fig. 6C). These data suggest that the induction of FGF1 expression and release by thrombin is dependent on the down-regulation of Notch signaling.

The role of PAR1-mediated signaling in thrombin-induced FGF1 release and expression

Since thrombin is known to mediate its biological responses predominantly through the activation of PARs (Vu et al., 1991), we sought to evaluate the role of PAR1 in thrombin-induced release of FGF1. We utilized embryonic fibroblasts obtained from PAR1 null mice, as well as control PAR1 null fibroblasts transfected with PAR1 (Connolly et al., 1996) that were transduced with the FGF1R136K adenovirus. Thrombin was unable to rapidly induce export of the FGF1R136K from PAR1 null cells (Fig. 7A). However, PAR1 null cells exported FGF1R136K in response to temperature stress (42°C), suggesting that they were not defective in mediating stress-induced non-classical FGF1 export. At the same time, thrombin induced rapid FGF1R136K release from control PAR1 null fibroblasts transfected with PAR1 (Fig. 7A, lower panel). Additionally, we assessed whether the PAR1-activating peptide, TRAP, which is devoid of proteolytic activity, was able to mimic the ability of thrombin to induce FGF1R136K release. TRAP rapidly induced the export of FGF1R136K (Fig. 7B), as well as the expression of the FGF1 transcript in NIH 3T3 (Fig. 7C) and Swiss 3T3 cells (data not shown). Thus, although thrombin produced biologically active sJ1 (39kDa), and thrombin-induced rapid stimulation of FGF1 expression and release were inhibited by the expression of caN1, these rapid effects of thrombin were dependent on PAR1.

Long-term thrombin incubation induces FGF1 release from PAR1 null cells, and overexpression of Jagged1 accelerates FGF1 release from PAR1 null cells in response to thrombin

Because thrombin failed to stimulate rapid FGF1 release from PAR1 null mouse embryonic fibroblasts, we were interested in determining whether long-term thrombin treatment results in FGF1 release from PAR1 null cells due to the accumulation of sJ1 (39kDa) in the conditioned medium. Indeed, we found that PAR1 null mouse embryonic fibroblasts expressed Jagged1 transcripts at levels similar to those in NIH 3T3 cells (Fig. 8A). PAR1 null cells were incubated with thrombin (1 U/ml) or TRAP (5.7 μM) in complete cell culture medium for 2 or 48 hours. In parallel, other PAR1 null cells were transduced with FGF1R136K adenovirus. Forty-eight hours after transduction, the cells were carefully washed in serum-free medium containing heparin (5 U/ml), and incubated for an additional 2 hours in the medium conditioned by untransduced PAR1 null cells treated with thrombin or TRAP. As shown in Fig. 8B, medium conditioned for 2 hours by thrombin-treated cells failed to induce FGF1 release. At the same time, the medium conditioned for 48 hours by cells treated with thrombin, but not with TRAP, was able to induce FGF1 release from PAR1 null cells. We hypothesized that the continuous presence of thrombin during 48 hours results in the accumulation in the extracellular compartment of sJ1 (39kDa), which induced FGF1 export. In order to further assess this hypothesis, we overexpressed Jagged1 in PAR1 null cells by adenoviral transduction. Cells transduced with FLJ1NV5 were stimulated with thrombin for 2 hours. Conditioned media were collected and added for 2 hours to PAR1 null cells transduced with FGF1R136K adenovirus. Conditioned medium from thrombin-treated PAR1 null cells overexpressing FLJ1 induced FGF1 release (Fig. 8C); however, conditioned medium from thrombin-treated control β–galactosidase-transduced cells did not exhibit such an effect. These data demonstrate that the accumulation of sJ1 (39kDa) in the medium conditioned by thrombin-treated cells can result in PAR1-independent FGF1 release.

Discussion

FGF1 participates in vascular remodeling (Bjornsson et al., 1991; Nabel et al., 1993) and promotes angiogenesis after vascular injury (Thompson et al., 1988; Bjornsson et al., 1991; Herbert, 1988). Tissue injury induced by stress as a result of physical trauma, infection or inflammation is often accompanied by a thrombotic event (Taubman et al., 1999). Based on our results, thrombin in sites of injury may play a critical role, not only in the formation of a temporary extracellular matrix of fibrin but also in the stimulation of expression and export of FGF1. FGF1 contains a thrombin cleavage site (Erzurum et al., 2003); however, this cleavage is inhibited by heparin (Rosengart et al., 1988). We suggest that extracellular heparan sulfates may protect the released FGF1 from thrombin-induced cleavage. On the other hand, saturation of heparan sulfate proteoglycan sites by exported FGF1 may assure that excess FGF1 is proteolytically-inactivated by thrombin (Erzurum et al., 2003; Lobb, 1988). As we demonstrated, thrombin's mitogenic activity was dependent on the activity of FGF signaling, since thrombin failed to stimulate cell proliferation when 3T3 cells expressed a dominant-negative form of FGFR1.

PAR1 is an important player in promoting inflammation and abnormal remodeling during restenosis, neointima formation after vascular injury, and fibrosis in the injured lung (Andrade-Gordon et al., 2001; Wahlgren, 2004; Howell, 2005). PAR1 may be a link between blood coagulation and inflammation in response to tissue injury. Here we reported that the rapid stimulation of FGF1 expression and release by thrombin was dependent on PAR1. Due to the presence of several PAR1-coupled G-proteins, each of which activates different pathways, the signaling network induced upon PAR1 activation is complex. In most cell types, thrombin modulates the activity of adenylyl cyclase (Berk et al., 1991; Kanthou, 1998; Dery et al., 1998), activates phospholipase C and A2, protein kinase C (PKC), Ras/mitogen-activated protein kinase (MAPK) signaling pathway, and also regulates the expression of a broad range of transcriptional factors. Specific inhibitors of PKC, Src, and phosphatidylinositol-3-kinase suppress PAR1-induced VEGF expression (Tsopanoglou and Maragoudakis, 1999). It remains to be elucidated which signaling pathway is involved in PAR1-mediated stimulation of FGF1 expression and release. Recently, we demonstrated that non-transformed cell cultures stimulated with FGF1 transit through only one cell cycle, and then are blocked in the G1 phase of the second cycle (Andreeva, 2004). Interestingly, this non-

proliferative state is characterized by activation of the Ras/MAPK signaling pathway and high levels of expression of cyclins D and E. It is possible that PAR1 stimulation induces expression and release of FGF1 needed for mitogenic response at the early stage of vascular repair response.

Thrombin also stimulated expression and release of FGF1 through its ability to cleave Jagged1 and to produce sJ1 (39kDa), which we demonstrated to induce the expression and release of FGF1 and inhibition of Notch signaling. We hypothesize that thrombin-induced FGF1 release proceeds through at least two temporally related stages. At the early stage, thrombin-induced FGF1 release is PAR1-dependent. This early stage is caused by tissue damage involving the activation of either the intrinsic or extrinsic coagulation pathways, providing fibrin deposition, as well as source of thrombin at the damage site and consequent PAR1 activation. Should the time period of the initial stress be extended after this early phase by additional physiologic or pathophysiologic stress or both, e.g. starvation, acidosis, and hypoxia and if the PAR1 population is desensitized, it is likely that the non-classical export of FGF1 is temporarily maintained by the function of the stress-induced pathway (Prudovsky et al., 2003). The export of FGF1 at the early stage of thrombin-induced response may contribute to the increase of Jagged1 expression level in thrombin-treated cells. Indeed, FGF1 stimulation induces Jagged1 transcription in endothelial cells (Zimrin et al., 1996). Therefore, the continued presence of thrombin in the extracellular compartment may result in the accumulation of sJ1 (39kDa), and the release of FGF1 at the latter stage of thrombin stimulation may be dependent upon the sJ1-induced downregulation of Notch signaling. The role of Notch signaling in formation and remodeling of the vasculature is reported in many publications. Indeed, mice with defects in genes encoding Notch, Notch ligands, and components of the Notch signaling cascade invariably display vascular defects (Iso et al., 2003). Furthermore, either smooth muscle or endothelial cells greatly increase the expression of Notch1, Jagged1, and Jagged2 (Lindner et al., 2001) after balloon catheter denudation of rat carotid arteries and aorta. Our results merge FGF1, Notch, and thrombin signaling pathways. The crosstalk between these pathways may have an important role in vascular repair and remodeling.

The mechanism involved in FGF1 translocation from the cytosol to the extracellular compartment remains to be determined. We previously demonstrated that at stress, FGF1 is released into the extracellular

compartment as a multiprotein complex including p40 Synaptotagmin1, S100A13, and a covalent Cys30-mediated FGF1 homodimer (Prudovsky et al., 2003). The Cu^{2+} ions are required for the assembly of this complex; however, the machinery that mediates the membrane translocation of these proteins remains unknown. Translocation of proteins across the lipid bilayer might require conformational changes that increase hydrophobicity. It was demonstrated that FGF1 is able to permeabilize membranes composed of acidic phospholipids, phosphatidylserine (pS), and phosphatidylglycerol (Mach and Middaugh, 1995). Moreover, FGF1, Synaptotagmins, and S100 proteins bind to pS, an acidic phospholipid (Donato, 1999; Tarantini et al., 1995) known to flip from the inner to outer leaflet of the lipid bilayer in response to stress (Arduini et al., 1989), suggesting that transmembrane translocation of FGF1 may be due to the interaction with acidic phospholipids. As mentioned in the introduction, Annexin 2, a protein known to exhibit a stress-induced flip-flop through the cell membrane (Kim and Hajjar, 2002), and to be associated with FGF1 in brain-derived FGF1-containing multiprotein complex (Soldi, R., Prudovsky, I. and Maciag, T. unpublished results) may participate in the export of the FGF1 release complex. However, it remains to be elucidated whether thrombin induces FGF1 translocation through a mechanism similar to FGF1 stress-induced release or through an alternative mechanism.

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Figure Legends

Fig. 1. Thrombin induces FGF1 expression. The expression of *fgf1* in thrombin-treated NIH 3T3 cells was evaluated by nuclear run-on analysis. Cells were harvested at various intervals after the addition of thrombin; the nuclei were isolated and the transcription rate for *fgf1* gene was determined as described (LaVallee et al., 1998). The bar graphs represent the normalized ratio of [^{32}P]-labeled *fgf1* to the *gapdh* transcript ± s.e.m. Mouse brain endothelial cells (MBEC) that express endogenous FGF1 served as a positive control.

Fig. 2. Thrombin induces non-classical release of FGF1. (A) Thrombin induces rapid release of FGF1 at 37°C. FGF1 immunoblot analysis was used to identify the presence of FGF1R136K in media conditioned by FGF1R136K NIH 3T3 cell stable transfectants at 37°C, following the addition of thrombin for 5, 15, 30, and 60 minutes. Media conditioned in the absence of thrombin at 37°C or by heat shocked cells (42°C, 110 minutes) served respectively, as negative and positive controls. The cell lysate (CL) from these cells is shown in the left panel (1/10 of total cell lysate was loaded). Bar graphs represent the percentage of FGF1 released from FGF1R136K NIH 3T3 cell transfectants upon thrombin treatment. Densitometric gel analysis was used to quantify FGF1 release. The densitometric values for different time points were normalized for total FGF1 expression levels. Each bar represents the mean of the normalized FGF1

release ± s.e.m. from three independent experiments. (B) Thrombin-induced FGF1 release is dose-dependent. FGF1 immunoblot analysis of media conditioned by FGF1R136K NIH 3T3 transfectants in response to 15 minutes treatment with 0.05, 0.25, 0.5, and 1 U/ml thrombin at 37°C is shown. (C) Thrombin induces redistribution of FGF1 to the cell membrane. Top panel: FGF1:HA cell transfectants stimulated for 30 minutes with thrombin (b) or untreated (a) were fixed and processed for immunofluorescence microscopy as described in "Material and Methods". Confocal images of median horizontal cell sections were taken using the 100X objective. Bar, 20 μm. Lower panel: FGF1R136K NIH 3T3 cells transfectants were treated with or without 1U/ml thrombin for 30 minutes, and cytosol and membrane fractions were prepared as described in the "Matherial and Methods".

Fig. 3. A dnFGFR1 mutant inhibits thrombin-induced DNA synthesis in Swiss 3T3 cells. Quiescent Swiss 3T3 cells transiently transfected with a FGFR1 deletion mutant that lacks the intracellular domain were stimulated with either 10% BCS (serum), 10 ng/ml FGF1 plus 10 U/ml of heparin or thrombin for 24 hours. DNA synthesis in control and dnFGFR1$^+$ cells was determined using [^{3}H]-thymidine incorporation assay combined with anti-FGFR1 immunoperoxidase staining, as described in "Materials and Methods". "Q" refers to quiescent cells.

Fig. 4. Thrombin cleaves Jagged1. (A) In vitro translated human Jagged1 is cleaved by thrombin. The FLJ1 transcript was in vitro translated in the presence of a [^{35}S]-Met/Cys mixture, and the translation product was incubated with or without thrombin for 15 minutes at 37°C. The reaction products were visualized by autoradiography. (B) Schematic diagram of FLJ1, FLJ1NV5, and sJ1 (39kDa). Top panel: Schematic diagram of FLJ1 and the position of the thrombin cleavage site. The Jagged1 amino acid sequence between residues 345 and 364 in context with the thrombin cleavage site (arrow) between the third and fourth EGF repeats is presented. Asterisks identify Cys residues utilized in the identification of the NH_2-terminal thrombin cleavage product by automated Edman sequencing of the [^{35}S]-Cys/Met-labeled Jagged1 translation product. Middle panel: The FLJ1NV5 construct sequence showing the V5 tag insert between the signal peptide and the DSL domain of the Jagged1. Lower panel: The sJ1 (39kDa)

deletion mutant corresponding to the thrombin cleavage product of the human Jagged1. Domains are as follows:
 : Signal Peptide,  : DSL domain,  : EGF Repeat domain,  : Cys-Rich (CR) domain, and  : transmembrane domain.

(C) Thrombin cleaves Jagged1 expressed in HEK 293 cells. HEK 293 cells were transfected with the FLJ1NV5 adenovirus. Forty-eight hours after transfection, the cells were treated either with thrombin or thrombin plus hirudin for 1 hour at 37°C. Control FLJ1NV5-transduced cells were incubated in serum-free media without thrombin. Cleaved Jagged1 was immunoprecipitated from the conditioned medium by utilizing anti-V5 antibodies. Immunoprecipitated 39 kDa Jagged1 fragment was visualized, using anti-V5 immunoblotting. The corresponding cell lysates from the FLJ1NV5 transfectant HEK 293 cells are shown on the top panel.

Fig. 5. The Jagged1 thrombin cleavage product (39kDa fragment) displays biological activities similar to sJ1 (117kDa). (A) sJ1 (39kDa) decreases the CSL-mediated transcription in NIH 3T3 transfectants. Vector control, FLJ1NV5, sJ1 (117kDa), and sJ1 (39kDa) NIH 3T3 stable transfectants were transiently cotransfected with luciferase and renilla constructs. The assay of CSL-mediated transcription was performed, as described in the "Materials and Methods". Renilla activity served as internal control for transfection efficiency. The data represent the normalized ratio of luciferase to renilla activity ± s.e.m. (B) sJ1 (39kDa) induces FGF1 expression. RT-PCR (top) and Q-RT-PCR (bottom) on total RNA extracted from untransfected NIH 3T3 cells, vector control, sJ1 (117kDa), and sJ1 (39kDa) transfectants were performed to assay *fgf*1 expression by using primers and conditions described in the "Materials and Methods". *gapdh* served as mRNA loading control. (C) sJ1 (39kDa) induces FGF1 release under normal growth conditions. Immunoblot analysis of FGF1 export into the extracellular compartment by vector control and sJ1 (39kDa) stable NIH 3T3 tranfectants transduced with FGF1 adenovirus for 48 hours, and then subjected to heat shock or maintained under normal growth conditions for 2 hours. Bar graphs represent the percentage of FGF1 released as described in Fig. 2A.

Fig. 6. Role of Notch signaling suppression in thrombin-induced FGF1 release and expression. (A) Thrombin attenuates CSL-dependent transcription in Jagged1 NIH 3T3 cell transfectants. Full-length Jagged1 transfectants were treated with thrombin or TRAP for 12 hours before and 48 hours after luciferase and renilla cotransfection. CSL1-mediated transcription was assayed, as described in the "Materials and Methods". The bar graphs represent the normalized ratio of luciferase to renilla activity, ± s.e.m. as a function of the concentration of thrombin or TRAP. The results from unstimulated NIH 3T3 cells served as a control. (B) Thrombin-induced release of FGF1 is repressed by the expression of constitutively active Notch1 (caN1). FGF1R136K NIH 3T3 cell transfectants were adenovirally transduced with caN1; the levels of the FGF1 in media conditioned by the addition of thrombin were assessed using immunoblot analysis. β-galactosidase-transduced cells were used as a control. The representative cell lysate (CL) from FGF1R136K-transduced cells is shown in the left panel. Bar graphs represent the percentage of FGF1 released as described in Fig. 2A. (C) Thrombin-induced expression of FGF1 is repressed by caN1. NIH 3T3 cells transduced with caN1 adenovirus and vector control were treated for 30 minutes with thrombin. RT-PCR experiments were performed as described in the "Materials and Methods". *gapdh* served as mRNA loading control.

Fig. 7. The role of PAR1-mediated signaling in the thrombin-induced FGF1 expression and release. (A) PAR1 mediates rapid thrombin-induced release of FGF1. FGF1 immunoblot analysis of media conditioned by PAR1 null or PAR1-transfected PAR1 null cells adenovirally transduced with FGF1R136K at 5 and 60 minutes, after the addition of thrombin is presented. FGF1 immunoblot analysis of media conditioned by heat shocked PAR1 null cells served as a positive control. The cell lysates (CL) from PAR1 null and control PAR1-transfected embryonic fibroblast are shown (left panel). Bar graphs represent the percentage of FGF1 released, as described in Fig. 2A. (B) TRAP rapidly induces FGF1 release. FGF1 immunoblot analysis of media conditioned by FGF1R136K NIH 3T3 cell transfectants in response to the addition of TRAP for 5 minutes is presented. The release of FGF1R136K in response to heat shock in the absence of TRAP, and a representative cell lysate (CL) are also shown. Bar graphs

represent the percentage of FGF1 released, as described in Fig. 2A (C) TRAP rapidly induces FGF1 expression. The expression of *fgf1* in TRAP-treated NIH 3T3 cells was evaluated by nuclear run-on analysis, as described for thrombin treatment (Fig.1). Unstimulated NIH 3T3 cells served as a negative control for FGF1 expression. The bar graphs represent the normalized ratio of [^{32}P]-labeled *fgf1*to the *gapdh* ± s.e.m.

Fig. 8. Long-term thrombin incubation induces FGF1 release from PAR1 null mouse embryonic fibroblasts, and overexpression of Jagged1 accelerates FGF1 release from PAR1 null cells in response to thrombin. (A) Jagged1 expression in PAR1 null mouse embryonic fibroblasts. The expression of *Jagged1* in PAR1 null, PAR1 wt control mouse embryonic fibroblast and NIH 3T3 cells was determined by RT-PCR, using primers and conditions described in the "Materials and Methods". *gapdh* served as control for mRNA loading. (B) Long-term thrombin incubation induces FGF1 release from PAR1 null cells. PAR1 null cells were stimulated either with thrombin or TRAP for 2 or 48 hours. Conditioned media were collected and added for 2 hours to PAR1 null cells transduced with FGF1R136K adenovirus. Detection of FGF1 in the conditioned media was performed as described in the "Materials and Methods". The cell lysate (CL) from these cells is shown in the left panel. Bar graphs represent the percentage of FGF1 released as described in Fig. 2A. (C) Short-term thrombin stimulation induces FGF1 release from PAR1 null cells overexpressing Jagged1. PAR1 null cells transduced with FLJ1NV5 or β–galactosidase adenoviruses for 48 hours were stimulated with thrombin for 2 hours. Conditioned media were collected and added for 2 hours to PAR1 null mouse embryonic fibroblasts transduced with FGF1R136K. Detection of FGF1 in the conditioned medium was performed, as described in the "Materials and Methods". The representative lysate (CL) from these cells is shown in the left panel. Bar graphs represent the percentage of FGF1 released as described in Fig. 2A.

Figure 1

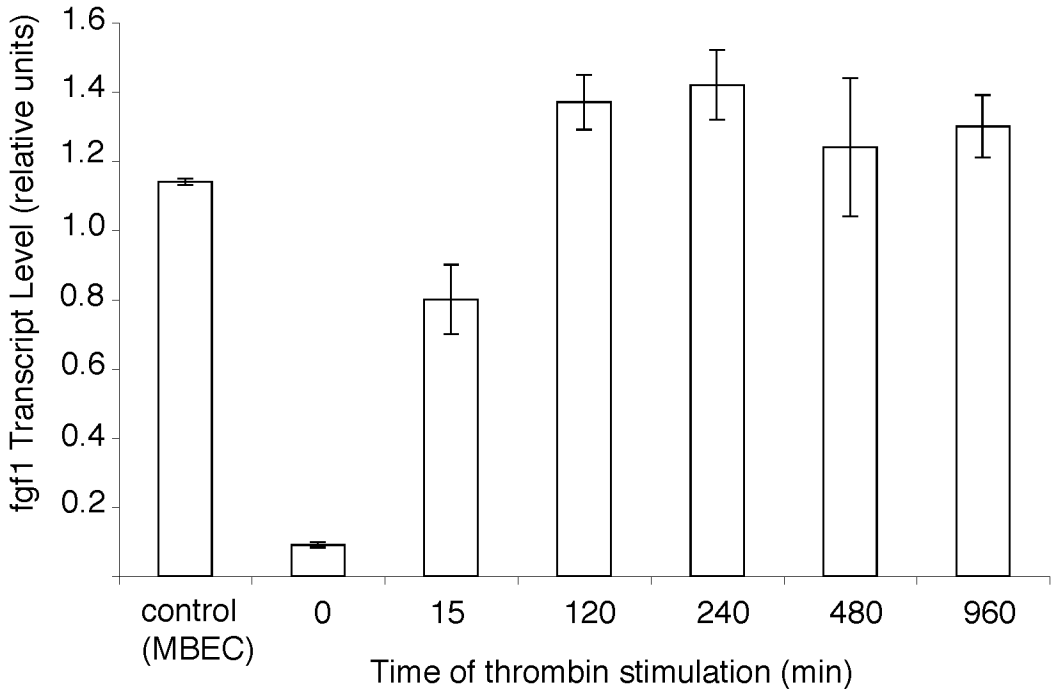

fgf1 Transcript Level (relative units)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
control (MBEC)
0
15
120
240
480
960
Time of thrombin stimulation (min)

