Syndecan-4 in cell signaling and membrane trafficking

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*Syndecan-4 in cell signaling and
membrane trafficking*

Syndecan-4 in cell signaling and membrane trafficking

DISSERTATION

To obtain the degree of Doctor
at the Maastricht University,
on the authority of the Rector Magnificus,
Prof. dr. G.P.M.F. Mols
in accordance with the decision of the Board of Deans,
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"Many will think that they can with reason blame me, alleging...that my proofs are contrary to the authority of certain men held in great reverence by their unexperineded judgements, not considering that my works are the issue of simple and plain experience which is the true mistress."

Leonardo da Vinci

Contents

<i>Introduction</i>	9
Chapter 1	13
Syndecans - new kids on the signaling block.	
Chapter 2	31
Modulation of Microvascular Signaling by Heparan Sulfate Matrix: Studies in Syndecan-4 Transgenic Mice.	
Chapter 3	41
Growth Factor-specific Modulation of Cellular Response by Syndecan-4.	
Chapter 4	57
Clustering induces redistribution of syndecan-4 core protein into raft membrane domains.	
Chapter 5	67
Fibroblast Growth Factor 2 Endocytosis In Endothelial Cells Proceed Via Syndecan-4-dependent Activation of Rac1 and a Cdc42-dependent Macropinocytic Pathway.	
Chapter 6	83
Syndecan-4 clustering induces migration in synectin dependent manner.	
Chapter 7	95
Summary	95
Samenvatting	99
 <i>Acknowledgments</i>	 103
 <i>Curriculum Vitae</i>	 104

List of abbreviations

BSA	bovine serum albumin
CS	chondroitin sulfate
DMEM	Dulbecco's modified Eagle's medium
GAG	glycosaminoglycan
GPI	glycosylphosphatidylinositol
EGF	epithelial growth factor
EGFP	enhanced green fluorescent protein
Erk	extracellular signal regulated protein kinase
FACS	fluorescence-assisted cell sorting
FBS	fetal bovine serum
FcR	Fc receptor Ia CD64
FcR-S4	Fc receptor-syndecan-4 chimera
FGF2	fibroblast growth factor 2
FLAER	Alexa-488-conjugated inactive variant of proaerolysin
GIPC	GAIP c-terminus interacting protein
HA	hemagglutinin
HGF	hepatocytes growth factor
HRP	horseradish peroxidase
HS	heparan sulfate
HUVEC	human umbilical vein endothelial cells
kD	kiloDalton
LDL	low density lipoprotein
NO	nitric oxide
OST	O-sulfotransferase
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDZ	postsynaptic density 95, disk large, zona occludens-1
PIP2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PP1/2A	protein phosphatase type 1/2A
RFPEC	rat fat pad endothelial cells
R-PE	red-phycoerythrin
TIRF	Total Internal Reflection Fluorescent Microscopy
WASP	Wiscott-Aldrich syndrome protein
S4	syndecan-4
TGF β	transforming growth factor β
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor

Introduction

The syndecan family contains four highly related transmembrane proteoglycans that are cellular signaling molecules in embryonic development, tumorigenesis and angiogenesis. Syndecan-4 is the most ubiquitously expressed member of this family. It is upregulated in various tumors as well as injured and ischemic tissues. A number of studies have concluded that syndecan-4 plays an important role in many cell signaling pathways. However, to the surprise of many, syndecan-4 null mice are viable and healthy. While lacking visible developmental abnormalities, these mice exhibited defects under various stress conditions. Those include a high mortality following septic shock, and slow wound healing and muscle regeneration. Thus, syndecan-4, while not needed for embryonic development, may play an important role in an acute response settings

The syndecan-4 extracellular domain carries heparan and chondroitin sulfate chains that can bind a number of ligands including cell matrix proteins and various heparin-binding growth factors. One area of research in our laboratory involves fibroblast growth factor 2 (FGF2), one of the heparan-binding growth factors capable of binding syndecans. Our group, and others, have previously demonstrated that removal of heparan from the cell surface results in perturbed FGF2-induced cell signaling.

Modulation of cell signaling by syndecan-4 is determined not only by heparan sulfate chains bound to the extracellular portion, but also by unique properties of its cytoplasmic domain. Portions of the cytoplasmic domain are conserved in all members of the syndecan family and bind common intracellular partners. These include Src kinase, cortactin and several PDZ-domain containing proteins. In addition to the cytoplasmic common regions, each of the syndecans has a variable region in the cytoplasmic domain and these differences are thought to underlie the unique properties of each of the syndecans. Syndecan-4 variable domain binds to PKC α , α -actinin and syndesmos. These variable domain interactions are responsible for the unique role played by syndecan-

4 among the syndecan family in controlling focal adhesion formations and regulating PKC α activity. The research in this thesis is aimed at identifying molecular mechanisms involving syndecan 4 that regulate the cellular responses important in post-natal vascular remodeling in endothelial cells.

To investigate the role of syndecan-4 in modulation of microvascular cell signaling we initially used a mouse transgenic model that overexpressed syndecan-4. We found that FGF2 treatment of microvessels isolated from these animals led to an increase in nitric oxide production. However, the increase in nitric oxide production is largely due to an increase in heparan and chondroitin sulfate mass and is not a specific syndecan-4 effect.

We then decided to investigate the cell signaling role of cytoplasmic tail of syndecan-4 by generating a series of constructs with mutagenized cytoplasmic tails. In these experiments we focused on syndecan-4's C-terminus PDZ-binding region and the middle variable domain region. Mutations that disrupted the PIP2/PKC α -binding region and the PDZ-binding region led to altered responsiveness of endothelial cells to FGF2 in migration, cell proliferation and tube formation assays. Thus, disruption of the syndecan-4 signaling complex can perturb FGF-2 induced cell response.

We hypothesized that the subcellular localization of syndecan-4 can modulate the growth factor responsiveness. Thus we looked at the properties of syndecan-4 containing membranes before and after FGF-2 treatment. FGF-2 induced syndecan-4 clustering results in a shift in syndecan to cholesterol rich membrane domains called lipid rafts. The exact role of lipid rafts is unclear; however, the fact that these regions are enriched in kinases and other signaling molecules suggest that they play a role in the assembly of signaling complexes.

Binding of a ligand to its receptor generally results in endocytosis of the receptor-induced signaling complex, leading to downregulation of cell signaling. Consequently, rate of receptor endocytosis may determine the strength of the

cell signaling. Thus we decided to investigate the mechanisms associated with syndecan-4 internalization that are induced by FGF2 binding. We found that FGF2 binding to syndecan-4 results in a shift of the FGF2/syndecan-4 complex to lipid rafts and this complex is then internalized via the constitutive macropinocytic pathway. This uptake requires syndecan-4 clustering-induced activation of Rac1 and basal activity of CDC42.

Both Rac1 and CDC42 are GTPases of the Rho-family that are important for migratory cell polarization. Thus, we hypothesized that the activation of Rac1 seen following syndecan-4 clustering may promote cell migration in addition to its role in the uptake of syndecan-4. To this end, we demonstrated, that in endothelial cells antibody clustering of syndecan-4 leads to polarization of Rac1 activity within the cell that results in increased cell migration. In contrast, cells expressing syndecan-4 with a disrupted PDZ-binding domain do not show an increase in clustering-induced cell migration. Surprisingly, the PDZ-binding domain mutants exhibited high levels of Rac1 activity even in non-stimulated cells. Active Rac1 in these cells is distributed uniformly, whereas "normal" migratory cells primarily localize active Rac1 along the leading edge of the cell. As expected, cells expressing the PDZ-binding domain mutant also failed to form the typical trailing edge region of the cell with minimal Rac1 activity. These results have led us to propose that syndecan-4 clustering locally releases inhibition of Rac1 via a PDZ-domain interacting protein, thus promoting migratory polarization of the cell.

Synectin is a PDZ-domain containing protein that is known to interact with the PDZ-binding domain of syndecan-4, and our group has previously demonstrated that synectin modulates cell migration in response to FGF2. We found that endothelial cells isolated from synectin knockout mice failed to migrate in response to antibody clustering of syndecan-4. They also exhibited a high basal level of Rac1 activity. Thus, our data suggest that syndecan-4 clustering induces cell migration by local activation of Rac1 in a synectin-dependent manner. This process is likely

to involve internalization of a Rac1-inhibiting signaling complex that would allow plasma-membrane targeting and activation of Rac1.

This thesis demonstrates that perturbing the expression of syndecan-4 results in an altered cellular response to FGF2. These changes in FGF2-dependent signaling in experiments performed here are a consequence of a number of syndecan-4 related events including changes in the cell surface heparan/chondroitin sulfate mass and perturbation of the FGF/syndecan signaling complex. It has been speculated before, that syndecan-4 regulates the localization of bound growth factors not only by shedding from the cell surface but also as a consequence of its plasma membrane localization and endocytosis. We, for the first time, demonstrate and characterize the FGF2 dependent shift of the syndecan-4/FGF2 complex into lipid rafts followed by internalization. One of the unique properties of this uptake is that syndecan-4 internalizes via constitutive macropinocytosis which is unusual for transmembrane proteins. Presumably, the large sulfate chains of syndecan-4 do not allow it to fit into smaller clathrin or caveolin coated vesicles. This work is the first evidence of the ligand induced targeting of a transmembrane protein to the macropinosomal endocytosis route.

Using antibody clustering, we have identified a novel migratory activity associated with syndecan-4. Clustering of syndecan-4 alone, in the absence of its associated growth factor tyrosine kinase receptors and integrin cell matrix proteins leads to cellular migration. This establishes syndecan-4 as an independent signaling molecule in addition to its previously described role as a co-receptor for other signaling molecules.

The main purpose of this thesis was to uncover molecular mechanisms of the role of syndecan-4 in vascular remodeling in the adult organism. We found that syndecan-4 expression leads to an increase of the heparan mass that effects NO production in microvessels. Ligand induced clustering of syndecan-4 creates a signaling complex via the scaffolding protein, synectin. This complex regulates Rac1 activity, thus influencing stability of cell-extracellular matrix interactions,

controlling membrane trafficking and promoting cellular migration; processes important in angiogenesis and tissue repair.

In this thesis

Chapter 1 is a general review of the role of syndecans in cell signaling.

Chapter 2 is a study of heparan sulfate matrix changes in syndecan-4 transgenic mice and their modulation of microvascular signaling events.

Chapter 3 describes the FGF-2 specific modulation of cell signaling by syndecan-4.

Chapter 4 is dedicated to the characterization of membrane structures containing syndecan-4 before and after ligand binding.

Chapter 5 characterizes the mechanism of syndecan-4 dependent FGF-2 endocytosis.

Chapter 6 is a paper about the mechanism of migration induced by syndecan-4 clustering.

Chapter 7 contains concluding remarks and future directions.

Chapter 1

Syndecans - new kids on the signaling block

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Abstract

Cell-associated proteoglycans provide highly complex and sophisticated systems to control interactions of extracellular cell matrix components and soluble ligands with the cell surface. Syndecans, a conserved family of heparan- and chondroitin-sulfate carrying transmembrane proteins, are emerging as central players in these interactions. Recent studies have demonstrated the essential role of syndecans in modulating cellular signaling in embryonic development, tumorigenesis and angiogenesis. In this review, we will focus on new advances in our understanding of syndecan-mediated cell signaling.

Introduction

Cells interactions with their environment are critical to a large number of processes including growth, migration, adhesion and apoptosis among many others. In addition to a variety of specialized receptor systems that have evolved to transmit signals from specific ligands, other systems have also evolved to inform cells of the broader extracellular context. Thus, adhesion receptors such as integrins can be activated by a number of extracellular matrix proteins while selectins and various cellular adhesion molecules participate in cell-cell interactions.

Over the last few years, it has become increasingly clear that cells possess yet another unique system that integrates signaling from circulating ligands such as growth factors and extracellular matrix proteins with other cellular receptor systems such as integrins. This unique function, carried out by the syndecan family of proteins, places them at the center of signal integration in the cell and has earned them the name of "tuners of transmembrane signaling"¹.

Syndecan structure

Syndecans are a family of transmembrane core proteins capable of carrying both heparan sulfate (HS) and chondroitin sulfate (CS) chains. While invertebrates have only one syndecan, four syndecan genes (syndecan-1,2,3,4) are present in vertebrates. Each syndecan has a short cytoplasmic domain, a single span transmembrane domain and an extracellular domain with attachment sites for three to five HS or CS chains (Fig 1). The presence of HS chains allows interactions with a large number of proteins including heparin-binding growth factors, such as fibroblast growth factors (FGFs), vascular endothelial growth factors (VEGFs), transforming growth factor

(TGF)- β , and platelet-derived growth factors (PDGFs). Furthermore, heparan sulfates facilitate interactions with various extracellular matrix proteins including fibronectin and plasma proteins such as antithrombin-1. The role of CS chains is less clear. A recent study has suggested that syndecan-1 and syndecan-4 CS chains cooperate with HS chains in binding to the heparin-binding growth factors, midkine and pleiotrophin² and to the extracellular matrix protein laminin³.

Extracellular domain

The structural diversity of both HS and CS chains results from a series of post-translational modifications beginning with the attachment of the first hexosamine to the linkage region tetrasaccharide which is *O*-linked to serine or threonine in the core transmembrane protein committing the oligosaccharide chain to either HS (*N*-acetylglucosamine, uronic acid) or CS (*N*-acetylgalactosamine, uronic acid) disaccharide polymer⁴. Then, HS and CS glycosaminoglycan (GAG) backbones are extended by different sets of polymerizing glycosyltransferases resulting in HS and CS chains of 20 to 80 disaccharides in average length. Then, these newly synthesized chains are modified by epimerization of glucuronic to iduronic acid residues and sulfation of the C2 position of glucuronic and/or iduronic acid. A myriad of specific CS (4-*O* and 6-*O* *N*-acetylgalactosaminyl) and HS (2-*O*, 3-*O* and 6-*O* *N*-acetylglucosaminyl and *N*-deacetylase/*N*-) sulfotransferases complete the decoration of these GAG chains (for further details see⁵).

Various combinations of enzymes involved in HS chain modifications produce unique binding motifs that selectively recognize different proteins. Thus, a specific combination of 2-*O* and 6-*O* sulfation is necessary for synthesis of the FGF2 binding site while 3-*O*-sulfotransferase 1 activity is needed for generation of the antithrombin-1 binding site. Modifications of sulfotransferase expression and activity can significantly modulate syndecans functions as demonstrated, for example, in the case of 2-*O*-sulfotransferase (2-OST) deficiency that results in marked abnormalities of FGF signaling⁶. On the other hand, augmentation of 2-OST expression, as occurs in the setting of hypoxia, leads to enhanced FGF responsiveness⁷.

The recent discovery of specific mammalian sulfatases (Sulf 1 and Sulf2) which can modify GAG chains extracellularly adds another layer of complexity to the system. Both Sulf1 and Sulf2 are secreted, HS specific 6-*O*-sulfatases^{8,9} and their activity can modify heparin binding growth factor signaling¹⁰. In quail, Qsulf1,

Figure 1. Syndecan structure.

MW- molecular weight,
kD- kilodalton,
GAG- glycosaminoglycan,
C1- conserved domain 1,
V- variable domain,
C2- conserved domain 2.

Upper panel.

Cartoon representation of the vertebrate syndecan structure.

Cytoplasmic domains are shown in red and glycosaminoglycan chains are shown in green.

Protein core MW	33 kD	23 kD	43 kD	22 kD
Syndecan 1				
Syndecan 2				
Syndecan 3				
Syndecan 4				

Cytoplasmic domains

Syndecan1	RMKKKDEGS Y SLEE PKQANGGAYQK- PTKQE EFYA
Syndecan3	RMKKKDEGS Y TL EE PKQA - SV TYQK- PDKQE EFYA
Syndecan2	RM R KK DE GS Y DLGERK - P S S AAYQKAPTK - - EFYA
Syndecan4	RMKKKDEGS Y DLG -KK - P - - IYKKAPT N - - EFYA
	<div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;"> <div style="width: 100px; border-top: 1px solid black; margin: 0 auto;"></div> <div>C1</div> </div> <div style="text-align: center;"> <div style="width: 100px; border-top: 1px solid black; margin: 0 auto;"></div> <div>V</div> </div> <div style="text-align: center;"> <div style="width: 100px; border-top: 1px solid black; margin: 0 auto;"></div> <div>C2</div> </div> </div>
Interacting Proteins	<div style="display: flex; justify-content: space-around;"> <div style="text-align: center;"> <i>cortactin, src, tubulin, ezrin</i> </div> <div style="text-align: center;"> <i>syndesmos,PKC-α, α-actinin (for syndecan-4 only)</i> </div> <div style="text-align: center;"> <i>synectin, syntenin, CASK, synbindin</i> </div> </div>

Lower panel.

Protein alignment of mouse syndecan cytoplasmic domains. Amino acids in color identify posttranslational or binding motifs: red - phosphorylation in situ, brown- phosphorylation in transfectants or a synthetic peptide, green - PDZ2 binding domains, dark blue - c-src or fyn kinase activity in a complex with cortactin, light blue - ezrin binding site.

the avian homolog of Sulf1 and Sulf2, has been shown to remodel HS on the cell surface to promote Wnt-1 signaling¹¹, while in human cancer cell lines Hsulf1 expression inhibited FGF2 and HGF stimulation of cell growth¹².

It has been generally assumed that all syndecan HS and CS chains are created equal, that is, there is no preference for specific HS/CS sequences on specific syndecans¹³. However, a recent study has suggested that CS and HS chains on syndecan-1 and syndecan-4 are structurally different². If confirmed, this will add yet more complexity to syndecan biology. In addition to interacting with extracellular protein via their HS and CS chains, the syndecan-4 core can directly engage in protein-protein interactions¹⁴. The extracellular domain of syndecan-4 binds specifically to human foreskin fibroblasts (IC₅₀ = 10⁻⁸ M), mouse aortic endothelial cells as well as other

cell types while same species syndecan family members did not efficiently compete with these interactions¹⁴.

Transmembrane and intracellular domains

While the degree of conservation in the extracellular domain of syndecans is fairly low, the transmembrane domain (TM) is highly conserved. As with classic type I membrane protein architecture, syndecans have a single-pass TM. Uniquely though, the TM and a small sequence adjacent to it have a high affinity for self-association which, in the case of syndecan-4, has been shown to be required for protein kinase C-α (PKCα) activation¹⁵.

The cytoplasmic domain, despite being relatively short, has a number of important regions. The domain is divided into three regions- conserved regions 1 and 2

(C1 and C2) and a variable region (V) (Figure 1). The C1 domain, immediately adjacent to the plasma cell membrane, is virtually identical in all four mammalian syndecans. It is thought to be involved in syndecan dimerization (all syndecans probably exist as homodimers and higher order oligomers) and in binding of several intracellular proteins including ezrin, tubulin, Src kinase and coractin¹⁶⁻¹⁸. The universally conserved C-terminal C2 domain contains a PDZ2-binding site at the C-terminal end and two tyrosine residues. There are four identified syndecan interacting PDZ domain-containing proteins - syntenin, synectin, synbindin and CASK¹⁹⁻²³.

The V domain is highly heterogeneous among the four mammalian syndecans. This region has been studied most extensively in syndecan-4 due to its unique features. The syndecan-4 V-region sequence includes a phosphatidylinositol-(4,5)-bisphosphate (PIP_2) binding site that is involved in syndecan-4 dimerization where two syndecan-4 cytoplasmic domains are linked by two PIP_2 molecules in anti-parallel fashion (Fig 2)²⁴.

In addition, PIP_2 binding to the variable region plays a critical role in binding and activation of $\text{PKC}\alpha$, an enzyme that plays a key role in syndecan-4 signaling (see below)^{15,25-29}. Indeed, this feature places $\text{PKC}\alpha$ in the class of receptor-activated kinases, and allows it to play a role not dissimilar from that of integrin-linked kinases. α -Actinin and $\text{PKC}\alpha$ compete for syndecan-4 binding, possibly providing a mechanism for regulation of syndecan-4-cytoskeletal interaction³⁰. Another syndecan-4-specific binding protein is syndesmos³¹. While the precise nature of its interaction with syndecan-4 has not been defined, it requires both C1 and V syndecan-4 domains for binding. Syndesmos binding to focal adhesion adaptor proteins paxillin and Hic-5 may be an alternative to an α -actinin mechanism, linking syndecan-4 to focal adhesions³².

Nuclear magnetic resonance structural analysis demonstrates that the C-terminal orientation of syndecan-4 changes upon PIP_2 binding implying a possible regulation of PDZ interactions either by PIP_2 or the PDZ binding partners (Fig 2)^{24,33}.

Regulation of syndecan expression

Each syndecan family member has a distinct temporal and spatial pattern of expression with every mammalian cell expressing at least one syndecan that is highly regulated during development³⁴. Syndecan-1 is expressed predominantly in epithelial and mesenchymal tissues, syndecan-2 in cells of mesenchymal origin, neuronal and epithelial cells, syndecan-3 almost exclusively in neuronal and musculoskeletal tissue while syndecan-4 is found in virtually every cell type³⁵.

Given their role in tuning numerous signaling events, it is not surprising that syndecan levels are tightly regulated. Growth factors play an important role in regulation of syndecan expression. Thus, $\text{TNF}\alpha$ upregulates syndecan-2 and downregulates syndecan-1 in endothelial cells³⁶. Similarly, $\text{TGF}\beta 2$ upregulates syndecan-4 and downregulates syndecan-1 in epithelial cells³⁷. In aortic smooth muscle cells, $\text{FGF}2$ induces syndecan-4 but not syndecan-1 or -2 expression³⁸. Mechanical stress is also a prominent inducer of syndecan-4 expression in smooth muscle during arteriogenesis³⁹. Syndecan-4 levels are markedly upregulated in several disease states including arterial injury⁴⁰ and acute myocardial infarction⁴¹. Another condition associated with a pronounced increase in syndecan-1 and -4 expression is wound healing^{42,43}. While there are probably numerous factors

Figure 2. NMR structure of syndecan-4 cytoplasmic domain in the presence and absence of PIP_2 .

C1- conserved domain 1,
C2- conserved domain 2.

Note that interaction with PIP_2 results in reorientation of PDZ binding domains.

Table I. Syndecans Function in Animal Models

Gene	Mutant	Species	Developmental processes affected	Signaling processes affected	Ref.
Syndecan	P-element insertion	<i>D.melanogaster</i>	Axonal and myotube guidance	Slit/Robo	64
	KO	<i>C. elegans</i>	Egg laying		154
Syndecan-1	KO	Mouse	Viable and fertile; slow re-epithalization during wound healing; mammary tumorigenesis with Wnt1 interaction; leukocyte-endothelial interactions.	Wnt1	86 54 53
	Transgenic	Mouse	Slow re-epithalization during wound healing	FGF2	79
Syndecan-2	Morpholinos	Zebrafish	Intersegmental vessel formation.	VEGF	92
	OE of Syndecan-2 Δ -cyto construct	Zebrafish	Intersegmental vessel formation. Randomized lefty expression	VEGF Lefty	56
Syndecan-3	KO	Mouse	Viable and fertile; feeding behavior; hippocampus-dependent memory function; muscular dystrophy.	HGF, FGF2	52 58 155
	OE of syndecan-3 in chondrocytes	Chicken	Chondrocyte differentiation.	Indian Hh	103
Syndecan-4	KO	Mouse	Viable and fertile; postnatal angiogenesis during wound healing; focal adhesion formation; sensitivity to lipopolysaccharide injection, susceptibility to carrageenan; muscular regeneration.	HGF, FGF2, TNF- β	63 122 62 61 52
	Transgenic in cardiac myocytes	Mouse	Microvessel relaxation	FGF2	156
	Transgenic in endothelium	Mouse	Microvessel relaxation	FGF2	157

Abbreviations used: KO- knockout, OE-overexpression, Δ -cyto- cytoplasmic domain truncation.

responsible for this increase, one interesting mediator is an inflammatory cell-derived peptide, PR39, that has a unique ability to markedly upregulate syndecan-1 and syndecan-4 expression *in vitro* and *in vivo*^{44,45}.

Very little is known about regulation of syndecans-2 and -3 expression. Syndecan-2 levels increase during transformation of fat cells into myofibroblasts, whereas the level of syndecan-1, -3 and -4 remains constant⁴⁶. Syndecan-3 expression is highly regulated during development. In rodents, its level in central nervous system rises at birth, peaks on day 7, and then declines to the level of adult⁴⁷. This period of high level of syndecan-3 expression corresponds with oligodendrocyte

differentiation and myelin formation in the central nervous system. The temporal dermal expression of syndecan-3 suggests the possibility of its involvement in feather development⁴⁸. During chick embryo limb development, syndecan-3 is transiently expressed the period of mesenchymal condensation⁴⁹.

Interestingly, changes in one syndecan family member's gene expression may affect others. For example, increased syndecan-3 expression in satellite cells leads to the downregulation of syndecan-4-transduced FGF2 and HGF signaling⁵⁰, while syndecan-3 expression is reduced in syndecan-4^{-/-} satellite cells.

Syndecans function in development

Recent studies have begun clarifying the roles played by various syndecans during the developmental process (Table 1). Homozygous disruption of the syndecan-1 gene in mice leads to viable offspring. The mice are grossly normal but demonstrate abnormally slow re-epithelialization following injury⁵¹ and increased leukocyte adhesion⁵². Syndecan-1 appears to be involved in modulation of Wnt-1 signaling as mammary gland-specific expression of Wnt-1 leads to development of tumors in wild type but not in the syndecan-1 knockout mice⁵³.

Studies in zebrafish using anti-syndecan-2 morpholino oligonucleotides demonstrated that this syndecan plays an important role in vascular development involving modulation of VEGF signaling. Syndecan-2 knockdown resulted in suppression of intersegmental vessels while formation of dorsal vessels (aorta, cardinal vein) was not affected⁵⁴. Overexpression of VEGF₁₆₅ in syndecan-2 morphants was not able to induce ectopic vessel formation, whereas co-expression of syndecan-2 and VEGF₁₆₅ resulted in an increase of vessel formation compared with VEGF alone. Application of moderate doses of syndecan-2 and VEGF morpholinos demonstrated synergistic inhibition of angiogenesis. Expression of cytoplasmic domain-truncated syndecan-2 mimics the phenotype produced by syndecan-2 knockdown. Based on these data, syndecan-2 appears to potentiate VEGF-induced capillary sprouting.

Syndecan-2 is also involved in left-right asymmetry regulation (reviewed in³⁵). In *Xenopus* syndecan-2 is phosphorylated by PKC- γ only in right animal cap ectodermal cells⁵⁵. Overexpression of the cytoplasmic domain truncated form of syndecan-2 results in perturbed determination of left-right asymmetry of embryo and also randomizes expression of *lefty*, a left/right symmetry regulator⁵⁵.

Syndecan-3 levels in hypothalamus have been demonstrated to physiologically regulate feeding behavior and mice with homozygous disruption of both syndecan-3 alleles displayed reduced feeding behavior in response to food deprivation⁵⁶ as well as impaired performance in tasks utilizing hippocampal functioning indicating learning and memory abnormalities⁵⁷. They develop muscular dystrophy characterized by fibrosis, deteriorated locomotion, and hyperplasia of myonuclei and satellite cells⁵⁰.

Syndecan-4 deficiency has generated much interest.

Null mice are viable and fertile but display a number of subtle defects. However, syndecan-4^{-/-} embryos demonstrate much more frequent thrombi formation in the vessels of the placental labyrinth than the littermate controls resulting in much higher embryo loss and implicating syndecan-4 in regulation of blood clotting⁵⁸. The adult syndecan-4^{-/-} mice have increased mortality following lipopolysaccharide injection suggesting their inability to clear pathogens⁵⁹ and to down regulate TNF β -induced suppression of interleukin-1 β expression in macrophages⁵⁷. The syndecan-4^{-/-} mice also have increased susceptibility to kappa-carrageenan induced renal damage presumably due to increased deposition in renal collecting ducts⁶⁰.