Figure 2

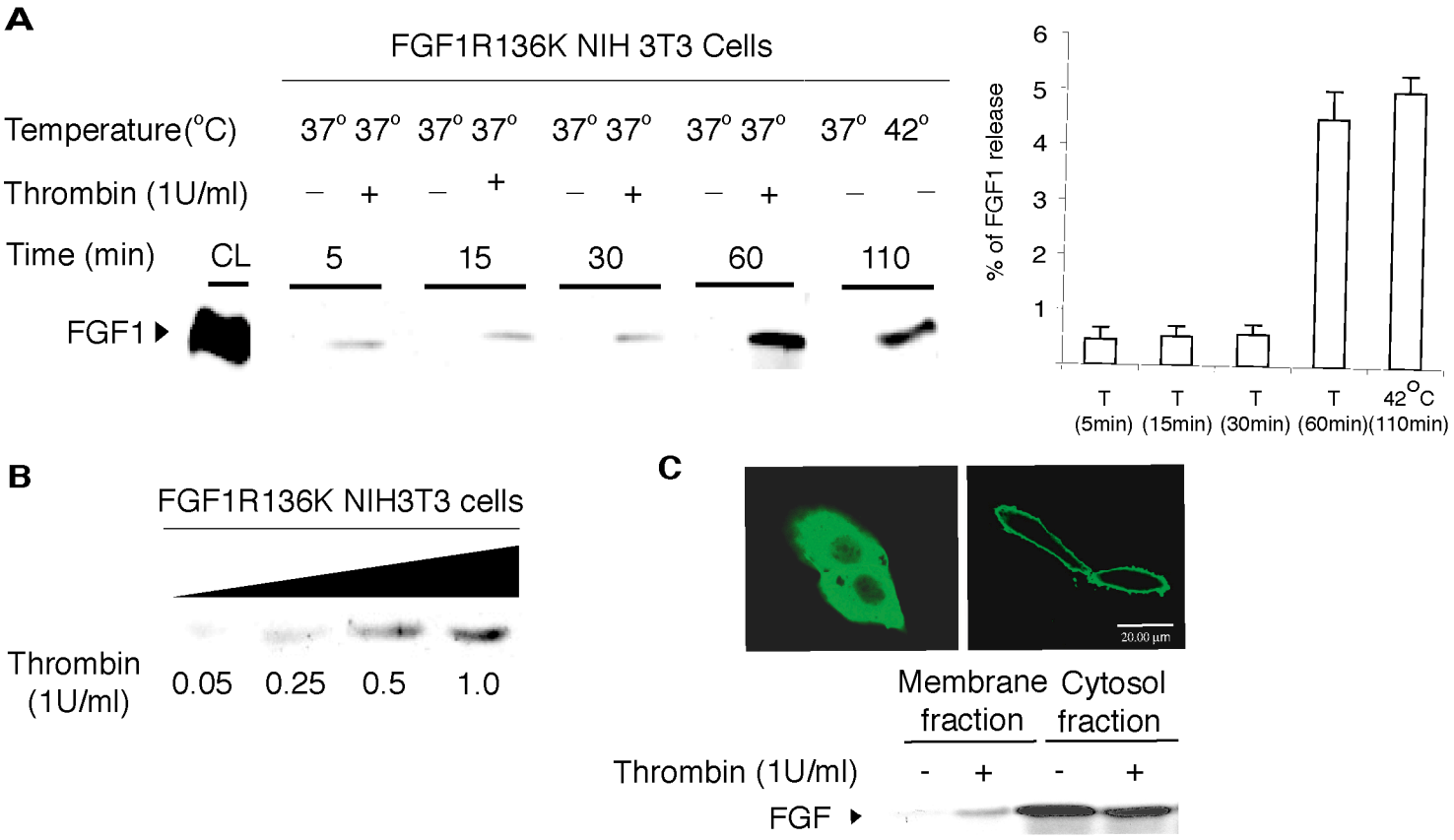

A
FGF1R136K NIH 3T3 Cells
Temperature(°C)
37° 37° 37° 37° 37° 37° 37° 37° 37° 42°
Thrombin (1U/ml)
– + – + – + – + – –
Time (min)
CL 5 15 30 60 110
FGF1
% of FGF1 release
6
5
4
3
2
1
T (5min) T (15min) T (30min) T (60min) 42°C (110min)
B
FGF1R136K NIH3T3 cells
Thrombin (1U/ml)
0.05 0.25 0.5 1.0
C
20.00 µm
Membrane fraction
Cytosol fraction
Thrombin (1U/ml)
- + - +
FGF

Figure 3

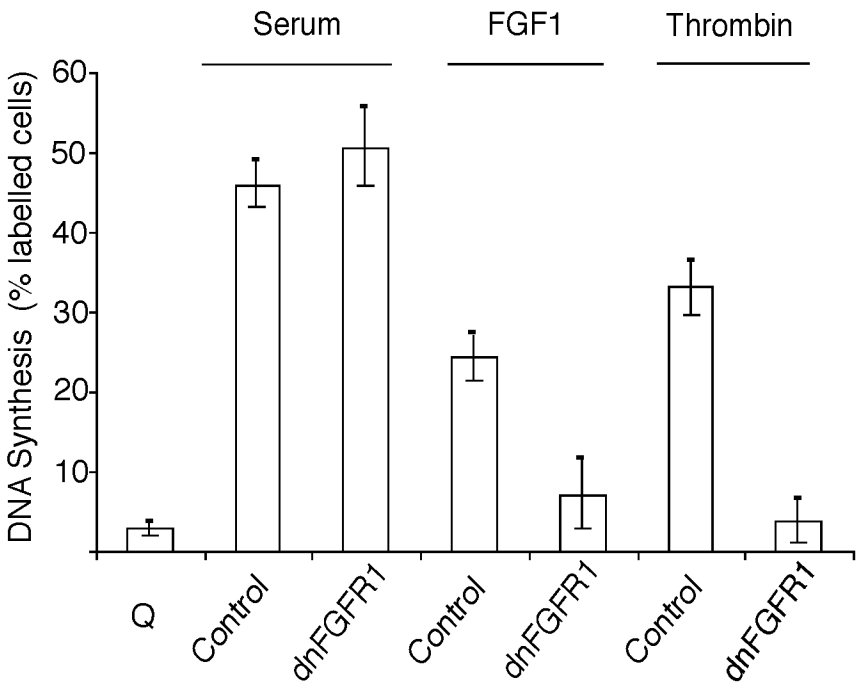

Serum
FGF1
Thrombin
DNA Synthesis (% labelled cells)
60
50
40
30
20
10
Q
Control
dnFGFR1
Control
dnFGFR1
Control
dnFGFR1

Figure 4

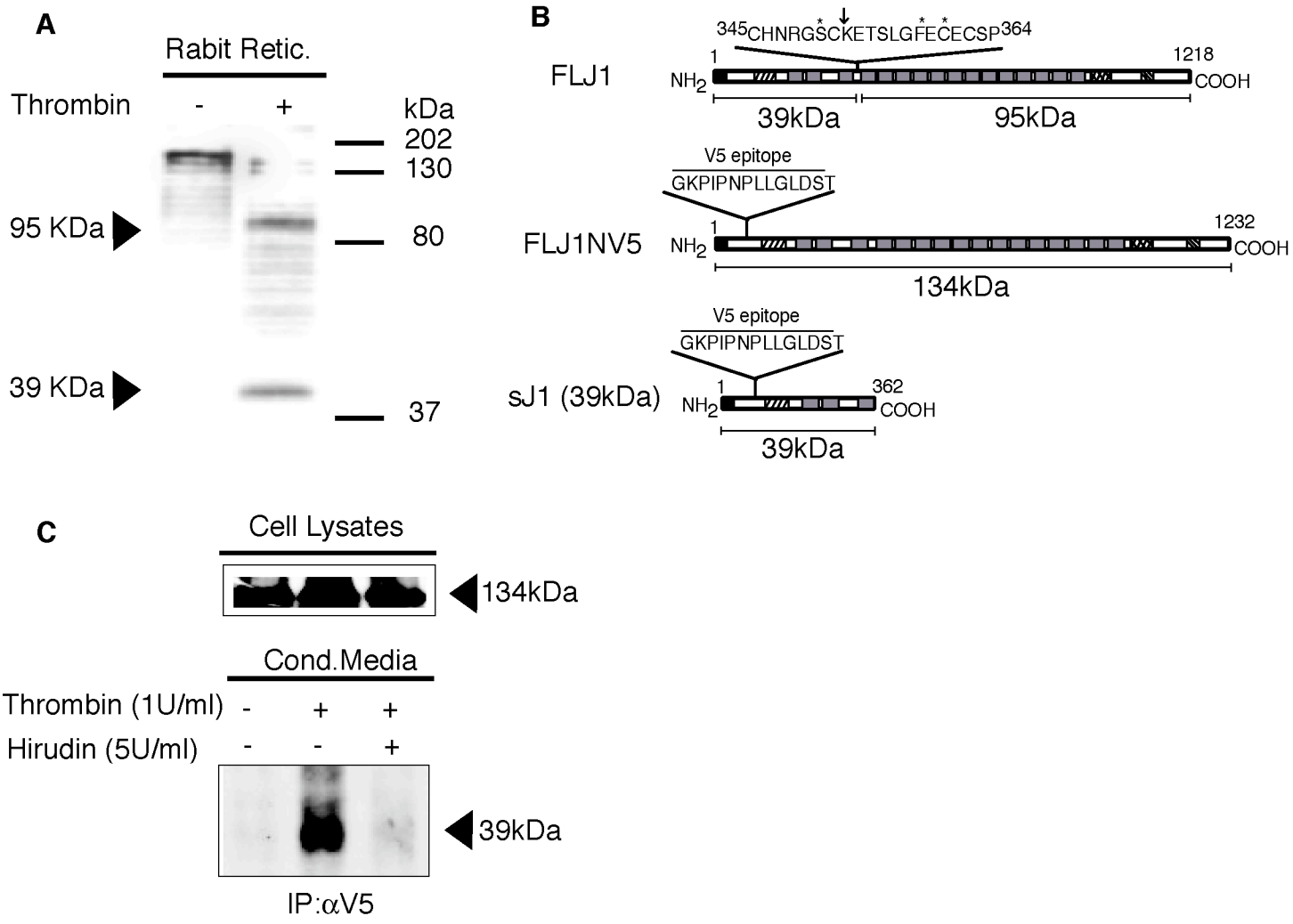
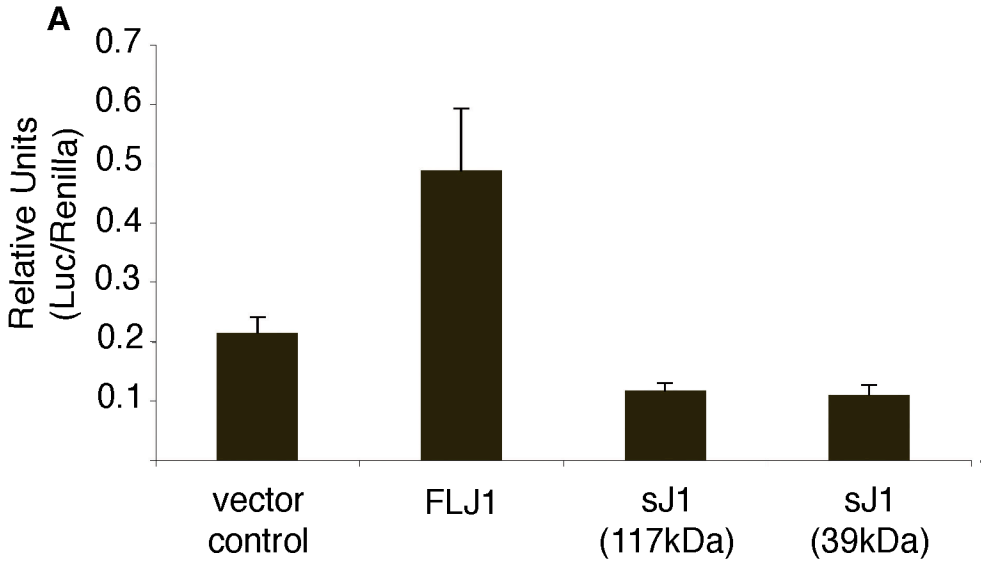
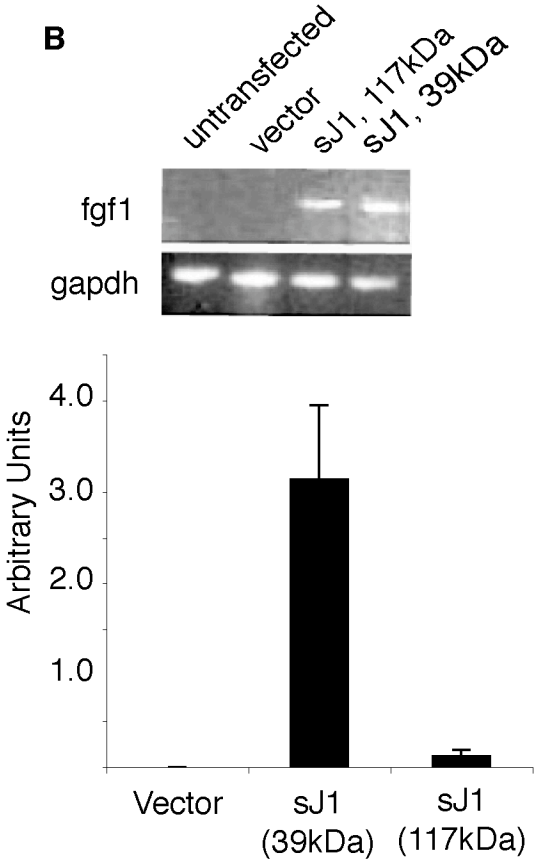

A
Rabit Retic.
Thrombin
-
+
kDa
202
130
95 KDa
80
39 KDa
37
B
345CHNRGSCKETSLGFECECSP364
FLJ1
1
1218
NH2
COOH
39kDa
95kDa
V5 epitope
GKPIPNPLLGLDST
FLJ1NV5
1
1232
NH2
COOH
134kDa
V5 epitope
GKPIPNPLLGLDST
sJ1 (39kDa)
1
362
NH2
COOH
39kDa
C
Cell Lysates
134kDa
Cond.Media
Thrombin (1U/ml)
-
+
+
Hirudin (5U/ml)
-
-
+
39kDa
IP:αV5

Figure 5


A
Relative Units (Luc/Renilla)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
vector control
FLJ1
sJ1 (117kDa)
sJ1 (39kDa)


B
untransfected
vector
sJ1, 117kDa
sJ1, 39kDa
fgf1
gapdh
Arbitrary Units
4.0
3.0
2.0
1.0
Vector
sJ1 (39kDa)
sJ1 (117kDa)

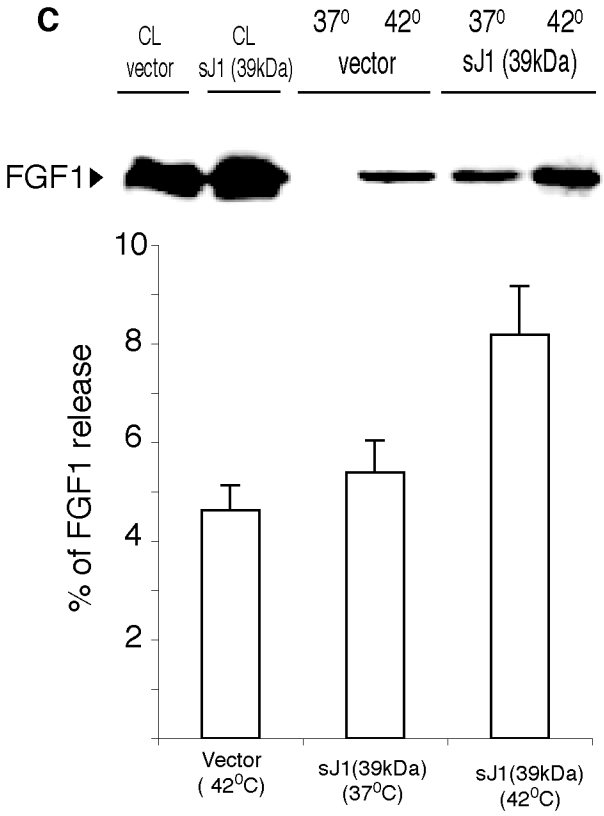

C
CL vector
CL sJ1 (39kDa)
37⁰
42⁰
vector
37⁰
42⁰
sJ1 (39kDa)
FGF1
% of FGF1 release
10
8
6
4
2
Vector (42⁰C)
sJ1(39kDa) (37⁰C)
sJ1(39kDa) (42⁰C)

Figure 6

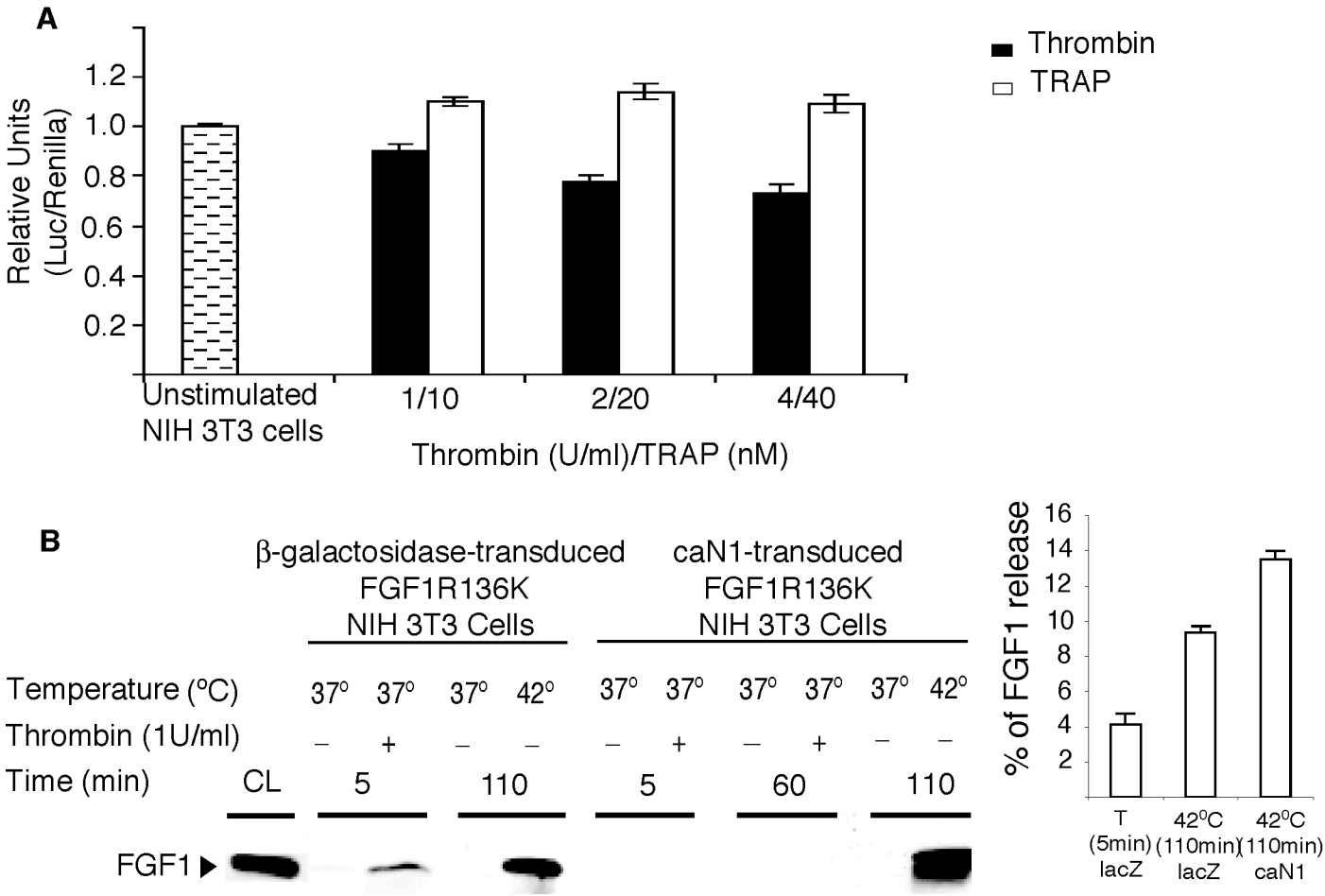

A
Thrombin
TRAP
Relative Units (Luc/Renilla)
1.2
1.0
0.8
0.6
0.4
0.2
Unstimulated NIH 3T3 cells
1/10
2/20
4/40
Thrombin (U/ml)/TRAP (nM)
B
β-galactosidase-transduced FGF1R136K NIH 3T3 Cells
caN1-transduced FGF1R136K NIH 3T3 Cells
Temperature (°C)
37° 37° 37° 42° 37° 37° 37° 37° 37° 42°
Thrombin (1U/ml)
– + – – – + – + – –
Time (min)
CL 5 110 5 60 110
FGF1
% of FGF1 release
16
14
12
10
8
6
4
2
T (5min) lacZ
42°C (110min) lacZ
42°C (110min) caN1
C
3T3/caN1
3T3/Vector Control
Thrombin
- + - +
fgf1
gapdh

Figure 7

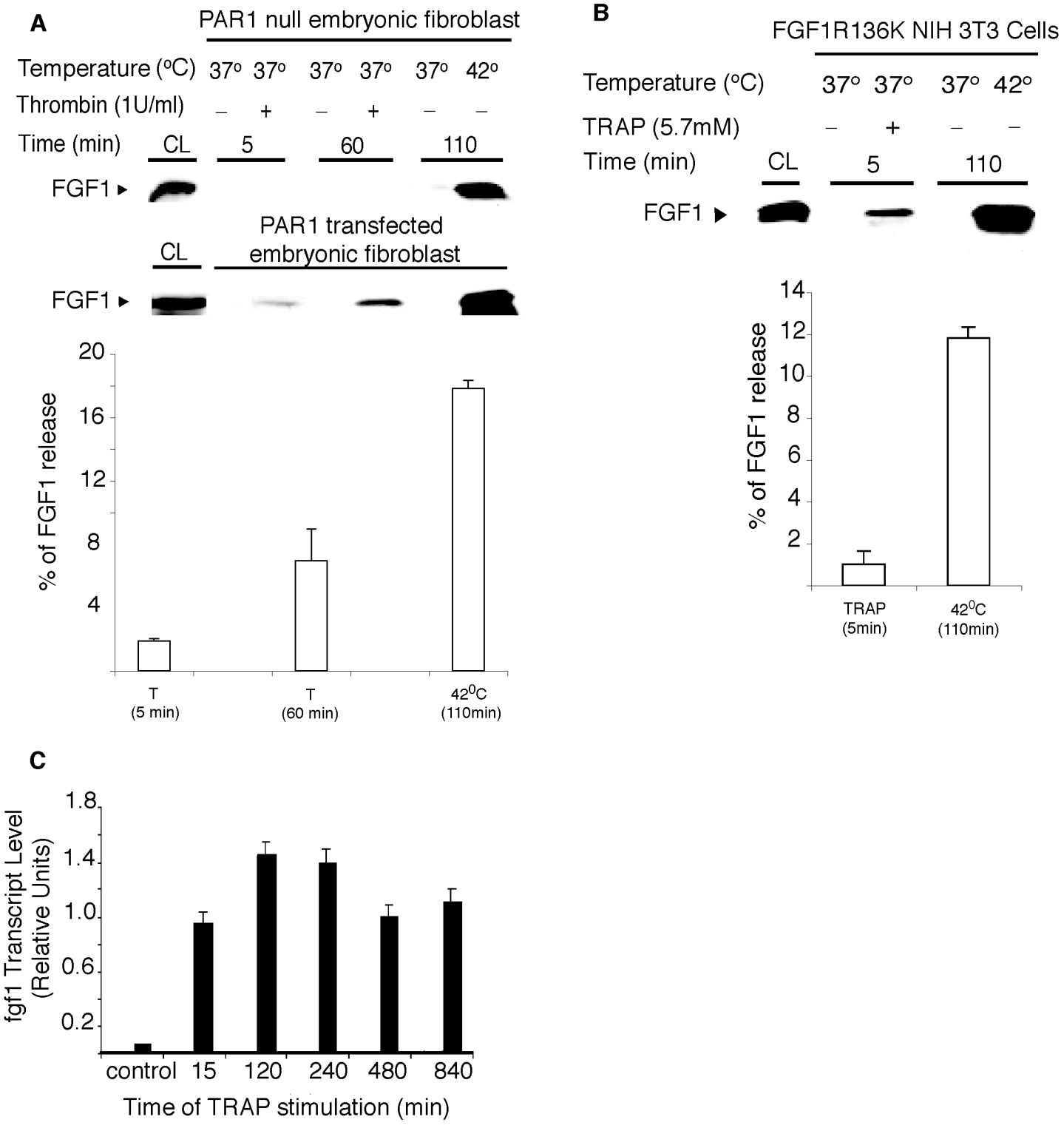
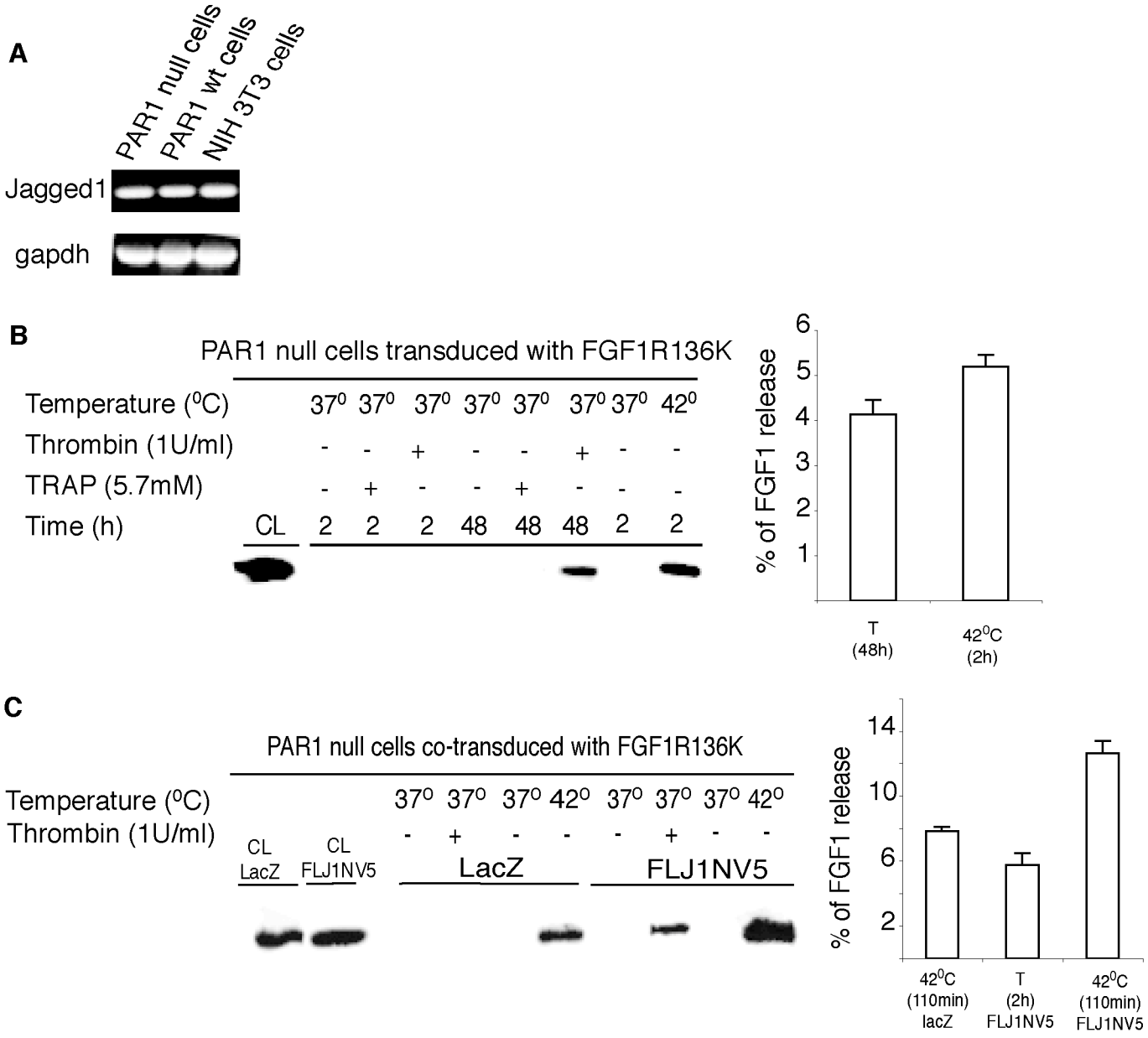