Other interesting observations include impaired skin wound healing that is thought to be secondary to defective angiogenesis⁶¹. However, no other angiogenesis deficiency phenotypes have been reported to date. Finally, satellite cells from syndecan-4^{-/-} mice fail to reconstitute damaged muscles suggesting that syndecan-4 presence is required for migration of these skeletal muscle progenitor cells⁵⁰. This may in part be due to the defective FGF2- and HGF-induced activation of Erk-1/2⁵⁰.

Modulation of outside-in signaling

The preceding section demonstrates that disruption of various syndecan genes leads to phenotypes consistent with the "tuning" role of syndecans in signal transduction. The precise mechanism of this tuning has generated much interest and appears to be different for different syndecans. In the discussion that follows, while we will consider signaling events associated with individual syndecans, it should be kept in mind that considerable overlap between various syndecan-mediated events probably exists and that the "specific" signaling events described for an individual syndecan may just as well reflect the limitation of our knowledge as the real biological specificities. Nevertheless, there is also a considerable degree of specificity. In particular, it is useful to think, in the signaling context, of syndecan-1 and syndecan-3 as one subfamily and syndecan-2 and 4 as another. This classification reflects both homology of variable domain between corresponding syndecans as well as functional similarities. For example, both syndecan-1 and syndecan-3 expression is mostly associated with inhibition of cell growth while syndecan-2 and syndecan-4 expression leads to its stimulation.

Heparan and Chondroitin Sulfate Chains

The way in which syndecans engage in signal transduction remains a matter of active research and controversy. The first and the oldest hypothesis postulates that syndecans serve as co-receptors for various heparin binding growth factors. This is thought to occur due to GAG chains binding growth factors, thereby, restricting their presence to the membrane surface and facilitating their subsequent interactions with corresponding high affinity receptors⁶². Numerous studies have shown that inhibition of HS chain synthesis grossly affects signaling. For example, heparinase treatment of cultured smooth muscle cells decreases their responsiveness to FGF2⁶³ while heparinase treatment *in vivo* inhibits angiogenesis⁶⁴.

Genetic data is equally compelling. Inhibition of HS formation due to mutations of EXT1 and EXT2 genes which catalyze polymerization of glucuronic acid and N-acetylglucosamine, the crucial step in HS synthesis, results in hereditary multiple exostoses, an autosomal skeletal disorder characterized by inappropriate chondrocyte proliferation and bone growth⁶⁵. The defect is thought to be due to abnormal diffusion of hedgehog proteins⁶⁶. A homozygous disruption of Ext-1 expression in mice results in gastrulation defects and early embryonic lethality⁶⁷.

More subtle changes in the HS chain composition also have profound effects. Sulfation of both 2-*O* and 6-*O* HS sites is thought to be required for formation of FGF binding sites. A homozygous deletion of 2-*O* ST results in mice that survive until birth, but die perinatally because of the complete failure of kidney formation⁶. Similarly, a knockdown of the 6-*O*-sulfotransferase in *Drosophila* severely perturbs tracheal development, an FGF-dependent process⁶⁸. Deletions of other genes involved in HS biosynthesis have equally profound phenotypic effects⁶⁹.

In addition to HS-dependent signaling, syndecans also clearly can engage in protein-protein interactions via their protein core ectodomains, an event that can also initiate intracellular signaling as will be discussed below. Furthermore, syndecan-growth factor interaction, whether accomplished via the chain or the core-based interaction, does not simply serve to present the factor to its high affinity receptor. Rather, such binding can initiate signaling events via the cytoplasmic syndecan domains that have their own unique aspects as will be further discussed. Finally, each of the syndecans has its own characteristic biology that we will briefly review.

Syndecan-1 and cell-matrix interaction

Syndecan-1 is an important regulator of cell-cell and cell-extracellular matrix interactions. Downregulation of its expression in epithelial cells by antisense mRNA results in loss of cell polarity associated with a reduced level of E-cadherin on the cell surface, suggesting involvement in the epithelial-mesenchymal switch during development and in wound healing (reviewed in⁷⁰). Overexpression of syndecan-1 or shedding of its ectodomain inhibits FGF2-induced cell proliferation⁷¹. Mice overexpressing syndecan-1 have delayed dermal wound repair due to the inhibitory effect of soluble syndecan-1 ectodomain⁷². However, under certain circumstances, heparinases can convert syndecan-1 ectodomain from an inhibitor to an activator of FGF2⁷³. This reaction enables FGF2 interaction with syndecan-4 (see below), although a specific protein sequence in the syndecan-1 ectodomain may also play a role⁷⁴. The ability of syndecan-1 overexpression to inhibit cell growth and migration could be an explanation of the aggressive invasive behavior of tumor cells lacking syndecan-1^{75,76}.

Interestingly, downregulation of syndecan-1 expression in carcinoma cells results in impaired cell spreading on vitronectin but not on fibronectin⁷⁷. This seems to be mediated by the ability of syndecan-1 to modulate vitronectin interaction with its $\alpha v \beta 3$ integrin receptor since GPI-syndecan-1 extracellular domain construct expression in syndecan-1^{-/-} cells converts their 'no spreading phenotype' to normal spreading.

Syndecan-1 plays an important role in regulation of inflammation⁷⁸ as suggested by increased leukocyte-endothelial interactions in syndecan-1 null mice⁷⁹. It also plays an important role in chemokine gradient formation for trans-endothelial and trans-epithelial migration of neutrophils (reviewed in⁷⁸). The former process is facilitated by an IL-8/syndecan-1 complex and regulated by shedding of syndecan-1⁸⁰. Trans-epithelial neutrophil migration is dependent on matrilysin-mediated shedding of syndecan-1 from the mucosal surface of epithelium⁸¹. As would be expected, matrix-metalloproteinase matrilysin deficient mice demonstrate impaired trans-epithelial migration of neutrophils.

Finally, syndecan-1 may also play a role in regulation of ephrin signaling that is involved in veno-arterial differentiation. Ephrin-B4 (EphB4) is expressed in venule endothelium. Homozygous disruption of EphB4 or one of its ligands, ephrin-B2, results in fatal vascular defects^{82,83}. Activation of EphB4-positive endothelial cells causes upregulation of syndecan-1⁸⁴ resulting in

suppression of FGF2 signaling. However, in certain settings, the role of syndecan-1 could be the opposite, depending on the presence of heparinases. Degradation of heparan by platelet heparanase, for example, produces heparin-like heparan sulfate fragments, capable of promoting FGF2 mitogenicity⁷³.

Syndecan-2 and TGF- β signaling

Syndecan-2 is the predominant syndecan expressed during embryonic development. Although its role in adult cells has not been well established, recent studies suggest its involvement in regulating TGF- β signaling. While TGF- β can bind to HS chains, syndecan-2, but not other syndecans, binds TGF- β directly via a protein-protein interaction. Furthermore, syndecan-2 co-immunoprecipitates with the TGF- β as well as with the type III TGF- β receptor (betaglycan)⁸⁵ and expression of a syndecan-2 mutant with a truncated cytoplasmic domain results in impaired response to TGF- β ⁸⁵. These data correlate with a previously reported inability of cells overexpressing this construct to assemble laminin or fibronectin into a fibrillar matrix⁸⁶.

The mechanism of syndecan-2/TGF- β interactions is complex and not entirely clear (Fig 3). TGF- β binding to betaglycan transfers it to the type II receptor which undergoes autophosphorylation and then transphosphorylates both betaglycan and the type I receptor. The phosphorylated type I/type II receptor complex then engages in downstream signaling. Betaglycan binds to syndectin (GIPC) that retains betaglycan on the cell surface thus preventing its degradation⁸⁷. However, syndectin also binds syndecan-2. As already mentioned, expression of a syndecan-2 mutant with a truncated cytoplasmic domain increases plasma membrane betaglycan levels⁸⁵. While an increase in syndecan-2 protein, increases type I and type II TGF- β receptors expression and decreases betaglycan, probably as the consequence of competition for syndectin binding⁸⁵. This decrease in betaglycan expression possibly leads to dysregulated activity of the type-I/type-II TGF- β receptor complex.

Similar to syndecan-1, syndecan-2 may also play a role in ephrin signaling. Cell surface ephrin-Eph signaling induces clustering of syndecan-2 and recruitment of cytoplasmic molecules, which leads to localized actin polymerization via Rho family GTPases, N-WASP, and the Arp2/3 complex⁸⁸.

Figure 3. Modulation of TGF- β signaling by syndecan-2.

Syndecan-2 effects on TGF- β signaling are shown for normal cells and cells overexpressing syndecan-2 constructs.

(Upper left) Under normal circumstances, TGF- β binds to betaglycan which then transfers it to the TGF β RI/R2 complex

(Upper right) Expression of Syndecan-2 PDZ- mutant results in increase of betaglycan on the cell surface and impaired signaling.

(Lower) In contrast, overexpression of full length syndecan-2 results in plasma membrane accumulation of TGF β RI and II, and reduced levels of betaglycan (TGF β RIII).

Syndecan-3 and growth factors signaling

Syndecan-3 plays an important role in regulation of skeletal muscle differentiation and development. Its levels are transiently elevated in the developing limb bud but are absent in adult skeletal muscle. Skeletal muscle myoblasts are held in an undifferentiated state until they receive signals to further differentiate. The maintenance of these cells in their undifferentiated state has been shown to be controlled by specific growth factors including FGF2, hepatocyte growth factor/scatter factor and TGF- β ⁸⁹. Syndecan-3 inhibition results in expression of myogenin, a master transcription factor for muscle differentiation, and in accelerated skeletal muscle differentiation and myoblast fusion⁹⁰.

In the model of skeletal muscle regeneration induced by barium chloride injection, syndecan-3 showed the earliest and largest increase in satellite cells⁹¹. Myoblast grafting of C₂C₁₂ cells transfected with antisense syndecan-3 resulted in cells with a normal proliferation rate but defective in fusion and formation of skeletal muscle fibers. Further, examination of syndecan-3 null mice demonstrates mislocalization of MyoD (a myocyte differentiation transcription factor) and an aberrant differentiation of skeletal muscle⁵⁰.

Syndecan-3 also appears to be involved in regulation of hedgehog signaling. Hedgehog (Hh) is a family of secreted signaling proteins that play an important role in embryogenesis and differentiation. One of the mammalian family of hedgehog (Hh) members, indian Hh (iHh), is produced by prehypertrophic chondrocytes and regulates chondrocyte proliferation. Proper spatial and temporal regulation of iHh is regulated by syndecan-3⁹².

Syndecan-4, FGF signaling and the extracellular matrix

Syndecan-4 has become the most extensively studied member of the syndecan family. The proteoglycan has a number of activities including modulation of FGF2 signaling, regulation of cell migration via cross-talk with β 1 integrin and control of adhesion via cytoskeletal modifications. All these various events are achieved via actions of a 33 amino acid cytoplasmic tail, one of the most overworked small proteins among the signaling giants.

FGF signaling and endocytosis

In addition to being able to bind FGF2 to HS chains and to present it to FGF tyrosine kinase receptors, syndecan-4 directly initiates a number of intracellular signaling events. The most direct demonstration of syndecan-4 signaling came from studies of syndecan-4/glypican chimeras in endothelial cells. Expression of a full-length syndecan-4 (S4), syndecan-1 (S1), glypican-1 (G1) or of chimera constructs consisting of the ectoplasmic domain of glypican-1 linked to the transmembrane/cytoplasmic domain of syndecan-4 (G1-S4c) or of the ectoplasmic domain of syndecan-4 linked to the glypican-1 glycosylphosphatidylinositol (GPI) anchor (S4-GPI) significantly increased cell-associated HS mass and the number of low affinity FGF2-binding sites. However, only cells expressing S4 and G1-S4c constructs but not G1, S1, S4-GPI cells demonstrated enhanced responsiveness to FGF2. Thus, the presence of syndecan-4 cytoplasmic domain and not simply an increase in the cell surface HS mass with a corresponding increase in the number of the low affinity FGF2 binding sites is required for signaling. However, removal of the syndecan-4 HS chains also blocked enhanced FGF2 responsiveness demonstrating that both syndecan-4 HS chains and the syndecan-4 cytoplasmic domain are required for FGF2 signaling⁹³. Syndecan-3 cannot replace syndecan-4 to promote FGF2 and HGF induced cell migration⁵⁰.

Additional evidence of direct syndecan-4 signaling ability comes from studies in vascular smooth muscle cells expressing dominant-negative fibroblast growth factor receptor (FGF-R). FGF2 was able to transiently stimulate Erk 1/2 activation and induce cell migration, activities that were inhibited by removal of HS chains⁹⁴. It should be noted that FGF2-induced Erk activation in the absence of functional FGF-R1 is transient and does not lead to cell proliferation.

The final piece of evidence demonstrating syndecan-4 modulation of FGF2 signaling comes from studies that explored the effect of dominant-negative syndecan-4 construct expression on endothelial cell responses to FGF2 and other growth factors. Introduction of syndecan-4 constructs with the mutated PDZ or PIP₂ binding regions that, respectively, eliminated syndecan-4 ability to bind PDZ proteins or PIP₂, inhibited FGF2 but not serum induced endothelial cell growth, migration, and the ability to form vascular structures on Matrigel²⁷.

While it is clear that syndecan-4 can modulate FGF2 signaling, the precise mechanism of this modulation is uncertain. A sustained activation of FGF2 signaling

requires internalization and perhaps nuclear transport of the ligand⁹⁵. Syndecan-4 is directly involved in FGF2 endocytosis that proceeds in a clathrin- and dynamin-independent fashion and requires syndecan-4-dependent activation of Rac1⁹⁶. The sensitivity of this process to amiloride as well as co-localization of internalized FGF2 with dextran suggests that FGF2 uptake proceeds via macropinocytosis⁹⁶. An interesting feature of this process is the fact that FGF2 uptake proceeds from lipid rafts, a specialized plasma membrane domain thought to be involved in signaling.

Endocytosis, lipid rafts and caveolae

Plasma membrane location of syndecan-4 and its participation in FGF2 endocytosis raises the issue of its relation to lipid rafts, plasma membrane domains that provide fluid platforms to segregate membrane components and dynamically compartmentalize membranes (reviewed in⁹⁷). Those phospholipid- and cholesterol-rich domains contain specific sets of proteins that include GPI-anchored proteins, doubly acetylated proteins (Src family tyrosine kinases), G_{α} subunit of heterotrimeric G proteins and palmitylated proteins including endothelial nitric oxide synthase (eNOS)^{98,99}.

While in unstimulated cells syndecan-4 is predominately present in the non-raft compartments, clustering with FGF2 or anti-syndecan-4 antibody induces its shift to lipid rafts¹⁰⁰. This shift is necessary for the initiation of FGF2 endocytosis since its blockade by cholesterol depletion from the plasma cell membrane completely blocks FGF2 uptake⁹⁶. Similarly, low density lipoprotein (LDL) causes clustering and clathrin independent uptake of syndecan-1, -2 and -4¹⁰¹. In the model where syndecan-1 was used, LDL clustering also induced a shift of syndecan into the lipid rafts¹⁰².

Caveolae are subset of lipid rafts. They are flask-shaped invaginations of the plasma membrane¹⁰³ which play a role in regulation of certain endocytic pathways and regulation of enzymes such as eNOS. A variety of protein and lipid signaling molecules are concentrated in caveolae. These include PKC, RhoGTPases and non-receptor tyrosine kinases such as c-Src, Yes, Fyn, PI 3-kinase, and phosphatidylinositol kinases among others¹⁰³⁻¹⁰⁵. Caveolins are the principal building blocks of caveolae and are usually accepted as its marker. Caveolin-1 oligomeric complex creates a stable membrane structure that transiently interacts with plasma membrane and endosomes.¹⁰⁶

Upon initiation of cell migration, pools of syndecans-1

and -4 and caveolin-1 are directed into the same retracting region of the moving cell¹⁰⁷⁻¹⁰⁹. Caveolin's property of inhibiting kinase activity of a variety of proteins involved in leading edge formation was speculated to play a key role in cell migration¹¹⁰. In contrast to the deactivating role of caveolin, syndecan-4 is needed for activation of several kinases including focal adhesion kinase (FAK), which results in increased turnover of focal adhesions¹¹¹⁻¹¹³. Silencing of caveolin-1 gene expression or introduction of a dominant negative mutant of syndecan-4 leads to slow migration and impaired tube formation of endothelial cells on Matrigel^{127,107}. Syndecan-4 and caveolin-1 knockout mice demonstrate reduced postnatal angiogenesis that may be related to the impairment of endothelial cell migration^{61,110}.

While syndecan-4 does not co-localize with caveolin-1 at the cell surface, syndecan-4- and caveolin-1-containing vesicles frequently move in tandem, presumably along microtubules¹⁰⁰. Remarkably, internalization of caveolae depends on Src and PKC α activation¹¹⁴. The ability of syndecan-4 to activate PKC α is well established and will be discussed in detail below. Similar to syndecan-3, it can also activate Src¹⁷. Thus, syndecan-4 may be responsible for modulation of caveolae pinching from the plasma membrane in response to heparin binding growth factors^{115,116}.

The association of syndecan-4 with fast moving caveolin-1-positive vesicles could also be used for a fast delivery of syndecan-4 pinosomal cargo to intracellular destinations. This process might be needed for proper distribution of macropinosome associated small GTPases leading to migratory polarization of the cell^{117,118}. This interaction could be also involved in extracellular matrix turnover. Downregulation of caveolin-1 results in inhibition of fibronectin internalization and degradation¹¹⁹, suggesting the participation of fibronectin receptors such as integrins or syndecans in caveolae dependent uptake of fibronectin.

Activation of PKC α

Another feature of syndecan-4 signaling is activation of PKC α in the absence of Ca^{2+} . This unique ability of syndecan-4 to activate a calcium-dependent PKC was first shown by Oh and colleagues to require core protein multimerization and the presence of PIP₂^{15,120}. Syndecan-4 oligomerization in the presence of PIP₂ depends on the phosphorylation status of the Ser¹⁸³ site in its cytoplasmic domain²⁷. Phosphorylation of this site markedly reduces its affinity for PIP₂²⁶ and prevents PKC α activation *in vitro* and *in vivo*^{26,121}. Interestingly, the Ser¹⁸³ site is

Figure 4. Syndecan-4-dependent modulation of PKC activity.

Under normal circumstances Ser183 site is phosphorylated by PKC δ . Exposure to FGF2 results in dephosphorylation of the site that in turn allows syndecan-4 oligomerization, binding of PIP2 and activation of PKC α . Any signal that would activate PKC α would impair syndecan-4 dependent PKC α activation.

phosphorylated by PKC δ ²⁸. This sets up a system that allows one PKC to regulate activity of another (Fig 4). The Ser dephosphorylation is carried out by as yet undefined Type I/IIa Serine phosphatase¹²¹.

The molecular details of syndecan-4-dependent PKC α activation have not been established. The results of yeast two hybrid screening demonstrated that while a full-length PKC α weakly binds the variable domain of syndecan-4 (4V), the PKC α constructs that lack the pseudosubstrate region or that encode only the whole catalytic domain interacted more strongly^{15,122}. A mutation of Tyr¹⁹² in the syndecan-4 cytoplasmic domain abolished this interaction¹²². Interestingly, the corresponding binding site in the catalytic domain of PKC α (amino acid sequence 513-672) encompasses the regulatory autophosphorylation sites implicated in control of PKC activation and stability¹²². On the other hand, in vitro surface plasmon resonance studies suggested that binding affinity of a full length PKC α to the syndecan-4 cytoplasmic domain is very low in the absence of PIP₂²⁶.

While the precise role of syndecan-4-dependent PKC α activation has not been defined, co-localization of the two proteins in focal adhesions¹²⁰ suggests a role in cell

adhesion, spreading and stress fiber formation. Studies in cultured fibroblasts have shown that overexpression of syndecan-4, but not a mutant lacking its cytoplasmic domain, specifically increases the level of endogenous PKC α and enhances the translocation of PKC α in the plasma cell membrane in general and in the membrane raft fractions in particular and increases the activity of membrane PKC α ¹²³.

Cell adhesion and the cytoskeleton

The involvement of members of the syndecan family in regulation of cell adhesion suggests that these proteins likely modulate cytoskeletal rearrangements. Indeed, fibronectin binds to syndecan-4 HS chains and this interaction plays an important role in regulation of cell adhesion and spreading¹²⁴. Fibroblasts use both integrin receptors and syndecan-4 to induce Rho-dependent spreading in fibronectin^{125,126}. Overexpression of syndecan-4 results in the flattening of CHO-K1 cells, promotion of focal adhesion formation and a decrease in cell migration¹²⁷. Syndecan-4 null fibroblasts plated on fibronectin exhibit enhanced lamellipodia formation and increased level of Rac1¹²⁸ and low Rho¹¹¹ activities compared with wild type cells. Expression of syndecan-4 in these knockout cells downregulates Rac1 activity¹²⁸. In endothelial cells also plated on fibronectin, clustering of syndecan-4 construct by antibody results in upregulation of Rac1 activity⁹⁶. Those seemingly contrary results might be due to the differences in localization changes followed by binding to matrix versus soluble ligand. In this way, growth factors might modulate cell-matrix interactions by removal of the syndecan component via endocytosis⁹⁶.

In vascular smooth muscle cells, shear stress causes syndecan-4 dissociation from the focal adhesions³⁹. Overexpression of syndecan-4 blocks this dissociation and also results in reduced mechanical stress-induced cell migration. This observation suggests that syndecan-4 might be involved in regulation of smooth muscle migration during arteriogenesis.

Another role of syndecan-4 involves regulation of fibronectin signaling and matrix contraction together with tenascin-C. Tenascin-C is an extracellular matrix protein involved in regulation of cellular response to fibronectin by preventing cell spreading (reviewed in¹²⁹). Syndecan-4 knockout fibroblasts, while spreading normally on a 2D matrix, fail to spread in a 3D fibronectin matrix, do not activate RhoA and,

consequently, do not form focal adhesions and stress fibers. Anti-syndecan-4 antibody treatment of wild type fibroblasts similarly prevents spreading on fibronectin, decreases RhoA and activates focal adhesion kinase. Stimulation of RhoA by lysophosphatidic acid in syndecan-4^{-/-} cells rescues syndecan functions by activating RhoA and inducing cell spreading and matrix contraction. Addition of tenascin-C blocks spreading of control but not syndecan-4^{-/-} fibroblasts on a 2D fibronectin matrix while syndecan-4 overexpression overcomes this tenascin-C effect¹³⁰.

Activated B-lymphocytes, when seeded on syndecan-4 antibodies, exhibit dramatic morphological changes¹³¹. The most profound being filopodial extensions, implying the possibility of CDC42 activation. Overexpression of a truncated form of syndecan-4 lacking an intracellular domain demonstrated that the extracellular domain is sufficient to generate a response, indicating the need for a transmembrane partner to transmit signal. One such potential partner is the chemokine receptor 4 (CXCR4). Syndecan-4, but neither syndecan-1 nor syndecan-2, co-immunoprecipitate with CXCR4¹³². This syndecan-4/CXCR4 complex is likely a functional unit involved in chemokine stromal cell-derived factor-1 (SDF-1) binding that may play a role in SDF-1-dependent stem cells recruitment.

Syndecan-2 and -3 overexpression also induce filopodia formation in COS-1 and CHO K1 cells^{133,134}. In the case of syndecan-2, this process is CDC42-dependent^{133,134}. The deletion of V or C2 domains but not entire cytoplasmic part of syndecan-3 results in more filopodia formation while extensive membrane blebbing is observed in cell expressing a C2-truncated mutant¹³⁴. The blebs are distributed uniformly over the cell surface rather than next to the leading edge suggesting improper targeting or activation of Rac1 resulted to bleb formations away from cell edges as a possible mechanism.

Members of the ezrin-radixin-moesin (ERM) family have NH₂- and COOH-terminal domains that associate with the plasma membrane and the actin cytoskeleton, respectively. Following overexpression of a constitutively active form of RhoA, association of syndecan-2 with actin cytoskeleton through ezrin binding was observed in COS-1 cells^{16,18}. Thus, ezrin provides RhoGTPases a regulated link between syndecan-2 and actin. The ezrin binding motif is identical between syndecan-2 and -4 implying the possibility of syndecan-4/ezrin interactions (Fig1)¹⁸. Such regulation of cytoskeleton by Rho GTPases promises to be one of the most important "tuning" functions of syndecans.

Summary

As we hope this review demonstrates, syndecans have been shown to function as potent and specific regulators of cell signaling in a variety of settings. This regulatory function is very complex due to the involvement of different family members, differences in level and cellular location of expression, GAG chains modifications, and extracellular domain shedding. Overall, given their ability to participate in signal transduction involving the extracellular matrix, cell surface molecules and soluble ligands; syndecans are emerging as conductors of the entire signaling orchestra.

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Chapter 2

Modulation of Microvascular Signaling by Heparan Sulfate Matrix: Studies in Syndecan-4 Transgenic Mice

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Abstract

The onset of tissue ischemia is associated with significant changes in the expression of heparan sulfate- (HS) carrying core proteins that, in turn, lead to alterations in composition of the extracellular HS matrix. Since HS can bind numerous growth factors and cytokines, such changes in the HS matrix content can have profound effects on the ability of these factors to interact with their target cells. To investigate the role of increased HS matrix content on microvascular function, we used myosin heavy chain (MHC) promoter to overexpress a HS-carrying core protein, syndecan-4, in cardiac myocytes in mice. Mice expressing the transgene (MHC-S4) demonstrated a significant increase in nitric oxide (NO) release in the coronary effluent in response to fibroblast growth factor 2 (FGF2, 1mg/mL) administration despite similar expression levels of NO synthase genes II and III (iNOS and eNOS, respectively). In vitro studies of coronary microvessels derived from MHC-S4 mice demonstrated increased relaxation response to FGF2 compared to control mice. At the same time, vasodilator response to adenosine diphosphate (ADP) was significantly impaired in MHC-S4 mice-derived microvessels. Addition of exogenous HS to microvessels derived from control mice enhanced FGF2-induced vasodilation while inhibiting ADP induced vasomotion. The vasomotor activity of the endothelial receptor-independent agent (A23187) and the endothelium-independent agent (sodium nitroprusside) was not affected by heparan sulfate. These results demonstrate that alterations in HS production have a profound and heterogeneous effect on endothelial receptor-dependent vasodilators and point to a novel role of the HS matrix in regulation of microvascular homeostasis.

Introduction

Cell surface heparan sulfates play an important role in control of signaling of a variety of heparin binding growth factors, including fibroblast growth factor 2 (FGF2) [1, 2]. This can be achieved either directly [3, 4] or by regulating presentation of these factor to their high-affinity receptors [5, 6]. In addition, in the case of FGF2 as well as of other heparin binding growth factors, HS in the extracellular matrix (ECM) can sequester these proteins, thus protecting them from degradation [7], and can regulate their transport through the matrix [8]. Thus, any alteration—increase or decrease—or change in composition of the extracellular matrix heparan sulfates may have a significant impact on microvascular

environment, including signal transduction in endothelial cells. In this study we set out to test whether increased expression of HS on cardiac myocytes and their subsequent shedding into the extracellular matrix in normal nonischemic hearts would alter vascular responses to endothelium-dependent and -independent agonists.

Heparan sulfates are carried on three major classes of core proteins—transmembrane syndecans, glycosyl-phosphatidylinositol (GPI) anchored glypicans, and the extracellular core protein perlecan. All three classes of core proteins are capable of carrying heparan sulfates and, to date, no difference in the HS chain composition derived from the different cores has been established [8]. Thus, theoretically, HS derived from any of these sources would have equal impact on the ECM-related processes. While perlecan HS chains are already present in the matrix due to the extracellular location of the core protein, glypicans and syndecans are equally capable of adding their HS chains to the mix due to shedding of their extracellular HS-carrying domains [9]. While regulation of the shedding processes is not fully understood, the presence of inflammatory cells and activation of extracellular proteases clearly enhance it [4, 20].

To explore the role of the ECM heparan sulfates in the regulation of microvasculature, we generated transgenic mice carrying syndecan-4 core protein gene under control of MHC promoter. Syndecan-4 is one of the four members of syndecan family members that is ubiquitously expressed in various cell types, including myocytes and endothelial cells. The onset of tissue injury or ischemia is associated with a rapid increase in syndecan-4 mRNA and protein levels that far exceeds changes in the levels of other HS-carrying proteoglycans, including glypican-1 and syndecan-1 [10]. Therefore, syndecan-4-derived HS is the principal type of HS chain deposited in the matrix under these conditions. We have recently demonstrated that, unlike other HS-carrying proteins, syndecan-4 has the ability to directly mediate FGF2 signaling in endothelial cells by virtue of its cytoplasmic domain [3]. Therefore, in order to study the effect of syndecan-4-derived matrix HS on microvascular environment, and, at the same time, to avoid the direct syndecan-4 signaling effects, we choose to target its expression to cardiac myocytes. In this setting, effects of the syndecan-4 transgene expression will be limited to the matrix/cell surface deposition of its shed extracellular domains that carry heparan sulfate chains.

To assess the effect of changes in the HS ECM composition, we examined the ability of several endothelium- dependent and -independent vasoactive

agents, including FGF2, adenosine diphosphate (ADP), and sodium nitroprusside (SNP), to affect microvascular responses. We find that increased HS content augmented FGF2-mediated vasodilation while decreasing ADP response. This change in the vasodilatory response was directly attributable to enhanced (FGF2) or reduced (ADP) ability to induce nitric oxide (NO) release. At the same time, vasomotor responses to other tested agonists were not affected. Thus, alteration in the HS ECM content exerts complex and heterogeneous effects on microvascular homeostasis.

Materials and methods

Generation of Transgenic Mice Lines

Full-length rat syndecan-4 cDNA was linked to the 3' end of mouse MHC promoter [11] (a gift of Dr. J. Robbins, University of Cincinnati) and the 5' end of human growth hormone polyadenylation signal sequence in the pBluescript SK(+). The construct was then microinjected into fertilized eggs of C57BL/6 mice. Polymerase chain reaction analysis of tail genomic DNA was carried out using a forward primer 5'-CCAAGGAACTGGAAGAGATGAGGTCATTC-3' corresponding to the rat syndecan-4 sequence and a reverse primer 5'-GCCAAGGTGGGTAATGACCTGAGATTAGG-3' corresponding to the 5' end sequence of human growth hormone. Four different transgenic lines were generated and used in the study.

In Vitro Microvascular Studies

The left anterior descending coronary artery from hearts of MHC-S4 (n=6) and age/strain matched controls (n=6) was dissected using a 10-60X dissecting microscope (Olympus Optical, Tokyo, Japan). Microvessels were placed in a Plexiglas microvessel chamber, cannulated with dual glass micropipettes measuring 40–80 μ m in diameter, and secured with a 10-0 nylon monofilament suture (Ethicon, Somerville, NJ) as previously described. For all measurements, the vessels were washed three times with a Krebs buffer solution and allowed to equilibrate in the buffer solution 15–30 min between interventions. Vessels were bathed in Mops buffer maintained at 37°C and aerated with room air. The microvessels (mean diameter = 125.2 ± 10.1 μ m) were imaged with the use of an inverted microscope connected to a video camera and the internal lumen diameter was measured with an electronic imaging apparatus (Living Systems, Burlington, VT). Vessels were precontracted with the thromboxane A2

analog U46619 by 30% of the baseline diameter prior to application of a vasodilator agent.

Vascular responses to FGF2 (109–104 M; R&D Systems, Minneapolis, MN) serotonin (5-HT, 109–104 M; Sigma, St. Louis, MO) adenosine-5-diphosphate (ADP, 109–104 M; Sigma), and SNP (109–104 M; Sigma) were examined. Selected experiments were performed following 20 min of pretreatment with 104 μ M/L NG-nitro-L-arginine (L-NNA, Sigma) or 1 g/mL of heparan sulfate (Sigma). All drugs were applied extraluminally. Measurements were made and recorded 2–3 min after drug administration, when the response was stabilized. One to four interventions were performed on each vessel. The order of drug administration was randomly selected.

RNA Analysis

For the RNA analysis of syndecan-4 expression in MHC-syndecan-4 mice, total RNA was isolated from the heart (n=12), brain, liver, lung, kidney, and intestine (n=4) using TriReagent (Sigma). For Northern blots, 10 g of total RNA were fractionated on 1.3% formaldehyde-agarose gels and transferred to GeneScreen Plus (DuPont) filters. The syndecan-4, FGF2, FGF-R1, and eNOS cDNA probes were labeled with 32 P-deoxycytidine triphosphate (New England Nuclear) with a random-priming labeling kit (Boehringer, Indianapolis, IN) and purified of unincorporated nucleotide using MicroSpin S-200 HR Columns (Amersham Pharmacia Biotech, Piscataway NJ). Following hybridizations, the blots were washed and the gene expression was determined using autoradiography. Isolated Heart Preparation For studies of NO release from the myocardium in response to FGF2, freshly excised hearts from MHC-syndecan-4 transgenic (n=6) and age-and-strain-matched control mice (n=6) were mounted on a Langendorff perfusion apparatus as previously described [12]. NO concentration in the coronary effluent was measured by using an amperometric sensor (ISONO, World Precision Instrument, Inc., Sarasota, FL). The principle of measurement and the electrode design have previously been described [13]. The probe was calibrated before each set of experiments using a standard chemical method which has been adapted to generate known concentrations of NO. To this end, KNO₂ (0.05 mM) was used as a generator of NO in the KI (0.1 M) and H₂SO₄ (0.1 M) mixture based on the following equation:

Following calibration and after 20 min of perfusion

with either vehicle or 1 $\mu\text{g}/\text{mL}$ FGF2 (Chiron Corp, Sunnyvale, CA) the electrode was positioned in the effluent to measure the amount of NO released from the coronary sinus.

Primary Cell Isolation and Culture

Primary microvascular endothelial cells were isolated from hearts of adult MHC-syndecan-4 (MHCS4) transgenic and control mice. Freshly extracted hearts were quickly dipped into ice-cold 70% ethanol for 20s and the atria were then discarded. The ventricular tissue was minced into 1-mm³ pieces and digested in Ca²⁺ and Mg²⁺ free Hank's solution containing 2 mg/mL collagenase II (Worthington Biochemical Co.) with agitation for 30 min at 37°. An additional digestion was performed by adding 0.3 mg/mL trypsin for 30 min at 37°. Following digestions, the cells were plated onto laminin coated dishes for 1 h and unattached cells were discarded. The remaining cells were washed with PBS three times and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS). Cells in the second passage were used for

experiment in this study.

For primary cardiac myocytes isolation, freshly extracted hearts from adult MHC-S4 transgenics and control mice were perfused with Tyrode solution (0.1 M NaCl, 5 mM KCl, 1 mM MgCl, 10 mM Hepes, and 10 mM glucose, pH 7.4) for 15 min at 37°. At that point, 1 mg/mL collagenase II (Worthington Biochemical Co.) was added to further digest the hearts for another 15 min at 37°. Following the completion of digestion, atria were discarded and the remaining tissue was quickly minced under a microscope into 1-mm³ pieces. Cells were harvested from the enzyme solution by centrifugation and the fresh culture medium (DMEM+15% FBS) was added to resuspend the cells. To enrich the myocyte population, a differential adhesion step was used. The dissociated heart cell suspensions were preincubated in 100-mm cell culture dishes for 60 min at 37°, allowing fibroblasts to attach. The remaining unattached cells (containing mostly myocytes) were collected and plated into 24-well cell culture dishes in DMEM+15% FBS and allowed to attach for 2 to 3 h and used immediately for the experiments.

Fig. 1. Syndecan-4 transgene expressed in MHC-syndecan-4 transgenic mice.

Northern blot analysis of syndecan-4 expression in the heart (A) and other organs (B). Note the presence of the transgene band in the heart of MHC-S4 mice. Effect of syndecan-4 expression in the heart on mRNA levels of FGF2, FGF-R1, and eNOS is shown in (C). Note similar levels of expression in both wild-type (WT) and MHC-S4 mice.

Results

Myocyte Syndecan-4 Expression Increased FGF2-Induced Nitric Oxide Release

To study the effect of syndecan-4 expression in normal cardiac myocytes on microvascular function, we generated four different transgenic mice lines carrying full-length rat syndecan-4 cDNA under control of MHC promoter. All transgenic lines grew and bred normally compared with the wild-type mice. As expected, syndecan-4 transgene expression was confined to the heart (Figs. 1A and 1B) and restricted to cardiac myocytes (not shown). The transgene expression had no effect on expression of basic fibroblast growth factor (bFGF or FGF2), its receptor 1 (FGF-R1), or endothelial nitric oxide synthase (eNOS; Fig. 1C).

FGF2 has been shown to dilate resistance vessels by inducing release of nitric oxide. To assess the effect of myocyte syndecan-4 expression on FGF2-induced NO

release, we first measured NO concentration in the coronary effluent from hearts of wild-type and MHC-S4 transgenic mice. While baseline levels of NO were similar in both groups, after perfusion with FGF2 (1 $\mu\text{g/mL}$), NO release increased more than 80% in MHC-S4 mice but only 20% in the control mice (control vs MHC-S4: 82.0 ± 22 vs 83.9 ± 16 nM/g tissue at baseline, $P=\text{ns}$; 135.3 ± 14.7 vs 103.0 ± 24 nM/g following FGF2 stimulation, $P<0.05$) (Fig. 2A).

To document that increased NO release was indeed secondary to syndecan-4 expression in cardiac myocytes, we carried out in vitro measurements of NO release from isolated primary endothelial cells and myocytes derived from hearts of control or MHC-S4 mice. While endothelial cells from both mice groups demonstrated a similar magnitude of NO release in response to FGF2 (16.5 ± 6.7 vs 20.4 ± 5.2 nM, control vs MHC-S4, $P=\text{ns}$), myocytes isolated from hearts of MHC-S4 mice had produced significantly greater amounts of NO compared to cardiac myocytes from the control mice (Fig. 2B).

Fig. 2. Effect of syndecan-4 expression on FGF2-induced nitric oxide release.

(A) The amount of NO release induced by FGF2 was tested in a Langendorff preparation of hearts from MHC-S4 (gray bars) and wild-type (WT, hatched bars) mice. Note a significant increase in NO release in MHC-S4 hearts (* $P<0.05$ FGF2 vs no FGF2 and MHC-S4 vs WT).

(B) NO release by primary cardiac myocytes (gray bars) and endothelial cells (stippled bars) from αMHC-S4 and wild-type mice. Note a significant increase in FGF2-induced NO release in myocytes but not endothelial cells (* $P<0.05$ with FGF2 vs without FGF2). Data are shown as the mean $\pm$ SD.

Changes in Heparan Sulfate Matrix Have Differential Effects on Endothelial Receptor-Dependent Responses

To assess the effect of myocyte syndecan expression on function of myocardial microvasculature, we studied *in vitro* responses of precontracted coronary arterial microvessels from MHC-S4 and wild-type mice hearts to various agonists. A direct vasodilator, nitroprusside, and an endothelial receptor-independent vasodilator, A23187, had similar magnitude effects on microvessels from both MHC-S4 and control mice hearts (Figs. 3A and 3B). Treatment with the endothelial receptor-dependent agonist serotonin also resulted in a similar extent of vasodilation in the MHC-S4 and control mice vessels (Fig. 3C). On the other hand, FGF2 was significantly more potent in vessels derived from MHC-S4 mice hearts (Fig. 4A), while ADP response was substantially inhibited in these mice (Fig. 4B). In both cases, vasodilation was inhibited by L-NNA (Figs. 4A and 4B), implying that NO release is responsible for this effect.

Since microvessels themselves do not express syndecan-4, the differences in these responses could have arisen from the presence of shed, endothelial-cell-surface-bound extracellular domains of syndecan-4 that would largely consist of heparan sulfate chains. To test the effect of the presence of shed HS chains on FGF2 and ADP responses, we assessed the ability of these agents to vasodilate coronary microvessels from normal (control) mice hearts in the presence or absence of exogenous HS chains in the buffer. We found that, similar to microvessels from MHC-S4 mice, the addition of HS to microvessels from the control mice potentiates vasodilatory response to FGF2 (Fig. 4C) while inhibiting ADP-induced vasodilation (Fig. 4B). Both FGF2 and ADP induce vasodilation by stimulating NO release. Therefore, we have examined the effect of exogenously added HS chains on NO release response to these agents by endothelial cells in culture. Similar to findings in the microvessels assays, addition of HS stimulated FGF2-induced NO release while inhibiting ADP-induced release (Fig. 4D).

Discussion

The major finding of this study is that an alteration in the extracellular matrix heparan sulfate content has a significant and heterogeneous impact on endothelial cell signaling. In particular, while FGF2 signaling was enhanced (as measured by NO release and microvascular

Fig. 3. *In vitro* response of mouse cardiac microvessels.

In vitro responses of precontracted cardiac microvessels from MHC-syndecan-4 (F) and wild-type (WT) (F) mice to the endothelium-independent vasodilator sodium nitroprusside (SNP) (A), endothelium-receptor-independent vasodilator A23187 (B), and endothelium-receptor-dependent agonist serotonin (C) are shown. Response is plotted as percentage change in diameter of precontracted vessels over a concentration range of the agonist. Note similar responses in microvessels from both WT and MHC-syndecan-4 mice hearts. Data are expressed as the mean $\pm$ SE.

vasodilation), microvasculature responses to ADP were inhibited and responses to serotonin, nitroprusside, and the calcium ionophore A23187 were unchanged. The ability of heparan sulfates to alter responses to heparin-binding growth factors and other molecules has been long acknowledged [14, 15]. In particular, FGF2 ability to induce NO release, critical to its ability to induce vasodilation [15], is fully dependent on HS-mediated binding to its high-affinity receptor [16–18]. At the same time, heparin can also induce vasodilation. This activity has been attributed to activation of NO release as well as activation of K[ATP] channels. However, the contribution of endogenous matrix HS to microvascular homeostasis and the effect of altered HS concentration on activity of other vasodilators such as VEGF or ADP have not been investigated.

In this study we have altered the myocyte cell surface

Fig. 4. Effect of syndecan-4 expression on FGF2 and ADP signaling.

In vitro response of microvessels derived from MHC-S4 (F) and wild-type (WT) (F) mice hearts to FGF2 (A, C) and ADP (B) was carried out in precontracted microvessels.

(A) Note significantly greater degree of vasodilation induced by FGF2 in microvessels from MHC-S4 compared to WT mice (* $P < 0.05$, MHC-S4 vs WT). Pretreatment with L-NNA fully blocks FGF2-induced vasodilation.

(B) In contrast to FGF2, ADP induced significantly less vasodilation in microvessels from MHC-S4 compared to WT hearts (* $P < 0.05$, MHC-S4 vs WT). Addition of exogenous heparan sulfate chains (HS, 1 g/mL) to microvessels from WT mice significantly inhibited ADP-induced vasodilation (* $P < 0.05$, WT + HS vs WT). Similarly to FGF2, L-NNA inhibits ADP-induced vasodilation.

(C) Addition of exogenous heparan sulfate chains (HS, 1 g/mL) to microvessels from WT mice significantly enhanced FGF2-induced vasodilation (* $P < 0.05$, HS vs vehicle).

(D) Effect of exogenous heparan sulfate (HS, 1 μg/mL) on FGF2 and ADP-induced nitric oxide release was in primary endothelial cells derived from control mice hearts. Note enhanced release of NO in the presence of HS chains in the case of FGF2 (hatched bars) and reduced NO release in the case of ADP (gray bars). Data are expressed as the mean $\pm$ SD. * $P < 0.05$ agonist vs/no agonist.

and the ECM HS content by transgenic expression of the syndecan-4 gene under the control of the cardiac myocyte-specific (MHC) promoter. Despite significantly increased syndecan-4 expression in MHC-S4 transgenics, baseline NO release from the hearts of these mice was not different from that of control mice when studied *in toto* on a Langendorff preparation. As expected, FGF2 induced greater NO release from MHC-S4 compared to control mice hearts. Furthermore, treatment of isolated primary cardiac myocytes derived from MHC-S4 mice with FGF2 resulted in greater NO release compared to NO release from cardiac myocytes isolated from control mice hearts. This finding is in accord with the above-stated expectations, since syndecan-4 expression on myocyte cell surface will enhance FGF2 binding and signaling [3]. At the same time, there was no difference in FGF2-induced NO release from primary endothelial cells derived from MHC-S4 or control mice. Both endothelial cells and myocytes derived from the hearts of MHC-S4 transgenics possess increased cell-surface HS content—myocytes due to cell-type-specific expression of syndecan-4 and endothelial cells due to syndecan-4 shedding from myocytes. However, since procedures employed for isolation of both cell types involve extensive enzymatic digestion of tissue, only the cells capable of regenerating HS-carrying core proteins, i.e., cardiac myocytes, would be expected to show enhanced FGF2 response. The fact that this response is, indeed, due to the increased presence of shed (extracellular) HS chains is illustrated by the fact that addition of exogenous HS chains to primary endothelial cells from control mice results in enhanced NO release.

The other evidence of the effect of cell surface HS on FGF2-induced NO release comes from studies of coronary microvasculature. Unlike the case of primary cell isolation, preparation of coronary vessels does not result in disruption of cell-surface-absorbed heparan sulfates, since it requires enzymatic digestion, and not simply washing, to remove cell-surface-associated HS chains. Thus, we have observed a significantly larger vasodilatory response to FGF2 in vessels derived from MHC-S4 compared to control mice. This conclusion is supported by the observation that addition of HS chains to microvessels from control mice hearts results in enhanced FGF2-induced vasodilation. Taken together, these experiments clearly demonstrate that increased expression of syndecan-4 core protein in cardiac myocytes results in amplified production of NO by these myocytes in response to FGF2 and that increased HS chain shedding accounts for increased responsiveness

of coronary microvessels in these hearts to FGF2. This conclusion is also supported by our previous observations documenting increased cell-surface-associated HS mass following expression of HS-carrying core proteins in general and syndecan-4 in particular [3].

To explore the effect of these changes in the matrix HS content on the ability of other vasoactive agents to induce microvascular responses, we have examined activity of ADP, serotonin, and A23187. Increased shedding of syndecan-4 derived HS produced a significant inhibition of ADP-induced vasodilation. This was demonstrated both in studies of MHC-S4 micederived microvessels and in control mice microvessels in the presence of exogenously added HS chains. Since both FGF2- and ADP-induced vasodilation requires NO release, it is logical to assume that the presence of shed HS chains has a differential effect on the ability of these molecules to stimulate NO production. In accordance with this hypothesis, the addition of HS chains to cultured primary endothelial cells stimulated FGF2-induced NO release while inhibiting ADP ability to generate NO. It is entirely unclear why alterations in the HS cell surface and/or matrix contents resulted in such a profound inhibition of ADP activity. One possibility is that HS chains block ADP binding to its receptor either directly or indirectly by altering its conformation. Alternatively, ADP may be sequestered by the HS chains away from its site of action.

These changes in the HS matrix content had little impact on the ability of another endothelial receptor-dependent agonist, serotonin, to induce vasodilation, nor have they affected the vasodilatory activity of the endothelium-receptor-independent agent A23187 or the endothelium-independent drug nitroprusside. In previous studies of microvascular responses in chronically ischemic porcine hearts, we have observed enhanced vasodilation in response to FGF2 that was attributed to increased FGF receptor expression [19]. At the same time, there were no changes in response to sodium nitroprusside and ADP response was suppressed. Since syndecan-4 expression is known to be increased in these settings, increased FGF2 responsiveness may have been due in equal measure to increased syndecan-4 as well as FGF receptor expression. Furthermore, decreased ADP responsiveness fits well with the data from the current study.

A number of factors can alter syndecan-4 expression, including ischemia and inflammation. In the postinfarct heart, the presence of blood-derived macrophages is required for increased syndecan-4 expression [10]. Similarly, arterial injury is also associated with enhanced

syndecan-4 expression [20] and both serum and FGF2 have been reported to increase syndecan-4 levels in smooth-muscle cells [21]. In addition, two other proteins have been shown to induce syndecan-4 levels: a peptide PR39 [10, 22] and TNF [23].

Expression of other HS-carrying core proteins is also altered in a number of disease states, including tissue injury and hypoxia [7]. Since HS chains found on different core proteins are thought to be structurally similar, alteration in expression of any these proteins can have a similar effect on growth factor signaling. In summary, alterations in the extracellular matrix heparan sulfate chain composition can have a significant and heterogeneous effect on endothelial cell responses and may account for changes in microvascular responses observed in the ischemic myocardium.

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Chapter 3

Growth Factor-specific Modulation of Cellular Response by Syndecan-4

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Abstract

Proteoglycans participate in growth factor interaction with the cell surface through their heparan sulfate chains (HS), but it is not known if they are otherwise involved in growth factor signaling. It appears now that the syndecan-4 core protein, a transmembrane proteoglycan previously shown to bind phosphatidylinositol 4,5-bisphosphate (PIP_2) and activate protein kinase C (PKC) α , participates in mediating the effects of fibroblast growth factor 2 (FGF2) on cell function. Mutations in the cytoplasmic tail of syndecan-4 that either reduced its affinity to PIP_2 (PIP_2^-), or disrupted its PDZ-dependent binding (PDZ^-), produced a FGF2-specific dominant-negative phenotype in endothelial cells, as evidenced by the marked decline of their migration and proliferation rates, and by the impairment of their capacity to form tubes. In both cases the molecular mechanism was determined to consist of a decrease in the syndecan-4-dependent activation of PKC α . This decrease was caused either by inhibition of FGF2-induced syndecan-4 dephosphorylation in the case of the PDZ^- mutation, or by disruption of basolateral targeting of syndecan-4 and its associated PDZ-dependent complex in the case of the PIP_2^- mutation. These results suggest that PKC α activation and PDZ-mediated formation of a serine/threonine phosphatase-containing complex by syndecan-4 are downstream events of FGF2 signaling.

Introduction

The participation of HS chains in binding numerous soluble ligands and extracellular matrix proteins (Bernfield et al., 1999) drew increased attention once HS presence on the cell surface was shown to be required for FGF2-dependent cell growth (Rapraeger et al., 1991). The list of HS-binding soluble ligands has grown significantly, culminating recently in the finding that syndecan-1-attached HS chains promote the tumorigenic response to Wnt-1 (Alexander et al., 2000).

The potential role of the HS-carrying core of the proteoglycans in mediating the cellular response to growth factors received little attention, however, despite earlier indications of their response to extracellular signals, such as the recruitment of syndecan-4 to focal adhesions (Baciu and Goetinck, 1995; Woods and Couchman, 1994). The possibility of fulfilling specific functional roles seems particularly relevant to the syndecan core proteins, all of

which share a distinct and highly conserved cytoplasmic tail. One of the motifs common to all the syndecans is a carboxy-terminal PDZ (Postsynaptic density 95, Disk large, Zona occludens-1)-binding motif, now known to bind at least four PDZ domain-containing partners (Cohen et al., 1998; Ethell et al., 2000; Gao et al., 2000; Grootjans et al., 1997; Hsueh et al., 1998). Similar to other PDZ proteins, these binding partners very likely serve as adaptors between the syndecans and additional members of larger complexes.

Syndecan-4, the most widely spread member of the family, differs in its sequence from the other three syndecans by a unique PIP_2 -binding 7-residue motif located in the middle of its 28-amino acid- long cytoplasmic tail (Horowitz et al., 1999; Lee et al., 1998). Syndecan-4 has been implicated in signal transduction (Volk et al., 1999) and in the activation of PKC α (Oh et al., 1997b). PIP_2 appears to underlie the signaling activity of the cytoplasmic tail of syndecan-4, serving as a binding interface (Horowitz et al., 1999) and an essential co-factor for PKC α activation (Oh et al., 1998), as well as a facilitator of the tail's multimerization (Oh et al., 1997a). These properties are regulated by the phosphorylation of S¹⁸³, located 4 residues away from the amino-terminus of the PIP_2 -binding motif (Horowitz and Simons, 1998a). Once phosphorylated, the affinity of the cytoplasmic tail for PIP_2 and its capacities to oligomerize and activate PKC α in the presence of PIP_2 are sharply reduced (Simons and Horowitz, 2001).

Given the HS-dependence of the activity of several growth factors, and the *in vivo*-observed increase in syndecan-4 expression during growth factor-regulated healing from injury (Gallo et al., 1994; Li et al., 1997; Nikkari et al., 1994), we asked whether the molecular attributes of syndecan-4 listed above are relevant to growth factor signaling. Using FGF2 as a HS-binding growth factor prototype, we found that disruption of either the PIP_2 or PDZ binding domains of syndecan-4 conferred a dominant negative phenotype in regard to this growth factor, but not to serum or to epithelial (EGF) and platelet-derived (PDGF) growth factor-induced response.

These data suggest, therefore, that syndecan-4 selectivity regulates FGF2 signaling in endothelial cells.

Results

Site-directed mutations in syndecan-4 confer dominant-negative effects on FGF2 signaling in endothelial cells

We addressed the potential role of syndecan-4 in regulating the cellular response to FGF2 by transfecting rat fat pad endothelial cells (RFPEC), which express syndecan-4 endogenously (Kojima et al., 1992), with hemagglutinin (HA)-tagged wild type (S4) syndecan-4, or with HA-tagged syndecan-4 constructs mutated at two key sites (Fig. 1a). In the first construct (PIP₂⁻), the three consecutive residues Y¹⁹²KK in the cytoplasmic tail of syndecan-4 were mutated to LQQ, a mutation that drastically reduces the PIP₂ affinity of the cytoplasmic tail and inhibits its PIP₂-mediated activation of PKCα (Horowitz et al., 1999). In another construct (PDZ⁻), the carboxy-terminal residue (A²⁰²) was deleted, thereby abolishing PDZ-dependent binding of syndecan-4 (Songyang et al., 1997). Finally, a PIP₂⁻/PDZ⁻ construct combined the features of the other two constructs.

In order to measure the expression level of the introduced constructs, as well as the effect of exogenous construct expression on endogenous syndecan-4 levels, we immunoblotted total cell lysates from each of the cell lines with antibodies to syndecan-4 and to HA (Fig. 1b). The syndecan-4 immunoblot represents the sum of endogenous and transfected syndecan-4 proteins, while the HA immunoblot assesses the amount of transfected syndecan-4. The immunoblots showed on average a 2 fold increase in the expression level of the exogenous constructs over the endogenous syndecan-4 protein in the transfected cell lines, excluding the PIP₂⁻ cells, where the mutated syndecan-4 variant is not recognized by the antibody against the cytoplasmic tail used in these experiments, since the YKK→LQQ mutation disrupts its epitope (data not shown).

To find whether these mutations perturbed FGF2 signaling, we examined several potentially susceptible cell functions. Migration rate in response to FGF2 treatment was measured by a wounding assay of confluent cell monolayers. The migration rates of cells expressing either PIP₂⁻ or PDZ⁻ syndecan-4 mutants were similar to each other and 3-fold lower than the migration rate of vector-transfected RFPEC (Fig. 2a). At the same time, migration of cells carrying the combined PIP₂⁻/PDZ⁻ mutation was not different from controls. In agreement with our previous observations (Volk et al., 1999), the migration

Fig. 1: (A) Schemes of syndecan-4 cytoplasmic tail constructs used in the study.

From left to right: WT cytoplasmic tail (S4; solid cylinder – PIP₂ binding domain, solid sphere – C-terminal A²⁰² residue), PIP₂⁻ (Y¹⁹²KK→LQQ) mutation (void cylinder), PDZ⁻ (deletion of the C-terminal A²⁰² residue) mutation, and PIP₂⁻/PDZ⁻ (combination of both mutations).