A
PAR1 null embryonic fibroblast
Temperature (°C) 37° 37° 37° 37° 37° 42°
Thrombin (1U/ml) – + – + – –
Time (min) CL 5 60 110
FGF1 ▸
PAR1 transfected embryonic fibroblast
CL
FGF1 ▸
% of FGF1 release
20
16
12
8
4
T (5 min)
T (60 min)
42°C (110min)
B
FGF1R136K NIH 3T3 Cells
Temperature (°C) 37° 37° 37° 42°
TRAP (5.7mM) – + – –
Time (min) CL 5 110
FGF1 ▸
% of FGF1 release
14
12
10
8
6
4
2
TRAP (5min)
42°C (110min)
C
fgf1 Transcript Level (Relative Units)
1.8
1.4
1.0
0.6
0.2
control 15 120 240 480 840
Time of TRAP stimulation (min)

Figure 8


A
PAR1 null cells
PAR1 wt cells
NIH 3T3 cells
Jagged1
gapdh
B
PAR1 null cells transduced with FGF1R136K
Temperature (°C) 37° 37° 37° 37° 37° 37° 37° 42°
Thrombin (1U/ml) - - + - - + - -
TRAP (5.7mM) - + - - + - - -
Time (h) CL 2 2 2 48 48 48 2 2
% of FGF1 release
6
5
4
3
2
1
T (48h)
42°C (2h)
C
PAR1 null cells co-transduced with FGF1R136K
Temperature (°C) 37° 37° 37° 42° 37° 37° 37° 42°
Thrombin (1U/ml) - + - - - + - -
CL LacZ
CL FLJ1NV5
LacZ
FLJ1NV5
% of FGF1 release
14
10
6
2
42°C (110min) lacZ
T (2h) FLJ1NV5
42°C (110min) FLJ1NV5

Paper 4

"The protease activated receptor (PAR) 1 selective antagonist SCH79797 inhibits cell proliferation at low doses and induces apoptosis at high doses, by a PAR1-independent mechanism"

Claudia Di Serio, Silvia Pellerito, **Maria Duarte**, Daniela Massi, Antonella Naldini, Isabella Micucci, Laura Doria, Patrizia Mirone, Giuseppe Cirino, Igor Prudovsky, Marco Santucci, Pierangelo Geppetti, Giulio Masotti, Francesca Tarantini

Journal of Pharmacology and Experimental Therapeutics
(Manuscript under revision)

To: Journal of Pharmacology and Experimental Therapeutics

The protease activated receptor (PAR) 1 selective antagonist SCH79797 inhibits cell proliferation at low doses and induces apoptosis at high doses, by a PAR1-independent mechanism.

Claudia Di Serio[1], Silvia Pellerito[1], Maria Duarte, Daniela Massi, Antonella Naldini, Isabella Micucci, Laura Doria, Patrizia Mirone, Giuseppe Cirino, Igor Prudovsky, Marco Santucci, Pierangelo Geppetti, Niccolò Marchionni, Giulio Masotti, Francesca Tarantini.

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Running title: SCH79797 inhibits cell growth and induces cell death.

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E-mail: taranf@unifi.it

[1] Claudia Di Serio and Silvia Pellerito participated equally to the study.

This study was financially supported by grants from MIUR (PRIN 2004) to G.C. (2004063945) and to M.S. (2004062950).

Text pages: *24*

Figures: *6*

Number of References: *35*

Number of words in the Abstract: *220*

Number of words in the Introduction: *535*

Number of words in the Discussion: *933*

Abbreviations: PAR, protease activated receptor; FGF, fibroblast growth factor; PDGF, platelet derived growth factor; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CL, cell lysate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MAPK, mitogen activated protein kinase.

Recommended section assignment: *Cellular and Molecular*

Abstract

Thrombin, a key mediator of blood coagulation, exerts a large series of cellular actions via activation of a specific G-protein coupled receptor, named protease activated receptor 1 (PAR1). Several studies in experimental animals have demonstrated the therapeutic potential of small molecules, with PAR1 antagonistic properties, in treating diseases such as vascular thrombosis and arterial restenosis. We studied the activity and specificity of one highly potent, selective PAR1 antagonist, SCH79797, *in vitro*. We found that this compound was able to interfere with the growth of several human and mouse cell lines (A375, NIH 3T3, HEK 293, B16/F10), in a concentration-dependent manner. In HEK 293 and NIH 3T3 cells, the ED_{50} for the anti-proliferative effect was 81 nM and 75 nM, respectively. The anti-proliferative effect was mediated by the ability of SCH79797 to inhibit serum-stimulated activation of p44/p42 Mitogen Activated Protein Kinase (MAPK). In addition, at higher doses SCH79797 induced apoptosis in NIH 3T3 cells. The anti-proliferative and pro-apoptotic effects of SCH79797 were obtained with concentrations very close to the reported IC_{50} for PAR1 inhibition (70 nM). However, these biological effects were not mediated by PAR1 antagonism, as they were also observed in embryonic fibroblasts derived from PAR1 null mice. In view of the development of PAR1 selective antagonists as therapeutic agents, effects potentially unrelated to PAR1 inhibition should be carefully scrutinized.

Introduction

Thrombin, a trypsin-like serine protease, is the most potent agonist for platelet aggregation and plays a central role in haemostatic processes (Strassen et al., 2004). Thrombin catalyzes the conversion of fibrinogen to fibrin by cleaving the peptide bond between an arginine and a glycine residue in the fibrinogen sequence (Walsh, 2004); it is also responsible for proteolytic activation of factors V, VIII, XI, XIII and protein C (Strassen et al., 2004). However, in addition to its role in blood coagulation, thrombin also stimulates mitogenic events in several cell types including fibroblasts, smooth muscle cells and astrocytes (Narayanan, 1999), therefore playing a central role in tissue repair, fibrosis, inflammation, neurodegeneration, atherosclerosis and restenosis (Derian et al., 2002; Junge et al., 2003; Suo et al., 2004; Viles-Gonzales et al., 2005).

All cellular actions of α-thrombin are mediated by specific G-protein coupled receptors, named protease activated receptors (PAR). Activation of PARs by thrombin and other trypsin-like serine proteases is based upon a novel mechanism: the protease cleaves part of the N-terminus domain of the receptor, releasing a "tethered ligand" that subsequently binds to an extracellular loop of the receptor and activates the G-protein coupled signal transduction (Trejo, 2003). Four protease activated receptors have now been cloned (Ossovskaya and Bunnet, 2003); in humans, PAR1 is considered the primary α-thrombin receptor, although thrombin can activate also PAR-3 and PAR-4 (Kataoka et al., 2003). Thrombin cleaves PAR1 between Arg_{41} and Ser_{42}, releasing an N-terminal peptide chain that carries the recognition motif "SFLLRN" (Coughlin, 1993).

Several small molecules capable of blocking α-thrombin active site have been characterized over the years as anti-thrombotic agents, starting with hirudin, a natural leech-derived peptide (Walsmann, 1991). However, the identification of the many

biological actions of α-thrombin mediated by PARs has encouraged the quest for compounds capable of blocking receptor activation without inhibiting thrombin protease activity. These compounds would still modulate platelet activation and aggregation but with minimal bleeding side-effects.

Potent and selective PAR1 antagonists have been synthesized in the last few years (Bernatowicz et al., 1996; Andrade-Gordon et al., 1999; Zhang et al., 2001). These non-peptide small molecules are designed to mimic the spatial constraints of the PAR1 recognition motif (SFLLRN) and interfere with the intra-molecular binding of the tethered-ligand to the receptor. PAR1 antagonists are now proposed as therapeutic options in several human diseases including thrombosis, atherosclerosis and restenosis after angioplasty (Ahn et al., 2003). Preclinical studies demonstrated that perivascular administration of a selective PAR1 antagonist significantly reduced neointimal thickness after balloon angioplasty in a rat model of restenosis (Andrade-Gordon et al., 2001) and intravenous administration of PAR1 antagonist prevented thrombotic occlusion of carotid arteries in a nonhuman primate model of vascular injury (Derian et al., 2003).

In view of their clinical application it is necessary to examine the full spectrum of activities of these compounds. Here we studied the cellular actions of a commercially available PAR1 selective antagonist SCH79797 (N-cyclopropyl-7-[4-(1-methylethyl)phenyl]-7*H*-pyrrolo [3,2-*f*] quinazoline-1,3-diamine dihydrochloride) and demonstrated that this molecule has biological effects that are unrelated to PAR1 inhibition. At low doses, SCH79797 directly affected cell proliferation stimulated by serum or growth factors other than α-thrombin, in several human and mouse cell lines, including PAR1 null cells. At higher doses, this compound was able to induce apoptosis.

Methods

Materials: fibroblast growth factor (FGF)-2 was obtained from SIGMA-Aldrich; platelet derived growth factor (PDGF) was purchased from PeproTech (PeproTech EC, London). Human recombinant FGF-1 was prepared as previously described (Tarantini et al., 1995). SCH79797 (N^3-Cyclopropyl-7- [[4-(1-methylethyl)phenyl]methyl] -7*H*-pyrrolo [3,2-*f*] quinazoline-1,3-diamine hydrochloride) (Ahn et al., 2000) was purchased from Tocris (Tocris Bioscience, Ellisville, MO, USA) and dissolved in 50 mM DMSO. Two different batches were used in the experiments (No. 1A/57193 and No. 1A/61853). Anti- p44/42 MAP kinases (total MAPK) and p44/42 phospho-MAPK (phospho Ser_{202}/Tyr_{204}) antibodies and anti-caspase-3 antibodies, directed against the large 35 kDa and the small 17 kDa fragments of caspase-3, were from Cell Signaling Technology (Beverly, MA, USA).

Cell culture*:* mouse NIH 3T3 and B16/F10 cells, and human HEK 293 and A375 cells, were obtained from ATCC (Rockville, MD, USA); PAR1 null mouse embryonic fibroblasts were a generous gift of Dr. S. Coughlin (University of California, S. Francisco) and were previously characterized (Connolly et al., 1996). All cells were grown in Dulbecco's modified Eagle's medium (DMEM) (EuroClone), supplemented with 10% (vol/vol) fetal bovine serum (FBS) (EuroClone), L-glutamine (EuroClone) and 1% (vol/vol) antibiotic/antimycotic solution (Gibco, Invitrogen S.R.L).

Cell proliferation assay: all cell lines were seeded at low density ($4x10^4$ cells/well) in 6-well cluster plates (Falcon). The next day, the cells were washed 3 times with PBS and than starved for 24 hours in DMEM containing 1% FBS. The next day the medium was changed with DMEM supplemented with 10% FBS, with PAR1 antagonist or vehicle alone (DMSO). When the cultures were stimulated with growth factors, the cells were starved for 24 hours in DMEM without serum; the next day the medium was supplemented with 25 ng/ml FGF-1, 25 ng/ml FGF-2 or 30 ng/ml PDGF. When FGF-1 and FGF-2 were

used to stimulate the culture, 10 U/ml heparin (Pfizer Italia S.r.l.) were added to the medium. Cells were fed every two days with fresh medium. When we studied the effect of cell density on SCH79797-mediated growth impairment, NIH 3T3 cells were plated at low ($4x10^4$ cells/well) or high ($2x10^5$ or $4x10^5$ cells/well) density in 6-well cluster plates and proliferation assay was carried out in 10% FBS, as described above. The number of viable cells was counted after trypsinization by hemacytometer at the indicated time, in triplicates, using two separate measurements per well. A statistical comparison between growth curves at each observation point was performed by using the Student *t* test. A value of $p<0.05$ was considered statistically significant.

RT-PCR: total RNA was extracted from embryonic fibroblasts isolated from PAR1 null mice ($PAR1^{-/-}$) and from $PAR1^{-/-}$ embryonic fibroblasts transfected with a mouse PAR1 gene construct ($PAR1^{+/+}$), using RNeasy kit (Quigen Inc. CA, USA), following manufacturer's instructions. Reverse transcription was performed with 1 μg total RNA, using the Platinum Taq One Step RT-PCR kit (Invitrogen). PCR amplification was performed with the following primers: *PAR1:* sense: 5'-CTGATTGGCAGTTCGGGTC-3', antisense: 5'-GAACAAAGCCCGCGACTTC-3'; *gapdh:* sense*:* 5'-CCACCCATGGCAAATTCCATGGCA -3, antisense: 5'-TCTAGACGGCAGGTCAGGTCCACC-3'. Amplification was performed by denaturation at 95°C for 45s, annealing at 54°C for 40s and elongation at 72°C for 110s, for 45 cycles.

Thymidine incorporation: $PAR1^{+/+}$ and $PAR1^{-/-}$ mouse embryonic fibroblasts were grown in 24-well cluster plates (Falcon) in 10% serum, until quiescence was started by exposing the cells to 0.25% serum for 48 hours. After quiescence was established, the culture was stimulated for 24 hours with fresh medium containing 0.25 % serum (quiescence), 20 ng/ml FGF-1 plus 10 U/ml heparin or 10% serum. ^{H3}thymidine at 1 mCi/ml was added to the media for 2 hours, and cells were processed for scintillation counting. A statistical

comparison between $PAR1^{+/+}$ and $PAR1^{-/-}$ cells at quiescence, 10% serum and FGF-1 stimulation was performed by using the Student *t* test.

Analysis of MAP kinase activation: a sub-confluent culture of NIH 3T3 cells was starved for 24 hours in DMEM without serum; the next day the cells were pre-treated with 150 nM SCH79797 or vehicle for 1 hour, then 10% FBS was added to the medium and the cells incubated for 5', 15' and 30', at 37^{o} C. At the indicated time points, cells were harvested and washed in ice-cold PBS, containing 1 mM sodium orthovanadate, 1 mM aprotinin and 2 mM leupeptin. Cells were then lysed in Triton X-100-based lysis buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, 2.5 mM NaPPi, 1% (vol/vol) Triton X-100, 5% (vol/vol) glycerol). Protein concentration was determined using BCA protein assay kit (Pierce, Rockford, IL, USA). Total protein samples (100 μg) were resolved by 10% (wt/vol) sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), transferred to a nitrocellulose membrane (Hybond C) (Amersham Pharmacia Biotech) and immunoblotted using anti-phospho MAPK polyclonal antibodies (1:1000) and goat anti-rabbit secondary antibodies (BioRad Laboratories S.r.l., Italy) (1:3000). Phospho-MAPK immunoreactive bands were visualized by chemiluminescence (ECL) (Amersham Pharmacia Biotech), following manufacturer's instructions. The membrane was then stripped and re-probed with anti-total MAPK polyclonal antibodies (1:1000) and goat anti-rabbit secondary antibodies (1:3000). The experiment was performed three times with similar results; the three immunoblots were scanned and quantitative image analysis performed using densitometry. Student's paired *t* test was used for statistical analysis.

Apoptosis detection: NIH 3T3 cells were seeded at low density ($4x10^4$ cells/well) in 6-well cluster plates (Falcon). The next day, the cells were washed 3 times with PBS and the medium was changed with DMEM supplemented with 10% FBS, with the indicated concentrations of PAR1 antagonist or vehicle alone (DMSO). At timed intervals, cells were resuspended with ice-cold PBS and centrifuge for 5' at 500xg, at 4°C. Cell pellets

were resuspended in ice-cold binding buffer and labelled with Annexin V-FICT solution and 7-AAD viability dye, following manufacturer's instructions (Beckman Coulter) and analyzed by flow cytometry (Coulter XL). Student's paired *t* test was used for statistical analysis.

Analysis of caspase-3 activation: a sub-confluent culture of NIH 3T3 cells was grown in DMEM containing 10% FBS, in the presence or absence of 300 nM SCH79797. At the indicated time points, cells were harvested and washed in ice-cold PBS, containing 1 mM aprotinin and 2 mM leupeptin. Cells were then lysed in Triton X-100-based lysis buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 1% (vol/vol) Triton X-100, 10% (vol/vol) glycerol). Protein concentration was determined using BCA protein assay kit. Total protein samples (100 μg) were resolved by 15% (wt/vol) SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted using anti-caspase-3 polyclonal antibodies (1:1000) and goat anti-rabbit secondary antibodies (1:3000). Caspase-3 immunoreactive bands were visualized by chemiluminescence, following manufacturer's instructions.

Results

PAR1 antagonist SCH79797 inhibits serum-dependent growth of several mouse and human cell lines:

NIH 3T3, A375, HEK 293 and B16/F10 plated at low density ($4x10^4$ cells/well), were grown in 10% serum, in the presence of increasing concentrations of PAR1 antagonist SCH79797. In all cell lines, SCH79797 produced a significant, concentration-dependent inhibition of serum-stimulated cell growth, compared to control cultures treated with vehicle alone (DMSO) (Figure 1). The ED_{50} for growth inhibition was 75 nM, 81 nM and 116 nM for NIH 3T3, HEK 293 and A375 cells, respectively; we also noticed that starting from 200 nM, SCH79797 induced a significant amount of cell death within the first 3 days in all cultures, except mouse melanoma cells, B16/F10, that were more resistant to the anti-proliferative effect (ED_{50} 160 nM) and to the pro-cell death effect of the antagonist and started to die at a concentration of 300 nM.

PAR1 antagonist SCH79797 inhibits proliferation of NIH 3T3 cells stimulated by FGF-1, FGF-2 and PDGF:

We also studied the effect of PAR1 antagonist on the growth rate of NIH 3T3 cells stimulated by three major growth factors, in serum-free medium: fibroblast growth factor (FGF)-1, FGF-2 and platelet-derived growth factor (PDGF) (Friesel and Maciag, 1999; Khachigian and Chesterman, 1992). Cells were plated at low density ($4x10^4$ cells/well) and FGF-1 (25 ng/ml), FGF-2 (25 ng/ml) or PDGF (30 ng/ml) were added to the medium, in the presence or absence of PAR1 antagonist. As shown in Figure 2A and B, the antagonist was able to inhibit cell proliferation stimulated by all three growth factors. To exclude the possibility that growth factors could indirectly induce activation of PAR1, thereby promoting cell proliferation, we stimulated the growth of $PAR1^{+/+}$ and $PAR1^{-/-}$ cells (Figure 3A) with FGF-1 and heparin. As shown in Figure 3B, $PAR1^{-/-}$ cells

responded to FGF-1 similarly to wild type cells, demonstrating that FGF-1-mediated thymidine incorporation was not dependent from activation of PAR1 receptors.

PAR1 antagonist SCH79797 inhibits proliferation of PAR1 null cells:

To confirm that the ability of the antagonist to block cell proliferation was not related to its PAR1 inhibitory action, we studied the effect of SCH79797 on the growth rate of embryonic fibroblasts isolated from PAR1 null ($PAR1^{-/-}$) mice (Connolly et al., 1996) (Figure 3C). As shown in Figure 3C, SCH79797 was able to slow the growth of $PAR1^{-/-}$ cells, in a concentration-dependent manner.

PAR1 antagonist SCH79797 inhibits serum-stimulated activation of p44/p42 MAPK in NIH 3T3 cells:

Since mitogen-activated protein kinase (MAPK) activity is essential for proliferation in many cell types (L'Allemain, 1994), we investigated whether SCH79797 was able to interfere with serum-stimulated induction of p44/p42 MAPK. NIH 3T3 cells, starved for 24 hours, were stimulated to grow by adding 10% serum, in the presence or absence of PAR1 antagonist. The cells were harvested at timed intervals (5', 15' and 30'). Because upon activation p44/p42 MAP kinases become phosphorylated on threonine and tyrosine residues, we analyzed the amount of phosphorylated p44/p42 compared to total protein content. As shown in Figure 4, the antagonist was able to significantly inhibit phosphorylation of p44/p42 MAPK; the inhibition was evident at 15' and was maintained at 30' time point.

PAR1 antagonist SCH79797 induces apoptosis in NIH 3T3 cells:

Since at high doses the antagonist induced cell death, we studied the ability of SCH79797 to trigger activation of caspase-3, an effector protein involved in regulation of apoptosis (Baker and Reddy, 1998). Activation of caspase-3 requires proteolytic processing of its p35 inactive zymogen into two small catalytic subunits, p19 and p17 (Fernandes-Alnemri et al., 1994). Once activated, caspase-3 is responsible for the

proteolytic cleavage of many key proteins of the apoptotic cascade, such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP). NIH 3T3 cells grown in the presence or absence of the inhibitor, were harvested at timed intervals (3, 6, 8, 12, 20 and 24 hours) and cell lysates analyzed for the presence of cleaved caspase-3. As demonstrated in Figure 5A, after 20 hours incubation with PAR1 antagonist, the small p17 catalytic subunit was detected inside the cells. No proteolytic modification of the pro-enzyme was noticed in vehicle control cells (DMSO).

In order to confirm that activation of caspase-3 was indeed related to programmed cell death, we studied apoptosis-associated phosphatidyl-serine (PS) exposure and 7-AAD viability in cells grown in the presence of the PAR1 antagonist compared to vehicle control cells. As shown in Figure 5B a statistically significant increase in the percentage of apoptotic cells was detected in cultures treated with 200 nM SCH79797 compared to vehicle (DMSO) (12.85±3 vs. 2.5±0.3 Annexin-V; 20.1±0.8 vs. 4.3±2 7-AAD).

The effect of PAR1 antagonist SCH79797 on NIH 3T3 cell growth and apoptosis is cell density dependent:

When we tested the effect of cell density on SCH79797-mediated growth inhibition, we found that the response of NIH 3T3 cells to the antagonist depended on the initial number of cells that were plated. Low density cultures ($4x10^4$ cells/well) were significantly inhibited at a concentration of 100 nM, whereas up to 200 nM concentration of the antagonist had no effect on high density cultures ($2x10^5$ cells/well) after 4 days (Figure 6A). However, NIH 3T3 cells plated at high density, still responded to an increase of 5 times in SCH79797 concentration (500 nM) (Figure 6A). The pro-apoptotic effect of PAR1 antagonist also depended on cell density (Figure 6B). NIH 3T3 cells plated at high density ($4x10^5$ cells/well) were insensitive to the effect of 300

nM SCH79797 and started to die only at a concentration 10 times higher (3 μM) (Figure 6B).

Discussion

Inhibition of thrombin receptor activation is a promising therapeutic approach for treatment of several major diseases such as stroke, myocardial infarction and restenosis after angioplasty (Andrade-Gordon et al., 2001; Derian et al., 2003; Ahn et al., 2003). Thrombin plays a central role in the cardiovascular system as it is the most potent agonist for platelet activation, triggers the coagulation cascade (Stassen et al., 2004) and stimulates vascular endothelial cells and smooth muscle cells, directly (Ossovskaya and Bunnet, 2003).

Starting from the sequence of the thrombin receptor activating peptide (TRAP), SFLLR-NH2, numerous peptidic and non peptidic antagonists have been synthesized in the last few years (Bernatowicz et al., 1996, Andrade-Gordon et al., 1999; Zhang et al., 2001). However, several limitations such as lack of potency and specificity, low affinity and partial agonist activity, have been reported for the majority of them. Only few PAR1 antagonists have been described in the literature as highly potent and specific (Elliott et al., 1999; Andrade-Gordon et al., 1999) and among them only SCH79797 [*N^3-Cyclopropyl-7-{[4-(1-methylethyl)phenyl]methyl}7H-pyrrolo[3,2-f]quinqzoline-1,3-diamine]*, is commercially available (Ahn et al., 1999; Ahn et al., 2000).