(B) Protein levels of exogenous and endogenous syndecan-4 in transfected cell clones.

Top: immunoblots for total syndecan-4 protein levels, using antiserum specific to the cytoplasmic tail of syndecan-4, and a histogram representing the densitometric values of each band (in arbitrary units).

Bottom: as above, using HA tag antibody. Aliquots of cell lysate containing equal masses of total protein were

immunoprecipitated from each cell group with antiserum specific to the ectoplasmic tail of syndecan-4. Glycosaminoglycan chains were digested prior to gel electrophoresis.

rate of RFPEC overexpressing S4 was even higher than vector-transfected cells (relative gap closure of 0.34 ± 0.09 , versus 0.27 ± 0.03 , respectively; $n=12$, $p=0.023$; see Fig. 2a). Cell growth in response to FGF2 over a period of 3 days was measured by proliferation assays with the same cell clones used in the migration assays. Cells expressing either the PIP₂⁻ or PDZ⁻ mutants had 3-5-fold lower proliferation rates in comparison to vector-transfected cells, while cells expressing the PIP₂⁻/PDZ⁻ mutation had the same proliferation rate as vector controls, a

pattern similar to the results of the migration assays (Fig. 2b). Finally, tube formation on extracellular matrix basement in response to FGF2 by cell clones expressing the PIP₂⁻ or PDZ⁻ syndecan-4 mutations, but not the PIP₂⁻/PDZ⁻ mutation, was also markedly impaired in comparison with the continuous and articulated tube networks formed by vector-transfected cells (Fig. 2c), or by S4-overexpressing RFPEC (data not shown).

The combined results of the migration, proliferation, and tube formation experiments indicate that both the Y¹⁹²KK → LQQ substitution and the A²⁰² deletion conferred a dominant-negative phenotype on RFPEC when co-expressed with endogenous syndecan-4. This effect was FGF2-specific, since when performed in the presence of 10% serum (Fig. 2a, 2c), or in the presence of either EGF or PDGF AB (25 ng/ml each; data not shown) migration and tube formation by PIP₂⁻ and by PDZ⁻ cells did not differ from those of vector-transfected cells.

PIP₂⁻ mutation impairs syndecan-4 targeting to the basolateral region

To elucidate the mechanism of the dominant-negative effect of the mutations in the cytoplasmic tail of syndecan-4, we compared the cellular distribution of each mutated syndecan-4 variant to that of the endogenous molecule. Staining of untransfected RFPEC with the antibody to

Fig. 2: Effects of syndecan-4 mutations on cell function.

(A) Migration of RFPEC as measured in "wounding" assays (inset). Cells were starved in 0.5% FBS for 24 hrs, scratched, and incubated for another 6 hrs, either with 0.5% FBS and 20 ng/ml FGF2 (solid bars), or with 10% FBS alone (void bars). Gap size was measured immediately before (W_0) and after (W_6) the 6 hr incubation period ($\Delta W = W_0 - W_6$). Data shown as mean $\pm$ S.D., $n=12+18$; * – $p<0.05$.

(B) Fold increase in cell number relative to day 0, as measured in RFPEC proliferation assays. Cells were starved as above, and then treated with 20 ng/ml FGF2 ($n=3$). Cells were counted immediately before FGF2 application, then at 24, 48, and 72 hrs after it. Data shown as mean $\pm$ S.D., $n=4$; * – $p<0.05$ (diamonds – vector-transfected control cells; triangles – PIP₂⁻ cells; squares – PDZ⁻ cells; circles – PIP₂⁻/PDZ⁻ cells).

(C) Tube formation assays on extracellular matrix basement (Matrigel™, Beckton Dickinson). Cells were starved as above, then treated for 24 hrs either with 0.5% FBS and 20 ng/ml FGF2, or with 10% FBS alone (insets), and imaged immediately. Each cell line was assayed in duplicate. Note that all cell lines formed normal tube networks when treated with 10% FBS.

Fig. 3: Cellular distribution of endogenous and transfected syndecan-4 in RFPEC.

Top, phase (left panel) and immunofluorescence (right panel) images of the same untransfected RFPEC labeled with 1:50 diluted syndecan-4 cytoplasmic tail antiserum. Arrows, Golgi apparatus, arrowheads, cell junctions. Nuclear staining in the right panel is due to non-specific immunolabeling.

Bottom, confocal immunofluorescence images of RFPEC doubly labeled with 1 μ g/ml HA antibody (Roche), and with 2.5 μ g/ml antibody to GM130 (Transduction Laboratories), showing the distribution of HA-tagged WT syndecan-4 (S4, left panel), GM-130 Golgi marker (center), and an overlay of both images (bars, 25 μ m). Note the distribution of both endogenous and WT overexpressed syndecan-4 (S4) along cell junctions and in the Golgi apparatus.

syndecan-4 demonstrated the presence of the proteoglycan along the cell borders and in the perinuclear region (Fig. 3, top panels). S4 expressed in RFPEC assumed a similar distribution (Fig. 3, bottom panels). The perinuclear location coincided with the Golgi apparatus, as shown by overlap with the staining for the Golgi scaffold protein GM130 (Nakamura et al., 1995).

Both the PIP_2^- and the $\text{PIP}_2^-/\text{PDZ}^-$ syndecan-4 variants maintained their Golgi localization, but were no longer present in the basolateral region (Fig. 4a and 4c, respectively). In contrast with the cellular distribution of the PIP_2^- and the $\text{PIP}_2^-/\text{PDZ}^-$ syndecan-4 mutants, the

distribution of the PDZ^- mutant (Fig. 4b) did not differ from that of the endogenous syndecan-4, indicating that syndecan-4 is targeted to the basolateral region by its association with PIP_2 , rather than with a PDZ protein.

To further examine the effect of the PIP_2^- mutation on the surface targeting of syndecan-4, the S4, PIP_2^- and $\text{PIP}_2^-/\text{PDZ}^-$ variants were transfected into RFPEC via a bicistronic EGFP-expressing plasmid. Each cell group was scanned at both 488 nm, the EGFP emission wavelength, and at 580 nm, the emission wavelength of the red-pycoerythrin fluorophore conjugated to the secondary antibody used to detect the HA-tagged exogenous

Fig. 4: The distribution of syndecan-4 mutants in RFPEC.

(A) PIP_2^- -expressing cells doubly immunolabeled with syndecan-4 cytoplasmic tail antiserum (left panel) and HA antibody (center). Note that the distribution of the PIP_2^- syndecan-4 (center panel) was restricted to the Golgi, while native syndecan-4 (left panel) is localized both to the Golgi and along cell borders, as in the top right panel of Fig. 3. Nuclei appear in this and in parts b and c due to non-specific binding of the syndecan-4 antiserum.

(B) PDZ^- cells doubly immunolabeled with syndecan-4 cytoplasmic tail antiserum (left panel) and HA antibody (center panel). Note that the distribution of the PDZ^- syndecan-4 was similar to that of HA-tagged WT syndecan-4 in Fig. 3, bottom left panel.

(C) $\text{PIP}_2^-/\text{PDZ}^-$ cells doubly immunolabeled with syndecan-4 cytoplasmic tail antiserum (left panel) and HA antibody (center panel). Note that the distribution of the $\text{PIP}_2^-/\text{PDZ}^-$ syndecan-4 was similar to that of PIP_2^- syndecan-4 in Fig. 4a, center panel.

Panels on the right are overlays of the left and center panels. Bars are 25 μ m.

syndecan-4. As shown in Fig. 5, cells transfected by S4 (panel b) had high counts at both wavelengths, while cells expressing the PIP_2^- and $\text{PIP}_2^-/\text{PDZ}^-$ mutants (panels c and d, respectively) had low counts at 580 nm, similar to the vector-transfected control cells (a). Note that though the fraction of EGFP-expressing cells out of the total PIP_2^- cell population is low relative to the EGFP expressing fractions in the other three the cell populations, the ratio between the cell fraction expressing both EGFP and PIP_2^- and the fraction expressing only EGFP (1:20) is similar to the corresponding ratio (1:25) in the $\text{PIP}_2^-/\text{PDZ}^-$ cell population, while the ratio in the S4 cell population is much higher (1:3). These results confirm the conclusion drawn from the immunofluorescence experiments that the PIP_2^- syndecan-4 mutant is not present on the cell surface.

The sequestration of the PIP_2^- mutated syndecan-4 to the Golgi apparatus may potentially reduce the

presence of endogenous syndecan-4 on the cell surface, if the two could form hetero-oligomers. In that case, the lower responses of the PIP_2^- cells to FGF2 observed in the functional assays (Fig. 2) may result not only from the presence of the mutated form of syndecan-4, but more trivially, from the resulting deficit in the available FGF binding sites along the syndecan-4-associated HS chains. To test this possibility, we quantified the amount of syndecan-4 present on the cell surface in vector-transfected and PIP_2^- cells by FACS, using an antibody against the ectoplasmic domain of syndecan-4. Since we have already shown (Figs. 4a and 5a) that the PIP_2^- variant of syndecan-4 is not present on the cell surface, the antibody would detect only the endogenous syndecan-4. The FACS results (Fig. 5b) show no significant differences between the amounts of syndecan-4 molecules present on the surface of vector-transfected and PIP_2^- cells.

Fig. 5: FACS analysis of exogenous and endogenous syndecan-4 expression levels.

(A) Distributions of pIRES2-EGFP vector transfected (a), HA-WT overexpressing (b), PIP_2^- -transfected (c), and $\text{PIP}_2^-/\text{PDZ}^-$ -transfected cells (d) as a function of their fluorescence intensities at 488 nm (EGFP, horizontal axis, arbitrary units, AU) and at 580 nm (R-PE, vertical axis), corresponding to the EGFP protein levels expressed either alone (a) or in tandem with each syndecan-4 variant (b, c, d), and to the cell surface expression levels of the syndecan-4 variants, respectively. The quadrants were drawn so as to enclose near to 100% of control non-transfected cells (not shown) in the lower right quadrant. Numbers in corners denote the percentage of the cell population in each quadrant. Quasi colors denote count density (red – high, blue – low).

(B) Intensity distribution (in arbitrary units) of cells labeled with antiserum to the syndecan-4 ectoplasmic domain followed by anti-rabbit IgG-Alexa 594, showing the levels of cell surface expression of endogenous syndecan-4 in vector-transfected (a) and PIP_2^- -expressing (b) cell lines.

PIP₂⁻ and PDZ⁻ mutations reduce syndecan-4-associated activity of PKC α

An established signaling role of syndecan-4 is the modulation of FGF2-stimulated PIP₂-dependent PKC α activity (Horowitz and Simons, 1998a; Oh et al., 1997b). Since the activity of this PKC isoenzyme has been previously linked to the promotion of cell growth (Besson and Yong, 2000; Cai et al., 1997; Kolch et al., 1993; Kolch et al., 1996; Lallena et al., 1999; Schonwasser et al., 1998), migration (Harrington et al., 1997), and tube formation (Wang et al., 2002), we studied the effect of PIP₂⁻ and PDZ⁻ mutations on PKC α activity in these cells. To this end, we assayed the activity of PKC α co-immunoprecipitated with HA-tagged syndecan-4 core proteins from the RFPEC clones expressing S4, PIP₂⁻, PDZ⁻ or PIP₂⁻/PDZ⁻ syndecan-4 constructs, before and after FGF2 stimulation.

FGF2 treatment increased syndecan-4/PIP₂-dependent PKC α activity 8-fold in syndecan-4-overexpressing RFPEC relative to untreated cells of the same type (Fig. 6a). On the other hand, syndecan-4-dependent PKC α activity in cells expressing PIP₂⁻, PDZ⁻, or PIP₂⁻/PDZ⁻ syndecan-4 constructs was not increased by FGF2. No significant FGF2-induced relative increases in syndecan-4-associated kinase activities were observed when instead of PIP₂ the assays were done in the presence of Ca²⁺, diacylglycerol, and phosphatidylserine. The absolute level of the Ca²⁺-dependent activity of PKC α is typically higher by 20% than its activity in the presence of PIP₂ and the cytoplasmic tail of syndecan-4 (Horowitz and Simons, 1998a). The fact that the Ca²⁺-dependent PKC α activities of all the cell lines tested did not significantly differ from each other (Fig. 6a), either with or without FGF2 treatment, indicates that similar amounts of PKC α were immunoprecipitated in all cases. Similar to the functional assays described above, the increase in the syndecan-4-associated kinase activity was specific to FGF2, as no significant differences were found between the syndecan-4-associated kinase activities in serum-treated versus untreated cells. Since the kinase activities were measured ratiometrically for each cell line (FGF2 or serum-treated vs. untreated cells), they were not affected by variations between different cell lines in the absolute amounts of syndecan-4-associated PKC α .

Though the binding of PKC α to the cytoplasmic tail of syndecan-4 is well documented (Horowitz and Simons, 1998a; Oh et al., 1997b), we verified that the measured kinase activity in these assays was produced by

Fig. 6: Syndecan-4-associated kinase activities.

(A) Relative kinase activities of HA immunoprecipitates from RFPEC starved in 0.5% FBS for 24 hrs, then incubated for another 20 min either without or with 20 ng/ml FGF2, or with 10% FBS. Data shown as mean $\pm$ S.D. (n=3) of the ratios between the kinase activity immunoprecipitated from FGF2- or FBS-treated cells, and from non-treated cells of the same type. Note that the kinase activity of S4 cells was increased significantly by FGF2 treatment relative to other cell lines, and that this relative increase was observed only when the immunoprecipitates were assayed in the presence of PIP₂, but not when assayed in the presence of Ca²⁺, phosphatidylserine, and dioleoin. Note also that the PIP₂-dependent kinase activities immunoprecipitated from S4 and PIP₂⁻-expressing cells did not significantly differ from each other when cells were treated with FBS instead of FGF2 (black bars – S4 cells; light gray – PIP₂⁻ cells; white – PDZ⁻ cells; dark gray – PIP₂⁻/PDZ⁻ cells).

(B) Effects of PKC autoinhibitor peptide (AIP, 100 nM), or of G66976 (10 nM), on kinase activities (mean $\pm$ S.D., n=3) of anti HA immunoprecipitates from RFPEC in the presence of Ca²⁺, phosphatidylserine, and dioleoin. Results are relative to the kinase activity of untreated control samples (insets in A and B: representative phosphoimages of PKC α optimal substrate peptide bands, used for quantifying the kinase activities of anti-HA precipitates).

a calcium-dependent PKC, and not by other unidentified syndecan-4-associated kinases. Both the highly specific PKC autoinhibitor peptide (House and Kemp, 1987) and the cPKC inhibitor Gö6976 (Martiny-Baron et al., 1993) effectively suppressed the syndecan-4 associated kinase activity (Fig. 6b).

PDZ⁻ syndecan-4 is hyperphosphorylated following FGF2 stimulation

Surprisingly, the FGF2-stimulated PKC α activity in cells transfected with the PDZ⁻ mutant was similar to that seen in cells transfected with the PIP₂⁻ mutant and much lower than in cells transfected with the native syndecan-4 construct (Fig. 6a).

Given that the capacity of syndecan-4 to activate PKC α is sharply reduced by the phosphorylation of S¹⁸³ in its cytoplasmic tail (Horowitz and Simons, 1998a), we compared the effect of FGF2 stimulation on the phosphorylation level of the PDZ⁻ mutant to its effect on the S4 variant. FGF2 administration lowered the phosphorylation levels of S4 in a statistically significant manner (Fig. 7) relative to its basal level, in agreement with previously described results (Horowitz and Simons, 1998a), but it did not lower those of the PDZ⁻ mutant. Moreover, the phosphorylation level of the PDZ⁻ mutant after FGF2 treatment was significantly higher than its basal level. This increase may reflect the dependence of the phosphorylation level on a dynamic balance between the opposing actions of a kinase and a phosphatase. The dephosphorylation of the PDZ⁻ mutant would be impaired if the association of syndecan-4 with the putative phosphatase were mediated by a PDZ adaptor protein. Consequently, the activity of the kinase that phosphorylates Ser¹⁸³, which could conceivably be elevated by FGF2, would be unopposed by the phosphatase, resulting in an effective increase in the phosphorylation level of syndecan-4.

Since it is likely that the PDZ⁻ mutant and the endogenous syndecan-4 copolymerize, the abolishment of the binding of the putative phosphatase may affect the phosphorylation levels of both syndecan-4 variants. As the currently available antibodies cannot separate between the endogenous and the exogenous syndecan-4 species, we were able to measure the effect of FGF2 on the phosphorylation level of only the total syndecan-4 population, which was not significantly changed (Fig. 7). This outcome could be attributed to opposing and mutually canceling changes in the phosphorylation levels

Fig. 7: Phosphorylation levels of syndecan-4.

Data shown as means $\pm$ S.D. (n=3) of syndecan-4 phosphorylation levels measured as described in Materials and Methods. Cells were incubated in 0.5% FBS either without (black bars), or with (white bars) 20 ng/ml FGF2 starting 2 hrs before radio labeling, for a total of 4 hrs. Note the significant difference (*-p<0.05) between the phosphorylation levels of S4 and PDZ⁻ before and after FGF2 treatment (tot = total syndecan-4 population in the cell). Insets, immunoblots of representative samples from each cell group and condition.

of the endogenous and PDZ syndecan-4 populations in response to FGF2, thus implying that the expression of the PDZ⁻ mutant did not significantly affect the phosphorylation level of endogenous syndecan-4.

PIP₂⁻ mutation reduces syndecan-4 PIP₂-dependent oligomerization and activation of PKC α

The capacity of syndecan-4 to activate PKC α is dependent on its affinity to PIP₂, and on its tendency to oligomerize in the presence of this phosphoinositide (Horowitz and Simons, 1998a). The potent suppression of PKC α activity and of several cell functions by the Y¹⁹²KK $\rightarrow$ LQQ mutation implies that the PIP₂⁻ mutant has a dominant negative effect when expressed on a background of endogenous syndecan-4. Therefore, we investigated the effect of mixing PIP₂⁻ mutated cytoplasmic syndecan-4 tail peptide with the WT tail peptide on the combined affinity of the mixture to PIP₂ *in vitro*. As little as 25% of PIP₂⁻ peptide in the total WT/PIP₂⁻ mixture increased the apparent K_D of the mixture by 3 orders of magnitude compared with that of the WT peptide alone, as measured by surface plasmon

	Peptide fraction (%)			
WT	100	75	50	0
PIP ₂ ⁻	0	25	50	100
K _D	10±2 nM	9.1±1.8 μM	24.3±0.0 μM	940±3.3 μM

Fig. 8: In vitro experiments with synthetic WT and mutated syndecan-4 cytoplasmic tail peptides.

(A) Dependence of surface plasmon resonance-measured apparent first order equilibrium constants (K_D) between PIP₂ and PIP₂⁻/WT peptide mixtures on PIP₂⁻ peptide fraction. Note the gradual elevation in K_D value upon increasing the PIP₂⁻ peptide fraction.

resonance (Fig. 8a).

Since syndecan-4-PIP₂ binding is thought to play a role in the oligomerization of syndecan-4 tails (Horowitz and Simons, 1998a; Oh et al., 1997a), we examined the oligomerization capacity of the PIP₂⁻ peptide. When resolved by column chromatography, the PIP₂⁻ peptides did not form high-order oligomers in the presence of PIP₂, unlike the WT peptides (Fig. 8b). Both peptides formed, however, smaller PIP₂-independent oligomers of a size between tetramers and hexamers. The capacity of the PIP₂ peptide to form low-order oligomers in the same manner as the WT peptide may explain the significantly higher K_D between PIP₂ and the 25%:75% PIP₂⁻:WT peptide mixture reported above, as the two peptides could form hetero-oligomers of a PIP₂ affinity much lower than that of WT homo-oligomers, due to the presence of the PIP₂⁻ peptide. The decreased affinity of the PIP₂⁻ peptide to PIP₂, and its lower tendency to oligomerize were accompanied by a large reduction in its capacity to activate PKCα in the presence of PIP₂, relative to the WT peptide (Fig. 8c). Though the PDZ⁻ peptide is shorter than the WT peptide only by a single residue, the C-terminal alanine, the PKCα activity in its presence at 2.5 μM ≤ [PIP₂] ≤ 10 μM was lower in a statistically significant manner than the corresponding activities in the presence of the WT peptide, but higher than those of the PIP₂⁻ mutant.

Discussion

We have described a novel mechanism regulating growth factor signaling, distinctive both for its specificity to FGF2 (*vs.* serum), as well as for the pivotal role of syndecan-4. The main feature of this signaling pathway is activation of PKCα by the syndecan-4/PIP₂ complex in response to FGF2 stimulation. This process is controlled by the phosphorylation of S¹⁸³ in the cytoplasmic tail

(B) Column elution profiles of WT (top) and PIP₂⁻ (bottom) cytoplasmic tail peptides with (continuous line) and without (dashed line) PIP₂. Note the formation of high order oligomers by the WT (arrow), but not by the PIP₂⁻ peptide in the presence of PIP₂. Note also that both peptides are not present as monomers but form smaller oligomers of similar sizes, both in the presence and absence of PIP₂.

(C) Dependence of the in vitro activity of recombinant PKCα on PIP₂ concentration, in the absence (squares) and in the presence of 1 μM WT (circles), PIP₂⁻ (up-turned triangles), or PDZ⁻ (down-turned triangles) cytoplasmic tail peptides (mean ± S.D., n=3-6; * - p<0.05). The mean kinase activity measured at 1 μM PIP₂ with 1 μM WT peptide was used as a unitary reference value.

of syndecan-4 that down regulates the response to FGF2 by preventing PIP₂-dependent oligomerization of syndecan-4 and the subsequent activation of PKC α .

Our working model of this mechanism (Fig. 9) consists of the following steps:

(1) FGF2 binding to its high affinity tyrosine kinase receptor induces the activation of a putative serine/threonine protein phosphatase type 1/2A (PP1/2A) (Horowitz and Simons, 1998b), which is associated with the cytoplasmic tail of syndecan-4 through a PDZ adaptor protein.

(2) The PP1/2A dephosphorylates Ser¹⁸³ in the membrane-proximal domain of the syndecan-4 cytoplasmic tail, which is normally maintained at a high basal phosphorylation level. As the heparan sulfate chains carried by proteoglycans can reach estimated lengths of 80 nm (Kato et al., 1994), the dephosphorylation is likely to occur in a *trans*, rather than a *cis* mode in regard to the syndecan-4 molecule carrying the FGF2-binding HS chain. Since syndecan-4 is a transmembrane protein, this dephosphorylation may similarly occur in the Golgi-residing syndecan-4.

(3) Dephosphorylation of syndecan-4 sharply increases its affinity to PIP₂ (Horowitz and Simons, 1998a). PIP₂ binding, in turn, facilitates the multimerization of syndecan-4.

(4) The clustered syndecan-4/PIP₂ complex activates PKC α , which associates with syndecan-4 through PIP₂ (Horowitz et al., 1999).

The existence of this regulatory mechanism is suggested by our observations that expression of PIP₂⁻ or PDZ⁻ syndecan-4 mutants on a background of endogenous syndecan-4 inhibits cell response to FGF2, but not to serum or to the HS binding EGF and PDGF AB, in a dominant-negative manner. In the case of the PIP₂⁻ mutation, the mistargeting of the mutant syndecan-4 leads to Golgi sequestration of its PDZ-binding partner. The critical role of this sequestration is emphasized by the lack of dominant-negative effects of the double PIP₂⁻/PDZ⁻ syndecan-4 mutation. In the case of the PDZ⁻ deletion, FGF2 treatment would lead to an increase in the extent of syndecan-4 phosphorylation, and, consequently, would decrease PKC α activation due to the low affinity of phosphorylated syndecan-4 for PIP₂ (Horowitz et al., 1999).

A recent study on syndecan-4 knockout mice reported that the proliferative response of skin fibroblasts extracted from these mice did not differ from the response of cells from wild type mice (Echtermeyer et al., 2001). The difference between this finding and the significantly

reduced proliferation rates of cells expressing the PIP₂⁻ or PDZ⁻ syndecan-4 mutants that we observed may stem from each of the following reasons: (a) as explained above, the expression of these mutants interferes with the function of endogenous syndecan-4. This active perturbation of a signaling pathway was not present in the mouse syndecan-4^{-/-} cells; (b) differences between cell types (fibroblasts versus endothelial cells) used in the two studies; (c) assuming that Echtermeyer et al. used the same medium as in their migration assays (the medium used in their proliferation assays is not described), the 2% serum present in this medium may have contained other growth factors that compensated for the impaired response to FGF2.

PIP₂⁻ mutation: effect on plasma membrane targeting of syndecan-4

One interesting and unexpected finding in this study is the fact that PIP₂ binding, rather than the interaction with a PDZ protein, targets syndecan-4 to the plasma membrane. The data supporting this observation include immunofluorescence staining showing reduced expression of the PIP₂⁻ and PIP₂⁻/PDZ⁻ mutants in the basolateral region and virtual absence of syndecan-4 variants containing the PIP₂⁻ mutation from the cell surface as demonstrated by FACS. Similarly, deletion of the PDZ motif in the syndecan-2 cytoplasmic tail did not eliminate its incorporation into the plasma membrane (Ethell and Yamaguchi, 1999).

The Golgi apparatus is a known site of PIP₂ synthesis (Godi et al., 1999; Jones et al., 2000), and the involvement of PIP₂ in protein sorting is suggested by preliminary observations (Morrow and Weidman, Mol. Biol. Cell, vol. 11 (suppl.), 280a, 2000). The actual targeting mechanism is, however, still unknown. The results of the FACS experiments (Fig. 5a) show that the PIP₂⁻ mutation precluded not only the basolateral targeting, but also the overall incorporation of syndecan-4 in the plasma membrane.

The dominant-negative phenotype exhibited by the PIP₂⁻ mutant suggests that it disrupts a syndecan-4-dependent protein complex involved in mediating FGF2 signaling. Interestingly, it is the presence of the PIP₂⁻ mutant and not a mere absence of syndecan-4 from the cell, that impairs cellular function. This conclusion is supported by studies of the PIP₂⁻/PDZ⁻ mutant that does not cause a similar dominant-negative phenotype, yet it is similarly absent from the plasma membrane (Fig. 4c), and by a recently described syndecan-4 knockout mice (Ishiguro et al., 2000). We propose, instead, that the

Fig. 9: Schematic representation of FGF2 signal transduction mediated by syndecan-4.

(FGFR – FGF2 tyrosine kinase receptor; HS/CS – heparan or chondroitin sulfate side chains). See Discussion for details.

dominant negative effect of the PIP_2^- mutation is caused by sequestration to the Golgi apparatus of a syndecan-4-binding PDZ protein specifically involved in FGF2 (and not serum, EGF, or PDGF) signaling. Assuming that FGF2 signaling requires the presence of this syndecan-4-bound PDZ protein in the basolateral region, the PIP_2^- mutant may compete with endogenous syndecan-4 for binding to the PDZ protein, thus capturing a large part of this protein population at the Golgi apparatus, interfering with its targeting to the plasma membrane and the formation of a syndecan-4-dependent complex.

PDZ binding regulates syndecan-4 phosphorylation in response to FGF2

We have shown that the dominant negative effect of the PDZ^- mutation is associated with increased phosphorylation of the PDZ^- syndecan-4 mutant following FGF2 treatment. The phosphorylation state of syndecan-4 is controlled on one hand by a novel PKC (nPKC) and, on the other, by a FGF2-activated PP1/2A (Horowitz and Simons, 1998b). Since the phosphorylation level of the PDZ^- mutant was significantly higher than that of S4 following FGF2 treatment, the most likely explanation for this observation is impaired dephosphorylation of the PDZ^- mutant, resulting in unopposed activity of the nPKC. This in turn suggests that the PP1/2A involved in syndecan-4 dephosphorylation forms a complex with syndecan-4 through a PDZ adaptor protein. In the absence of the PDZ-binding motif of syndecan-4 and, hence, PDZ adaptor binding, the phosphatase would no longer dephosphorylate the PDZ^- mutant. This explanation applies both to the membrane and the Golgi-associated syndecan-4 pools, since the cytoplasmic tail of the latter would protrude into the cytoplasm and be accessible to

the phosphatase and kinase.

The hyperphosphorylated PDZ^- mutant has reduced tendencies to oligomerize and bind PIP_2 , resulting in a diminished capacity to activate PKCα (Horowitz and Simons, 1998a). Moreover, its presence in the plasma membrane may interfere with the oligomerization, PIP_2 binding, and activation of PKCα by dephosphorylated endogenous syndecan-4. This loss of syndecan-4-dependent activation of PKCα in response to FGF2 may then account for the dominant negative effects of the PDZ^- mutation.

It should be noted that the lower activity of PKCα in the presence of the PDZ^- peptide compared to the WT peptide (Fig. 8c) leaves open the possibility that the induction of a dominant negative phenotype by the PDZ^- mutation can be caused by reduction in PKCα activity, similar to the PIP_2^- cells. While it is not obvious how deletion of a single C-terminal residue in the cytoplasmic tail of syndecan-4 produces this effect, the deletion could conceivably affect the conformation of the PIP_2 -binding motif and hence reduce its affinity to PIP_2 .

FGF2-specificity of the syndecan-4 signaling pathway

Another intriguing observation in this study is the apparent FGF2-specificity (*vs.* serum, EGF, and PDGF) of the syndecan-4 signaling pathway. This specificity may stem in part from the choice of functional assays used in this study, as both cell migration (Harrington et al., 1997) and proliferation (Besson and Yong, 2000; Schonwasser et al., 1998) have been linked to the activation of PKCα, the kinase regulated by syndecan-4. However, not all FGF2-induced signaling events may necessarily be regulated in this fashion. The fact that unlike FGF2, serum stimulation of cell growth and proliferation is not affected by the PIP_2^- and PDZ^- syndecan-4 mutants,

suggests that other growth factors present in serum, such as EGF or PDGF AB, may stimulate cell growth and migration independent of PKC α . The lack of increase in PIP₂-dependent PKC α activation following serum stimulation (Fig. 6a) agrees with this observation.

Though all syndecans and glypicans can carry heparan sulfate chains and may, therefore, bind FGF2, only syndecan-4 selectively modulates FGF2 signaling (Simons and Horowitz, 2001; Volk et al., 1999). The unique nature of the syndecan-4-dependent amplification of FGF2 signaling is related to the presence of its cytoplasmic tail, since the expression of chimeras consisting of either glypican-1 (Volk et al., 1999) or syndecan-1 (Volk and Simons, unpublished data) ectoplasmic domain fused to the transmembrane and cytoplasmic domains of syndecan-4 mimics the positive effects of intact syndecan-4 overexpression on cell proliferation and migration. These findings, together with the results of the current study, suggest that PIP₂ binding, and the concomitant ability to activate PKC α , underlie the unique capacity of syndecan-4 among the cell surface proteoglycans to regulate FGF2 signaling.

In summary, we describe a novel signal transduction pathway that involves selective regulation of endothelial cell migration and proliferation by FGF2 *via* syndecan-4.

Materials and methods

cDNA constructs, mutagenesis, and transfection

Syndecan-4 cDNA containing an ectoplasmic HA tag (Shworak et al., 1994) was subcloned into MSCV retroviral vector (Hawley et al., 1994) between its EcoRI and BglII sites. Site-directed mutations were introduced into syndecan-4 cDNA by PCR-mediated oligo mutagenesis (QuikChange, Stratagene). The vector was delivered into RFPEC by either retroviral (as described, ref. (Volk et al., 1999)) or liposome-mediated transfection (LipofectAmine Plus, Invitrogen). In both cases, stably transfected cells were selected by neomycin resistance (0.4 mg/ml Geneticin, Invitrogen). Syndecan-4 expression levels were assessed by immunoblotting cell lysates as described (Horowitz and Simons, 1998a), with 50 mU/ml horseradish peroxidase-conjugated 3F10 HA antibody (Roche).

Alternatively, the HA-tagged syndecan-4 cDNA constructs were inserted between the XhoI and EcoRI restriction sites of a bicistronic mammalian expression plasmid (pIRES2-EGFP, Clontech) that co-expresses

enhanced green fluorescent protein (EGFP) and the inserted cDNA under a single promoter. RFPEC were stably transfected by LipofectAmine 2000 (Invitrogen) and selected by neomycin resistance as a pooled population. To enrich each pool with cells having high expression levels of the transfected protein, only cells in the top 2-5% of the population as determined by 2-3 consecutive sorting rounds (see below), were retained and expanded.

Cell function assays

Cells were incubated as described (Horowitz and Simons, 1998a) in M199 medium (Invitrogen) with the indicated supplements. Cell migration was measured by "wounding" assays (Tang et al., 1997) in which cells were grown to sub-confluence in 6-well plates, then starved for 24 hrs in 0.5% serum. The cell layer was scratched with a pipette tip, producing a gap approximately 2 mm wide. The gap width was measured at marked locations from images taken by inverted microscope (TMS-F, Nikon) immediately after the scratching, and again 6 hrs later at the same locations. Proliferation and tube formation assays were done as described (Volk et al., 1999). All experiments were repeated with two clones of cells stably transfected with each syndecan-4 variant. Neomycin was withdrawn from the culture medium at least 24 hrs before the cell function assays, as well as before all other experiments with stably transfected RFPEC, to prevent artifacts caused by neomycin sequestration of PIP₂ (Gabev et al., 1989).

Fluorescence-assisted cell sorting (FACS)

To measure the amount of exogenous syndecan-4 present on the cell surface, cells were dissociated from plates (non-enzymatic solution, Sigma), labeled with 1 μ g/ml antibody to HA (Roche), followed by 10 μ g/ml of anti-rat IgG conjugated to red-pycoerythrin (R-PE; Jackson Immunologicals) and sorted automatically (FACScan, Beckton Dickinson). Detection of the surface expression of both exogenous and endogenous syndecan-4 was done similarly, using a 1:50 dilution of antiserum to the ectoplasmic domain of syndecan-4 (Shworak et al., 1994), and 10 μ g/ml of anti-rabbit IgG conjugated to Alexa-594 (Molecular Probes). To detect the overall cellular expression of exogenous syndecan-4, cells were scanned at the EGFP-emitted wavelength (488 nm).

Immunofluorescence

RFPEC were plated in chamber slides (Nalge

Nunc), fixed with 2% formaldehyde in phosphate buffered saline (PBS; 137 mM NaCl, 4.3 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 2.7 mM KCl, 1.4 mM KH_2PO_4 , pH 7.3), for 10 min at room temperature, washed twice with PBS, permeabilized with 0.1% Triton X-100/PBS for 10 min, washed again as above, blocked with 3% bovine serum albumin (Invitrogen) in PBS for 30 min, and incubated with primary antibodies at the indicated concentrations in 1% bovine serum albumin in PBS for 3 hrs. The slides were washed 4 times as above, and incubated for 1 hr with 10 $\mu\text{g}/\text{ml}$ of the appropriate secondary antibodies conjugated either to Alexa 488, or Alexa 594 (Molecular Probes), washed again as before, and mounted with ProLong medium (Molecular Probes). Slides were imaged by laser-scanning confocal microscopy (Radiance2000, Bio-Rad).

Immunoprecipitation

Cells were lysed and immunoprecipitated as described (Horowitz and Simons, 1998a; Horowitz and Simons, 1998b), using 80 μl anti-HA affinity matrix (Roche) suspension per 750 μl cell lysate. Alternatively, endogenous or HA-tagged syndecan-4 was immunoprecipitated with 10 μl cytoplasmic tail antiserum (Shworak et al., 1994), or with 5 μg HA antibody (3F10, Roche) per 750 μl cell lysate, respectively, and 40 μl suspension of protein G Plus/protein A agarose (Oncogene). Where indicated, glycosaminoglycan chains were digested as described (Horowitz and Simons, 1998b).

Surface plasmon resonance

Experiments were carried out as described (Horowitz et al., 1999), using 28 amino acid-long (Horowitz and Simons, 1998a) syndecan-4 cytoplasmic tail peptides (Genemed Synthesis) as ligands, and PIP_2 (Sigma) as analyte.

Column chromatography

Synthetic syndecan-4 cytoplasmic tail peptides (1 μM), either alone, or mixed with PIP_2 (2 μM) in 0.1 M phosphate pH 7.4 and 20% acetonitrile, were injected (22.5 μl) into a 300 $\times$ 6 mm silica (5 μm spheres, 60 Å pores) HPLC (Waters, 515 Pump, 2487 Absorbance Detector)-mounted column (YMC). Elution profiles corresponding to light absorbance at 210 nm were recorded digitally (Millenium³², Waters).

Kinase assays

With recombinant PKC α . These were performed either in the presence of PIP_2 , or Ca^{2+} , phosphatidylserine, and diolein, as described (Horowitz and Simons, 1998a), using recombinant PKC α (120 ng/ml). Kinase activity was quantified by phospho-imaging (Molecular Dynamics) of gel-resolved PKC βI optimal substrate peptide bands. PKC autoinhibitor peptide and G66976 were purchased from Calbiochem.

With immunoprecipitated syndecan-4. Syndecan-4 complexes immunoprecipitated as described above with 5 μg HA antibody per 750 μl cell lysate and 40 μl protein G Plus/protein A agarose suspension were used for in vitro kinase assays. After washing in 25 mM Tris-HCl (pH 7.4) buffer, the beads were sedimented, the buffer removed and replaced with 30 ml kinase assay buffer containing either PIP_2 , or Ca^{2+} , phosphatidylserine, and diolein, as described (Horowitz and Simons, 1998a). The assay was stopped by adding 10 μl $\times 4$ Laemmli sample buffer (final concentration 2% SDS, 10% glycerol, 0.5% β -mercaptoethanol, 0.004% bromophenol blue, 50 mM Tris-HCl, pH 6.8) and boiling for 4 min. Kinase activity was quantified as in the recombinant PKC α assays.

Measurement of syndecan-4 phosphorylation level

RFPEC in 100 mm plates were grown for 24 hrs in phosphate-free DMEM (Invitrogen) supplemented with 0.5% fetal bovine serum (FBS), and radiolabeled for 4 hrs with 0.5 mCi/ml ^{32}P -orthophosphoric acid (New England Nuclear). Cells were washed with tris-buffered saline (137 mM NaCl, 25 mM Tris-HCl, pH 7.4), and scraped off in 0.5 ml lysis buffer (150 mM NaCl, 20 mM NaF, 20 mM $\text{Na}_4\text{P}_2\text{O}_7$, 5 mM EDTA, 5 mM EGTA, 1 mM Na_3VO_4 , 1 mM PMSF, 1% Triton X-100, 50 mM HEPES, pH 7.4) supplemented with protease inhibitor cocktail (Complete, Boehringer). Cell lysates were pre-cleared by incubation with 1 μg non-immune rat IgG (Sigma) and 20 μl Protein G Plus/ Protein A agarose bead suspension at 4 °C for 1 hr. Following agarose bead sedimentation, the cleared samples were supplemented with 80 μl of anti-HA affinity matrix bead suspension (Roche) or with 10 μl cytoplasmic tail antiserum (Shworak et al., 1994) and 40 μl suspension of protein G Plus/protein A agarose (Oncogene) and incubated in rotating tubes over night at 4 °C. The beads were sedimented, washed three times in heparinase digestion buffer

(50 mM NaCl, 4 mM CaCl₂, 20 mM Tris-HCl, pH 7.4), and glycosaminoglycan chains were digested as described (Horowitz and Simons, 1998b). The immunoprecipitated syndecan-4 core protein was dissociated from the beads by a 10 min incubation in 40 μ l Laemmli sample buffer at 95 °C. Samples were resolved on 12% Tris-Glycine gels (BioRad), and the bands corresponding to the cytoplasmic tail of syndecan-4 were identified by immunoblotting with a peroxidase-conjugated antibody to the HA tag (Roche). The bands were excised and the ³²P level was measured by scintillation counter (Beckman).

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Chapter 4

Clustering induces redistribution of syndecan-4 core protein into raft membrane domains

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Abstract

Syndecan-4 is a heparan sulfate carrying core protein that has been directly implicated in fibroblast growth factor 2 (FGF2) signaling. Recent studies have suggested that most signaling proteins localize to the raft compartment of the plasma cell membrane. To establish if syndecan-4 is present in the raft compartment, we have studied the distribution of the core protein and an Fc receptor (FcR)-syndecan-4 chimera prior to and following clustering with FGF2 or antibodies. While unclustered syndecan-4 was present predominantly in the non-raft membrane compartment, clustering induced extensive redistribution to the rafts as demonstrated by the sucrose gradient centrifugation and live confocal microscopy. At the same time, while syndecan-4 and caveolin-1 moved in tandem, syndecan-4 was not present in caveolae, a major subset of raft compartments. We conclude that syndecan-4 clustering induces its own redistribution to the non-caveolae raft compartment. This process may play an important role in syndecan-4 mediation of FGF2 signaling.

Introduction

Syndecans, a four member family of transmembrane proteoglycan core proteins, are found in a wide spectrum of cells and engage in a variety of interactions including binding growth factors, growth factor receptors, matrix proteins such as fibronectin and vitronectin, lipids and other with biologically active molecules (1,2). All syndecans carry both heparan sulfate and chondroitin sulfate chains on their extracellular domain and engage in PDZ-dependent interaction via conserved intracellular domains (1,2). Syndecan-4 is a unique member of the syndecan core protein family. Unlike other syndecans, it possesses a PIP2 binding site in its cytoplasmic tail that allows it to bind and activate PKC α (3,4). Syndecan-4 is found on endothelial cells and is directly involved in regulation of FGF2-induced cell growth and migration (5).

While previous studies have shown that syndecan-4 is predominantly found on the baso-lateral plasma cell membrane and focal adhesions (6,7), little information is available regarding its association with various membrane sub-domains. Recent studies have suggested that rafts, lipid-ordered microdomains enriched by cholesterol and sphingolipids, act as platforms for conducting a variety of cellular functions, such as vesicular trafficking and signal transduction (8,9). We have conducted this study, therefore, to determine whether activation of syndecan-4

signalings initiated by its oligomerization on the plasma cell membrane, leads to its appearance in the membrane rafts.

Several protein families have been reported to modify lipid rafts structurally and functionally. These include integral membrane proteins such as caveolins and flotillins, exoplasmic GPI-linked proteins such as Thy-1 and alkaline phosphatase, and receptor tyrosine kinases among others (9). Caveolin integration into the microenvironment of a lipid raft leads to raft's invagination and formation of caveolae (9-11). Caveolae are formed in many cell types, including endothelial cells (12) and they may play a role in modulation of cell signaling. One hypothesis suggests that interactions of caveolins with signaling molecules regulate their activation status.

Since FGF2-induced syndecans-4 dependent signaling requires the presence of heparan sulfate chains on core protein's extracellular domain (13), it is reasonable to hypothesize that FGF2 induces aggregation of syndecans-4 complexes in the plasma cell membrane. However, since FGF2 induces a number of other signaling events that may affect syndecans-4 movement in the plasma cell membrane in manner independent of oligomerization of its extracellular domains, we linked the extracellular domain of the human Fc receptor (FcR) to the transmembrane/cytoplasmic domains of syndecans-4. The resultant chimera can then be aggregated with an immunoglobulin, mimicking FGF2-induced syndecans-4 oligomerization. We found that such an aggregation of syndecan-4 cores leads to a shift of syndecans-4 complexes from the non-raft to raft microdomains of the plasma cell membrane. At the same time, while syndecans-4 motion was synchronous with movements of caveolin-1, syndecans-4 was not present in the caveolae portion of the plasma membrane rafts.

Materials and Methods

Antibodies and Reagents

Polyclonal rabbit antisera against cytoplasmic and extracellular domains of syndecan-4 were described elsewhere (14). Polyclonal chicken IgY against extracellular domains of syndecan-4 was produced (Aves Labs) using the same peptide as previously used for generation in rabbits (14). Anti-FcR (CD64) monoclonal antibody was purchased from Abcam (UK). Cy-3 and biotin-SP conjugated non-immune human IgG, Cy-3 conjugated goat anti-human F(ab')₂ fragment, Cy-5 conjugated streptavidin and goat anti-chicken F(ab')₂ fragment were

purchased from Jackson ImmunoResearch. Secondary antibodies conjugated to Alexa-594, and a nuclear stain ToPro3 were from Molecular Probes. Alexa-488-labelled proaerolysin variant (FLAER) was purchased from Protox Biotech (Victoria, BC). Secondary antibodies conjugated to horseradish peroxidase (HRP) were purchased from Vector Labs and immobilized streptavidin and HRP-conjugated streptavidin were from Pierce. Protein Cholera toxin β subunit conjugated HRP conjugated was purchased from Sigma. HRP-conjugated cholera toxin β subunit was purchased from Sigma.

cDNA constructs & transfection

Fc receptor/syndecan-4 chimera (FcR-S4) was constructed by linking the ectoplasmic domain (amino acids 1-288) of human Fc receptor Ia (CD64) cDNA and the transmembrane and cytoplasmic domains of rat syndecan-4 (amino acids 150-202). The chimera and full-length Syndecan-4 construct were inserted into pCR 3.1-Uni expression vector (Invitrogen). Caveolin-1-EGFP (caveolin-1 fused by its C-term to EGFP) cDNA construct (15), was a gift of Dr. L. Pelkmans (Institute of Biochemistry, Swiss Federal Institute of Technology).

RFPEC were cultured in DMEM medium (Life Technologies) as described (16). Stable expression of FcR-S4 chimera was achieved by transfecting the cDNA construct into wild type RFPEC using LipofectAmine 2000 (Life Technologies) according to the manufacture protocol. Cells were selected for neomycin resistance (0.4 mg/ml Geneticin, Life Technologies) and pooled populations were used for all studies. To enrich each pool with cells having high expression levels of FcR-S4 protein, cells were subjected to two fluorescent-activated sorting (see below) to select cells in the top 10% of the population.

Transient expression of caveolin-1-EGFP and pEGFP-N1 (Clontech) in RFPEC was achieved by transfection using LipofectAmine 2000 (Life Technologies). Cells were used within 48-72 hours after transfection.

Fluorescence-assisted cell sorting (FACS)

Cells were dissociated from plates using non-enzymatic solution (Sigma) and labeled for 20 min with FITC-conjugated human non-immune IgG (0.1 μ g/ml, Rockland) in Dulbecco's phosphate buffered saline (DPBS) with 1% bovine serum albumin (BSA), thus specifically detecting the transfected Fc receptor, which is not endogenously expressed in endothelial cells.

To detect endogenous syndecan-4, cells were incubated with 1 μ g/ml chicken antibody against extracellular domain of syndecan-4 for 20 min at room temperature, washed twice in 1%BSA-DMEM and then incubated with a secondary antibody (Cy-5-conjugated F(ab')₂ fragment, 1 μ g/ml) for 20 min at room temperature, followed by another wash with 1%BSA-DMEM. Cell sorting was carried out on a MoFlo sorter (Cytomation) with a FACScan (Becton Dickinson, San Jose, CA) and analyzed using WinMDI v2.8 (Scripps, CA) software.

Cell surface biotinylation

The cells were washed twice with ice-cold DMEM and incubated with 0.5 mg/ml sulfo-N-hydroxysuccinimide ester-long chain-biotin (Pierce Chemical Co.) in PBS, pH 7.4 for 15 min at 40°C. The biotinylation reaction was quenched with ice-cold 1% BSA-DMEM.

Syndecan clustering

For antibody clustering studies, RFPEC expressing FcR-S4 construct were grown overnight in 10% FBS-DMEM. The cells were washed twice in DMEM and then incubated with human non-immune IgG (1 μ g/ml) for 15 minutes in 1%BSA-DMEM at 37°C. The cells were then washed again (x2) with DMEM and incubated for another 15 minutes at 37°C with anti-human F(ab')₂ (2 μ g/ml) in 1% BSA-DMEM.

For FGF2 clustering, RFPEC cultured in 10%FBS-DMEM were washed twice with DMEM and then incubated with 25 ng/ml FGF2 (Chiron Corp, Sunnyvale, CA) in 1%BSA-DMEM for 15 minutes at 37°C.

Isolation of lipid raft membrane fractions

Raft membrane microdomains from cell surface biotinylated RFPEC were isolated using flotation on discontinuous sucrose gradients as described (17). In brief, adherent cells in confluent 15 cm dish were washed with ice-cold PBS and lysed for 30min on ice in 1% Triton X-100 in MNE buffer (150mM NaCl/2mM EDTA/25mM, pH 6.5) containing a protease inhibitor mixture (Roche Diagnostics). The lysis solution was homogenized, and nuclei and cellular debris were pelleted by centrifugation at 10,000g for 30min. For sucrose-gradient centrifugation, 1ml of the cleared supernatant was mixed with 1ml of 85% sucrose in MNE buffer and transferred to the bottom of a centrifuge tube. The diluted lysate was overlaid with 2ml of 30% sucrose and 1ml of 5% sucrose in MNE buffer. The samples were

centrifuged in an SW55 rotor at 200,000g at 4°C for 16h. Seven fractions of 700µl were collected from the top of the gradient and studied using immunoblotting (see below).

Detergent extraction of lipid rafts was carried as described (18). Briefly, cells were grown to confluence in a 6-well plate, rinsed with 1 ml ice-cold TNE (Tris/NaCl/EDTA) buffer (25 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA), placed on ice and then lysed by addition 1% Triton-X100 and protease inhibitor mixture in TNE buffer for 20 min. Lysates were scraped and centrifuged for 5 min at 4°C and the supernatant was transferred to the fresh tube. To recover membrane rafts proteins, Triton X-100 pellet was resuspended in 100 µl pellet lysis buffer (50 mM Tris-HCl, pH 8.8, 5 mM EDTA, 1% (w/v) SDS). The mixture was diluted with 1ml TNE-1% Triton X-100 and centrifuged for 2 min at 4°C. Supernatant was then analyzed by immunoprecipitation, SDS-PAGE and immunoblotting. Glycosaminoglycan chains were digested as described (4).

Plasma membrane cholesterol depletion was accomplished by pretreatment of cultured cells with β -cyclodextrin (10µM), which removes unesterified cholesterol from the cell membrane (19), for 30 min at 37°C, immediately followed by chilling the cells to 4°C.

Immunoprecipitation, immunoblotting and dot blotting

Immunoprecipitation (IP) of syndecan-4 from cell lysates was carried by incubation of 500 µl of total cell lysates with 10 µl of anti-syndecan-4 cytoplasmic tail antiserum in the presence of Protein G/ Protein A agarose (30 µl). Prior to immunoblotting, glycosaminoglycan chains were digested as described (4). The immunoprecipitated material was then subjected to SDS-PAGE and transferred to the polyvinylidene difluoride (PVDF) membrane (Millipore). Syndecan-4 detection was performed using HRP-conjugated streptavidin (0.2 µg/ml) for cell surface biotinylated samples or by anti-syndecan-4 antiserum (1µl/ml) followed by incubation with a secondary HRP-conjugated goat antibody specific for the primary anti-syndecan-4 antibody.

Immunoprecipitation of syndecan-4 from sucrose gradient fractions was performed in the similar manner. The precipitated material was then dot blotted on the nitrocellulose membrane (Schleicher&Schuell) and syndecan-4 was detected by HRP-conjugated streptavidin.

For GM1 sphingolipid detection in sucrose gradient fractions, 100 µl of each fraction were applied to the

nitrocellulose membrane and GM1 was then detected using HRP-conjugated cholera toxin subunit β (0.2 µg/ml). GM1 detection in Triton X-100 soluble and insoluble fractions was carried out by blotting 20 or 200 µl of each fraction on the nitrocellulose filter, which was then probed with HRP-conjugated cholera toxin subunit β (0.2 µg/ml). All blots were developed using enhanced SuperSignal Wet Pico chemiluminescent substrate (Pierce).

Live fluorescent microscopy

For live fluorescent microscopy, cells were grown on gelatin 0.1% PBS coated cover slips (Fisher Scientific.) to confluence. Staining for FcR-S4 was done using biotinylated non-immune IgG and the biotin label was then visualized by incubation with Cy-5 conjugated streptavidin (5µg/ml) at 37°C for 15 min. Glycosylphosphatidylinositol (GPI) anchored proteins were detected by cell incubation with 10-8 M of Alexa-488 conjugated inactive variant of the protein proaerolysin (FLAER) at 37°C for 1 h. FLAER binds selectively to GPI anchors (20).

Cover slips were removed from the staining solution and mounted on a microscope slide. All microscopy imaging was done using Bio-Rad (Hercules, CA) MRC-1024 Krypton/Argon laser confocal system microscope using 63x lens by Zeiss. Time lapse microscopy was carried out at 20°C. Image analysis was done using Adobe Photoshop 6.0 (Adobe Systems Inc.) and ImageJ software (NIH).

Results

To study the distribution of syndecan-4 core protein in the plasma cell membrane and examine the effect of ligand clustering on its spatial localization, we linked the cytoplasmic and the transmembrane domains of syndecan-4 to the extracellular domain of the Fc receptor. To demonstrate the expression of the FcR-S4 chimera on the cell surface, FcR-S4 construct and empty vector-transfected cells were subjected to FACS using FITC-labeled non-immune IgG that specifically recognizes Fc receptor, and anti-syndecan-4 ectoplasmic domain antibodies. FcR expression was noted only on the FcR-S4 construct transfected cells (Fig. 1A). Furthermore, expression of the native syndecan-4 was not affected by FcR-S4 expression (Fig. 1A). To demonstrate that the cytoplasmic tails of FcR-S4 chimeras interact with native syndecan-4 proteins, RFPEC cells expressing FcR-S4 construct were decorated with non-immune

Figure 1. FcR-syndecan-4 chimera interaction with endogenous syndecan-4 on the cell surface.

(A) Flow cytometry analysis of FcR-S4 and native syndecan-4 expression in RFPEC.

Fluorescent intensity of antibody staining for the FcR and native syndecan-4 in cells transfected with the FcR-S4 construct (red) or vector control (green). Note the absence of signal for the FcR (non-immune IgG) in the vector transfected cells (shown in green). The expression of the native syndecan-4 is the same in vector-transfected (orange) and FcR-S4 transfected (blue) cells.

(B). FcR-S4 forms complexes with native syndecan-4. Total cell lysates from cells transfected with FcR-S4 construct prior to (-) or after (+) antibody clustering of FcR-S4 chimeras, were subjected to immunoprecipitation with the anti-ectoplasmic domain syndecan-4 antibody, followed by Western blotting of the precipitate with anti-FcR (CD64) antibody. Note the presence of bands corresponding to FcR-S4-syndecan-4 monomer and dimer.

IgG. Clustering of FcR-syndecan-4 complexes was then performed with the anti-IgG antibody. Clustered and unclustered RFPEC were then lysed and subjected to immunoprecipitation with the antibody against the ectoplasmic domain of syndecan-4 followed by Western blotting with an anti-FcR (CD64) antibody. Both clustered and unclustered cells demonstrated the presence of FcR-S4-syndecan-4 complexes (Fig. 1B).

The distribution of syndecan-4 in the plasma cell membrane was examined by subjecting whole cells lysates of RFPEC expressing FcR-S4 construct to sucrose density gradient centrifugation. Blotting of the syndecan-4 antibody immunoprecipitated material from various sucrose gradient fractions demonstrated the core protein presence in both heavy (40% sucrose) and light (10-15% sucrose) fractions (Fig. 2A, right panel). The lighter fractions contain plasma membrane raft proteins as shown by blotting with HRP-conjugated cholera

toxin subunit that specifically binds to the raft marker GM1 (Fig. 2A, left panel). In the absence of antibody clustering, most of syndecan-4 appeared in the non-raft membrane fractions. Antibody clustering of FcR-S4 chimeras induces a pronounced shift towards the raft-containing fractions (Fig. 2A, right panel). The specificity of HRP-conjugated cholera toxin subunit β GM1 binding for detection of raft fractions of the membrane was tested by dot blot analysis of Triton X-100 soluble and insoluble portion of the plasma cell membrane (Fig. 2B). As expected, only the Triton X-100-insoluble fraction was labeled with the cholera toxin.