SCH79797 was characterized initially on human platelets as a competitive inhibitor of the PAR-1-selective agonist TRAP for binding to the receptor, with IC_{50} values of 70 nM. Functionally, SCH79797 blocked platelet aggregation induced by TRAP, but not by ADP or collagen (IC_{50} 300 nM), inhibited thrombin and TRAP-mediated thymidine-incorporation in human coronary artery smooth muscle cells (hCASMC), (K_i values of 82 and 55 nM, respectively), but it did not affect platelet aggregation induced by PAR-4 activating peptide and calcium mobilization induced by PAR-2 agonists in hCASMC.

These latter observations indicated selectivity of SCH79797 for PAR1 (Ahn et al., 2000). SCH79797 was also used to characterize the role of PAR1 in complex pathophysiological models, including angiogenesis (Ma et al., 2005), as it inhibited VEGF and endostatin release mediated by PAR1 activation. Consistent with a role of PAR1 in regulation of angiogenesis and wound healing, treatment with SCH79797 for one week in rats with established gastric ulcers, resulted in wound healing impairment (Ma et al., 2005).

We have demonstrated that SCH79797 has a remarkable anti-proliferative effect in numerous cell lines, from mouse to human. SCH79797 was able to reduce growth factor and serum-stimulated cell growth and serum-stimulated activation of p44/p42 MAPK. In addition, the anti-proliferative effect of SCH79797 seemed to depend on cell density: at higher density the cultures were less sensitive to the effect of the antagonist and required higher concentrations of SCH79797 to reach the same level of cell growth inhibition, suggesting the existence of a dilution effect in the presence of an increased number of functional binding sites. When the cells were grown in the presence of higher doses of the antagonist, we noticed a rapid change in cell shape which was suggestive of apoptosis (Jellinger, 2001). Indeed after 24 hours incubation with higher concentrations of the antagonist, we could detect activation of programmed cell death. Once more, induction of apoptosis was inversely associated to cell density, suggesting that it may depend from a dilution-related effect.

Because SCH79797 has been characterized as selective for PAR1 at the concentrations we used (Ahn et al., 2000; Ma et al., 2005), as it was unable to block PAR2, PAR3 and PAR4, and because cell density modulates the response of the culture to the antagonist, it is likely that SCH79797 interacts with a receptor system different than PAR. Alternatively, this compound may interfere directly with intracellular signaling pathway(s), resulting in a proliferation block.

IC_{50} of SCH79797 for inhibition of TRAP binding to PAR1 is 70 nM (Ahn et al., 2000), which corresponds to the concentration that induced a significant anti-proliferative effect in our system. However, the actions we described are likely not mediated by PAR1, as demonstrated by the ability of SCH79797 to slow the proliferation rate of mouse PAR1 null cells as well. These data suggest that this molecule has multiple biological effects, which include PAR1 receptor inhibition and PAR1-unrelated actions. It is worth mentioning that the concentration of SCH79797 able to induce cell death in the majority of cell types we tested (150-200 nM) was very closed to the concentration that induced growth inhibition (100 nM) and to the reported IC_{50} for platelet aggregation (70 nM), indicating a small PAR1-specific therapeutic range for this compound.

Whether or not other PAR1 antagonists have similar dual biological effects needs to be determined. Here, we studied a second PAR1 antagonist which was based on the published chemical structure of RWJ-56110 (Andrade-Gordon et al., 1999). This compound produced an anti-proliferative effect only at concentrations (300 μM) well over the IC_{50} for platelet aggregation by α-thrombin (0.8 - 8.0 ± 2.0 μM) (data not shown) (Andrade-Gordon et al., 2001). These latter finding supports the view that the anti-proliferative effect of SCH79797 is independent from PAR1 inhibition and may be specific for this type of molecule.

Thrombin antagonism has been proposed as an attractive new therapeutic option for treatment of patients with cardiovascular diseases, such as angina, acute myocardial infarction, stroke and patients undergoing coronary angioplasty to minimize restenosis. Moreover, PAR1 and PAR4 modulation could be beneficial in inflammatory diseases, wound healing and inhibition of tumor growth (Ahn et al., 2003; Ma et al., 2005). The PAR1 unrelated actions of SCH79797 described here, could be consider toxic or else, beneficial for certain therapeutic purposes. However, they should be carefully

considered for any application of the antagonist to the field of PAR1 related research or, even more important, for clinical development of this drug. The present data also suggest that other PAR1 antagonists should be carefully scrutinized for PAR1 unrelated actions.

Figure Legends:

Figure 1. Growth curves of NIH 3T3, A375, HEK 293 and B16/F10 stimulated by 10% serum in the presence of SCH79797 or vehicle (DMSO). Cells were counted at the indicated time points (days in culture). Statistical analysis demonstrated a significant reduction of cell number ($p \leq 0.001$) at all time points for all concentrations of SCH79797, compared to vehicle alone, except for 100 nM at day 3 in HEK 293.

Figure 2. Effect of SCH79797 on NIH 3T3 cell growth stimulated by FGF-1 (A), FGF-2 or PDGF (B). Cells were counted at day 3. $*p \leq 0.001$.

Figure 3. A. PAR1 RT-PCR of cDNA derived from $PAR1^{-/-}$ (lanes 1 and 2) and $PAR1^{+/+}$ (lane 3) embryonic fibroblasts. GAPDH was used as control. B. $PAR1^{+/+}$ and $PAR1^{-/-}$ cells were stimulated with 10% serum or FGF-1 (25 ng/ml). DNA synthesis was detected as ^{3H}thymidine incorporation and expressed as arbitrary units. No statistically significant difference was detected between $PAR1^{+/+}$ and $PAR1^{-/-}$ cells. C. Growth curve of $PAR1^{-/-}$ stimulated by 10% serum, in the presence of SCH79797 or vehicle (DMSO). Cells were counted at the indicated time points (days in culture). $p \leq 0.001$ for all concentrations of SCH79797, at day 5 and 7 compared to vehicle.

Figure 4. Effect of SCH79797 on serum-stimulated phosphorylation of MAPK (p44/p42), in NIH 3T3 cells. Cells starved for 24 h (C) were pre-incubated with vehicle (DMSO) (-) or 150 nM SCH79797 (+) for 1 h, followed by stimulation with 10% serum, for the indicated times. Cell lysates were analyzed with anti- phospho p44/p42 ($p44/p42^{P}$) and anti- total p44/p42 ($p44/p42^{t}$) MAPK antibodies (A). The experiment was performed three times. The immunoblots were scanned and quantitative image analysis performed using densitometry (B). Data are expressed as times of MAPK activation in samples treated with SCH79797 or vehicle, compared to control.

Figure 5. A. Activation of caspase-3 by SCH79797 in NIH 3T3 cells. Cells were incubated with 300 nM SCH79797 or vehicle (DMSO) for the indicated time. Cell lysates were

analyzed with anti-caspase 3 antibodies. The p35 inactive zymogen and the p17 small catalytic subunit are indicated as caspase-3 and cleaved caspase-3, respectively. B. Activation of apoptosis by SCH79797 in NIH 3T3 cells. Apoptosis-associated pS exposure and cell viability were determined by FICT conjugated Annexin-V and DNA specific viability dye 7-AAD respectively, in NIH 3T3 cells incubated with 150 nM and 200 nM SCH79797 or vehicle (DMSO), for the indicated times. Results are expressed as percentage of positive cells. *$p \leq 0.001$.

Figure 6. The effect of SCH79797 on cell growth and apoptosis in NIH 3T3 cells is dependent on cell density. A. Cells plated at low ($4x10^4$ cells/well) or high ($2x10^5$ cells/well) density were grown in 10% serum, in the presence of SCH79797 or vehicle (DMSO). Cells were counted at day 4. *$p \leq 0.001$. B. Cells plated at high density ($4x10^5$ cells/well) were grown in 10% serum, in the presence of SCH79797 or vehicle (DMSO). Cells were counted at the indicated time (days in culture). *$p \leq 0.001$.

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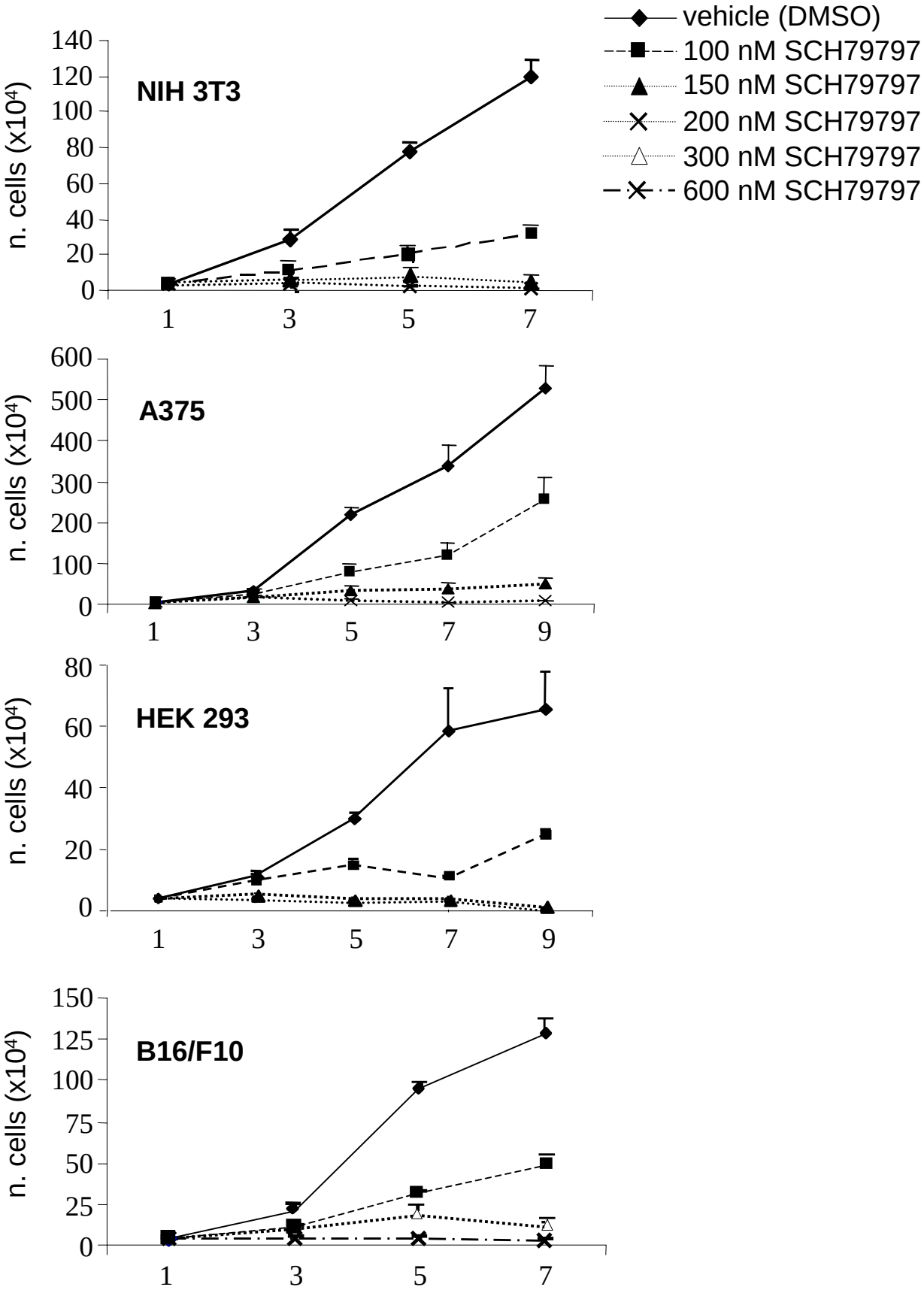
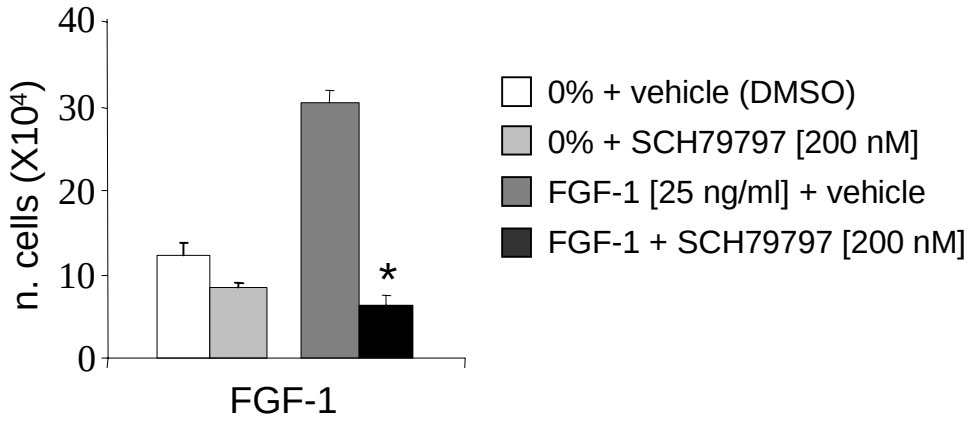

vehicle (DMSO)
100 nM SCH79797
150 nM SCH79797
200 nM SCH79797
300 nM SCH79797
600 nM SCH79797
NIH 3T3
A375
HEK 293
B16/F10
n. cells (x10⁴)
(days in culture)

Figure1

A.


40
30
20
10
0
n. cells (X10⁴)
*
FGF-1
0% + vehicle (DMSO)
0% + SCH79797 [200 nM]
FGF-1 [25 ng/ml] + vehicle
FGF-1 + SCH79797 [200 nM]

B.

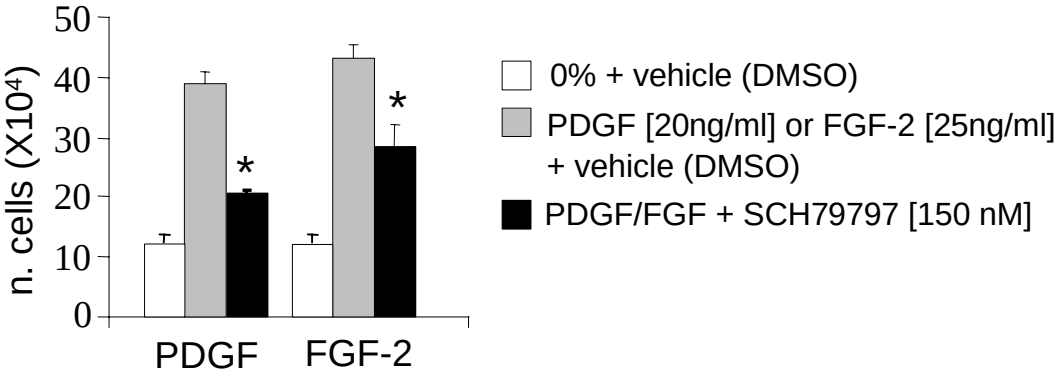
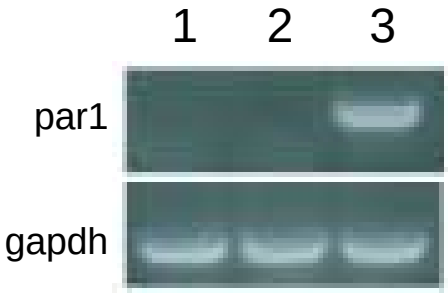
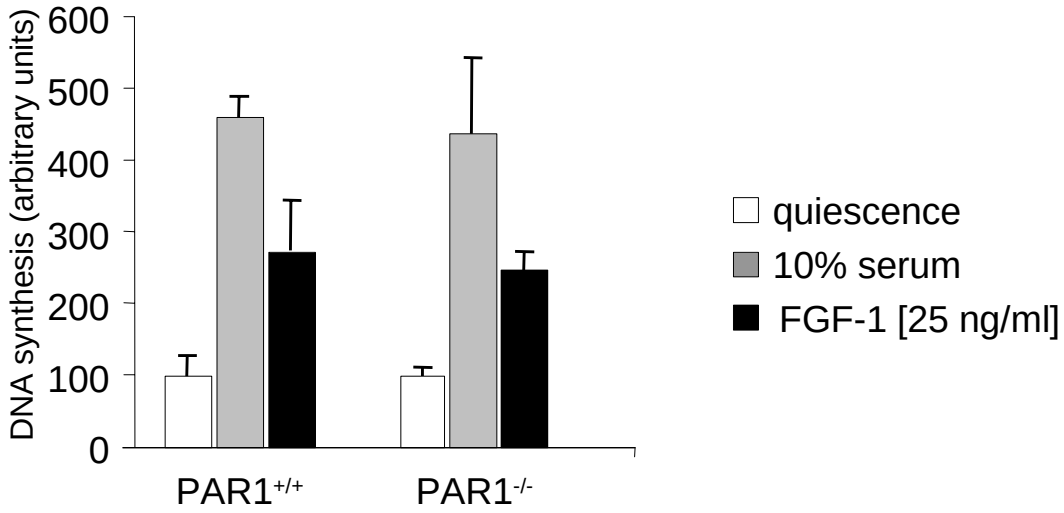

50
40
30
20
10
0
n. cells (X10⁴)
*
*
PDGF
FGF-2
0% + vehicle (DMSO)
PDGF [20ng/ml] or FGF-2 [25ng/ml]
+ vehicle (DMSO)
PDGF/FGF + SCH79797 [150 nM]

Figure 2

A.


1
2
3
par1
gapdh

B.


DNA synthesis (arbitrary units)
600
500
400
300
200
100
0
PAR1+/+
PAR1-/-
quiescence
10% serum
FGF-1 [25 ng/ml]

C.

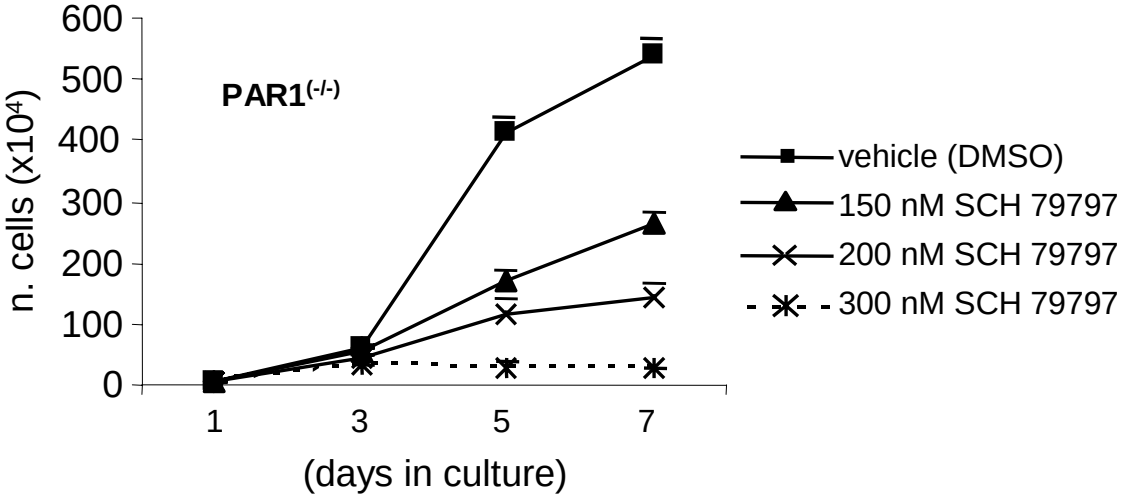
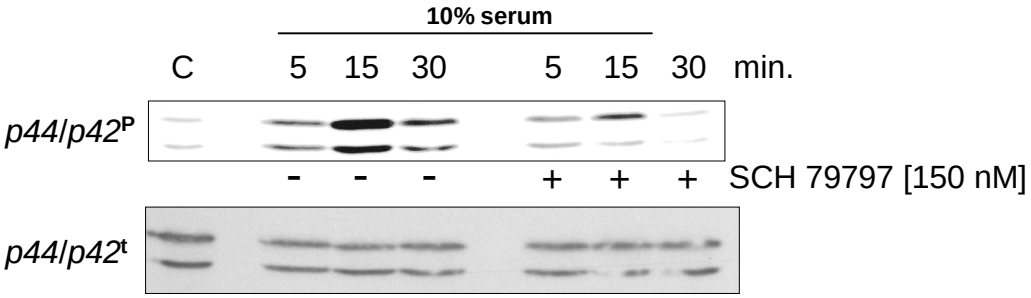

PAR1(-/-)
n. cells (x10⁴)
600
500
400
300
200
100
0
1
3
5
7
(days in culture)
vehicle (DMSO)
150 nM SCH 79797
200 nM SCH 79797
300 nM SCH 79797

Figure 3

A.


10% serum
C
5
15
30
5
15
30
min.
p44/p42P
- - - + + + SCH 79797 [150 nM]
p44/p42t

B.

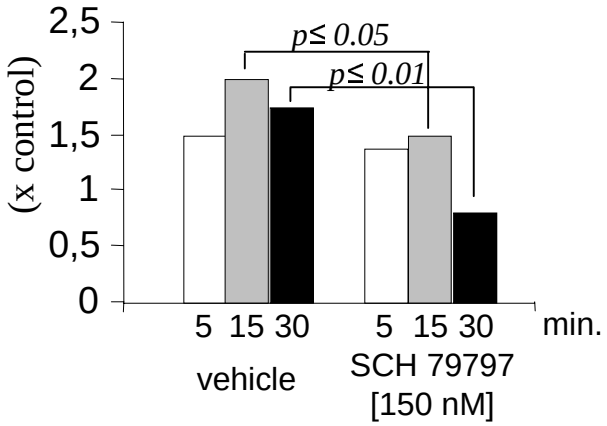
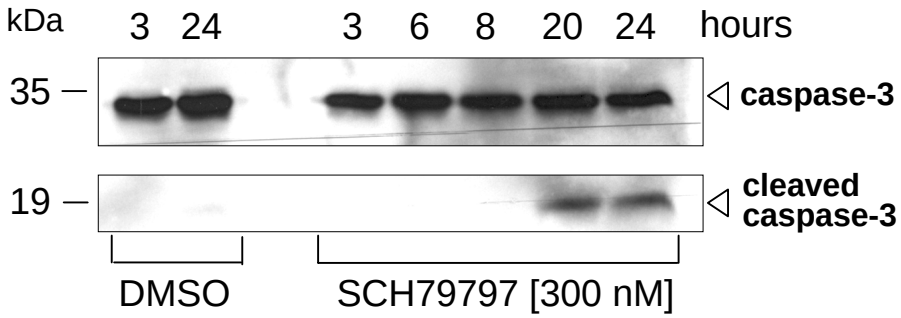

2,5
2
1,5
1
0,5
0
(x control)
p≤ 0.05
p≤ 0.01
5 15 30
5 15 30
min.
vehicle
SCH 79797
[150 nM]

Figure 4

A.


kDa
3 24 3 6 8 20 24 hours
35
caspase-3
19
cleaved caspase-3
DMSO
SCH79797 [300 nM]

B.