To further explore the relationship between clustering and syndecan-4 redistribution into the raft compartment, we studied syndecan-4 appearance in the Triton X100-insoluble (raft) and soluble (non-raft) fractions of plasma cell membranes. Equal numbers of FcR-S4 expressing cells were subjected to lysis in Triton X-100 as described

Figure 2. Clustering induces syndecan-4 redistribution into the lipid rafts.

(A) Sucrose gradient analysis. Raft membrane microdomains from cell surface biotinylated RFPEC were isolated using flotation on discontinuous sucrose gradients. Gradient fractions containing rafts were detected using HRP-conjugated cholera toxin subunit β that identifies the presence of the raft marker GM1 (left panel). The distribution of FcR-S4 in the gradient fractions was determined by immunoprecipitation of each fraction with the anti-syndecan-4 cytoplasmic domain antibody. The precipitated material was then dot blotted, and syndecan-4 detected by HRP-conjugated streptavidin (right panel). Note that prior to clustering most of syndecan-4 is present in the non-raft sucrose fractions with a shift toward the raft fraction following antibody clustering of FcR-S4 constructs.

(B). Raft microdomains are present in Triton X-100 insoluble fractions. To demonstrate the presence of raft microdomains in the Triton X-100-insoluble fraction, RFPEC were subjected to lysis in Triton X-100 at 4°C as described in the Methods. 20 or 200 µl of each fraction were blotted on the nitrocellulose filter, which was then probed with the HRP-conjugated cholera toxin subunit β . Note the presence of GM1 signal only in Triton X-100-insoluble fractions.

(C). Antibody clustering induces the shift of FcR-S4 chimeras from non-raft to raft membrane microdomains. RFPEC expressing FcR-syndecan-4 decorated with non-immune IgG were subjected to cold Triton X-100 extraction followed by IP with antibodies against the cytoplasmic domain of syndecans-4 and Western blotting with HRP-streptavidin. Prior to clustering (-), syndecan-4 is predominantly found in the soluble (non-raft) fraction (lanes 1,2) while antibody clustering of FcR-syndecan-4 chimeras (+) induces pronounced shift towards the insoluble (raft) fraction (lanes 3,4) lipid rafts. Cholesterol depletion of the plasma cell membrane with β -cyclodextrin led to a pronounced reduction in clustering induced shift of syndecan-4 towards the raft fraction (lanes 5,6).

(D) FGF2 treatment shifts native syndecan-4 to the raft microdomains. RFPEC treated with FGF-2 were cell surface biotinylated and then subjected to Triton X-100 extraction at 4°C followed by IP with antibodies against the cytoplasmic domain of syndecans-4 and Western blotting with HRP-streptavidin. Note paucity of syndecan-4 protein in the raft fraction prior to FGF2-induced clustering (lanes 1,2) and a pronounced shift towards the raft fraction following FGF2 treatment (lanes 3,4)

in the Methods. The Triton X100-soluble and insoluble fractions were then immunoprecipitated with anti-syndecan-4 antibody, separated on SDS-PAGE and subjected to Western blotting with HRP-streptavidin. Prior to antibody clustering of FcR-S4 chimeras, essentially all of syndecan-4 was in the Triton-X100 soluble fraction (Fig. 2C). Antibody clustering induced redistribution of ~50% of the total cellular syndecan-4 (cell surface only) to the Triton X-100 insoluble fraction. Since the formation of membrane rafts depends on high local cholesterol ester content, we disrupted raft formation by depleted membrane cholesterol by treating cells with β -cyclodextrin. Such cholesterol depletion almost fully prevented clustering-induced syndecan-4 redistribution into the rafts compartment (Fig. 2C).

Since clustering of the native syndecan-4 with its natural ligands, such as FGF2, may potentially have a different effect on its plasma membrane dispersal than antibody

clustering of FcR-S4 chimeras, we have examined the effect of FGF2 treatment on syndecan-4 distribution in RFPEC cells. Similar to the FcR-S4 chimera antibody clustering studies, FGF2 induced a significant redistribution of syndecan-4 from Triton-X100 soluble to insoluble fraction (Fig. 2D).

To confirm localization of clustered syndecan-4 to plasma membrane rafts, we used vital confocal microscopy with anti-syndecan-4 antibody and the GPI-anchor marker FLAER in RFPEC. Following antibody clustering of FcR-S4 chimeras, FLAER imaging demonstrated prominent raft clusters on the apical plasma cell membrane (Fig. 3, top left panel). Staining with a labeled non-immune IgG that detects FcR-S4 chimeras demonstrated equally prominent FcR-S4 clusters on the apical plasma cell membrane (Fig. 3, top middle panel). The merged image demonstrates that essentially all FcR-S4 chimeras overlapped with FLAER-labeling (Fig. 3,

Figure 3. FcR-Syndecan-4 co-localizes with the GPI-anchored protein marker FLAER in RFPEC.

Confocal images of live RFPEC transfected with the FcR-S4 construct (top) or vector control (bottom). Prior to immunolabeling, cells were subjected to antibody clustering of FcR-S4 chimeras as described in the Methods. Labeling with the GPI-anchored proteins marker FLAER (green) is shown in the left panel while labeling of FcR-S4 using biotinylated non-immune IgG visualized by Cy-5 conjugated streptavidin is shown in the middle panel. The merged image is shown in the right panel. Note a virtually complete overlap of FcR-S4 signal with the FLAER signal. Since there is a large excess of GPI proteins compared to FcR-S4 chimeras, not all FLAER signal demonstrates co-localization with the FcR-S4 signal. Note the absence of FcR-S4 signal in vector-transfected cells (bottom panels).

Figure 4. FcR-Syndecan-4 associates with caveolae.

(A) Syndecan-4 association with caveolae. Differential Interference Contrast (DIC) image of live RFPEC co-transfected with caveolin-1-EGFP and FcR-S4 constructs is overlaid by images of caveolin-1-EGFP (green) and clustered FcR-S4 (red). Note a lack of overlap of caveolin-1-EGFP and FcR-S4 signals. Inset: digitally magnified view of the indicated region. An associated movie file demonstrates co-movement of syndecan and caveolin-1. Time-lapse series of 48 images from the inset were acquired with 2.5 second interval. Note association (but not an overlap) of syndecan-4 and caveolin-1 signals.

(B) Spatial analysis of caveolin-syndecan-4 movement. Movement of the caveolin-1-EGFP (green) and syndecan (red) signals were tracked over time in X and Y planes. The graphs display the relative motion along the Y (top) and X (bottom) axis over time. Note the tandem movement but lack of overlap, of the two proteins.

(C) EGFP expression in RFPEC. To control for the effect of EGFP expression on the behavior of caveolin-1-EGFP construct, RFPEC cells were transfected with EGFP vector. The DIC image of live RFPEC expressing EGFP is overlaid with the EGFP (green) and FcR-S4 (red) signals. Note a diffuse cytoplasmic distribution of EGFP, quite distinct from the appearance of caveolin-1-EGFP. Syndecan-4 staining (red) is present along the cell border.

top right panel), consistent with syndecan-4 presence in the FLAER-labeled rafts. The specificity of staining was confirmed by FLAER and non-immune IgG labeling of the vector-transfected RFPEC (Fig. 3, bottom panels) that demonstrated the absence of FcR-S4 signal.

Caveolae constitute a distinct subpopulation of cholesterol-rich rafts. In order to see if syndecan-4 is present in caveolae, we generated RFPEC cells expressing EGFP-tagged caveolin-1. Live confocal microscopy showed no co-localization between syndecan-4 and caveolin-1 (Fig. 4A) and coordinated movement of the two proteins (movie file). To explore the effect of syndecan-4 clustering on its association with caveolae, we used time-lapse microscopy to visualize syndecan-4 movement in caveolin-1-EGFP expressing RFPEC cells. Reconstruction of time stacks of syndecan-4-caveolin-1 images in the XY plane demonstrated that while both proteins moved together, they did not localize to the same cellular compartment (Fig. 4B). Western blots with an anti-caveolin-1 antibody failed to detect its presence among proteins present in the syndecan-4 immunoprecipitations (not shown). To control for the effect of EGFP expression on the cellular distribution of caveolin-1-EGFP chimeric protein, RFPEC were transiently transfected with an EGFP construct. Visualization of EGFP demonstrated diffuse distribution of the protein in the cytoplasm (Fig. 4C), quite distinct from the appearance of caveolin-1-EGFP protein. Syndecan-4 staining is present along the cell border (Fig. 4C).

Discussion

While syndecans have largely been considered structural proteins, recent data suggest that they play a variety of roles including lipoprotein uptake (21), cell adhesion (6) and regulation of FGF2 signaling (5,22). However, the molecular events involved in these activities are not well understood. Previous studies have shown that in CHO cells syndecan-1 is found, upon clustering, in the Triton X-100-insoluble regions of the membrane (23), and that this event may play a role in syndecan-1-mediated lipid endocytosis (21,24). While syndecan-1 and syndecan-4 share significant homologies in the transmembrane and cytoplasmic regions domains, the regions thought to play a role in clustering-induced aggregation in membrane rafts, there are also significant differences. Syndecan-4 is a unique member of the syndecan core protein family because of its ability to engage in FGF2 signal transduction. Furthermore, no data have previously been available regarding the effect of FGF2 binding on syndecan-4 clustering and the occurrence of this event in polarized

endothelial cells.

Since FGF2 treatment induces a number of cellular events that can potentially affect membrane syndecan-4 trafficking, we employed FcR-syndecan-4 chimeras to study the effect of syndecan clustering on its localization in the plasma cell membrane. The use of extracellular domain of Fc receptor is particularly convenient because Fc receptor is not expressed in endothelial cells and non-immune IgG can be used to decorate cells expressing Fc receptor. The chimera protein behaves in a mode analogous to the native syndecan-4: it is present on the cell surface, it forms complexes with the native syndecan-4 and its expression does not affect expression of the native protein. Therefore, FcR-S4 clustering behavior can for appear representative of ligand clustering of the native syndecan-4.

We find that in quiescent endothelial cells, FcR-S4 chimeras as well as native syndecan-4 are present mostly in the non-raft fraction of the membrane and that aggregation of FcR-S4 chimeras with antibodies, or syndecan-4 with FGF2, leads to a rapid redistribution of the proteins to the membrane rafts. Several independent pieces of data are consistent with this interpretation, including sucrose gradients, Western blotting of various membrane fractions, and live confocal microscopy.

Two principle raft sub-domains are caveolae-containing and caveolae-free rafts. Interestingly, syndecan-4 did not co-localize with caveolin-1, suggesting that it was not present in the caveolae but rather in the non-caveolae rafts. The absence of caveolin-1 in the syndecan-4 immunoprecipitated material further argues against direct interaction between these proteins. At the same time, clustering of FcR-S4 chimeras, led to close apposition (but, apparently, not binding) of syndecan-4 and caveolae and a complex coordinated movement of both proteins.

While we have not examined which portion of the syndecan-4 molecule mediates its association with plasma membrane rafts, by analogy with syndecan-1, the transmembrane domain and the immediately adjacent highly cationic cytoplasmic domain are the likely candidates. Of note, the presence of the PIP₂ binding domain in syndecan-4 (absent in syndecan-1) did not inhibit its localization to the raft subdomain. The presence of plasma membrane cholesterol is clearly required for this event as its depletion led to a virtually complete loss of syndecan-4 enrichment in Triton X-100-insoluble regions.

While syndecan-4 clustering with its signaling agonist, FGF2, induces its redistribution to non-caveolae rafts,

the functional significance of this event is unclear. One possibility is that this serves to bring syndecan-4 in close contact with FGF receptors. Since FGF receptor-dependent activation of a protein phosphatase is needed for activation of syndecan-4 signaling (16), this would serve to facilitate this connection. Alternatively, raft localization of syndecan-4 complexes may bring it in close contact with other signaling complexes thereby promoting cross-talk between various receptor pathways.

In summary, ligand-dependent clustering of syndecan-4 in primary endothelial cells leads to its concentration in non-caveolae rafts. This event may play a role in regulation of syndecan-4 signaling.

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Chapter 5

Fibroblast Growth Factor 2 Endocytosis In Endothelial Cells Proceed Via Syndecan-4-dependent Activation of Rac1 and a Cdc42-dependent Macropinocytic Pathway

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Abstract

Full activity of fibroblast growth factors (FGFs) requires their internalization in addition to the interaction with cell surface receptors. Recent studies have suggested that the transmembrane proteoglycan syndecan-4 functions as a FGF2 receptor. In this study we investigated molecular details of syndecan endocytosis and its role in FGF2 internalization in endothelial cells. We find that syndecan-4 uptake, induced either by treatment with FGF2 or by antibody clustering, requires the integrity of plasma membrane lipid rafts for its initiation, occurs in a non-clathrin, non-dynamin dependent manner and involves Rac1 which is activated by syndecan-4. FGF2 is internalized in a complex with syndecan-4 in 70 kD dextran-containing endocytic vesicles. FGF2 and syndecan-4 but not dextran endocytosis is blocked by the dominant negative Rac1 while amiloride and the dominant-negative Cdc42 blocked internalization of dextran in addition to FGF2 and syndecan-4. Taken together, these results demonstrate that FGF2 endocytosis requires syndecan-4 clustering-dependent activation of Rac1 and the intact CDC42-dependent macropinocytic pathway.

Introduction

Fibroblast growth factors (FGFs) play a multitude of roles in modulation of cell growth, migration and differentiation (Ornitz and Itoh, 2001). The signaling activity of FGFs involves interaction with high affinity tyrosine kinase receptors and cell surface heparan sulfates. The latter molecules have long been thought to play a role as co-receptors for heparin binding growth factors such as FGFs. In particular, studies of FGF2 signaling established its dependence on the presence of cell surface heparan sulfates (Rapraeger et al., 1994) that are carried on membrane-bound core proteins, syndecans and glypicans (Rosenberg et al., 1997; Zimmermann and David, 1999). However, more recent studies have suggested that some core proteins themselves, and not just their heparan sulfate chains, may play an important role in signal transduction. Of these, syndecan-4 has emerged as a signaling receptor because of its ability to bind and activate protein kinase C (PKC) upon FGF2-induced oligomerization (Simons and Horowitz, 2001). This association of syndecan-4 with PKC puts the proteoglycan in a category similar to other transmembrane receptors, such as integrins, that do not

possess intrinsic catalytic activity, but are associated with kinases or phosphatases.

A substantial body of literature suggests that full activity of FGFs requires not only receptor interactions but also their internalization (Goldfarb, 2001) and that the latter may proceed via heparan sulfate proteoglycans-dependent and -independent pathways (Citores et al., 1999; Gleizes et al., 1995; Grieb and Burgess, 2000; Roghani and Moscatelli, 1992). Internalization of transmembrane proteins plays an important role in multiple cellular processes. In particular, syndecan endocytosis has been suggested as an important regulator of lipoprotein uptake (Fuki et al., 1997; Williams, 2001). At the same time, the details of FGF endocytosis and the involvement of specific proteoglycans have not been established.

Recent studies demonstrating direct syndecan-4 involvement in FGF2 signaling (Horowitz et al., 2002; Volk et al., 1999) raise the possibility that this proteoglycan may also play a role in its internalization thereby providing an additional avenue for regulation of its activity. This possibility is bolstered by observations that syndecan-4 binding to soluble (e.g. growth factors) or extracellular matrix ligand or cell exposure to mechanical stress results in its endocytosis (Li and Chaikof, 2002; Tkachenko and Simons, 2002). However, the link between syndecan-4 endocytosis and FGF2 internalization as well as molecular mechanisms of this process have not been defined.

To gain further insight into syndecan-4 function, we studied its clustering-induced endocytosis in endothelial cells. We find that following oligomerization, the core protein is internalized from the plasma membrane in a lipid raft-dependent, clathrin- and dynamin-independent manner by a process that is amiloride sensitive and requires activation of the small GTPase Rac1. Furthermore, syndecan-4 clustering induced by FGF2 resulted in FGF2 internalization together with syndecan-4 and 70 kDa dextran in the same endocytic vesicles. FGF2 and syndecan-4 but not dextran internalization was blocked by dominant negative Rac1, while amiloride and dominant-negative Cdc42 blocked internalization of not only FGF2 and syndecan-4 but also of 70 kDa dextran. This set of conditions shows that FGF2-induced syndecan-4 clustering leads to internalization of both proteins that proceeds via a macropinocytic pathway. Activation of Rac1 is required for the initiation of syndecan-4 endocytosis that also requires a fully functional macropinocytic pathway.

Materials and Methods

Antibodies and reagents

Human non-immune IgG and F(ab')₂ fragment-specific goat anti-human IgG F(ab')₂ antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Alexa-488 and Alexa-647 labeling kits, anti-HA epitope antibodies directly labeled by Alexa-488 and Zenon Alexa Fluor-488 mouse IgG1 labeling kit, BODIPY[®] FL C5-ganglioside GM1 complexed to bovine serum albumin (BSA) and dextran-rhodamineB 70 MW were purchased from Molecular Probes. Alexa-488-conjugated inactive variant of the protein proaerolysin (FLAER) was purchased from Protox Biotech (Victoria, British Columbia, Canada). Mouse anti-c-myc epitope-tagged monoclonal antibody (mAb) was purchased from Sigma. Chicken IgY anti-extracellular domain of syndecan-4 was fluorescently labeled with Alexa-488 according to the manufacturer's protocol (Molecular Probes). Human recombinant FGF2 was courtesy of Chiron Corporation (Sunnyvale, CA, USA). All other reagents were purchased from Sigma.

cDNA constructs and transfections

Fc receptor-syndecan-4 chimera (FcR-S4) construct was previously described (Tkachenko and Simons, 2002), eGFP-Cdc42-N17 (dominant negative), eGFP-Cdc42-I61 (constitutively active), eGFP-Rac1b-N17 (dominant negative), eGFP-Rac1b-I61 (constitutively positive) and GPI-GFP plasmids were provided by M. Way (Cancer Research UK) and S. Mayor (Sabharanjak et al., 2002) (National Centre for Biological Sciences, Bangalore, India). RhoA T19N (dominant negative) and RhoA G14V (constitutively active) were purchased from Guthrie Research Institute and were recloned into pIRES2-eGFP bicistronic vector (Clontech, CA, USA). eGFP-clathrin light chain construct was a kind gift from J. Keen (Thomas Jefferson University, Philadelphia, PA, USA), AP180C was from H. McMahon, dynamin1K44A from S. Schmid (Scripps Research Institute, La Jolla, CA, USA) and dynamin2k44a construct was from M. A. McNiven (Cao et al., 1998). Rat fat pad endothelial cells (RFPEC) were cultured in DMEM (Invitrogen, CA, USA) as described previously (Horowitz et al., 2002).

Endocytosis assays

To label vesicles that undergo macropinocytosis, rat fat pad endothelial cells (RFPEC) were incubated on ice in 1% BSA-DMEM for 30 minutes. Neutral 70-

kDa Dextran (rDx)-Rhodamine B was added to a final concentration of 0.5 mg/ml and incubated with cells at 37°C in 5% CO₂ for 20 minutes. To label clathrin-coated endocytic vesicles cells were incubated in 1% BSA-DMEM and then loaded with 1 µg/ml of DiI-LDL or 25 µg/ml of transferrin-Alexa647 (TfR) followed by incubation for 30 minutes on ice, washed once and then incubated at 37°C for 20 minutes in 5% CO₂. Labeling of endogenous GPI-anchored proteins by FLAER was done as previously described (Tkachenko and Simons, 2002).

FGF2 labeling with Alexa Fluor 647

In order to protect the syndecan-4 binding sites, FGF2 (1 mg) was adsorbed to 0.5 ml bed-volume heparin-Sepharose beads (Amersham Biosciences). The beads were washed with ice-cold 0.1 M NaHCO₃, resuspended in 1 ml of ice-cold 0.1 M NaHCO₃, pH 8.2. AlexaFluor647 succinimidyl ester was added (0.5 mg in 0.2 ml 0.1 M NaHCO₃) and the mixture was shaken for 1 hour at 4°C. The supernatant was drained and the column washed with 4 ml TBS and 2 ml 10 mM Tris/0.3 M NaCl pH 7.5. Labeled FGF2 was eluted with 2 ml 10 mM Tris/2 M NaCl pH 7.5 and desalted using two Hi-Trap desalting cartridges connected in series and elution with Tris-buffered saline. The FGF2-containing fraction was concentrated in an Amicon 10-kDa centrifugal filter (Millipore) and protein concentration was determined by spectrophotometry. The labeling ratio dye:FGF2 was 0.5:1, which was intended to be low as not to interfere with the biological function of FGF2.

Syndecan clustering

Antibody and FGF2 clustering were performed as described previously (Tkachenko and Simons, 2002). Briefly, RFPEC stably expressing FcR-syndecan-4 were plated and grown overnight in 10% FBS-DMEM. For antibody clustering, cells were washed with DMEM and incubated in the presence of biotinylated non-immune IgG (1 µg/ml) for 15 minutes in DMEM supplemented with 1% BSA at 37°C. Then cells were washed with ice-cold DMEM and incubated for another 30 minutes on ice with anti-human F(ab')₂-Cy3 (3 µg/ml) in DMEM-1% BSA followed by wash as above and incubation at 37°C with 5% CO₂ in DMEM 1% BSA for various times. To distinguish plasma membrane syndecan-4 from internalized syndecan-4, the cells were then washed with ice cold medium and incubated with streptavidin-Cy5 or streptavidin-Alexa488 (1:400 dilution) for 30 minutes at 4°C. At this temperature, streptavidin has

access only to the surface bound biotinylated IgG, or the IgG that is in vesicles open to the cell surface. For FGF2 labeling, cells were washed with ice-cold DMEM twice and incubated for 30 minutes on ice in DMEM in the presence of 50 ng/ml of FGF2-Alexa Fluor. After washing cells were incubated in DMEM-1% BSA for various times. To remove remaining cell surface FGF2, cells were placed on ice and incubated with 10 mg/ml of heparin in DMEM.

Immunofluorescence microscopy

RFPEC were plated in chamber slides (Nalge Nunc), fixed with 2% paraformaldehyde for 10 minutes at room temperature, then washed twice with phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100/PBS for 10 minutes, washed again as above, blocked with 3% BSA/PBS for 30 minutes, and incubated with primary antibodies (1 µg/ml) in 1% BSA/PBS overnight at 4°C. The slides were then washed four times as above, and incubated for 1 hour with 10 µg/ml of the appropriate reporter-conjugated secondary antibodies, washed again as before, and mounted with ProLong medium (Molecular Probes). Fluorescence microscopy was carried out on live cells as described previously (Tkachenko and Simons, 2002). Imaging was carried out using Bio-Rad MRC-1024 and Zeiss LSM 510 Meta laser confocal system microscopes with Zeiss 63x objectives. Image analysis was done using Adobe Photoshop 7.0 (Adobe Systems Inc.), AutoDeblur 9.1 (Auto Quant Imaging Inc.) and Image J software (NIH).

Toxin treatment and plasma membrane cholesterol depletion

RFPEC were incubated with 20 ng/ml of Clostridium difficile toxin B for 3 hours or 100 ng/ml exotransferase C3 for 12-16 hours in the normal growth medium. 5-(N-ethyl-N-isopropyl) amiloride treatment of RFPEC was performed as described previously (Muro et al., 2003). Plasma membrane cholesterol depletion was accomplished by pretreatment of cultured cells with 10 mM methyl β-cyclodextrin (MβCD), for 30 minutes at 37°C followed by a wash with DMEM and standard clustering procedure. MβCD removes unesterified cholesterol from the cell membrane (Rodriguez et al., 1997).

Quantitative measurements of endocytic uptake

Endocytic uptake of dextran, transferrin, GPI-

GFP, GM1-BODIPY, FcR-syndecan 4 and FGF2 was quantified as previously described (Sabharanjak et al., 2002). In brief, 10-15 images were acquired from two wells of the same experiment and the number of cells showing high level of uptake relative to untransfected cells was scored. The data are presented as mean of three or four independent experiments, each with 20 or more cells.

In addition, the effect of various toxin treatments on syndecan-4 and transferrin uptake was measured by flow cytometry. For these experiments (n=3) cells were grown to 70-80% confluence on 24-well plates. Toxin treatments, FcR-syndecan 4 and transferrin uptake assays were performed as described above. At the end of the experiment plates were placed on ice, washed once with ice-cold PBS pH 7.4 and then twice with ice-cold acidic PBS pH 2.5 for 30 seconds to remove cell-surface bound fluorescent ligands (Fuki et al., 2000). Cells were then trypsinized, washed once with PBS pH 7.4 and resuspended in DPBS pH 7.4 with 1% BSA. Flow cytometry was performed and analyzed as previously described (Tkachenko and Simons, 2002).

Rho GTPase pulldown assays

To determine activation of Rho, Rac and Cdc42 after FcR-S4 clustering, EZ-detect Rho and Rac1 activation kits (Pierce) were used. In brief, cells were seeded on fibronectin-coated 100 mm dishes and grown to 80% confluency then starved for 24 hours in DMEM 0.5% FBS. Following antibody clustering performed as described above, cells were lysed in 250 µl lysis/binding/washing buffer including proteinase inhibitors (Pierce). As positive and negative controls, four clarified non-clustered cell lysates (500 µg) were treated with either 0.1 mM GTP S or 1.0 mM GDP in the presence of 10 mM EDTA, pH 8.0 at 30°C for either 15 minutes (to activate or inactivate Rac1 and Cdc42) or 30 minutes (to activate or inactivate Rho). The nucleotide exchange reaction was terminated by adding MgCl₂ and placing samples on ice.

The cell lysates (500 µg) were then incubated with either GST-Pak1-PBD (to pull down active Cdc42 or Rac1) or GST-Rhotekin-RBD (to pull down active Rho) in the presence of SwellGel-immobilized glutathione at 4°C for 1 hour in a spin column. After incubation, the mixture was centrifuged at 8,000 g to remove the unbound proteins. The resins were washed three times with lysis/binding/wash buffer and the sample was eluted by adding 50 µl of 2x SDS sample buffer and boiling at 95°C for 5 minutes. Half (25 µl) of the sample volumes

Figure 1. Internalization of the native and chimera syndecan-4 proteins

(A) RFPEC cells expressing FcR-S4 construct were decorated with Cy3 (red) labeled human non-immune IgG (nilgG) and then clustered with AlexaFluor 647 (green) labeled FGF2. IgG decorated FcR-S4 chimeras are present on the cells surface and in the cytoplasm 20 min after FGF2 clustering (red). FGF2 is present both on the cell surface and in the cytoplasm. Note co-localization (yellow) of internalized FGF2 and FcR-S4 chimeras (merged images). This observation is consistent with FGF2-induced oligomerization of FcR-S4- native S4 heterodimers. Scale bars, 10 μm.

(B) FcR-S4 expressing cells decorated with biotinylated nilgG followed by clustering with Cy3 labeled clustering antibodies (red). Biotinylated nilgG remained on the cell surface was visualized by streptavidin-Cy5 (green). Dual stain on the merged image shows cell surface localized FcR-S4 whereas single red stain shows internalized one. Right figure shows merged colors image overlaid with DIC. Lower figure shows Z-plane projection of the area in white rectangle.

were analyzed by SDS-PAGE and transferred to a PDVF membrane. The active Rac1 and Rho were detected by western blotting using a specific mouse monoclonal antibody. A goat anti-mouse antibody conjugated with HRP was used as the secondary antibody. For the detection of Cdc42, anti-Cdc42 monoclonal antibody (Upstate) was labeled using the Zenon Alexa-488 mouse IgG1 labeling kit and applied to the rac1/cdc42 PVDF membrane. Detection was performed using West Pico Chemiluminescent Substrate (Rho and Rac1) (Pierce) or fluorometry (Cdc42) followed by exposure to X-ray film or detection of fluorescence in a Typhoon 9410 (Amersham Biosciences) detector, respectively.