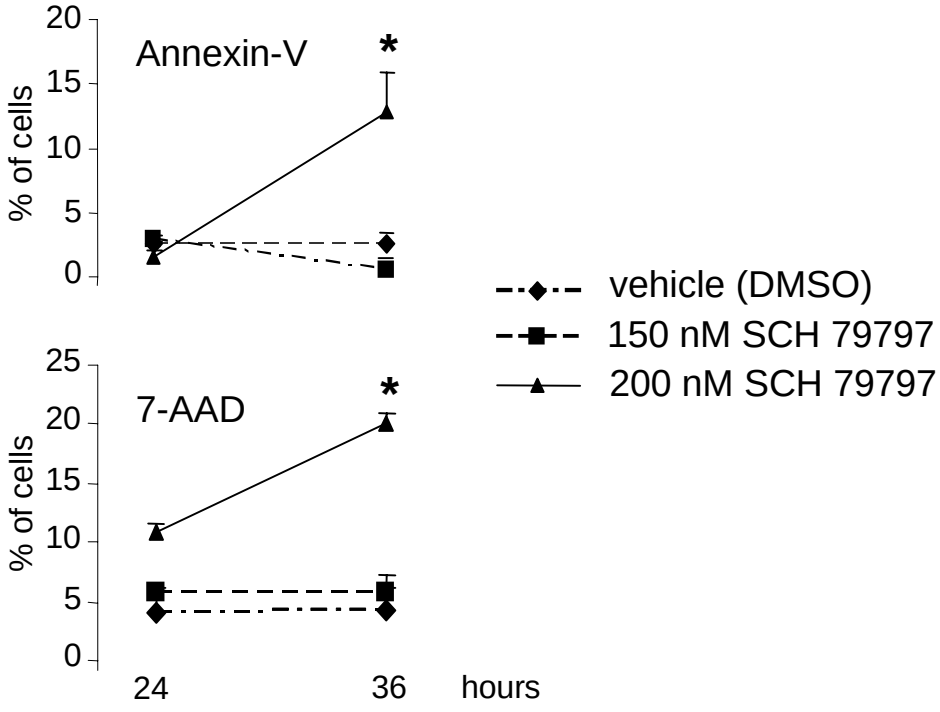
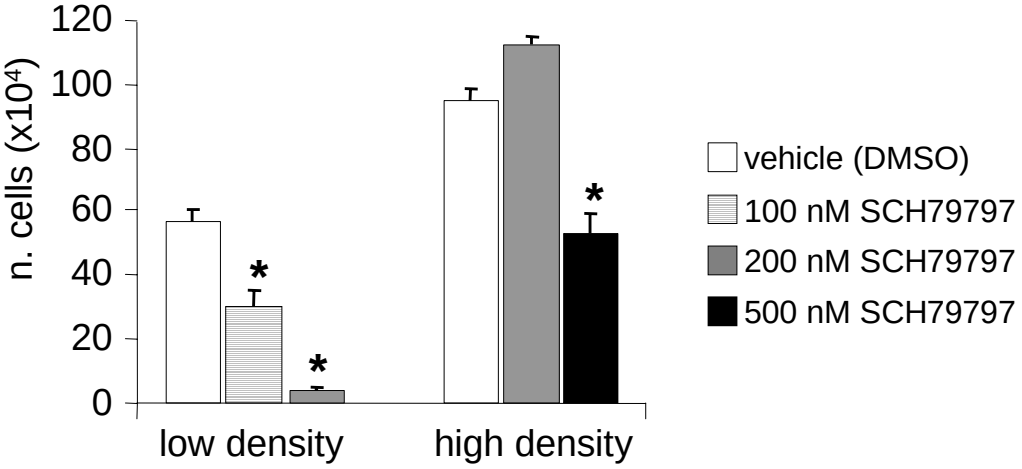

Annexin-V
% of cells
20
15
10
5
0
*
7-AAD
% of cells
25
20
15
10
5
0
*
24
36
hours
vehicle (DMSO)
150 nM SCH 79797
200 nM SCH 79797

Figure 5

A.


120
100
80
60
40
20
0
n. cells (x10⁴)
*
*
*
low density
high density
vehicle (DMSO)
100 nM SCH79797
200 nM SCH79797
500 nM SCH79797

B.

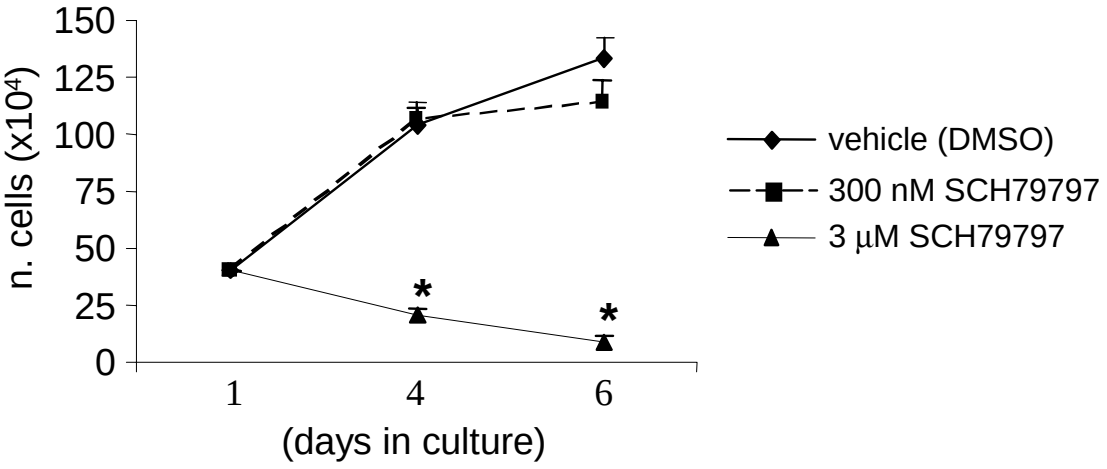

150
125
100
75
50
25
0
n. cells (x10⁴)
*
*
1
4
6
(days in culture)
vehicle (DMSO)
300 nM SCH79797
3 μM SCH79797

Figure 6

Paper 5

"Sphingosine Kinase 1 Is a Critical Component of the Copper-Dependent FGF1 Export Pathway"

R. Soldi, A. Mandinova, K. Venkataraman, T. Hla, M.A. Vadas, S.M. Pitson, **M. Duarte**, I. Graziani, V. Kolev, D. Kacer, O. Sideleva, T. Maciag and I. Prudovsky

FASEB
(Manuscript under revision)

To: FASEB, 2005

Sphingosine Kinase 1 Is a Critical Component of the Copper-Dependent FGF1 Export Pathway.

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Running Title: Non-Classical Export of FGF1 and Sphingosine Kinase 1

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[γ] This work was performed by MD from the Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, Braga, Portugal in partial fulfillment of the requirements for Ph.D. degree.

This article is dedicated to the memory of Tom Maciag, scientist, mentor, and friend.

Non-Classical Export of FGF1 and Sphingosine Kinase 1

Summary

Sphingosine kinase 1 catalyzes the formation of sphingosine-1-phosphate which is involved in the regulation of angiogenesis. Sphingosine kinase 1 lacks a classical signal peptide sequence but is constitutively released from cells. Since the copper-dependent non-classical stress-induced release of FGF1 also regulates angiogenesis, we questioned whether sphingosine kinase 1 is involved in the FGF1 release pathway. We report that (i) the coexpression of sphingosine kinase 1 with FGF1 inhibits the release of sphingosine kinase 1 at 37°C; (ii) sphingosine kinase 1 is released at 42°C in complex with FGF1; (iii) sphingosine kinase 1 associates with S100A13 and p40 Syt1, which are critical components of the FGF1 export pathway; (iv) sphingosine kinase 1 knockout cells fail to release FGF1 at stress; (v) sphingosine kinase 1 is a high affinity copper-binding protein which forms a complex with FGF1 *ex vivo*, and (vi) sphingosine kinase 1 overexpression rescues the release of FGF1 from inhibition by the copper chelator tetrathiomolybdate. We propose that sphingosine kinase 1 is a component of the copper-dependent FGF1 release pathway.

Introduction

Sphingosine-1-phosphate (S1P), a lipid mediator produced by sphingosine kinase 1 (SK1), is implicated in a variety of biological processes (1, 2). While high levels of intracellular S1P induce calcium mobilization and enhance cell proliferation and survival (3-5), extracellular S1P acts through specific G-protein-coupled receptors to promote cytoskeletal rearrangement, cell migration, vascular maturation, and angiogenesis (6-11). It is suggested that these activities are regulated by a dynamic balance between the levels of the sphingolipid metabolites, ceramide and S1P, described as the "sphingolipid rheostat" (12).

SK1 is both a cytosolic and a membrane-associated enzyme. It is activated by several biological regulators including tumor necrosis factor-α (13), platelet-derived growth factor (14), nerve growth factor (1, 15), muscarinic acetylcholine receptor agonist (16), serum (14) and phorbol esters (17). The expression of SK1 promotes the G_1-S transition in NIH 3T3 cells, and protects these cells against apoptosis induced by serum deprivation (18). In addition, it is suggested that SK1 may act as an oncoprotein (19).

Interestingly, SK1 is exported from human umbilical vein endothelial cells (20), SK1-transfected human embryonic kidney 293 cells, and lung smooth muscle cells (21) through a non-classical pathway of release (20). A number of other extracellular proteins have been demonstrated to follow various non-classical export routes (for review see (22, 23). Among them are such potent pro-angiogenic and pro-inflammatory polypeptides as FGF1 (24), FGF2 (25-27), IL1α (28), and IL1β (29, 30). The release of SK1 shares several similarities with FGF1, which is also an important regulator of cell proliferation and migration (31). Indeed, like FGF1, SK1 lacks a classical signal peptide sequence required for release through the endoplasmic reticulum and Golgi apparatus (20, 24), and its export into the extracellular compartment is brefeldin A-insensitive (20, 24), ATP-dependent (20, 32), and requires an intact actin cytoskeleton (20, 33). FGF1 is released as a copper-dependent multiprotein complex, which is known to include the EF hand-containing protein, S100A13, and the p40 form of synaptotagmin 1 (Syt1) (22). Interestingly, all known members of the FGF1 release complex are copper-binding proteins, and we demonstrated the ability of FGF1 to form high molecular weight aggregates with both S100A13 and p40 Syt1 in presence of exogenous copper in a cell free system (34). Moreover, we reported that the copper chelator tetrathiomolybdate (TTM) inhibits the temperature dependent release of FGF1 (35) . Because (i) FGF1 is exported in response to temperature stress

(36), (ii) in yeast, SK1 is implicated in the heat shock response by its ability to enhance cell survival upon severe heat stress (37, 38), and (iii) since the export of SK1 (20) exhibits similar pharmacologic properties to those described for FGF1 release (33, 35), we sought to determine whether SK1 is a component of the FGF1 release pathway. We report that SK1 is released from NIH 3T3 cells in response to cellular stress as a component of the FGF1 multiprotein release complex, and that SK1 knockout results in the blockage of FGF1 release. Our data also suggest that SK1 may act as a copper donor facilitating the formation of the FGF1-containing multiprotein release complex.

Experimental Procedures

Cell Culture. Murine NIH 3T3 cells (ATCC) and stable FGF1 NIH 3T3 (24) cell transfectants were maintained in DMEM (Cellgro) supplemented with 10% bovine calf serum (HyClone) on human fibronectin-coated dishes (10 $\mu g/cm^2$) (24). Stable transfectants were also supplemented with 0.4 g/l Geneticin (G418 Life Technologies, Inc.). SK1 +/+ mouse embryo fibroblast cells and SK1 knockout mouse embryo fibroblast cells were maintained in DMEM supplemented with 10% fetal bovine serum (HyClone) on human fibronectin-coated dishes (10 $\mu g/cm^2$) (24).

Preparation of Adenoviral Constructs and Cell Infection. The SK1 construct cloned into the V5-His-pcDNA3.1 eukaryotic expression vector (Invitrogen) was excised from the plasmid by digestion with HindIII and PmeI (New England Biolab); and the fragment was subcloned in the multiple cloning site of the adenovirus shuttle vector, pAdlox, using HindIII and SmaI sites. The resulting plasmid was digested with SfiI, resolved by 1% agarose gel electrophoresis, excised and purified using the QIAquick Gel Extraction kit (Quiagen). The fragment containing the SK1 cDNA was co-transfected with ψ5 helper virus DNA into mouse epithelial kidney CRE 8 cells; and the recombinant viral product was purified by two sequential CsCl gradient centrifugations as previously described (39). The adenoviral S100A13:Myc and the S100A13ΔBR:Myc constructs each containing six Myc epitopes, were obtained as previously described (35). NIH 3T3 cells and stable FGF1 NIH 3T3 cell transfectants cells were infected as previously described (40).

SK1 Knockout Mouse Embryo Fibroblast Cells Immortalization. SK1 knockout mouse embryo fibroblast cells, generously donated by Dr. R. Proia (NIH, Bethesda, MD), were immortalized by stable transfection with SV40 T-antigen in the psg65 vector (gift of Dr. James DeCaprio, Harvard University) in parallel with normal mouse embryo fibroblast cells. The resulting colonies were tested for lack of SK1 expression by RT PCR analysis using specific SK1 primers as described (41).

Heat Shock and Processing of Conditioned Media. Heat shock induced non-classical protein release was studied as previously described (33, 35). NIH 3T3 cell transfectants were grown to 70% confluency ($7X10^6$ cells per 15 cm Petri dish), and prior to heat shock, the culture medium was changed to DMEM containing 5 U/ml of heparin (Sigma). Following temperature stress, the conditioned media were collected, filtered, treated with 0.1% DTT (Sigma) for 2 hours at 37°C, and adsorbed to a 1 ml heparin-Sepharose CL-6B column (Amersham Pharmacia, Biotech.), pre-equilibrated with 50 mM Tris pH 7.4 containing 10 mM EDTA (TEB). The adsorbed proteins were washed with TEB, eluted with TEB containing 1.5 M NaCl, and concentrated (Centricon 10; Amicon). The samples were resolved by 15% SDS-PAGE and immunoblotted with a rabbit polyclonal anti-FGF1 antibody (42). The flow through media from the heparin-Sepharose CL-6B column was collected, concentrated using Ultrafree-15 Centrifugal

filter device (Millipore), and incubated with a monoclonal anti-V5 antibody (Invitrogen) overnight at 4°C. Protein G–Sepharose (Amersham Pharmacia, Biotech) was added and the mixture was incubated for 2 hours at 4°C. Immunoprecipitates were resolved by 12% SDS-PAGE and immunoblotted with the monoclonal anti-V5 antibody. Total cell lysates were obtained from the individual populations of cells, as previously described (33). The loading of cell lysates and conditioned medium on electrophoretic gels was standard in all experiments. Each experimental point corresponded to one 15 cm Petri dish of cell culture. Total processed conditioned medium or 1/10 of cell lysate were loaded on one electrophoretic lane. The activity of lactate dehydrogenase (Sigma) in conditioned media was utilized as an assessment of cell lysis in all experiments, as previously described (43).

Immunoprecipitation Experiments. 20 ml of conditioned media from $7x10^6$ heat shocked or control NIH 3T3 cells were collected, filtered, and concentrated to the volume of 1 ml by using Ultrafree-15 centrifugal filter device (Millipore) for 1 hour at 2000 g at 4°C. To prepare cell lysates, the cells were washed twice with PBS and lysed in NPB buffer (20 mM Tris HCl, pH 7.5, containing 300 mM sucrose, 60 mM KCl, 15 mM NaCl, 5% glycerol, 2 mM EDTA, 1% Triton X-100, 1 μg/ml leupeptin, and 0.2% deoxycholate). In some experiments, the cells were hypotonically lysed in 5 mM Tris HCl pH 7.4 containing protease inhibitors (50 μg/ml pepstatin, 50 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF), using Dounce homogenization. The lysates were centrifuged at 10,000 g for 15 minutes at 4°C, and the protein concentration was measured by the BCA method (Pierce). Equal amount of lysates and conditioned media were precleared by incubation for 1 hour at 4°C with protein G-Sepharose (Amersham, Pharmacia) and mouse IgG (1 μg/ml). After centrifugation at 10,000 g for 10 minutes, the supernatants were immunoprecipitated with a monoclonal anti-FGF1 antibody, a monoclonal anti-Myc antibody (Oncogene Research), a polyclonal anti-p40 Syt1 antibody (44), or a monoclonal anti-V5 antibody (Invitrogen) overnight at 4°C. The immunoprecipitates were recovered using protein G-Sepharose affinity, washed four times with NPB buffer, the pellets were solubilized in 60 μl of boiling Laemmlie buffer, resolved by 12% SDS-PAGE, and analyzed using the monoclonal antibodies described above.

Copper Affinity of SK1 and Gel Shift Analysis. In order to evaluate the affinity of SK1 for copper, it was necessary to delete the His tag sequence from the SK1 construct. This was accompanied by the introduction of a termination codon upstream at nucleotide 1054, which is located at 5′ of the His sequence within the V5-His epitope tag. The mutated V5-His-pCDNA3.1 expression plasmid was translated *in vitro* using the T7 Quick *in vitro* transcription and translation system (Promega). The reaction product was adsorbed to a HiTrap chelating column (Amersham Pharmacia, Biotech.) which was pre-adsorbed with 0.1 M $CuCl_2$ in 20 mM sodium phosphate buffer, pH 7.2 containing 1 M NaCl. The column was eluted with increasing concentrations of imidazole (Sigma) in the equilibration buffer. The eluted fractions were concentrated by using Centricon 10 (Amicon), resolved by 10% SDS-PAGE and subjected to V5 immunoblot analysis.

To analyze the interactions among the recombinant forms of SK1 (45) and FGF1 (34, 46, 47), the recombinant proteins were mixed at a molar ratio of 1:1, as previously described (34), lyophilized, and resuspended in 50 μl of phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS), in the presence or absence of 1 mM $CuCl_2$. The samples were incubated at 42°C for 30 minutes, resuspended in non-reducing SDS-PAGE loading buffer, and resolved by non-reducing/limited denaturing SDS-PAGE system containing 0.1% SDS within the running gel as

previously described (35). The samples were then immunoblotted using polyclonal antibodies against FGF1 and SK1. When indicated, recombinant SK1 was incubated with 500 mM imidazole for 30 minutes at 37°C, dialyzed against PBS, and incubated in a mixture containing FGF1 in the presence or absence of $CuCl_2$ as described above. The samples were resuspended in a non-reducing SDS-PAGE loading buffer, and resolved by non-reducing/limited denaturing SDS-PAGE.

Assay of Sphingosine kinase activity. Sphingosine kinase activity was determined as described previously (48) . Briefly, conditioned media and lysates were obtained from heat shocked Flag:FGF1 tagged cells co-transfected with SK1:V5 as described above, and, after equalization of the amount of protein per sample, subjected to immunoprecipitation with antibodies against flag or V5 tags. The immunoprecipitated proteins were incubated with 20 μM of sphingosine, 500 μM [^{32}P] ATP (10 μCi), 5 mM MgCl2, 15 mM NaF, 0.5 mM 40-deoxypyridoxine, and 40 mM β-glycerophosphate for 30 min at 37°C. After chlorophorm extraction, [^{32}P]S1P was resolved by TLC on Silica Gel G60 using 1-butanol/acetic acid/water (60/20/20) buffer. The bands corresponding to S1P were also scraped from the plates and counted in a scintillation counter.

Results

FGF1 represses the constitutive release of SK1 from NIH 3T3 cells. We previously demonstrated that the coexpression of FGF1 with the small calcium-binding protein, S100A13, which also lacks a signal peptide, resulted in the blockage of S100A13 release at 37°C but not at 42°C. These data were the first indication that S100A13 is a member of the FGF1 release complex (34). Thus, we used a similar coexpression approach to evaluate the possibility of SK1 participation in the FGF1 release pathway. Since initially FGF1 release was studied in NIH 3T3 cells, we verified the release of SK1 from these cells. Because SK1 is released from human endothelial cells and from SK1-transfected 293 cells *in vitro* at 37°C, and its export is enhanced at 42°C (20), we investigated whether the release of SK1 is heat shock-dependent in NIH 3T3 cells. Equal amount of media conditioned by control and heat shocked NIH 3T3 cells transduced with adenovirus encoding V5-tagged SK1 were collected, and analyzed using immunoprecipitation with antibodies against the V5 epitope. As shown in Figure 1A, SK1 is constitutively released from NIH 3T3 cells at 37°C, and its export is enhanced at 42°C. To assess the cell damage, we measured the activity of lactate dehydrogenase (43) in both 37°C and 42°C conditioned media, and this test demonstrated the absence of any significant cell damage under normal or stress conditions. Thus, the results previously obtained using endothelial cells and SK1-transfected 293 cells (20) were confirmed. In contrast, analysis of medium conditioned by NIH 3T3 cells coexpressing SK1 and FGF1 revealed that under these conditions the release of SK1 at 37°C is drastically reduced, while it normally occurs upon temperature stress (Figure 1A). Noteworthy, the level of expression of SK1 was equal at 37° and 42°C (data not shown). In addition, heparin-binding analysis of this conditioned medium from NIH 3T3 cells coexpressing SK1 and FGF1 showed that SK1 associated with heparin-Sepharose (Figure 1B). Conversely, although SK1 was released at 37°C and 42°C from FGF1-free NIH 3T3 cells (39) (Figure 1A), the released SK1 did not adsorb to heparin-Sepharose (Figure 1B). These results suggested that at stress, SK1 and FGF1 are released as a complex able to bind heparin due to the heparin affinity of FGF1.

SK1 is associated with FGF1. The observation that SK1, in media conditioned by heat shock, binds heparin in a FGF1-dependent manner (Figure 1B) is consistent with other FGF1-binding proteins, S100A13 (49) and p40 Syt1 (47) that are involved in non-classical FGF1 export. Therefore, we evaluated the association between SK1 and FGF1 in the co-expressed cells and in the conditioned medium. We performed coimmunoprecipitation experiments utilizing NIH 3T3 cells coexpressing FGF1 and V5-tagged SK1. Cell lysates and equal amount of conditioned media were obtained from control and heat shocked cells, and after equalization of protein concentration, the samples were incubated with an anti-FGF1 monoclonal antibody. The immunoprecipitates were resolved by SDS-PAGE, and analyzed by V5 immunoblot. As shown in Figures 2A and 2B, SK1 and FGF1 were efficiently coprecipitated from both the cell lysate and the conditioned medium derived from temperature-stressed cells. Much less SK1 was detected in the anti-FGF1 immunoprecipitate of the cell lysate derived from non-stressed cells (Figure 2A). As expected, FGF1 antibodies did not precipitate FGF1 or SK1 from medium conditioned by non-stressed cells. These data indicate that SK1 and FGF1 display intracellular association, which is significantly enhanced by stress conditions, and results in the release of a complex including both FGF1 and SK1.

SK1 knockout abolishes stress-induced FGF1 release. To evaluate the role of SK1 in FGF1 release, we performed experiments with SK1 knockout mouse embryo fibroblasts (41) immortalized using the SV40 large T antigen as described in the Materials and Methods. Immortalized fibroblasts from SK1 knockout and control mice were transduced with adenovirus encoding FGF1. We observed that SK1 knockout fibroblasts exhibited a drastic inhibition of FGF1 release under heat shock conditions (Figure 3). Thus, SK1 appears to be a critical component of the FGF1 multiprotein release complex. Interestingly, it was reported that SK1 knockout mice do not exhibit a severely abnormal phenotype apparently as a result of normal S1P levels in these animals (41) . It has been hypothesized that SK2 and possibly other kinases, such autaxin (50, 51) may be alternative sources of S1P production (41). These data are intriguing, since they suggest the specificity of the interaction FGF1 and SK1, as well as the independency of FGF1 release from S1P production. Indeed, we observed that S1P treatment failed to either enhance or inhibit the release of FGF1 from SK1:V5 and FGF1 co-transfectant NIH 3T3 cells both at 37° and 42° C (data not shown). To verify whether released SK1 is catalytically active during heat shock, we performed the kinase assay of SK1 from both lysates and media conditioned by SK1:V5 and FGF1 co-transfectant NIH 3T3 cells at 37° and 42° C (20). We were unable to detect any S1P production from SK1 released in association with FGF1 into the conditioned media (Fig..). However, SK1 displayed catalytic activity when associated with FGF1 inside the cells (Fig..).

SK1 associates with S100A13, and rescues FGF1 release from inhibition by S100A13ΔBR. We previously reported the important role of S100A13 in FGF1 release (34). To be exported, FGF1 requires homodimerization mediated by its cysteine 30 residue; and the Cys-free FGF1 mutant is not released (36). However, the overexpression of S100A13 is able to rescue the stress-induced release of Cys-free FGF1 (34). Another indication of the role of S100A13 in FGF1 export is that the expression of the S100A13ΔBR mutant lacking a C-terminal basic amino acid rich (BR) domain inhibits the stress-induced release of FGF1 (34). Interestingly, unlike S100A13, SK1 failed to rescue the release of Cys-free FGF1 mutant (data not shown). Thus, apparently SK1 is not a substitute of S100A13 but is an additional member of the FGF1 release

complex. We sought to examine whether SK1 was able to interact with S100A13. S100A13, and FGF1 were co-expressed in NIH 3T3 cells with and without the overexpression of SK1, and the cells were subjected to heat shock. Under these conditions, the expression of SK1 did not alter the stress-induced release of FGF1 (Figure 4A). As shown previously, the expression of S100A13ΔBR, in the absence of SK1, inhibited FGF1 export (15). Interestingly, however, in the presence of SK1 expression the S100A13ΔBR mutant was unable to repress the stress-induced release of FGF1 into the extracellular compartment (Figure 4A). The stress-induced release of SK1 was also unaltered by S100A13ΔBR (Figure 4B). These data demonstrate that the expression of SK1 compensated for the inhibitory effect of the S100A13ΔBR mutant on FGF1 export in response to temperature stress. In addition, these data imply that there are interactions between SK1 and S100A13ΔBR, which are mediated by the association of SK1 with a S100A13 domain other than the BR sequence.