Results

Clustering initiates syndecan-4 endocytosis

To study plasma membrane syndecan-4 internalization, we used live confocal laser microscopy to track the core protein in endothelial cells before and after FGF2 and/or antibody-induced clustering. To help visualize the fate of the native syndecan-4, we took advantage of heterodimerization between the wild-type syndecan-4 and the FcR-S4 chimeras previously demonstrated in the FcR-S4-expressing RFPEC (Tkachenko and Simons,

2002). In quiescent endothelial cells both the FcR-S4 chimera and the wild-type syndecan-4 were similarly expressed on the plasma membrane (Fig. 1A). FcR-S4-expressing RFPEC were treated with Cy3-labeled IgG. This decorates chimeras expressed on the cell plasma membranes but does not initiate their internalization (not shown). The cells were then treated with FGF2. Twenty minutes after FGF2 exposure, there was a substantial internalization of FcR-S4 chimeras (Fig. 1A, left panel) and of FGF2 itself (Fig. 1A, middle panel). The merged image demonstrates significant intracellular co-localization of FGF2 and FcR-S4 signals (Fig. 1A, right panel). This suggests that FcR-S4 chimeras that have formed heterodimers with the native syndecan-4 core protein, were internalized when the latter was oligomerized by the FGF2 treatment, as we have shown previously (Tkachenko and Simons, 2002).

Similarly, F(ab')₂-mediated clustering of IgG-decorated FcR-S4 chimeras also induced their internalization (Fig. 1B). Overall, kinetics of FcR-S4 chimera endocytosis was similar to that of the endogenous syndecan-4 with 50% of both proteins internalized within 20 minutes of antibody-mediated clustering.

Syndecan-4 endocytosis depends on the integrity of the lipid rafts and occurs in a clathrin and dynamin-independent manner

We have previously shown that FGF2-induced syndecan-4 or antibody-induced FcR-S4 chimera oligomerization result in redistribution of approximately 50% of the total plasma membrane content of these proteins to the lipid rafts (Tkachenko and Simons, 2002). To determine whether syndecan-4 endocytosis proceeds from the rafts or a non-raft portion of the plasma membrane, we used confocal microscopy of live cells to track internalized FcR-S4 chimeras as well as BODIPY-GM1, a lipid raft marker.

Within 5 minutes after clustering with F(ab')₂ fragments, most if not all internalized FcR-S4 chimeras co-localized with BODIPY-GM1 (Fig. 2a-e). To confirm this finding, we examined syndecan-4 endocytosis in RFPEC-expressing GPI-anchored GFP. Following antibody clustering of the FcR-S4 chimera, confocal microscopy of live cells demonstrated considerable co-localization between the internalized syndecan-4 chimeras and GPI-GFP (Fig. 2f-i). To confirm co-localization of internalized syndecan-4 with rafts proteins, we used FLAER to detect GPI-anchored proteins (Brodsky et al.,

2000 ; Tkachenko and Simons, 2002). As in the GPI-GFP studies, we observed a high level of colocalization of internalized syndecan-4 with FLAER-labeled endogenous GPI-anchored proteins (Fig. 3). These results suggest, therefore, that essentially all internalized syndecan-4 originated from a region rich in lipid rafts. To confirm this conclusion, we used methyl β -cyclodextrin (M β CD) to deplete plasma membrane cholesterol content thereby preventing formation of lipid rafts. As expected, M β CD treatment completely prevented antibody-induced internalization of FcR-S4 chimeras (Fig. 2j-m).

Internalization of plasma membrane receptors can occur via clathrin-dependent or independent pathways. To determine the role played by clathrin in syndecan-4 internalization, we examined co-localization of the endocytosed syndecan-4 and clathrin-LC-eGFP and studied the effect of expression of the AP180C construct that has been shown to selectively block clathrin-dependent endocytosis (Ford et al., 2001) on syndecan-4 uptake. Confocal microscopy demonstrated no co-localization between syndecan-4 and clathrin (Fig. 4a-c). After transient transfection of AP180C into RFPEC, cells demonstrating AP180C expression showed, as expected, inhibition of clathrin-dependent transferrin uptake (a). Syndecan-4 endocytosis, however, was not affected by AP180C expression (Fig. 4d-h, Fig. 5a). These results suggest that syndecan-4 endocytosis occurs via a clathrin-independent pathway.

To further define the endosomal pathway involved in syndecan-4 trafficking, dynamin dominant-negative constructs specific for both dynamins, dyn1K44A and dyn2K44A were transiently expressed in RFPEC. While expression of either dynamin construct had little effect on syndecan-4 endocytosis (Fig. 4i-l, Fig. 5a), both dynamin dominant negatives inhibited clathrin-dependent transferrin uptake (Fig. 5a).

Syndecan-4 endocytosis requires Rac1

Rho GTPases are involved in a number of endocytic processes. To examine the involvement of these GTPases in oligomerization-induced syndecan-4 uptake, we first studied the effect of syndecan oligomerization on activation of different Rho family members. To this end, we examined the time course of activation of RhoA, Cdc42 and Rac1 in RFPEC expressing the FcR-S4 construct following antibody-induced chimera oligomerization. While there was no change in RhoA or Cdc42 activity, within 5 minutes there was a significant activation of Rac1 that gradually declined to baseline

Figure 2. Syndecan-4 is internalized from lipid rafts region of the plasma membrane.

Confocal microscopy analysis of syndecan-4 endocytosis was carried out in RFPEC cells expressing FcR-S4 chimera. The rafts portion of the plasma cell membrane was labeled with BODIPY GM1 (blue) (Panel a) or by transient expression of GPI-GFP construct (Panel f). FcR-S4 chimeras were decorated with biotinylated nilgG and clustering was then initiated with the addition of Cy3-F(ab)₂ fragments (red). Five minutes after the initiation antibody clustering all internalized FcR-S4 chimeras (Panel b) were noted in close association with GM1-BODIPY (magenta, Panels a,d,c) membrane domains or with GPI-GFP (yellow, Panels h and i). Merged colors image (Panels d and h) overlaid with DIC are shown in panels e and i, respectively. The depletion of membrane cholesterol with M β CD fully blocks clustering-induced FcR-S4 internalization (Panels j,k,l,m). Scale bars, 10 μ m.

Figure 3. Syndecan-4 is internalized with endogenous GPI-anchored proteins.

FcR-S4 expressing RFPES were labeled with FLAER, decorated by biotinylated nilgG and clustered by Cy-3-F(ab)₂ (Panel a). Five minutes after clustering cells were placed on ice and cell surface remained FcR-S4 stained with streptavidin-Cy5 (Panel b). FLAER-labeled GPI-anchored proteins are shown in panel c. Cell surface (Panels d and f) and internalized (Panels e and f) FcR-S4 demonstrate high level of colocalization with GPI-anchored proteins. Arrows (panel f) point to examples of FcR-S4 and FLAER labeled internalized vesicles. Scale bars, 10 μ m.

Figure 4. Syndecan-4 is internalized in the clathrin- and dynamin-independent manner.

Syndecan-4 endocytosis was studied in FcR-S4 expressing RFPEC transiently transfected with various constructs.

Antibody clustering of FcR-S4 chimeras carried out as described in Fig 1 and 2 previously leads to internalization of syndecan-4 (Panel a). Note the absence of co-localization of internalized syndecan-4 with clathrin in cells expressing clathrin-eGFP (Panels b and c) 5 min after clustering

The effect of clathrin dominant negative was studied in cells transiently expressing c-myc tagged AP180C construct (Panel d). Cell expressing AP180C demonstrates the same FcR-S4 internalization as non-transfected cells (Panels e,f,g,h). In the panels FcR-S4 remaining on the cell surface is shown in green and internalized in red.

The role of dynamin was studied in cells transiently expressing a dominant-negative HA-tagged dyn^{2K44A} construct (Panel i). Note that the cell expressing Dyn^{2K44A} demonstrates high level of FcR-S4 internalization (Panels j,i,l,m). Panels h and m show merged color images g and l, respectively overlaid with DIC.

Panels g,h,i,l: red color shows internalized FcR-S4.

Panels f,g,h,k,l: yellow and green colors correspond to FcR-S4 constructs remaining on the cell surface. Scale bars, 10 μ m.

levels over the next 60 minutes (Fig. 6).

Since Rac1 activation preceded the activation of syndecan-4 endocytosis and reverted to baseline levels when its endocytosis was complete, we next examined whether Rac1 activation is indeed required for this process. Treatment of cells with *C. difficile* toxin B, inhibitor of all Rho GTPases, completely abolished all FcR-S4 endocytosis (Fig. 7a). At the same time, treatment with a RhoA inhibitor, C3 exotransferase, had no effect on this process (Fig. 7b). To further investigate which Rho family member is involved in syndecan endocytosis, dominant

negative Rac1 and RhoA as well as constitutively active mutants of Rac1, Cdc42 and RhoA were transiently expressed in RFPEC expressing FcR-S4 chimeras and their effect on clustering-induced syndecan-4 endocytosis was then examined. Transient expression of a Rac1 DN construct completely blocked FcR-S4 endocytosis while the expression of a Rac1 constitutively active construct markedly accelerated it (Fig. 8a, Fig. 5b). Transient expression of a Cdc42 dominant also downregulated syndecan-4 endocytosis while a Cdc42 constitutively active construct had no effect (Fig. 8b, Fig. 5b). At the

Figure 5. Quantitative analysis of syndecan-4 endocytosis

Quantitative analysis of syndecan-4 endocytosis was carried out using confocal microscopy as described in Methods. The results are presented as a fold change from baseline for each substance.

(A) Effect of dominant negative constructs inhibited clathrin-dependent uptake (AP180c) and dynamin 1 and 2 function (Dyn1^{K44A} and Dyn2^{K44A}) on syndecan-4 (black bars) and transferrin (grey bars). Note inhibition of transferrin but not syndecan-4 uptake. * $p < 0.05$ vs. baseline.

(B) The effects of Rac1 and Cdc42 dominant negative and constitutively active constructs as well as amiloride treatment on FcR-syndecan-4 (black bars), FGF2 (grey bars) and dextran (striped bars) uptake was examined in RFPEC. Note a significant inhibition of syndecan-4 and FGF2 uptake in Rac1-DN expressing cells and increased uptake in Rac1-CA expressing cells. Cdc42-DN and amiloride inhibited uptake of all three substances.

* $p < 0.05$ vs. baseline endocytosis.

same time, expression of RhoA dominant negative or constitutively active constructs had no effect on FcR-S4 internalization (Fig. 8c, Fig. 5b).

Syndecan-4 and FGF2 internalization proceeds via macropinocytosis

The involvement of Rac1 in syndecan-4 endocytosis suggests that the latter might proceed via a macropinocytic pathway. To evaluate this possibility FcR-S4-expressing RFPEC were treated with fluorescently labeled 70 kDa dextran and then syndecan clustering was induced by an antibody or by addition of fluorescently labeled FGF2 (Fig. 9a-c). Confocal microscopy of live cells demonstrated partial co-localization of internalized

Figure 6. Rac1 activation by syndecan-4 clustering

Time course of Rac1, RhoA and Cdc42 activation following antibody oligomerization of FcR-S4 chimeras. GST pull down assays with specific substrates were performed as described in the Methods. Note activation of Rac1 5 min following initiation of syndecan-4 oligomerization while neither RhoA nor Cdc42 activity is changed.

dextran and FcR-S4 (Fig. 9d) following antibody clustering, a finding consistent with the presence of FcR-S4 chimera in macropinocytic vesicles. Similarly, following FGF2 treatment of endothelial cells exposed to dextran, there was considerable co-localization of dextran and FGF2 signals (Fig. 9e) and both FGF2 and dextran were observed in syndecan-4-containing vesicles (Fig. 9f).

Since macropinocytosis is sensitive to amiloride, we examined the effect of this inhibitor on both syndecan-4 and FGF2 endocytosis. Pretreatment of cells with amiloride completely inhibited internalization of dextran, as expected (Fig. 5b). At the same time, it also inhibited both the antibody clustering-induced FcR-S4 chimera endocytosis (Fig. 9g-j) and FGF2 endocytosis (not shown, see Fig. 5b for quantification). However, amiloride did not inhibit uptake of GM1 and GPI-GFP (not shown).

Since modulation of Rac1 activity affected syndecan-4 endocytosis, we examined its effect on FGF2 uptake as well as macropinocytosis as defined by dextran internalization. Transient expression of a dominant negative Rac1 construct significantly inhibited FGF2 endocytosis while a constitutively active Rac1 increased it (Fig. 10a,b). At the same time neither construct affected dextran uptake (Fig. 5b).

To contrast effects of membrane cholesterol depletion and amiloride and clathrin-dependent uptake of transferrin and clathrin-independent endocytosis of syndecan-4 and to corroborate confocal microscopy data, we used flow cytometry to quantitate these processes. In agreement with the confocal microscopy

Figure 7. Role of Rho family GTPases in syndecan-4 endocytosis.

The effect of pre-treatment with *C. difficile* toxin B (A) and C3 exotransferase (B) on syndecan-4 endocytosis was examined in antibody-clustered FcR-S4 chimeras. Note that while toxin B treatment completely inhibited FcR-S4 endocytosis, C3 exotransferase had no effect. Scale bars, 10 μ m.

results, cyclodextrin treatment virtually completely inhibited syndecan-4 uptake while reducing transferrin endocytosis to a lesser degree (Fig. 11). This is consistent with similar published results (Rodal et al., 1999 ; Subtil et al., 1999). However, the possibility of a yet undefined effect of cyclodextrin on macropinocytosis cannot be excluded. Amiloride also fully inhibited syndecan-4 uptake as expected for a macropinocytic process, while mildly stimulating transferrin entry into cells (Fig. 11). Finally, to explore whether syndecan-4 clustering affects the rate of macropinocytosis, we measured dextran uptake following antibody clustering of FcR-S4 chimeras, native syndecan-4 or following FGF2 treatment. In all cases, dextran uptake was not affected (not shown) suggesting that syndecan-4 clustering or FGF2 treatment does not stimulate macropinocytosis.

Discussion

The results of this study demonstrate that FGF2 internalization proceeds in a syndecan-4-dependent manner and document the endocytic pathway of syndecan-4 uptake. The pathway is initiated by the cell plasma membrane syndecan-4 clustering that results in Rac1 activation and proceeds in a caveolae-, clathrin- and dynamin-independent manner. Moreover, it is amiloride and Cdc42 sensitive and results in dextran and FGF2 internalization in the syndecan-4-containing vesicles. These data are consistent with the notion that syndecan-4 clustering initiates its endocytosis via activation of Rac1 that allows syndecan-4 to detach from the actin cytoskeleton, enter the macropinocytic pathway and to internalize FGF2.

Receptor endocytosis can proceed in a clathrin-dependent and -independent manner (Conner and

Figure 8. Specific role of Rho GTPases in syndecan-4 endocytosis

The contributions of Rac1, Cdc42 and RhoA GTPases to syndecan-4 endocytosis were studied in FcR-S4 expressing RFPEC transiently transfected with respective eGFP-tagged dominant negative and constitutively active constructs.

(A) dominant negative Rac1 completely inhibited (left panels) while a constitutively active Rac1 stimulated (right panels) FcR-S4 endocytosis.

(B) The expression of a dominant negative Cdc42 construct (left panels) somewhat inhibited FcR-S4 endocytosis while a constitutively active (right panels) Cdc42 constructs had no effect.

(C) The expression of either dominant negative (left panels) or constitutively active (right panels) RhoA constructs had no effect on FcR-S4 endocytosis.

CA- constitutively active. DN- dominant negative. Scale bars, 10 μ m.

Schmid, 2003). While endocytosis of most receptors, including G protein-coupled (Pierce et al., 2000) and tyrosine kinase receptors (Vieira et al., 1996) was originally described as clathrin-dependent, recent studies have clearly shown that clathrin-independent receptor endocytosis plays an equally important role (Nichols and Lippincott-Schwartz, 2001). To date a number of different clathrin-independent internalization pathways have been described that typically involve GTPase dynamin, different Rho GTPases and either caveolae or non-caveolar lipid raft portion of the plasma cell

membrane. One such pathway is the internalization of interleukin-2 receptor from the plasma membrane rafts. The process is rapid and requires the GTPase dynamin and a small GTPase RhoA in an activated state (Lamaze et al., 2001). The latter observation is in marked contrast to the clathrin-mediated endocytosis where RhoA and Rac1 act as negative regulators.

An example of another pathway is folate uptake via its receptor, a GPI-anchored protein, that is accomplished in a clathrin, dynamin- and caveolae-independent manner and is regulated by Cdc42 (Sabharanjak et al., 2002).

This pathway bears considerable similarity to FGF2/syndecan-4 endocytosis including its origin from the plasma membrane rafts, lack of dependence on dynamin and clathrin and inhibition by a Cdc42 dominant negative.

The major difference between the two processes arises from FGF2/syndecan-4 uptake dependence on activation of Rac1 that has not been reported for the folate/GPI protein endocytosis. The involvement of Rac1 in a non-clathrin-dependent endocytosis has previously been described but it has been limited to fluid phase pinocytosis that seems also to involve Pak1 (Dharmawardhane et al., 2000). Rac1 is also involved in type 1 phagocytosis where it acts in conjunction with Cdc42 (Ellis and Mellor, 2000). However, Rac1 function is very complex as shown by studies in epithelial cells where expression of the dominant negative Rac1 decreased the rates of apical and basolateral endocytosis and inhibited apical recycling of IgA whereas the constitutive active Rac1 mutant had the opposite effect (Jou et al., 2000).

In the case of syndecan-4, Rac1 activation is required for its endocytosis while the activity of RhoA does not affect this process. This conclusion is supported by several observations. First, is the observation that a pan-Rho GTPase inhibitor, C. difficile toxin B, inhibited all syndecan-4 uptake while a RhoA specific inhibitor, C3 exotransferase, had no detectable effect on its endocytosis. Second, a dominant negative Rac1 mutant completely inhibited syndecan-4 uptake while a constitutive active Rac1 mutant markedly accelerated it. At the same time, dominant negative and constitutive active forms of RhoA had no effect on syndecan-4 endocytosis. Of note, the time course of Rac1 activation by syndecan-4 oligomerization is in keeping with its above described role in syndecan-4 uptake since its activation precedes the initiation of the core protein internalization and its activity returns to normal when the process is complete. The capacity of syndecan-4 clustering to activate Rac1 is in keeping with the known syndecan-4 oligomerization-dependent activation of PKC (Horowitz and Simons, 1998; Oh et al., 1997) and the ability of PKC agonist PMA to activate Rac1 and to induce macropinocytosis (Grimmer et al., 2002).

There are several possible explanations for the role of Rac1 in syndecan-4-dependent endocytosis. In quiescent cells plated on fibronectin syndecan-4 participates in formation of focal adhesions and is found in association with the actin cytoskeleton (Saoncella et al., 1999). This linkage to actin may involve recently described syndecan-4 binding to α -actinin (Greene et al., 2003). Prior to

Figure 11. Flow cytometry analysis of syndecan-4 endocytosis.

The effects of membrane cholesterol depletion by treatment with methyl- β -cyclodextrin (M β CD) and amiloride on uptake of transferrin and FcR-syndecan-4 were analyzed using flow cytometry as described in Methods. The data are presented as fold change from baseline. M β CD treatment almost fully blocked syndecan uptake while significantly reducing the level of transferrin internalization. Amiloride treatment inhibited syndecan uptake but increased the rate of transferrin endocytosis.

* $p < 0.05$ vs. baseline.

clustering, syndecan-4 is absent from the raft plasma membrane domain and it moves there in response to FGF2 or antibody-induced oligomerization (Tkachenko and Simons, 2002). This data is in agreement with the finding that more than 50% of cell surface bound FGF2 is found in lipid rafts (Chu et al., 2004). Such a movement probably requires breaking of the syndecan-4-actin cytoskeleton association, an event accomplished by Rac1 activation. Once translocated to the raft domain, syndecan-4 endocytosis proceeds in a manner similar to that of the folate receptor. This is suggested by co-localization of syndecan-4 and BODIPY-GM1 and GPI-GFP markers of raft region proteins and by the effect of dominant-negative CDC42. This finding is also in agreement with a previously reported co-localization of syndecan-4 and the inactive variant of proaerolysin, a marker of GPI-associated proteins (Tkachenko and Simons, 2002).

The sensitivity of syndecan-4 uptake to inhibition with amiloride and the presence of dextran in syndecan-4-containing vesicles suggest that it proceeds via macropinocytosis, a process that proceeds by formation of large endocytic vesicles brought about by the closure of lamellipodia at ruffling membrane domains and is usually thought of in the context of bulk fluid uptake. A recent study demonstrated that dynamin 2 is required for PDGF-induced but not constitutive macropinocytosis

(Schlunck et al., 2004). This finding suggests that there are at least two different mechanisms of macropinocytic uptakes. This distinction is also emphasized by the fact that while syndecan-4 is associated with GM1 and GFP-GPI on the cells surface and in endosomes they do not follow the same endocytic pathway.

It has been suggested that macropinocytosis may be involved in internalization of large multimeric complexes and may play a role, for example, in viral entry into cells (Liu et al., 2002 ; Marechal et al., 2001) as well as endocytosis of several endothelial plasma membrane proteins including ICAM-1, PECAM-1 and E-cadherin (Muro et al., 2003 ; Paterson et al., 2003). Finally, macropinocytosis is an attractive pathway for syndecan internalization given the typical large sizes of its heparan sulfate chains. This role of rafts in recruitment for macropinocytosis may be similar to the recruitment of cholera and anthrax toxins for uptake via the clathrin pathway (Abrami et al., 2001 ; Shogomori and Futerman, 2001).

FGF2 internalization is an important consequence of syndecan-4 endocytosis. In the cell type under study the majority of FGF2 uptake occurred via the syndecan-4 pathway. The dependence of FGF2 uptake on syndecan-4 endocytosis is in keeping with previously published results that suggested that both FGF1 and FGF2 enter cells via an endocytic process involving heparan sulfate proteoglycans (Citores et al., 1999 ; Gleizes et al., 1995 ; Roghani and Moscatelli, 1992). Such proteoglycan-dependent uptake has been localized to uncoated pits, in keeping with our finding of non-clathrin-dependent uptake, and proceed at a relatively slow pace, once again consistent with the kinetics of syndecan-4 internalization observed in this study (Gleizes et al., 1995). The functional significance of syndecan-4-dependent FGF2 internalization has not been established but previous studies have suggested that FGF1 uptake is required for its full mitogenic effect (Grieb and Burgess, 2000).

In summary, we describe macropinocytosis of syndecan-4 triggered by its oligomerization on endothelial cell membranes that results in internalization of the clustering agent, in this case FGF2, and involves both Rac1 and Cdc42 GTPases. The biological function of this pathway is the subject of future studies.

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Chapter 6

Syndecan-4 clustering induces migration in a synectin-dependent manner

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(submitted for publication)

Abstract

Cell migration is a dynamic process involving formation of leading edge in the direction of migration and adhesion points from which tension is generated to move the cell body forward. At the same time, disassembly of adhesion points occurs at the back of the cell, a region known as the trailing edge. Syndecan-4 (S4) is a transmembrane proteoglycan thought to be involved in formation of focal adhesions. Recent studies have shown that its cytoplasmic domain can engage in signal transduction, making S4 a bona fide receptor. Here we show that ligand clustering of cell surface S4 on endothelial cells initiates a signaling cascade that results in activation of Rac1, induction of cell polarization and stimulation of cell migration that depends on S4 interaction with its PDZ binding partner synectin. Expression of a S4 mutant lacking its PDZ-binding region (S4-PDZ-) leads to decreased cell motility and a failure to form a trailing edge. Similarly, cells lacking synectin, a S4-binding PDZ domain-containing protein, also fail to migrate in response to S4 clustering. Upon clustering, both the full length S4 and S4-PDZ- forms co-localize with the active form of Rac1. However, S4, but not S4-PDZ-, targets activated Rac1 to the leading edge of live cells in a synectin-dependent manner. Thus, our data suggest that syndecan-4 promotes endothelial cell migration in response to ligand binding by activating Rac1 and localizing it to the leading edge, and that these processes are dependent on its interaction with synectin.

Introduction

Syndecans are a family of four transmembrane proteoglycans carrying both heparan sulfate and chondroitin sulfate chains. Different family members are expressed in various cell types with syndecan-4 demonstrating a nearly ubiquitous expression. Syndecans have been thought to play the role of co-receptors for various heparin-binding growth factors such as fibroblast growth factors (FGFs) and vascular endothelial growth factors (VEGFs) or fibronectin-binding integrins (Couchman et al., 2001; Simons and Horowitz, 2001; Couchman, 2003; Tkachenko et al., 2005). However, recent studies have suggested that syndecans' intracellular domains, and in particular, the syndecan-4 intracellular domain, can directly engage in signal transduction (Oh et al., 1997b; Oh et al., 1997a; Volk et al., 1999; Horowitz et al., 2002; Keum et al., 2004).

The syndecans' cytoplasmic tail contains two highly conserved domains. The first (C1) is the membrane-

proximal region that binds tubulin, Src kinase, ezrin and cortactin (Tkachenko et al., 2005). The second (C2) is a C-terminal region that contains a PDZ-domain binding motif. The part of the molecule between the two conserved domains has been termed the variable domain and its sequence is unique to each syndecan family member.

The variable domain of syndecan-4 binds to the PIP2/PKC α complex, α -actinin and syndesmos (Tkachenko et al., 2005). These interactions are responsible for the previously demonstrated syndecan-4 role in cytoskeleton regulation that includes formation of focal adhesions, of dynamic of stress fibers and of cell protrusions (Baciu and Goetinck, 1995; Couchman and Woods, 1999; Longley et al., 1999; Baciu et al., 2000; Keum et al., 2004; Midwood et al., 2004; Saoncella et al., 2004).

Syndecan-4 is an acute response molecule, highly expressed in ischemic tissues, vascular tissues after injury (Geary et al., 1995; Li et al., 1997) and in a variety of solid tumors (Geary et al., 1995; Li et al., 1997; Oh and Couchman, 2004). Its potential role in postnatal angiogenesis is supported by delayed dermal wound healing observed in syndecan-4 null mice (Echtermeyer et al., 2001). Several reports have implicated syndecan-4 as a mediator of growth factor-induced migration (Volk et al., 1999; Horowitz et al., 2002; Cornelison et al., 2004; Charnaux et al., 2005). For example, mutations of either PIP2 or PDZ-interacting regions resulted in impaired endothelial cell migration and proliferation in response to FGF2 (Horowitz et al., 2002), and the presence of the syndecan-4 cytoplasmic tail was necessary for FGF2 responsiveness (Volk et al., 1999). Similarly, regeneration of muscles in syndecan-4 null mice is impaired because of the inability of satellite cells to migrate in response to FGF2 or HGF (Cornelison et al., 2004).

Evidence of direct proteoglycan signaling ability also comes from studies in smooth muscle cells where, despite the presence of a dominant-negative FGF receptor 1, FGF2 activated Erk1/2 and promoted cell migration (Chu et al., 2004). Using a more direct approach in the investigation of S4 induced signaling that involved antibody clustering, we previously demonstrated syndecan-4-dependent activation of Rac1, a Rho-GTPase, in endothelial cells (Tkachenko et al., 2004). Also, antibody clustering of syndecan-4 facilitates cell spreading in a Rho-dependent manner (Saoncella et al., 1999). Finally, increased baseline Rac1 activity was reported in syndecan-4/- fibroblasts, thus suggesting syndecan's role in its regulation (Saoncella et al., 2004).