We further sought to determine whether SK1 associates with both S100A13 and S100A13ΔBR by utilizing FGF1-free NIH 3T3 cells (40) coexpressing V5-tagged SK1, and either myc-tagged S100A13 or myc-tagged S100A13ΔBR for the immunoprecipitation experiments. As shown in Figure 5A and B, SK1 coimmunoprecipitated with both S100A13 and S100A13ΔBR in a heat shock-dependent manner from conditioned media. SK1 also associated with S100A13 in the cytosol in a heat shock-dependent manner (Figure 5A). Interestingly, however, we observed a significant association of SK1 and the S100A13ΔBR mutant in the cytosol at 37°C (Figure 5B). These data suggest that the mechanisms utilized by S100A13 and SK1 to constitutively enter the extracellular compartment at 37°C are likely to be different from the stress-induced release, and do not involve the participation of the BR domain of S100A13.

p40 Syt1 is associated with SK1. Since p40 Syt1 is a critical component of the FGF1 stress-induced release pathway (47), we questioned whether p40 Syt1 is also able to associate with SK1. As shown in Figure 5C, p40 Syt1 coimmunoprecipitated with SK1 from both cell lysates and conditioned media from heat shocked NIH 3T3 cells coexpressing these two proteins. Interestingly, unlike FGF1 and wild type S100A13, the association between p40 Syt1 and SK1 in cell lysates obtained from non-stressed cells is as intensive as it is in heat shocked cells. These data suggest that SK1 and p40 Syt1 may interact in the cytosol in a temperature stress-independent manner, while the association of SK1 with FGF1 and S100A13 is drastically enhanced by stress.

SK1 is an avid copper-binding protein, yet its release is not blocked by copper chelation. The FGF1 release pathway requires intracellular copper, which facilitates the assembly of the FGF1:S100A13:p40Syt1 multiprotein release complex (35). Because FGF1 (46), S100A13 (35), and p40 Syt1 (35) have all been characterized as copper-binding proteins, and SK1 appears to be a component of the FGF1 release pathway, we hypothesized that SK1 may also bind copper. We demonstrated that the *in vitro* translation product of the SK1 transcript is an avid copper-binding protein, since it requires 0.5 M imidazole for elution from a copper affinity column (Figure 6A). Notably, p40 Syt1, FGF1, and S100A13 are eluted from this column at 0.05 M imidazole (35). Furthermore, the analysis of the copper-binding affinity of FGF1 and SK1 from lysates of NIH 3T3 cells coexpressing these proteins demonstrated that a portion of FGF1 is able to gain higher affinity for copper, when co-expressed with SK1 (Figure 6B). These results indicate that the intracellular complex of FGF1:SK1 exhibited copper affinity characteristic of SK1.

Since we previously reported that the copper chelator tetrathiomolybdate (TM) is able to repress the heat shock-induced release of FGF1 (35), we investigated whether the constitutive and stress-induced release of SK1 were also copper-dependent. Surprisingly, TM treatment was unable to inhibit the release of SK1 at 42°C in the presence or absence of FGF1 (Figure 7A), although some decrease of SK1 release at 37°C was observed. In addition, the coexpression of SK1 and FGF1 abolished the ability of TM to repress FGF1 export in response to heat shock (Figure 7B). These data suggest that that SK1 may be involved in facilitating the intracellular trafficking of copper needed for the formation of the FGF1 release complex.

SK1 associates with recombinant FGF1 in a cell-free system. Since SK1, like FGF1, S100A13, and p40 Syt1 is a copper-binding protein, and copper is able to mediate *in vitro* interactions between FGF1, S100A13, and p40 Syt1 (35), we questioned whether SK1 is able to form copper-dependent complexes with FGF1. Recombinant FGF1 and SK1 were mixed at equimolar ratio in the presence or absence of 1 mM $CuCl_2$. After 30 minutes of incubation at 42°C, the reaction products were resolved by non-reducing/limited denaturing (0.1% SDS) SDS-PAGE. We utilized this electrophoretic system, since it enables the resolution and detection by immunoblot analysis of high molecular weight multiprotein aggregates at the top of the stacking gel (35). FGF1 immunoblot analysis revealed the appearance of a band at the top of the stacking gel when SK1 was mixed with FGF1 (Figure 8A). Interestingly, however, the appearance of the high molecular weight aggregate was observed even in the absence of 1 mM $CuCl_2$, suggesting that SK1 does not require the addition of exogenous copper to associate with FGF1 in a cell-free system.

Because SK1 may facilitate the copper-induced formation of the FGF1 release complex, we sought to assess whether the interactions of SK1 with FGF1 were mediated by endogenous copper bound to SK1. Incubation of recombinant FGF1 with SK1 at 42°C for 30 minutes in the absence of $CuCl_2$ and in the presence of 500 mM imidazole, significantly reduced the appearance of the FGF1 band resolved at the top of the stacking gel (Figure 8A). These data, and the observation that the coexpression of SK1 rescued the export of FGF1 when incubated with TM, suggest that SK1 may be involved in the redistribution of intracellular copper required for the assembly of the FGF1 multiprotein release complex.

Discussion

FGF1 contributes to the regulation of cell proliferation, migration, and differentiation during angiogenesis and inflammation. Although FGF1 mediates its biological activity through interactions with cell surface receptors (31), it lacks a conventional signal peptide sequence that is necessary to access the endoplasmic reticulum/Golgi apparatus (24). However, it was demonstrated that FGF1 is released into the extracellular compartment through a stress- and copper-dependent non-classical pathway. The stress-dependent release of FGF1 requires the formation of a multiprotein complex containing the calcium- and acidic phospholipid-binding proteins, S100A13 and the p40 form of p65 Syt1.

Like FGF1 (31), SK1 is a regulator of angiogenesis (52, 53) through its ability to synthesize S1P (20). Interestingly, S1P may be involved in the determination of cell fate (54), and may also play a role in the inhibition of apoptosis (55), and in the regulation of the allergic response (56). Similar to FGF1, SK1 participates in the regulation of cell proliferation, migration, and vascular maturation (7). Our data suggest that SK1 is also a component of the FGF1 multiprotein release complex, and that it is involved in the regulation of the stress-induced

non-classical export of FGF1. Indeed, non-classical export of SK1 shares pharmacologic properties with the FGF1 release pathway (20, 24, 32). The expression of FGF1 is able to repress the constitutive release of SK1 from NIH 3T3 cells, and it restricts the export of SK1 to the stress-induced pathway. In addition, the observation that SK1 is associated with FGF1 in heat shock-conditioned medium suggests that SK1 participates in the FGF1 multiprotein release complex. In fact, SK1 also associates with S100A13 and p40 Syt1. Further, the BR domain of S100A13 is involved in binding FGF1, since deletion of the BR domain of S100A13 acts as a dominant-negative for FGF1 export in response to stress (34). Our observation that SK1 expression is able to rescue the release of FGF1 in the presence of the dominant-negative S100A13ΔBR mutant suggests that SK1 may associate with the FGF1:S100A13 heterotetramer at a site in S100A13 other than the BR domain. Furthermore, the ability of the S100A13ΔBR mutant to coprecipitate with SK1 is consistent with this premise. Most importantly, the critical role of SK1 in the non-classical stress-induced release of FGF1 is clearly demonstrated in the experiments with SK1 knockout mouse embryo fibroblasts.

It is noteworthy that the expression of SK1 is able to overcome the TM-induced inhibition of FGF1 release, implying a functional role for SK1 in the copper-dependent assembly of the FGF1 multiprotein release complex. Interestingly, SK1 exhibits an avidity for copper which is significantly higher than that of FGF1 (46), S100A13 (35), or p40 Syt1 (35). The observation that SK1 is able to alter the electrophoretic mobility of FGF1, in an imidazole-sensitive manner, in a cell-free and copper-free system is significant. Indeed, prior electrophoretic analysis of FGF1, S100A13 and p40 Syt1 complex formation revealed a mobility shift in response to the presence of exogenous copper (35). Thus we anticipate that SK1 may be able to function as an intracellular "copper sink", and SK1 may be responsible for the distribution of intracellular copper to the polypeptide components of the FGF1 non-classical export pathway. Our data demonstrate that SK1 released into the conditioned media upon heat shock was catalytically inactive when associated to FGF1. Moreover, significantly reduced enzymatic activity of SK1 was observed in presence of copper (45). The lack of biological activity appears to be a common characteristic of at least some of the proteins of the FGF1 release complex. Indeed, it has been reported that FGF1 dimer released upon temperature stress (32) in association with the other components of the FGF1 release complex (47, 49, 57), does not exhibit biological activity (46). However, the capacity to induce mitogenesis is re-gained once the FGF1 dimer is reduced to monomer (46) . Apparently, SK1 released as a member of the FGF1 export complex shares a similar behavior.

On the other hand, we observed SK1 catalytic activity when it was associated with FGF1 inside the cells. The observation that S1P was unable to interfere with FGF1 secretion, suggests the possibility of a new role of SK1 in the angiogenic processes. It is interesting to note that the enzymatic activity of SK1 is significantly enhanced by interactions with acidic phospholipids, particularly phosphatidylserine (pS) and phosphatidylinositol (pI) (45). Although specific acidic phospholipid-binding domains within the structure of SK1 have not been identified, it was reported that the interaction with pS may double the k_{cat} of the enzyme (45). It is noteworthy that FGF1 is characterized by the presence of a consensus sequence for acidic phospholipid-binding in the carboxy-terminal domain (36, 58). Moreover, the ability of FGF1 (58) to assume molten globule character in the presence of acidic phospholipids suggests that the FGF1 multiprotein release complex may utilize the flipping of pS or other acidic phospholipids for their export through the plasma membrane (22, 59). This premise is particularly interesting since pS, as well

as other acidic phospholipids, are known to exhibit electrostatic interactions with sphingosine, which is largely protonated at a physiological pH (60).

Although the biological significance of SK1 as a component of the FGF1 multiprotein release complex is not fully understood, recently the biological relevance of copper as a mediator of the assembly of this complex was deduced. Indeed, the response to injury in the rat aorta is sensitive to TM treatment by its ability to repress the release of IL1α, FGF1, and S100A13, as well as reducing the appearance of pS on the surface of cells involved in mediating this response (61). Since S1P is a mediator of angiogenic response *in vivo* (10, 53, 62), it is interesting that SK1, which is responsible for the production of S1P, is a component of the pro-angiogenic FGF1 release complex. It is not known whether the regulation of tissue response to injury *in vivo* involves the function of S1P generated in the extracellular compartment by SK1 released through stress-induced pathways. However, it is likely that at least one of the functions of intracellular cytosolic FGF1 may be to assure that SK1 remains within the cytosol, since its constitutive release may lead to premature angiogenic and inflammatory responses *in vivo*.

Acknowledgements

The authors thank R. Proia (NIH, Bethesda) for SK1 knockout mouse embryo fibroblasts, I. Kehayov (Institute of Immunology, Sophia, Bulgaria) for the monoclonal FGF1 antibody, D.B. Baldwin (University of Pittsburgh) for the pAdlox shuttle vector, and James DeCaprio (Harvard University) for the T antigen constructs. This work was supported in part by NIH grants HL67330 and HL60694 to TH and HL32348, HL 35627 and RR15555 to IP. This work was performed by MD to partially fulfill the requirements for the Ph.D. from the University of Porto, Portugal.

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Figure Legends

Figure 1. Release of SK1 in Response to Temperature Stress *In Vitro*. NIH 3T3 cells and FGF1 NIH 3T3 cell transfectants were adenovirally transduced with AdSK1:V5, and subjected to heat shock. After treatment with 0.1% DTT, conditioned media were subjected to immunoprecipitaion with V5 antibodies (A) or adsorbed to and eluted from a heparin-Sepharose column (B), as described in the Material and Methods. The samples were resolved in a 12% acrylamide SDS-PAGE, and subjected to immunoblot analysis with V5 (A, upper panel of B) or FGF1 antibodies (lower panel of B).

Figure 2. SK1 is Able to Coprecipitate with FGF1. Conditioned media (CM) and cell lysates (Lys) from heat shocked SK1:V5-transduced FGF1 NIH 3T3 cell transfectants were collected,

filtered, and subjected to immunoprecipitation with a monoclonal FGF1 antibody. (A) FGF1 immunoprecipitates were resolved by 12% acrylamide SDS-PAGE, and subjected to V5 immunoblot analysis. (B) The FGF1 immunoprecipitates from (A) were subjected to FGF1 immunoblot analysis.

Figure 3. SK1 Knockout Blocks the Release of FGF1. FGF1 was adenovirally transduced in immortalized SK1 knockout and normal mouse embryo fibroblast cells, as previously described (40). The heat shock-conditioned media were adsorbed to heparin-Sepharose, eluted by 1.5 M NaCl, resolved by 15% acrylamide SDS-PAGE, and subjected to FGF1 immunoblotting (A). (B) SK1 expression in knockout and control cells was monitored with specific SK1 primers by RT PCR.

Figure 4. The Expression of SK1 is Able to Rescue the S100A13ΔBR-Induced Inhibition of FGF1 Export. Media conditioned by heat shocked and control SK1:V5- and S100A13-transduced FGF1 NIH 3T3 cell transfectants or by heat shocked and control SK1:V5- and S100ABΔBR-transduced FGF1 NIH 3T3 cell transfectants. The media were filtered, and either (A) adsorbed to heparin-Sepharose columns, and eluted with 1.5 M NaCl and the eluates were resolved by 15% acrylamide SDS-PAGE, and subjected to FGF1 immunoblot analysis, or (B) subjected to immunoprecipitation with monoclonal V5 antibodies, and the immunoprecipitates were resolved by 12% acrylamide SDS-PAGE and subjected to V5 immunoblot analysis.

Figure 5. The Association of SK1 with S100A13, S100A13ΔBR and p40 Syt1. NIH 3T3 cells cotransduced with either SK1:V5 and S100A13:Myc (Panel A), SK1:V5 and S100A13ΔBR:Myc (Panel B) or SK1:V5 and p40 Syt1 (Panel C) were subjected to heat shock for 110 minutes at 42°C. Conditioned media (CM) and cell lysates (Lys) were collected and concentrated as described in the Materials and Methods. (A) and (B) The samples were immunoprecipitated with a monoclonal Myc antibody, resolved by 12% acrylamide SDS-PAGE, and subjected to V5 immunoblot analysis. (C) The samples were immunoprecipitated with a polyclonal p40 Syt1 antibody, resolved by 10% acrylamide SDS-PAGE and subjected to V5 immunoblot analysis.

Figure 6. The Ability of SK1 to Bind Copper. (A) The SK1:V5 translation product from an *in vitro* translation reaction was adsorbed to a HiTrap chelating column previously loaded with 0.1 M $CuCl_2$. The column was washed with 1 M NaCl, and eluted with increasing concentrations of imidazole (0.1 mM to 1.0 M). The eluted fractions were subjected to V5 immunoblot analysis. (B) Copper-binding affinity shift of FGF1 and SK1 in NIH 3T3 cells. Lysates of FGF1 NIH 3T3 transfectant cells (top left), FGF1 NIH 3T3 transfectants cells transduced with AdSK1:V5 (top right; bottom right), and NIH 3T3 cells transduced with AdSK1:V5 (bottom left) were adsorbed to copper columns, and eluted with increased concentrations of imidazole, as described in the Materials and Methods. The eluates were resolved by 12% acrylamide SDS-PAGE, and subjected to FGF1 immunoblot analysis (top) or to V5 immunoblot analysis (bottom).

Figure 7. The Effect of Tetrathiomolybdate on SK1 Release In Response to Heat Shock. AdSK1:V5-transduced NIH 3T3 cells and AdSK1:V5-transduced FGF1 NIH 3T3 cell transfectants were incubated for 18 h at 37°C in the presence or absence of 250 nM TM. After incubation, the cells were subjected to heat shock for 110 minutes at 42°C in DMEM with or without 250 nM TM. (A) Conditioned media from AdSK1:V5-transduced NIH 3T3 cells, and

AdSK1V5-transduced FGF1 NIH 3T3 transfectant cells were subjected to immunoprecipitation and V5 immunoblot analysis. (B) Conditioned media from either FGF1 NIH 3T3 cells or AdSK1:V5-transduced FGF1 NIH 3T3 cell transfectants were processed for heparin-Sepharose chromatography, as described in Materials and Methods. The heparin-Sepharose column was eluted with 1.5 M NaCl, and the fractions were resolved in a 15% acrylamide SDS-PAGE, and subjected to FGF1 immunoblot analysis.

Figure 8. Evaluation of the Cell-Free Interaction Between the Recombinant Forms of SK1 and FGF1. The recombinant forms of FGF1 (100 ng) and SK1 (250 ng) were resuspended in 50 μl of PBS in the presence or absence of 1 mM $CuCl_2$, incubated for 30 minutes at 42°C, diluted in non-reducing SDS-PAGE loading buffer, resolved by 12% acrylamide non-reducing/limited denaturing SDS-PAGE (35), and subjected to immunoblot analysis, as described in the Material and Methods. SK1 facilitates the aggregation of FGF1 independent of the presence of copper. FGF1 was resuspended with or without an equimolar amount of SK1 in PBS in the presence or absence of 1 mM $CuCl_2$. Note the multiprotein aggregate band (at the top of the stacking gel) resolved by FGF1 (top panel) and V5 (bottom panel) immunoblot analysis.

Figure 1

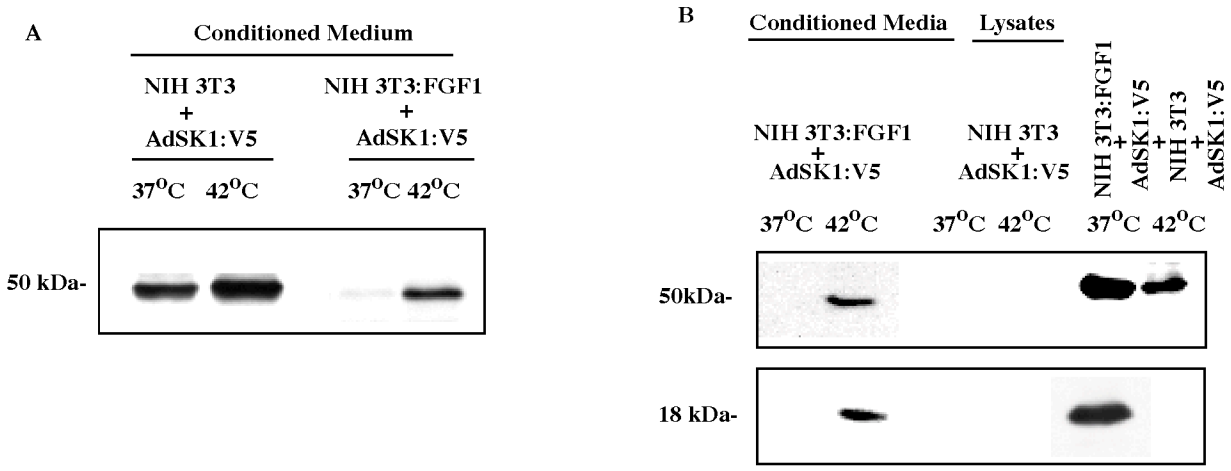

A
Conditioned Medium
NIH 3T3
+
AdSK1:V5
NIH 3T3:FGF1
+
AdSK1:V5
37°C 42°C
37°C 42°C
50 kDa-
B
Conditioned Media
Lysates
NIH 3T3:FGF1
+
AdSK1:V5
NIH 3T3
+
AdSK1:V5
NIH 3T3:FGF1
+
AdSK1:V5
NIH 3T3
+
AdSK1:V5
37°C 42°C
37°C 42°C
37°C 42°C
50kDa-
18 kDa-

Figure 2

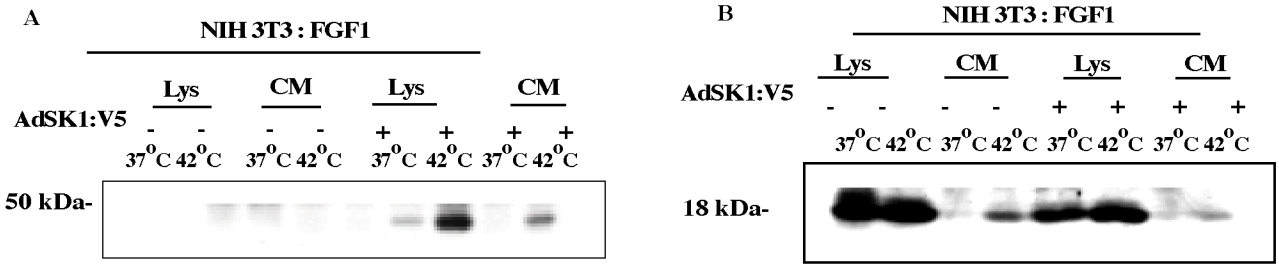

A
NIH 3T3 : FGF1
Lys
CM
Lys
CM
AdSK1:V5
- - - - + + + +
37°C 42°C 37°C 42°C 37°C 42°C 37°C 42°C
50 kDa-
B
NIH 3T3 : FGF1
Lys
CM
Lys
CM
AdSK1:V5
- - - - + + + +
37°C 42°C 37°C 42°C 37°C 42°C 37°C 42°C
18 kDa-

Figure 3

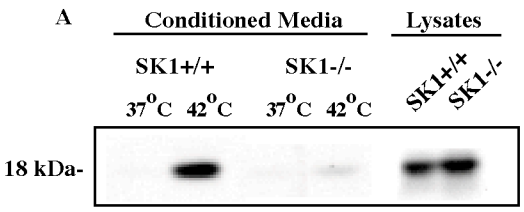

A
Conditioned Media
Lysates
SK1+/+
SK1-/-
37°C
42°C
37°C
42°C
SK1+/+
SK1-/-
18 kDa-

Figure 4

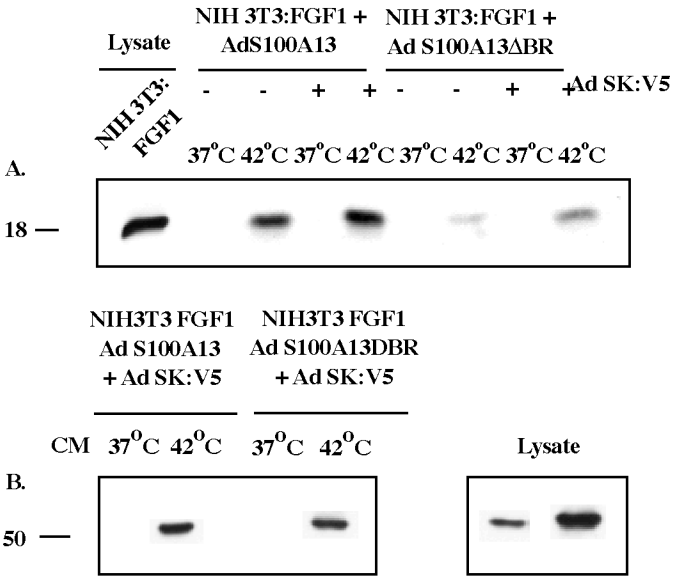

Lysate
NIH 3T3:FGF1 + AdS100A13
NIH 3T3:FGF1 + Ad S100A13ΔBR
NIH 3T3: FGF1
- - + + - - + + Ad SK:V5
37°C 42°C 37°C 42°C 37°C 42°C 37°C 42°C
A.
18 —
NIH3T3 FGF1 Ad S100A13 + Ad SK:V5
NIH3T3 FGF1 Ad S100A13DBR + Ad SK:V5
CM 37°C 42°C 37°C 42°C
Lysate
B.
50 —

Figure 5

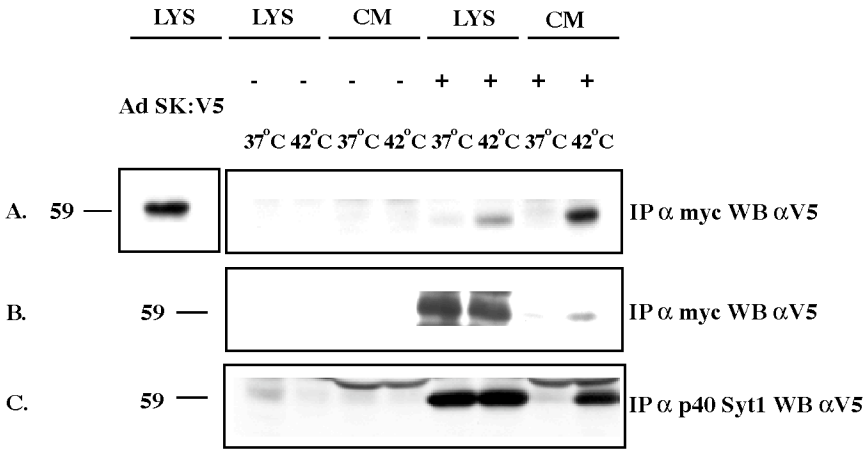

LYS
LYS
CM
LYS
CM
- - - - + + + +
Ad SK:V5
37°C 42°C 37°C 42°C 37°C 42°C 37°C 42°C
A. 59
IP α myc WB αV5
B. 59
IP α myc WB αV5
C. 59
IP α p40 Syt1 WB αV5

Figure 6

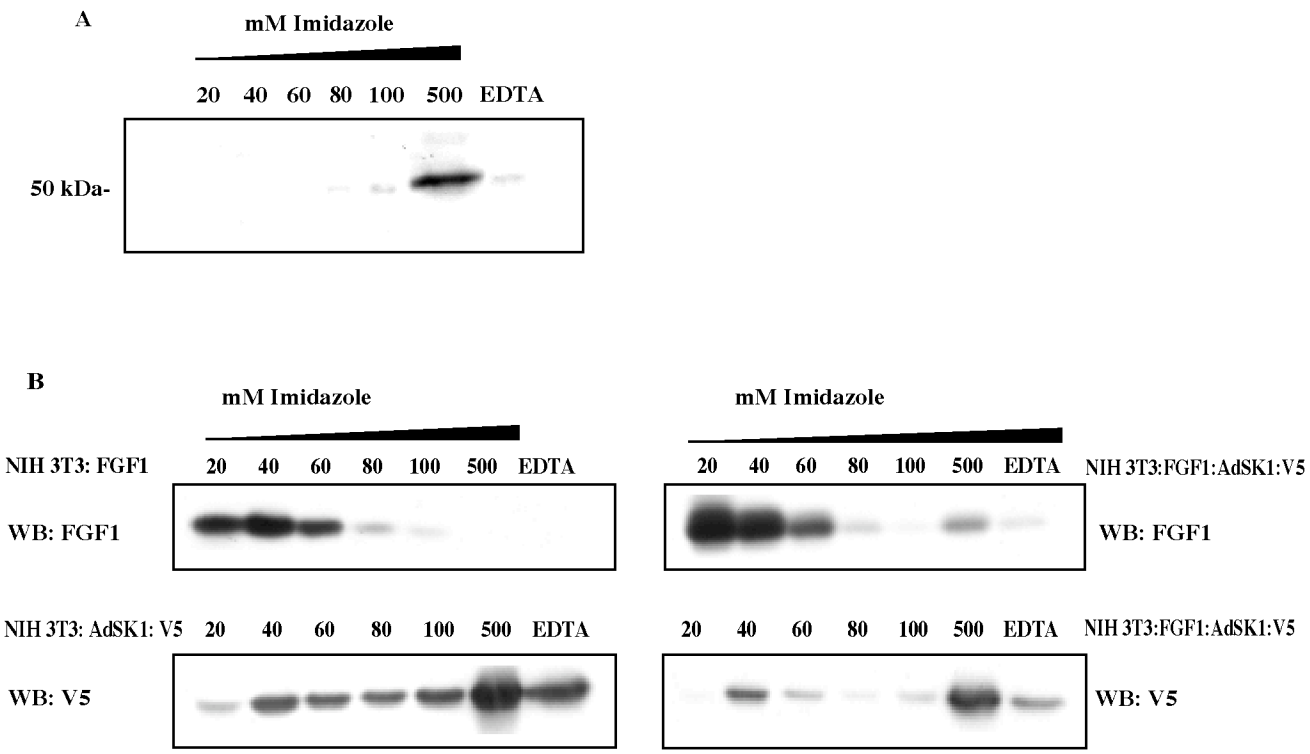
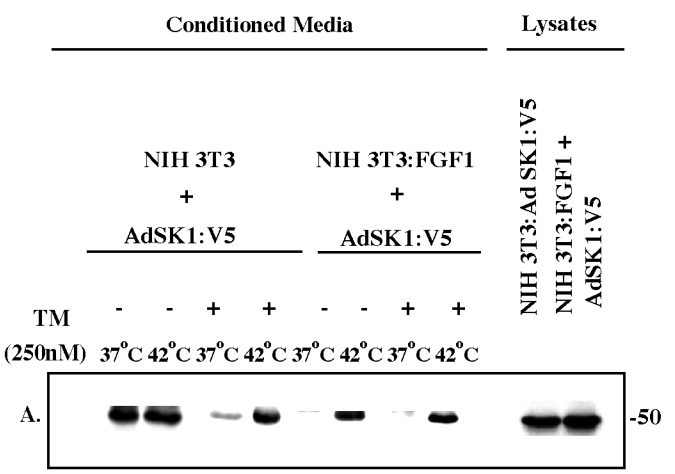

A
mM Imidazole
20 40 60 80 100 500 EDTA
50 kDa-
B
mM Imidazole
mM Imidazole
NIH 3T3: FGF1
20 40 60 80 100 500 EDTA
20 40 60 80 100 500 EDTA
NIH 3T3:FGF1:AdSK1:V5
WB: FGF1
WB: FGF1
NIH 3T3: AdSK1: V5
20 40 60 80 100 500 EDTA
20 40 60 80 100 500 EDTA
NIH 3T3:FGF1:AdSK1:V5
WB: V5
WB: V5

Figure 7


Conditioned Media
Lysates
NIH 3T3
+
AdSK1:V5
NIH 3T3:FGF1
+
AdSK1:V5
NIH 3T3:Ad SK1:V5
NIH 3T3:FGF1 +
AdSK1:V5
TM
(250nM)
- - + + - - + +
37°C 42°C 37°C 42°C 37°C 42°C 37°C 42°C
A.
-50

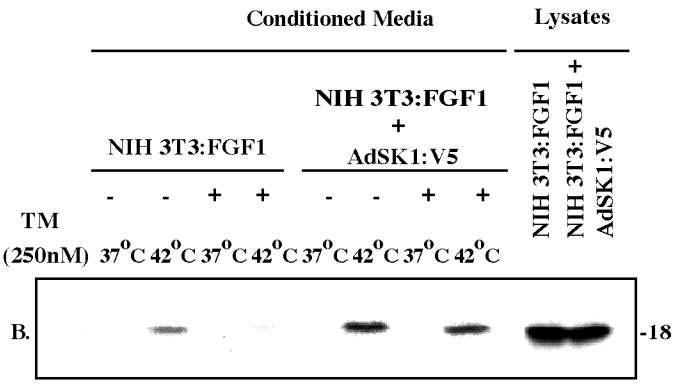
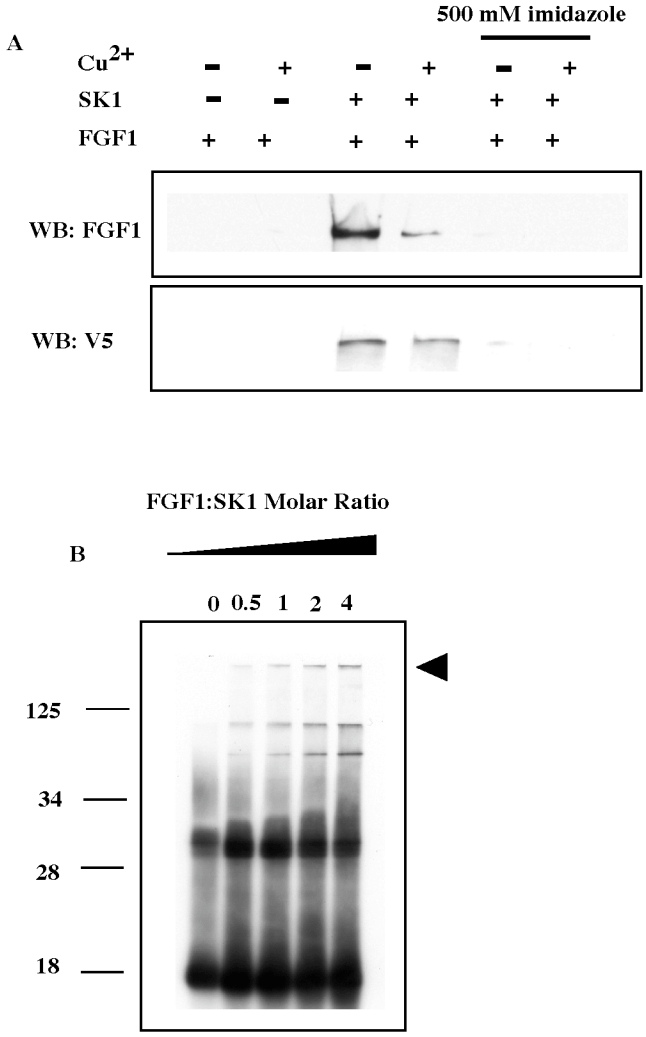

Conditioned Media
Lysates
NIH 3T3:FGF1
NIH 3T3:FGF1
+
AdSK1:V5
NIH 3T3:FGF1 +
AdSK1:V5
- - + + - - + +
TM
(250nM)
37°C 42°C 37°C 42°C 37°C 42°C 37°C 42°C
B.
-18

Figure 8


A
500 mM imidazole
Cu2+
SK1
FGF1
WB: FGF1
WB: V5
B
FGF1:SK1 Molar Ratio
0 0.5 1 2 4
125
34
28
18

Paper 6

"Members of the FGF-1 release complex permeabilize membranes composed of acidic phospholipids"

Irene Graziani, Cinzia Bagala', **Maria Duarte**, Raffaella Soldi, Vihren Kolev, Francesca Tarantini, Thallapuranam Krishnaswarny Suresh Kumar, David Neivandt, Chin Yu, and Igor Prudovsky

Journal of Biochemistry
(Manuscript under revision)

Members of the FGF1 release complex permeabilize membranes composed of acidic phospholipids †

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Running title: Non-Classical Release and Membrane Permeabilization

† This work was supported in part by NIH grants, HL 32348, HL 35627 and P20 RR 15555 (Project # 4) to IP and by the NSF grant 0132384 to DN. It was also partially supported by the following grants to CY: NIH NCRR COBRE (P20 RR15569), Department of Energy (DE-FG02-01ER15161), The Arkansas Bioscience Institute and the National Science

Council, Taiwan (NSC 94-2320-B007-005 and NSC-94-2113-M007-036). The work was performed by IG and MD in partial fulfillment of the requirements for Ph.D. degree from the Department of Critical Care Medicine and Surgery, Gerontology and Geriatrics Unit, University of Florence, Florence, Italy, and from the Life and Health Science University of Minho in Braga, Portugal, respectively.

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Abbreviations: Fibroblast growth factor (FGF), Synaptotagmin (Syt), phospholipids (pL), phosphatidylinositol (pI), phosphatidylserine (pS), phosphatidylglycerol (pG), phosphatidylcholine (pC), 5,6 carboxyfluorescein (CF).

The article is dedicated to the memory of Tom Maciag, friend, scientist, and mentor.

ABSTRACT

Fibroblast growth factor (FGF)1 is released from cells as a constituent of a multiprotein complex that contains the calcium binding proteins, S100A13, and the p40 kDa form of Synaptotagmin (Syt)1, through an ER-Golgi-independent stress-induced pathway. FGF1 and the other components of its secretory complex are signal peptide-less proteins, and their secretion mechanism is not fully understood. Since, when co-expressed in NIH 3T3 cells, S100A13, FGF1 and p40 Syt1 accumulated during stress in the vicinity of the cell membrane, we examined their capability to directly permeabilize lipid bilayers by studying protein-induced carboxyfluorescein (CF) release from liposomes of different phospholipid (pL) composition. FGF1 and S100A13 permeabilized acidic pL liposomes in a dose-dependent manner. The extent of CF released was more significant from phosphatidylinositol (pI) and phosphatidylglycerol than from phosphatidylserine liposomes. Surprisingly, p40 Syt1 exhibited a dose-dependent permeabilizing activity towards pI liposomes only. None of the proteins induced CF leakage from zwitterionic liposomes consisting of phosphatidylcholine. In order to identify the domain of p40 Syt1 involved in liposome permeabilization, we produced a mutant in which three lysine residues (K326, K327 and K331) in the C2B domain of p40 Syt1 were substituted with glutamines, and demonstrated that the ability of the mutant to induce pI liposomes permeabilization was strongly attenuated, and it did not exhibit either spontaneous or stress-induced release. Our results suggest that specific acidic pL in the inner leaflet of the cell membrane and basic amino acid-rich domains of constituent proteins are involved in the non-classical export of FGF1.

Fibroblast growth factor (FGF)1 regulates embryonic development of vertebrates (*1*) and plays important roles in angiogenesis, inflammation, wound healing, and as a neurotrophic factor (*2, 3*). Similar to FGF2, FGF1 belongs to a large group of proteins that lack a conventional signal sequence and are able to gain access to the extracellular compartment, independently of the endoplasmic reticulum (ER)-Golgi apparatus (*3-9*). Indeed, FGF1 release is insensitive to Brefeldin A, which blocks ER-to-Golgi vesicular transport (*10*), and FGF1 does not appear to be present in the cytoplasmic vesicles (*11*). Thus, FGF1 export through exocytotic fusion of secretory vesicles with the cell membrane is unlikely. FGF1 is secreted from cells upon stress stimuli such as heat shock (*12*), hypoxia (*13*), serum starvation (*14*), and treatment with oxidized LDL (*15*). The availability of free intracellular copper ions is necessary for FGF1 release, and *in vitro* data suggest the formation of a copper- and stress-dependent multiprotein export complex (*16*) containing calcium-binding proteins, S100A13 and p40 Syt1 (*17, 18*). p40 Syt1 is a non-transmembrane isoform of the integral component of secretory vesicles, Synaptotagmin (Syt)1, that is involved in the calcium (Ca^{2+})-triggered fusion of vesicles with the plasma membrane (*19*). Our laboratory demonstrated that p40 Syt1 is produced by alternative initiation of translation of p65 Syt1 mRNA (*20*).

Both S100A13 and p40 Syt1 are components of the heparin-binding aggregate containing FGF1 that was isolated from the brain (*21-23*). p40 Syt1 and S100A13 display a constitutive as well as a stress-induced release from transfected NIH 3T3 cells. Their constitutive release is blocked when they are cotransfected into the cells along with FGF1; however upon stress, they are released in a complex with FGF1 (*17, 18*). Specific deletion mutants of S100A13 and Syt1 block stress-induced FGF1 release, demonstrating that these proteins are necessary for FGF1 export (*17, 18, 24*).

Although significant progress has been achieved in the study of non-classical protein release, the final step that allows these polypeptides to translocate across the cell membrane remains unknown. Confocal immunofluorescence microscopy studies demonstrated that, in response to stress, cytosolic FGF1, S100A13, and p40 Syt1 traffic to the inner surface of the plasma membrane (*11*). It has been suggested that the assembly of the FGF1 release complex may occur in this locale through the interaction of the individual polypeptide components with membrane phospholipids (pL). Indeed, it is known that: (i) FGF1 is able to bind acidic pL in a solid phase pL assay (*25*), (ii) Syt1 can bind pS through its C2A domain and phosphorylated forms of phosphatidylinositol (pI) through its C2B domain (*26*), and (iii) some members of the S100 family, such as S100A6 and S100A10, bind pL and they are important regulators of the functions of pL-binding proteins (*27, 28*). However, the ability of S100A13 to interact with pL has not yet been evaluated.

It is known that FGF1 disrupts acidic pL-containing liposome integrity (*29*), is able to deform lipid bilayers (*30*), and it exhibits molten globule character at temperatures above 30°C (*31, 32*), at acidic pH, and also in the presence of anionic pL (*29*). Indeed, molten globule is a partially unfolded conformation that allows the protein to penetrate lipid bilayers (*33*). Given the pL binding capability and pL-induced molten globule state of FGF1 (*25, 29*), we investigated whether membrane pL play a role in FGF1 non-classical release. To this end, we studied the interaction of FGF1, S100A13, and p40 Syt1 with liposomes of various pL compositions, and we produced a p40 Syt1 mutant with reduced pL binding activity. Our results indicate that all three studied members of the FGF1 release complex permeabilized membranes composed of acidic pL. In addition, the mutation of specific basic amino acid residues in the C2B domain of p40 Syt1 blocked its ability to permeabilize liposomes and its release from the cells.

MATERIALS AND METHODS

Materials. Dioleoylphosphatidylinositol (pI), dioleoylphosphatidylglycerol (pG), dioleoylphosphatidylserine (pS), and dioleoylphosphatidylcholine (pC) were purchased from Avanti Polar Lipid (Alabaster, AL). The fluorescent dye, 5,6-carboxyfluorescein (CF), was purchased from Molecular Probes, Inc (Eugene, OR).

Plasmids and mutagenesis. The plasmids for eukaryotic expression of FGF1 (pXZ38) and p40 Syt1 (p40-Syt1:Myc pMEX hygro) were prepared as previously described (*12, 17, 18*). The K326,327,331Q mutant of p40 Syt1 was produced from the p40 Syt1:Myc pMEX Hygro and GST-p40 Syt1-pGEX-KG original plasmids (*18, 24*) by mutagenesis, using the QuickChange Site-directed Mutagenesis kit (Stratagene, Cedar Creek TX), and the following primers: 5' G CTG AAG AAG GAA GAG ACG ACG ATT GAG AAG AAC ACA CTC 3' (sense), 5' GT GTT CTT CTC AAT CGT CGT CTC TTC CTT CTT CAG CCT C 3' (antisense).

Recombinant protein purification. The recombinant FGF1, S100A13, and p40 Syt1 were prepared as previously described (*17, 24, 34*). Briefly, DH5α *E. coli* cells containing plasmids for the prokaryotic expression of FGF1 (FGF1-pET3C), S100A13 (GST-S100A13-pGEX-KG), or p40 Syt1 (GST-p40 Syt1-pGEX-KG), were grown at 37°C in selective medium containing ampicillin (150 μg/ml) to OD ~ 0.5 (wavelength: 600 nm); then 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to induce protein expression. Bacteria were harvested by centrifugation (6000 g), lysed in a lysis buffer (1X phosphate buffered saline, 2 mM EDTA, 0.05% Tween 20 for S100A13 and p40 Syt1 and 50 mM Tris pH 8.8, 10 mM EDTA, 10 mM glucose for FGF1) containing lysozyme (10 μg/ml), sonicated on ice (4 times X 15 sec), and centrifuged (10000 g for 10 minutes). The supernatant

containing the recombinant protein was incubated for 2 hours at 4°C with heparin sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) for FGF1, and glutathione sepharose (Amersham Pharmacia Biotech) for p40 Syt1 and S100A13. FGF1 was eluted from the heparin sepharose with 1.5 M NaCl in 50 mM Tris-HCl, 10 mM EDTA, pH 7.4 (TE), and dialyzed overnight against 1X phosphate buffered saline (PBS). Additionally, FGF1 was purified by High Pressure Liquid Chromatography (HPLC), using a cationic exchange column (Mono S HR 5/5, Amersham Pharmacia Biotech). For p40 Syt1 and S100A13 preparation, sepharose was treated with cleavage buffer (50 mM Tris pH 8.0, 150 mM NaCl, 2,5 mM $CaCl_2$) containing thrombin (3 μg/ml) for 45 minutes at room temperature. p40 Syt1 and S100A13 present in the supernatant were separated by HPLC, using respectively a cationic exchange column (Mono S HR 5/5, Amersham Pharmacia Biotech) and a reverse phase column (Luna C8, Phenomenex). p40 Syt1 K326,327,331Q mutant was purified by the same method as used for the wild type (wt) form of p40 Syt1.

Liposome Preparation. 1 ml of pL dissolved in chloroform (10 mg/ml) was dried under a nitrogen stream and resuspended in 100 mM CF solution at pH 7.0. The lipid suspension was sonicated for 30 minutes at 40 KHz, extruded 10 times through an Avanti Polar Lipids Mini-extruder, and passed through a 10 ml dextran desalting column (Pierce, Rockford, IL) pre-equilibrated with 10 mM HEPES, 150 mM NaCl at pH 7.0. The eluate was collected in several aliquots. The pL concentration of the liposome suspension in each aliquot was assessed by phosphorus assay according to Avanti Polar Lipids, Inc. instructions. Briefly, different amounts of phosphorus standard (Sigma, St. Louis, MO), 0 μmoles (0 μl) blank, 0.0325 μmoles (50 μl), 0.065 μmoles (100 μl), 0.114 μmoles (175 μl), 0.163 μmoles (250 μl), and 0.228 μmoles (350 μl), and 100 μl from each liposome fraction were placed in different tubes. 8.9 N H_2SO_4 was added, and the tubes were heated at 200-215°C for 25

minutes. 150 μl of H_2O_2 was added to the bottom of the tubes, and the tubes were heated for an additional 30 minutes. Sequentially, 3.9 ml deionized water, 0.5 ml of 2.5% ammonium molybdate tetrahydrate solution, and 0.5 ml of 10% ascorbic acid solution were added to all the tubes. The tubes were heated at 100°C for 7 minutes. Spectrophotometric analysis of the samples and standards was performed at 820 nm. A calibration curve of the absorbance generated using the standards was used to determine phosphorus concentration in the samples.

Fluorescence measurement. The fluorescence of liposomes resuspended in 10 mM HEPES, 150 mM NaCl (pH 7.0 unless differently indicated) was monitored for 10 minutes by a Fluorolog-3 spectrofluorimeter (Jobin Yvon Horiba, Edison, NJ) at an excitation wavelength 470 nm and an emission wavelength 520 nm. The temperature of the sample was controlled by a Peltier system. Different concentrations of FGF1, S100A13 and p40 Syt1 recombinant proteins were added to the cuvette at the second minute of the experiment. α-chymotrypsin (Sigma), at the maximal concentration employed for studied proteins, was used as a negative control and 0.1% Triton X-100 served as a positive control for complete liposome permeabilization (maximal CF release).

Cell culture. NIH 3T3 cells were grown to 70% confluence in Dulbecco's-modified Eagle's medium (DMEM; HyClone, Logan, UT) supplemented with 10% bovine calf serum (v/v) (BCS, HyClone) and 1X antibiotic/antimycotic mixture (Gibco, Grand Island, NY) on human fibronectin-coated (10 μg/cm^2) 10 cm dishes (Corning). The cells were transiently transfected with 10 μg p40 Syt1 wt or p40 K326,327,331Q Syt1 DNA, using JetPEI transfectant reagent according to the manufacturer's instructions (Qbiogene Inc.). For transient co-transfections, 5 μg of either p40 Syt1:Myc wild type (wt) DNA or p40

K326,327,331Q Syt1:Myc DNA, in combination with 5 μg of FGF1 DNA were added to a 10 cm dish. Twenty-four hours later, the cells were plated on 15 cm dishes.

Heat shock, conditioned media processing and immunoblot analysis. Transiently transfected NIH 3T3 cells grown to 70-80 % confluency were washed with DMEM containing 5 units/ml heparin (Sigma), and heat shock was performed as previously described (*12*) in DMEM containing 5 U/ml heparin (Sigma) for 110 minutes at 42°C; control cultures were incubated at 37°C in the same medium. Following heat shock, the media were collected, filtered through a 0.22 μm filter, and adsorbed to a heparin-sepharose CL-6B (1 ml) column (Amersham Pharmacia Biotech) preequilibrated with TE. The adsorbed proteins were washed with TE buffer, eluted with 1.5 M NaCl-TE buffer, and concentrated by Amicon Ultra (Millipore). The samples were resuspended in Laemmlie buffer, resolved by 12% (w/v) sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE), transferred to a nitrocellulose membrane, and immunoblotted with rabbit anti-FGF1 or anti-Syt1 antibody, followed by a goat anti-rabbit peroxidase-conjugated secondary antibody (Biorad). Proteins in the blots were detected using an ECL kit (Amersham Bioscences).

Confocal microscopy analysis. NIH 3T3 cells were plated on fibronectin-coated glass coverslips in 6-well plates, grown to 70% confluence in DMEM supplemented with 10% BCS and 1X antibiotic/antimycotic mixture, and then transiently transfected as described above with 1 μg per well of p40 Syt1:Myc or p40 Syt1:Myc K326,327,331Q DNA. After 24 hours, the culture medium was substituted with DMEM containing 5 U/ml heparin (Sigma), and heat shock was performed as described above. Cells were fixed with 4% formaldehyde and immunostained for Myc tag. Briefly, after fixation for 10 minutes, the cells were washed twice with PBS, blocked for 1 hour in blocking buffer (PBS containing 10% bovine serum albumin, 0.1% Tween 20, 0.1% Triton X-100, and 0.1% NaN_3), incubated for 1 hour with a

monoclonal anti-Myc antibody (Oncogene, 1 μg/ml), washed three times with PBS, incubated for 1 hour with a 1:100 dilution of fluorescein-conjugated anti–mouse IgG antibody (Molecular Probes) and 1 μg/ml Hoechst 34580 (Molecular Probes), washed three times with PBS, and embedded in Vectashield mounting medium (Vector Laboratories Inc, Burlingame CA). A LTCS-SP confocal system (Leica) was used for these studies. Cells were examined using a 100x objective and the 237 μM confocal pinhole of the Leica 2000 confocal software program.

RESULTS

Proteins of the FGF1 release complex induce liposome permeabilization that is dependent on liposome composition and protein concentration.

CF-loaded liposomes present a convenient model for studying the membrane permeabilizing activity of proteins. FGF1 was previously demonstrated to permeabilize mixed pG/pC liposomes (*29*). To investigate the interaction of FGF1 with various plasma membrane pL, we compared the permeabilizing effect of FGF1 on liposomes consisting of several acidic (pS, pI, pG) and a zwitterionic pL (pC). We also evaluated whether the other components of FGF1 release pathway, i.e. S100A13 and p40 Syt1, induce liposome permeabilization (Figure 1A, B, C). The recombinant forms of FGF1, S100A13, and p40 Syt1 were added to the pL liposome suspension in the cuvette, and the CF release was detected fluorimetrically. The final protein concentrations in the cuvette were 1 μM, 0.5 μM, 0.25 μM, 0.125 μM, and the pL concentration was 2 μM. A temperature of 50°C was chosen in accordance with published data regarding FGF1-induced liposome permeabilization (*29*). The dependence of the ability of FGF1 to induce liposome permeabilization upon pL

composition may be represented by the following relationship: pI > pG > pS > pC (Figure 1A). Interestingly, we observed that S100A13 also exhibited permeabilizing effects on liposomes with a similar relationship as FGF1: pG > pI > pS > pC (Fig. 1B). Conversely, p40 Syt1 was not able to induce any CF release from pC, pS, and pG liposomes, but very efficiently permeabilized pI liposomes (Figure 1C). It is important to stress that none of the studied proteins was able to induce a CF release from zwitterionic (pC) liposomes (Figure 1A, B, C). The extent of CF release was proportional to protein concentration. S100A13 at 1 μM induced a maximal CF release from pG liposomes almost equivalent to complete liposome permeabilization induced by 0.1% Triton X-100 (Figure 1B).

Thus we conclude that all the studied proteins of the FGF1 release complex were able to induce permeabilization of liposomes composed of acidic pL, but not zwitterionic pL, in a protein concentration-dependent manner. In addition, at least for p40 Syt1, this ability exhibited selectivity towards a specific acidic pL (pI).

S100A13- and p40 Syt1-induced permeabilization of acidic phospholipid liposomes is temperature- and pH-dependent.

Since the export of the FGF1 complex from cells (*17, 24*) and FGF1-induced liposome permeabilization are temperature-dependent (*29*), we evaluated the effect of temperature upon S100A13- and p40 Syt1-induced CF release from liposomes (Figure 2A). 125 nM S100A13 was added to a 1 μM pG liposome suspension, and fluorescence measurements were performed at 27°, 32°, 37°, 42°, 47° and 52°C. The release induced by the protein at each specific temperature, and, in further experiments, at each specific pH, was estimated as percentage of the maximal CF release obtained by subtracting the spontaneous liposomes leakage (untreated liposomes), which was equivalent to the leakage of liposomes in the presence of 125 nM α-chymotrypsin (data not shown), from the release induced by

0.1% Triton X-100. S100A13 started to induce CF release at 37°C, with the maximum release observed at 47°C. The temperature-dependent liposome permeabilization showed biphasic behavior. We evaluated the effect of temperature on p40 Syt1-induced CF release similarly to S100A13, however, because we observed that p40 Syt1-induced liposome permeabilization was pI-selective (Figure 1C), we employed pI liposomes (Figure 2B). Like S100A13, p40 Syt1 started to induce CF release at 37°C, but the permeabilizing activity increased progressively with temperature; in this case, the dependence of CF release upon temperature exhibited monophasic features. Interestingly, Mach et al. have previously demonstrated a similar monophasic temperature dependence for FGF1, that induced maximal release at 50°C (*29*).

Since the intracellular pH changes with specific physiological conditions, such as stress, (*35*), we considered the possibility that this factor may also influence the structure of the FGF1 release complex components and their interaction with pL. In order to evaluate the effect of pH upon S100A13-induced liposome permeabilization, 125 nM S100A13 was applied to 1 μM pI liposomes at 37°C and at pH 6.0, 7.0, and 8.0. The maximal CF release occurred at pH 6.0 (Figure 2C). The analysis of the pH-dependence of p40 Syt1 permeabilizing effect revealed an enhancement of the release at pH 6.0, similar to S100A13 (Figure 2D), suggesting that changes in intracellular pH may play a role in non-classical protein release. Interestingly, the FGF1-induced CF leakage from liposomes was also demonstrated to be enhanced at acidic pH (*29*).

p40 Syt1 K326,327,331Q mutant is not released by NIH 3T3 cells.

The ability of all three members of the FGF1 release complex to permeabilize acidic pL membranes prompted us to use mutagenesis in order to investigate whether this property is related to the ability to exhibit non-classical release. To that end, we have chosen Syt1,

whose interaction with membranes is well studied (*26*). A loss of pI binding capability was described for Syt2 after substitution of three closely located lysine residues in the C2B domain (*36*). Particularly, it was demonstrated for the binding of PIP_2, which is present only at the plasma membrane (*37*). Since these residues in the C2B domain are highly conserved among the Syt family members, we mutated them in p40 Syt1, in order to evaluate the release of the resultant mutant during stress and at normal conditions. Heat shock experiments performed on NIH 3T3 cells transiently transfected with either the mutant or the wt form of p40 Syt1 demonstrated that the p40 Syt1 K326,327,331Q mutant was not released either at 37º or 42ºC, unlike the wt form (Figure 3A). This suggests that the lysine residues at position 326, 327, 331 are required for both stress-induced and constitutive release of p40 Syt1 from cells. However, unlike the deletion mutant of p65 Syt1 lacking most of the C2A domain (*18*) p40 Syt1 K326,327,331Q was not able to prevent FGF1 release from NIH 3T3 cells upon heat shock when it was co-expressed with FGF1 (Figure 3B).

p40 Syt1 K326,327,331Q does not display perimembrane localization in transfected cell.

Previously, by confocal microscopy, we demonstrated that when co-expressed with FGF1, the wt form of p40 Syt1, like the other members of the FGF1 release complex, migrates to the plasma membrane under stress conditions (*11*). Since p40 Syt1 K326,327,331Q is not released by cells, we evaluated its localization by confocal fluorescence microscopy during heat shock and at normal conditions in transiently transfected NIH 3T3. Our study demonstrated that p40 Syt1 K326,327,331Q presented a diffuse cytosolic distribution pattern both at 37º (Figure 4C) and at 42ºC (Figure 4D) unlike the wt form which displayed a perimembrane localization under both conditions (Fig. 4A, B). Additionally, while p40 Syt1 wt exhibited nuclear and cytoplasmic localization, p40 Syt1 K326,327,331Q was localized only in the cytoplasm. Apparently lysine residues 326, 327, 331 of p40 Syt1,

which are necessary for its interaction with the cell membrane, can alternatively serve as a nuclear localization sequence.

K326,327,331Q mutations in the p40 Syt1 C2B domain lead to a sharp decrease of pI liposome permeabilization activity.

The inability of p40 Syt1 K326,327,331Q to be released by NIH 3T3 cells and to exhibit localization at the plasma membrane, prompted us to explore its liposome permeabilizing activity. As shown in Figure 5, the p40 Syt1 K326,327,331Q-induced CF release from pI liposomes was significantly (approximately 70%) reduced compared to wt p40 Syt1. Taken together, these data suggest that the lysine residues at positions 326, 327, and 331 play a crucial role in the ability of p40 Syt1 to bind the plasma membrane, permeabilize it, and exit to the extracellular compartment.

DISCUSSION

It is known that, despite the absence of a classical signal sequence in its structure, FGF1 exhibits stress-induced release (*12*). However the mechanism used by this growth factor to cross the cell membrane is still not sufficiently understood. Previously it was demonstrated that FGF1 binds acidic pL (*25*), and assumes a partially unfolded molten globule conformation in the presence of acidic pL (*29*). In this conformation, FGF1 maintains its secondary structure but loses almost completely its tertiary structure. It acquires hydrophobic characteristics that, theoretically, can allow it to gain solubility in lipid membranes and to permeabilize them (*29, 33*). However, since FGF1 is released in association with S100A13 and p40 Syt1 (*17, 18*), the question arises whether the two latter proteins could also display membrane permeabilizing properties. It is known that Syt1, similarly to FGF1 (*25*), is able to associate with pL, and particularly pS and pI (*26*). In

addition, some S100 family members have been demonstrated to bind pL or pL-binding proteins (*27, 28*). These previous observations prompted us to undertake a comparative study of the interaction of FGF1, p40 Syt1, and S100A13 with artificial membranes. We observed that the three proteins were able to permeabilize lipid membranes composed of acidic but not of zwitterionic pL. While FGF1 and S100A13 exhibited permeabilizing activity towards pG, pS, and pI membranes, p40 Syt1 induced selective permeabilization of only pI liposomes.

We previously demonstrated that when the components of the FGF1 release complex are co-expressed, they localize at the plasma membrane in response to stress (*11*). The observation that FGF1 release complex members are able to permeabilize acidic pL liposomes led us to hypothesize a critical role of plasma membrane pL in the assembly and export of the FGF1 release complex. Particularly, the preference exhibited by p40 Syt1 in permeabilizing pI liposomes suggests the possibility of the existence of specific pL sites, which recruit different constituents of the FGF1 release complex at the inner leaflet of the plasma membrane. Therefore, it is likely that specific pLs may function as anchors for individual proteins, facilitating the formation of the FGF1 multiprotein release complex at the inner face of the cell membrane. Additionally, acidic pLs might be responsible for inducing tertiary structure modifications that allow the proteins to translocate across the plasma membrane. This might be the case for p40 Syt1, which is known to bind pS and pI respectively through the C2A and C2B domains (*26*), but exhibits permeabilizing activity only towards pI liposomes. It is also important to remark that the well-known capability of some pLs, such as pS, to flip from the inner to the outer face of the plasma membrane in response to stress (*38, 39*) might be involved in this translocation process.

As already mentioned, conformational changes of FGF1 induced by acidic pL may play a crucial role in the translocation of the protein across the cell membrane (*29*). The calcium-binding protein, S1001A13, which is released from cells in association with FGF1,

and exhibited permeabilizing activity upon acidic pL liposomes similarly to FGF1, is a member of the S100 proteins family. S100 proteins are known to regulate partner proteins functions by inducing their structural modification, i.e. acting as chaperones; in particular, S100A10 regulates the process of insertion of annexin II into the membrane (*40*). Similarly to S100A10, S100A13 might promote FGF1 and p40 Syt1 plasma membrane penetration and translocation.

The establishment of membrane permeabilizing properties of the FGF1 release complex components prompted us to use mutational analysis to identify the permeabilizing domain of one of them, p40 Syt1. In these studies, we used the previously published information about the pI-binding residues of Synaptotagmin (*36, 37*). We found that mutation of lysines 326, 327 and 331 to glutamines resulted in the inability of p40 Syt1 to be released from NIH 3T3 cells both at 37º and at 42ºC, and in a strong decrease of pI liposome permeabilization. Additionally, we found that, while p40 Syt1 wt exhibited perimembrane localization, p40 Syt1 K326,327,331Q was distributed diffusely in the cytoplasm of transfected cells. Thus, lysines K326, 327, and 331 (i) may be responsible for the interaction of p40 Syt1 with plasma membrane pI, (ii) are required for its release, (iii) are at least partially responsible for the permeabilization of artificial pI membranes. Interestingly, p40 Syt1 K326,327,331Q did not inhibit the stress-induced release of FGF1. Apparently, the inability of the mutant p40 Syt1 to associate with the cell membrane both at stress and at normal conditions precludes its interference with FGF1 release.

In conclusion, the results of this work demonstrate that the members of non-classical FGF1 release complex exhibited the ability to permeabilize artificial membranes composed of acidic pL, which are known to be preferentially localized in the inner leaflet of the cell membrane (*41, 42*). Moreover, at least for p40 Syt1, its permeabilizing ability correlated with its non-classical release from NIH 3T3 cells. Further identification of the membrane

permeabilizing domains of FGF1 and S100A13 and structural characterization of the interaction of the FGF1 release complex components with lipid membranes is important for the understanding of the mechanism of non-classical protein release and may lead to design of compounds to interfere with this process for therapeutic applications.

ACKNOWLEDGMENT

The authors wish to thank the laboratory of R. Middaugh at the University of Kansas for their help in the design of the liposome studies.

FIGURE LEGENDS

Figure 1: The release of carboxyfluorescein (CF) from acidic and zwitterionic phospholipid liposomes by FGF1, S100A13, and p40 Syt1. CF-containing liposomes were added to the cuvette to produce a 2 μM final pL concentration. CF release was fluorimetrically detected at 520 nm emission wavelength for 600 seconds. Following an equilibration time (120 seconds), the recombinant forms of FGF1 (A), S100A13 (B), and p40 Syt1 (C) were independently added to the cuvette at concentrations of 1 μM (o-o), 500 nM (▲-▲), 250 nM (x-x) and 125 nM (◊-◊). The data are reported in fluorescence units as a function of time in seconds. The addition of 0.1% Triton X-100 (●-●) served as a positive control of permeabilization, the addition of 1 μM α-chymotrypsin (—) served as a negative control.

Figure 2: Effect of temperature and pH upon protein-induced permeabilization of pG liposomes. (A) 125 nM S100A13 was added to 1 μM suspension of CF-containing pG liposome suspension. The S100A13-induced pG liposome permeabilization was studied at the following temperatures: 27°C, 32°C, 37°C, 42°C, 47°C and 52°C. (B) 125 nM p40 Syt1

was added to 1 μM suspension of CF-containing pI liposomes. The effect of temperature on p40 Syt1-induced pI liposome permeabilization was studied as described in A. (C) 125 nM S100A13 was added to 1 μM suspension of pI liposomes at pH 6.0, 7.0, and 8.0 at 37°C. The effect of pH upon pG liposome permeabilization induced by S100A13 was studied. (D) 125 nM p40 Syt1 was added to 1 μM suspension of pI liposome at pH 6.0, 7.0, and 8.0. The effect of pH upon pI liposomes permeabilization induced by p40 Syt1 was studied. The protein-induced CF release from liposomes in A, B, C, and D was evaluated as a percentage of the maximal release induced by 0.1% Triton-X100 at each specific temperature and pH (see in "Results").

Figure 3: The release of p40 Syt1 K326,327,331Q from NIH 3T3 cells. (A) NIH 3T3 cells transfected either with p40 Syt1 wt or with p40 Syt1 K326,327,331Q were heat shocked. Lane 1 - p40 Syt1 wt transfectant cells lysate. Lane 2 - p40 Syt1 K326,327,331Q transfectant cells lysate. Lanes 3 and 4 - conditioned media from p40 Syt1 wt transfectant cells respectively at 37°C and at 42°C. Lanes 5 and 6 - conditioned media from the p40 Syt1 K326,327,331Q transfectant cells respectively at 37°C and at 42°C. (B) Heat shock was performed on NIH 3T3 cells co-transfected with FGF1, and either p40 Syt1 wt or p40 Syt1 K326,327,331Q. Lane 1 - FGF1/p40 Syt1 wt co-transfectant cells lysate. Lane 2 - FGF1/p40 Syt1 K326,327,331Q co-transfectant cells lysate. Lanes 3 and 4 - conditioned media from the FGF1/p40 Syt1 wt co-transfectant cells at 37°C and at 42°C. Lanes 5 and 6 - conditioned media from the FGF1/p40 Syt1 K326,327,331Q co-transfectant cells at 37°C and at 42°C.

Figure 4: Confocal fluorescence microscopy analysis of p40 Syt1 K326,327,331Q cellular localization at normal and stress conditions. NIH 3T3 transfected with either p40 Syt1 wt or

with p40 Syt1 K326,327,331Q were heat shocked. (A) and (C) represent the control cells respectively transfected with p40 Syt1:Myc wt and p40 Syt1:Myc K326,327,331Q maintained at normal conditions (37°C). (B) and (D) represent heat shocked (42°C) cells respectively transfected with p40 Syt1:Myc wt and p40 Syt1:Myc K326,327,331Q.

Figure 5: Analysis of CF release from pI liposomes induced by the p40 Syt1 K326,327,331Q mutant. 1 μM p40 Syt1 wt (o-o), or p40 Syt1 K326,327,331Q (▲-▲) was added to 2 μM pI liposome suspension in the cuvette, and CF release from liposomes was monitored. 0.1% Triton X-100 served as a positive control (●-●), and 1μM α-chymotrypsin as a negative control (—).

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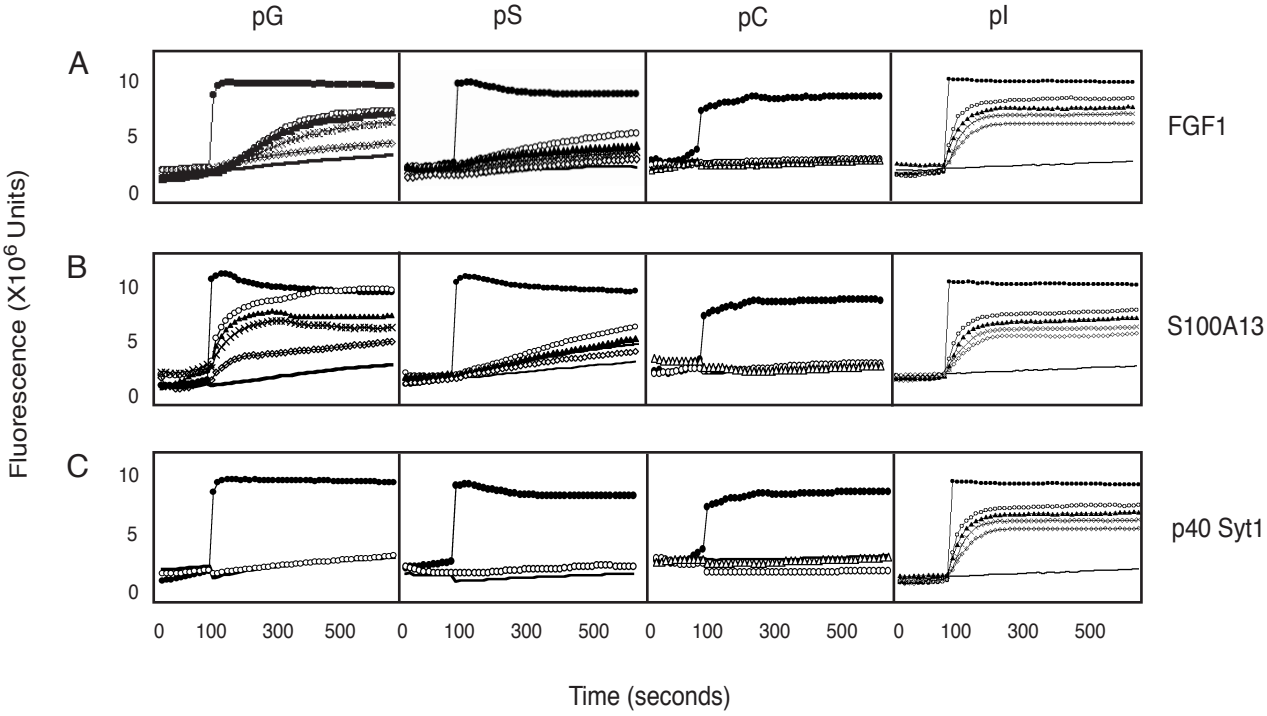
Figure 1


pG
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FGF1
S100A13
p40 Syt1
Fluorescence (X10^6 Units)
10
5
0
0
100
300
500
Time (seconds)

Figure 2

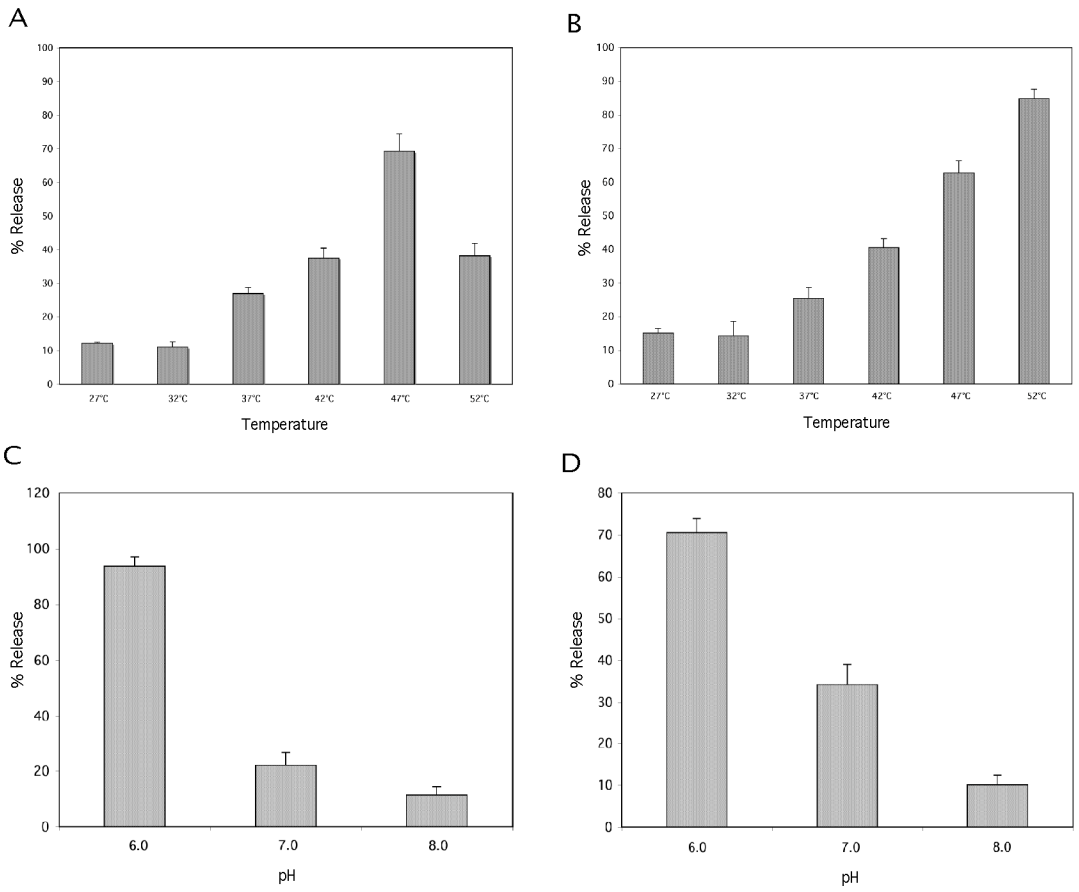

A
% Release
Temperature
27°C
32°C
37°C
42°C
47°C
52°C
B
% Release
Temperature
C
% Release
pH
6.0
7.0
8.0
D
% Release
pH

Figure 3

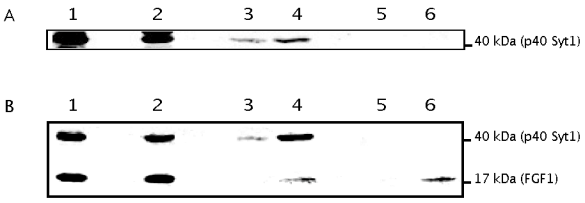


Figure 4

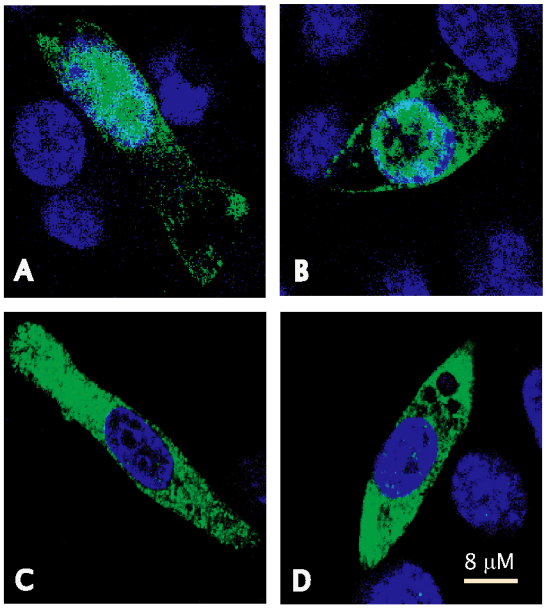
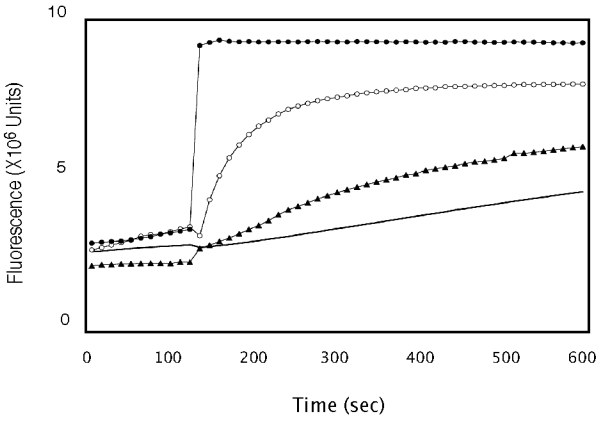


Figure 5


10
5
0
Fluorescence (X10⁶ Units)
0
100
200
300
400
500
600
Time (sec)

CHAPTER VII

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