Since formation of the leading edge of migrating

cells is known to be associated with activation of Rac1 (Waterman-Storer and Salmon, 1999; Ridley, 2001), we have set out to explore the role of syndecan-4-dependent activation of Rac1 in cell migration and the contribution of its PDZ-dependent interactions to this process. The PDZ domain-containing protein synectin was considered as a potential critical partner in S4-induced migration because of its ability to bind to S4 and to mediate the migratory response of endothelial cells (Gao et al., 2000). We found that syndecan-4 induced migration of endothelial cells require localization of active form of Rac1 in a manner dependent on syndecan-4 interaction with synectin.

Materials and methods

Antibodies and Reagents. Polyclonal goat IgG against extracellular domains of syndecan-4 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HA-Fluorescein and high affinity Fab fragments of IgG1 were purchase from Roche (Indianapolis, IN). Anti-FAK antibodies were from Cell Signaling Technology (Beverly, MA). Anti-FcR (CD64) monoclonal antibody was purchased from Abcam (Cambridge, United Kingdom). Non-immune human IgG, Cy-3-conjugated and unlabelled goat anti-human F(ab')₂ fragment and Cy-5-conjugated streptavidin were purchased from Jackson ImmunoResearch (West Grove, PA). Secondary antibodies conjugated to horseradish peroxidase (HRP) were purchased from Vector Laboratories (Burlingame, CA). Fluorescently labeled secondary antibodies were provided by Molecular Probes (Eugene, OR).

cDNA Constructs. Fc receptor-syndecan-4 chimera (FcR-S4) was previously described (Tkachenko and Simons, 2002). Mutation of PDZ binding sites of the chimera and the full length constructs by removal of the C-terminal alanine 202 was done using a PCR 3'-end primer TCA CTC CTC TGG GTT GGA GTC A. PCR reaction and cloning of the chimera into pCR3.1-Uni vector (Invitrogen) were done as previously described (Tkachenko and Simons, 2002). Full length syndecan-4 and syndecan-4 PDZ⁻ used to build adenoviral constructs were previously described (Horowitz et al., 2002). Adenoviruses were prepared by Harvard Gene Therapy Initiative (Boston, MA). Constructs encoding CFP-PAK-YFP (monitor of Rac1/CDC42 activity), and CFP-PAK-Rac1-YFP (monitor of Rac1 activity) were a gift from Michiyuki Matsuda (Osaka University, Japan).

Cell culture, transfection and transduction. HUVECs (Cambrex Corporation, East Rutherford, NJ) were cultured in EGM-2 medium (Cambrex). RFPEC were

cultured as previously described (Horowitz and Simons, 1998). One day prior to experiments, cells were plated on fibronectin coated dishes. A solution of 10 µg/ml of fibronectin in PBS was used to coat dishes for 30 minutes at room temperature. Stable expression of FcR-S4 and FcR-PDZ- chimeras was done as previously described (Tkachenko and Simons, 2002). Transient transfections were performed using GeneJammer (Stratagene, La Jolla, CA) according to manufacture's protocol. For transduction of HUVECs, we used 10 MOI of adenovirus.

Murine endothelial cells from lung tissue were isolated as previously described (Allport et al., 2002).

Cell "wounding" assay. For all experiments, cells were seeded on fibronectin coated tissue culture dishes (Corning, Acton, MA). Cell migration was measured by 3 to 4 independent "wounding" assays as described previously (Horowitz et al., 2002).

Syndecan clustering. Antibody clustering of FcR-Syndecan-4 chimeras and FGF2 treatment were performed as described previously (Tkachenko and Simons, 2002; Tkachenko et al., 2004). For clustering of endogenous syndecan-4, cells were starved prior to stimulation overnight in high glucose DMEM containing 0.5% serum. 3 µg/ml of anti-syndecan-4 antibodies (Santa Cruz) were then added to the starvation media.

Rho GTPase pull-down assays was done as described previously (Tkachenko et al., 2004).

Live Fluorescent Microscopy was done using the previously described microscopy system with climate control (Stan et al., 2004). To measure cell velocity, virally transduced cells were seeded on glassbottom dishes (MatTek, Ashland MA) for 12 hours and then labeled by adding 3 µg/ml of anti-HA FITC-Fab antibodies. After 15 min of incubation at 37°C, cells were washed with prewarmed culture media. Images were acquired every 5 minutes using a 20x 0.5 NA phase objective. Individual cell velocity was measured using the ImageJ program (NIH, Bethesda). In every experiment, velocities of 12 to 20 cells were analyzed. For FRET experiments, a dual splitter from Optical Insights (Tucson, AZ) with Chroma (Rockingham, VT) filter set (505 dcxr, HQ 465/30m, HQ 560/55m) was used. Microscope excitation and dichroic filter for CFP (brightlight FF458) was from Semrock (Rochester, NY). For FRET experiments, 20x 0.75 NA and 60x 1.45 NA TIRF objectives from Olympus (Japan) were used. Ration analysis was performed using ImagePro software from Media Cybernetics (Silver Spring, MD) using a method that was previously described (Yoshizaki et al., 2003). For TIRF acquisition, a 15 watt argon 488 laser from

Spectra-Physics (Irvine, CA) and illumination sideport from Olympus (Japan) were used. For TIRF experiments, 60x 1.45 NA TIRF objective from Olympus (Japan) was used. For colocalization studies, we used 100x 1.4 NA objective from Olympus (Japan).

Online Supplemental Material

Movie file Fig3Video1.mov shows syndecan-4 redistribution into the leading edge of RFPEC upon clustering by antibody. One frame per minute was acquired using TIRF microscopy.

Results

A variety of growth factors are able to bind and cluster syndecan-4. The resulting signaling events combine the elements of syndecan-4 signaling proper with the growth factors tyrosine kinase receptors signaling. Similarly, cell binding to extracellular matrix proteins combines the elements of syndecan-4 and integrin-dependent signaling. In order to completely isolate signaling events dependent solely on the syndecan-4 ligand binding and clustering, we used an anti-syndecan-4 extracellular domain antibody to cluster syndecan-4 on the surface of microvascular endothelial cells derived from the wild type and syndecan-4^{-/-} mice. Anti-syndecan-4 antibody clustering of wild type endothelial cells induced a significant migratory response while syndecan-4^{-/-} endothelial cells showed no increased migration (Figure 1A). The magnitude of syndecan-4-clustering induced migration was comparable to that induced by FGF2 (Figure 1A).

To further demonstrate that the observed stimulation of migration was due to syndecan-4 cytoplasmic domain oligomerization-initiated signaling, we employed an FcR-S4 chimera construct consisting of an FcR extracellular and transmembrane domains linked to syndecan-4 cytoplasmic domain (Tkachenko and Simons, 2002) stably expressed in a rat fat pad endothelial cell (RFPEC) line. Treatment of cells with an IgG by itself did not increase cell migration (not shown). However, subsequent oligomerization of IgG-decorated FcR-S4 expressing cells with an F(ab)2, but not F(ab) stimulated cell migration (Figure 1B). To explore the role of PDZ-dependent interaction in the initiation of syndecan-4-mediated cell migration, we expressed an FcR-S4-PDZ⁻ chimera in the same RFPEC line. Following antibody clustering, only the FcR-S4 chimera induced migration above basal levels, implying that the PDZ-binding domain is required to effect syndecan-4-dependent migration (Figure 1B).

To further examine the role of PDZ interactions in

Figure 1. Syndecan-4 clustering induces migration of endothelial cells in a PDZ-dependent manner. A. Primary lung endothelial cells from wild type (WT) and syndecan-4^{-/-} mice (S4^{-/-}) were stimulated with FGF2 or by antibody clustering of native S4. Note migration response induced by anti-S4 antibody similar in magnitude to that of FGF2 response in WT endothelial cells. S4^{-/-} endothelial cells do not migrate in response to anti-S4 antibody (*p=0.018). B. RFPEC expressing FcR-S4 or FcR-PDZ⁻ constructs were decorated by human nilgG followed by binding to Fab or clustering by F(ab)2 portions of anti-human IgG. Note significantly enhanced migration response in FcR-S4 vs. FcR-PDZ⁻ expressing cells (*p=0.015). C. Primary lung endothelial cells from WT and synectin^{-/-} mice were stimulated with anti-S4 antibody clustering. Note significantly better migratory response in WT cells (*p=0.03). All data are shown as mean±SE of the fold change from baseline.

Figure 2. Syndecan-4 matrix binding induces PDZ-dependent cell polarization.

A. HUVEC overexpressing syndecan-4 were plated on fibronectin-coated cover slips for 3 hr. Phase image overlaid with the syndecan-4 stain (red) is shown in the left panel, and the syndecan-4 only stain is shown in the right panel only. Arrows

syndecan-4-dependent migration, we evaluated the response of primary endothelial cells from syndecan-4^{-/-} mice. Unlike endothelial cells from syndecan-4^{+/+} littermate controls, cells from syndecan-4^{-/-} mice failed to enhance migration above basal levels upon antibody syndecan-4 clustering (Figure 1C). These data suggest that syndecan-4 promotes cell migration in response to clustering by a ligand and that this is specifically dependent upon its interaction with syndecan-4.

In order to monitor endothelial cell migration following binding to fibronectin, a native syndecan-4 ligand, full-length syndecan-4 and S4-PDZ⁻ constructs were transiently expressed in human umbilical vein endothelial cells (HUVEC). Upon attachment to a fibronectin-coated surface, full-length syndecan-4 became concentrated in lamellipodia (Figure 2A). In contrast, cells expressing S4-PDZ⁻ while also forming lamellipodia, failed to form trailing edges and exhibited a significant decrease in random migration velocity when compared with cells expressing GFP or syndecan-4 (Figure 2B-C).

In unstimulated endothelial cells, syndecan-4 is diffusely distributed over the cell surface. Following antibody clustering, a punctate distribution is observed (Tkachenko and Simons, 2002; Tkachenko et al., 2004). To further study cellular distribution of S4 following antibody clustering, we used total internal reflection fluorescence (TIRF) microscopy, a technique that allows detection of a fluorescent signal that originates only from the basal surface of the cell. Following antibody clustering, the FcR-S4 chimera localized to cell protrusions on the leading edge (Figure 3, upper panels; Fig3Video1). This is in contrast to cells expressing the FcR-S4-PDZ⁻ chimera, where increased basal surface concentrations of the fusion protein are found subsequent to clustering, although without the accompanying formation of cell protrusions (Figure 3, lower panels).

We have previously demonstrated that antibody clustering of FcR-S4 chimeras leads to Rac1 activation (Tkachenko et al., 2004). However, the role of the syndecan-4 PDZ domain in this process has not been established. Therefore, we have studied Rac1 activation in FcR-S4 and FcR-S4-PDZ⁻-expressing RFPEC. In

indicate regions of syndecan-4 concentration on the lamellopodia. B. Phase contrast image of HUVEC overexpressing syndecan-4 PDZ⁻ (red). Note the failure to form trailing edges (arrows).

C. Cell velocity of HUVEC expressing S4 or S4-PDZ⁻ constructs. Note decreased velocity of S4-PDZ⁻-expressing cells (*p=0.0017, S4 PDZ⁻ vs. control).

Figure 3. Syndecan-4 clustering induces PDZ-dependent lamellipodia outgrowth.

RFPEC expressing FcR-S4-PDZ⁻ (left panels) or FcR-S4 (right panels) were F(ab)₂ clustered as described. Note the chimera construct redistribution into the leading edge (arrows) of the cell upon antibody clustering (Fig3Vigeo1 time-lapse movie is in the supplementary file) in the FcR-S4 but not FcR-S4-PDZ⁻ cells. Insets magnify regions of lamellipodia protrusion formation.

contrast to FcR-S4 expressing cells, clustering of FcR-S4-PDZ⁻ expressing cells did not significantly increase the amount of GTP-bound Rac1 (Figure 4a,b). Interestingly, baseline RFPEC expressing the PDZ⁻ mutant construct had much higher levels of activated Rac1 than cells expressing FcR-S4. Similarly, syndecan-4^{-/-} cells also exhibit a high basal level of Rac1 activity and no activation in response to clustering (Figure 4c). These observations are in agreement with the reported high level of activated Rac1 in syndecan-4^{-/-} fibroblasts (Saoncella et al., 2004). Thus, syndecan-4-syndecan-4 interaction inhibits

Rac1 activity, and clustering of syndecan-4 removes this inhibition.

We hypothesized that high level of Rac1 activity in cells expressing PDZ⁻ mutant does not result in a pro-migratory phenotype because of incorrect localization of the active form of Rac1. To study localization of active Rac1 after syndecan-4 clustering, two independent monitors of Rac1 activity (Yoshizaki et al., 2003) were transiently overexpressed in FcR-S4 and FcR-S4 PDZ⁻ cell lines (Figure 5A). Using these constructs we observed that antibody clustering of FcR-S4 results in polarization

of Rac1 activity within a cell, an event that preceded formation of the trailing and leading edges of the cell (Figure 5B-C). In contrast, FcR-S4-PDZ⁻ cells did not exhibit a comparable cellular polarization of endothelial cell migration.

Discussion

The principal findings of this study are that syndecan-4 clustering on the endothelial cell surface that leads to the activation of Rac1, results in cell polarization and migration. The presence of the dominant-negative syndecan-4 construct lacking a PDZ binding domain or the absence of the syndecan-4 PDZ binding partner syndectin results in mislocalized high basal level activity of Rac1 and cell inability to polarize and initiate migration.

Syndecans have recently been recognized as signaling molecules in their own right (Tkachenko et al., 2005) but the extent of their signaling abilities and their molecular mechanism are poorly understood. Syndecan-4 in particular has been reported to be involved in mediation of cell migration in response to FGF2 (Volk et al., 1999; Horowitz et al., 2002) as well as in focal adhesion formation (Couchman and Woods, 1999; Woods and Couchman, 2001). Furthermore, we have previously demonstrated that syndecan-4 clustering induces Rac1 activation in endothelial cells (Tkachenko et al., 2004) while its absence has been reported to also result in increased Rac1 activity.

The present study was carried out to reconcile these findings and to explore the functional effect of syndecan-4

Figure 4. Rac1 activation following syndecan-4 clustering is PDZ-dependent.

Rac1 activity was measured by GST pull-down assays. Total Rac1 expression was used as a control for gel loading.

A. Rac1 activity at the indicated time points after clustering of FcR-S4 (left) and FcR-S4 PDZ⁻ (right) constructs in RFPEC.

B. Quantitative analysis of Rac1 GST pull-down assays (n=3). Data are shown mean±SE of the fold change of the unstimulated FcR-S4 expressing RFPEC. *p=0.054 and **p=0.06 (t-test)

Note the high basal level of active Rac1 in S4 PDZ⁻ cells.

C. Rac1 activity in lung endothelial cells from the wild type (+/+) and syndectin^{-/-} mice 10 min after treatment with anti-S4 (clustering) or non-immune (no clustering) antibody.

Note the high basal Rac1 activity in syndectin^{-/-} cells and its activation in WT cells.

clustering on endothelial cell migration. Since syndecan-4 clustering with its natural ligands such as FGF2 or fibronectin results not only in "pure" syndecan-4 signaling but also in signaling events mediated by FGF-R1 and $\alpha 5 \beta 1$ integrin respectively, we have used antibodies against either the extracellular domain of syndecan-4 or against the FcR portion of the FcR-S4 chimera. In both cases, syndecan-4 clustering induced endothelial cell migration that was comparable in magnitude to that induced by FGF2, a potent stimulator of cell motility.

The prominent feature of syndecan-4 clustering was rapid cell polarization, including formation of leading and trailing edges and syndecan-4 concentration on the leading edge and in the lamellipodia within 10 min of cell stimulation. This did not occur in cells expressing the S4-PDZ⁻ chimera suggesting that syndecan-4-PDZ-domain containing protein interaction was required for this event. Furthermore, while in wild type cells syndecan-4 clustering led to Rac1 activation and its concentration near the leading edge, in S4-PDZ⁻ cells Rac1 remained diffusely distributed. Taken together, these results demonstrate that syndecan-4-PDZ-domain containing protein interaction is required for transport of the activated Rac1 to the leading edge and initiation of cell migration.

Our results are similar to previously reported effects of dynamin-2 on the cellular localization of active Rac1 (Schlunck et al., 2004). In that study, expression of a dominant negative mutant of dynamin-2 (dynamin-2^{K44A}) affected the trafficking of Rac1, resulting in high basal level of Rac1 activity due to an improper targeting of the active enzyme for recycling. Furthermore, microinjection of dynamin-2^{K44A} impairs epithelial cell

Figure 5. Localization of active Rho GTPases following syndecan clustering.

FcR-S4 (left panels) and FcR-S4 PDZ⁻ (right panels) containing cells were transiently transfected with PAK (upper panels) and Rac1-PAK (lower panels) constructs. FRET signal intensity from 1.5 to 2.5 is displayed using pseudocolor as indicated by the scale bar. Blue represents low and red high FRET signal.

Note polarization of the GTPase activity in the stimulated FcR-S4 but not in the FcR-S4 PDZ⁻ cells.

ability to form the trailing edge during wound healing (Ezratty et al., 2005). Interestingly, dynamin has been shown to associate with macropinosomal vesicles containing the active form of Rac1 (Schlunck et al., 2004) while we have found that syndecan-4 is also present in macropinosomes (Tkachenko et al., 2004). Finally, a recent study has shown a direct interaction between syndecan-4 and dynamin-2 (Yoo et al., 2005).

One possible explanation for the similar phenotypes observed in syndecan-4-PDZ⁻ or dynamin-2^{K44A}-expressing cells may be that intracellular targeting of dynamin-2 necessary for migratory cell polarization (Schlunck et al., 2004; Ezratty et al., 2005) is dependent on syndecan-4-synectin interaction.

Another interesting finding of this study is that syndecan-4 clustering is required for Rac1 activation in wild type, but not in synectin^{-/-} or S4-PDZ⁻ expressing endothelial cells with high basal level of Rac1 activity. The mechanism of syndecan-4 clustering-dependent activation of Rac1 has not been established. One possible explanation of both syndecan-4 clustering-dependent Rac1 activation and the high baseline Rac1 activity in synectin^{-/-} and S4-PDZ⁻ cells is that syndecan-4 dissociation from synectin upon clustering results in removal of a synectin-associated Rac1 GAP.

Other proteins, in addition to fibronectin that have been implicated in promotion of cell spreading in a syndecan-4 dependent manner, are disintegrins from the ADAMs family (Thodeti et al., 2003). ADAM12 uses syndecan-4 as a primary cell surface receptor to trigger focal adhesions disassembly and β 1 integrin-mediated cell spreading. Clustering of another syndecan family member, syndecan-1, also results in the enhanced cell spreading and formation of lamellipodia, but in independent of PDZ-binding domain interactions (Chakravarti et al., 2005).

In summary, our results suggest that a syndecan-4-synectin protein complex inhibits Rac1 activation. Clustering of syndecan-4 locally releases this inhibition resulting in targeting of the active Rac1 to the leading edge of the cell thus promoting cell migration. These findings, therefore, establish syndecan-4 as a regulator of endothelial cell migration.

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Chapter 7

Summary

To date, syndecan-4 is the best described member of the syndecan family. Cell signaling events regulated by ligand binding to the extracellular part of this protein trigger actin cytoskeleton rearrangement resulting in the modulation of cell functions, such as cell migration and proliferation. Furthermore, syndecan-4 trafficking has been implicated in the uptake of natural ligands, that include growth factors, cell matrix proteins, LDL, and infectious agents including retroviruses.

Chapter 1 contains a review of signaling events regulated by syndecan. It also describes phenotypes of animal models lacking one of the syndecan family proteins. It appears that one syndecan subfamily (syndecan-1,-3) plays a mainly negative role in promotion of growth factor response, whereas another (syndecan-2,-4) enhances those responses. The main difference between syndecan-2 and -4 functions is in the time and location of their expression. Generally, syndecan-2 plays a major role in development, while syndecan-4 in acute response to tissue injury. For this reason, we studied syndecan-4's role in tissue injury-induced angiogenesis.

In Chapter 2, we investigated the effect of increased heparan sulfate mass due to overexpression of syndecan-4 in cardiomyocytes, on heart microvascular function. We found that the expressing animals demonstrated a significant increase in nitric oxide release in the coronary effluent in response to FGF-2. *In vitro*, coronary microvessels derived from those transgenic mice demonstrated increased relaxation response to FGF2 compared to control mice. Addition of exogenous heparan sulfate enhanced FGF-2 induced vasodilation in microvessels from control mice. Thus, we demonstrated that alteration of heparan sulfate production has a profound effect on microvascular homeostasis.

Further chapters are almost exclusively dedicated to the role of syndecan-4 cytoplasmic tail in the cell signaling.

In Chapter 3, the role of different cytoplasmic domains in FGF-2 signal modulation was investigated. Mutations in the cytoplasmic tail that either reduced its affinity to PIP_2 , or disrupted its PDZ-binding motif, produced an FGF2-specific dominant-negative phenotype in endothelial cells that demonstrated a marked decline in their migration and proliferation rates. Those cells also exhibited impaired capacity to form tubes on Matrigel. This molecular mechanism was determined to consist of a decrease in the syndecan-4-dependent activation of $\text{PKC}\alpha$.

Figure 1. Dominant-negative effect of syndecan-4 mutants. Overexpression of the mutant with disrupted PIP_2 binding site (left) results in its accumulation in the Golgi region and leads to the sequestering of PDZ-interacting protein(s) from the plasma membrane. In contrast, syndecan-4 with a mutated PDZ-binding motif (right) forms oligomers with endogenous protein thus preventing its binding to PDZ-domain containing protein(s).

Thus, PDZ-binding partner(s) of syndecan-4 play a critical role in the modulation of FGF2 signaling. Interestingly, a syndecan-4 mutant lacking the PIP_2 binding motif failed to be expressed on the cell surface. The mutant accumulates in the Golgi region, instead. Therefore, we hypothesized that depletion of the PDZ-binding partner by competitive binding due to the overexpression of the Golgi-retained syndecan mutant resulted in dominant negative cell phenotype (Figure 1). Indeed, in contrast to single mutations, mutations of the syndecan-4 cytoplasmic tail that resulted in the reduced affinity of the mutant to both PDZ domain and PIP_2 , generated a phenotype that respond normally to FGF-2 stimulation. The role of the PDZ-binding motif as well as an identification of the critical interacting protein is further discussed in Chapter 6.

In Chapter 4, we studied the membrane distribution of the core protein using an Fc receptor (FcR)-syndecan-4 chimera prior to and following FGF2- or antibodies-induced clustering. Previously described cholesterol rich compartments called lipid rafts were shown to contain a variety of signaling proteins that include syndecan-4 binding partners Src and $\text{PKC}\alpha$. We found that while unclustered syndecan-4 was present predominantly in the non-raft membrane compartment, clustering induced extensive redistribution to the non-caveolae lipid rafts. Clustering of syndecan-4 also induces its endocytosis. Interestingly, syndecan-4 positive endocytic vesicles can travel in tandem with caveolae vesicles. The role of this interaction remains unknown. FGF2 induced syndecan-4 uptake may play an important role in modulation of growth factor signaling.

Figure 2. Mechanism of syndecan-4 induced endocytosis.

FGF2 binding to syndecan-4 results in a shift of the FGF2/syndecan-4 complex to lipid rafts and this complex is then internalized via the constitutive CDC-42 dependent macropinocytic pathway.

In Chapter 5, the initial steps of syndecan-4 endocytosis and its role in FGF2 internalization were characterized. We found that syndecan-4 uptake, induced either by treatment with FGF2 or by antibody clustering, requires the integrity of plasma membrane lipid rafts for its initiation and occurs in a non-clathrin, non-dynamin dependent manner. Our results demonstrate that FGF2 endocytosis needs syndecan-4 clustering-dependent activation of Rac1 and the intact CDC42-dependent macropinocytic pathway. Therefore, we have two steps in syndecan-4 uptake. First, syndecan is shifted to the lipid rafts followed by clustering. As a result of this shift, syndecan assembles a signaling complex that activates Rac1. Second, syndecan-4 containing lipid rafts undergo endocytosis using constitutive, CDC42 driven macropinocytosis (Figure 2).

Figure 3. Mechanism of syndecan-4 induced cell migration.

The syndecan-induced signaling complex is capable of activating Rac1. Interaction of syndecan-4 with PDZ-domain-containing protein inhibits this activation. Syndecan clustering results in activation of Rac1 by selective removal of this inhibition which leads to the increase of cell migration (*left*). PDZ-binding domain mutation leads to inability of the PDZ-domain containing protein to inhibit Rac1 activation and results in the high basal level of Rac-1 activity (*right*).

Activation of Rac1 is a critical step in migratory polarization of cells. In Chapter 6, we investigated the migratory response of endothelial cells to clustering of syndecan-4. We showed that syndecan-4 promotes cell migration upon ligand binding by activating Rac1 and localizing it to the leading edge of the cell. Cells expressing syndecan-4 with a mutated PDZ-binding region or cells lacking synectin, a PDZ domain containing protein that interacts with syndecan-4, fail to migrate in response to syndecan-4 clustering (Figure 3). Thus, the syndecan-4/synectin interaction is critical in clustering-induced cell migration.

In conclusion, our studies show regulation of cellular and organ functions by syndecan-4 ranging from effects due to the simple increase of its heparan sulfate mass to complex intracellular interactions. The described trafficking and signaling properties of syndecan-4 contribute to the understanding of growth factor and cell matrix induced angiogenesis.

Chapter 7

Samenvatting

Syndecan 4 is het best beschreven lid van de syndecan-familie. De signaaltransductie van syndecan-4, die gereguleerd wordt door binding van het ligand aan het extracellulaire deel van het molecuul, induceert herschikking van het actine-cytoskelet hetgeen cellulaire functies, zoals celmigratie en -proliferatie, moduleert. Verder is syndecan-4 betrokken bij de opname van 'natuurlijke liganden' waaronder groeifactoren, celmatrix eiwitten en LDL, alsmede infectieuze agentia zoals retrovirussen.

In hoofdstuk 1 worden signaaltransductie-pathways die door syndecan-4 wordt gereguleerd beschreven. Verder worden fenotypes van de diermodellen beschreven die deficient zijn voor een van de syndecan-familieleden. Een van de syndecan subfamilies (syndecan-1 en 3) speelt een negatieve rol in het activeren van de respons op groeifactoren, terwijl een andere subfamilie (syndecan 2 en 4) deze net stimuleert. Het voornaamste verschil tussen syndecan 2 en 4 betreft tijdsspanne en localisatie. Syndecan-2 speelt een rol in de ontwikkeling, terwijl syndecan-4 een rol speelt in de acute respons na weefselbeschadiging. Het doel van dit proefschrift was dan ook het bestuderen van de rol van syndecan-4 in de angiogenese die optreedt na weefselchade.

In hoofdstuk 2 hebben we het effect van een toegenomen heparan-sulfaat massa op de microvasculatuur van het hart onderzocht. Dit is uitgevoerd door syndecan-4 tot overexpressie te brengen in de cardiomyocyten. Het bleek dat de dieren die syndecan-4 tot overexpressie brachten, meer NO tot expressie brachten in het coronair effluent na toediening van FGF2. In vitro toonden de coronaire microvasculatuur van deze muizen een toegenomen relaxatie na FGF2 toediening in vergelijking met controle muizen. Toediening van exogeen heparansulfaat versterkte de FGF-2 geïnduceerde vasodilatatie in microvaten van controle muizen. In dit hoofdstuk hebben we laten zien dat verschillen in heparansulfaatproductie een duidelijk effect heeft op de homeostase van de microvasculatuur.

De volgende hoofdstukken hebben betrekking op de rol van de C-terminale deel van syndecan-4 in de cel signaaltransductie.

In hoofdstuk 3 hebben we de rol van verschillende cytoplasmatische domeinen van syndecan-4 onderzocht tijdens de modulatie van FGF-2 signaaltransductie. Mutaties in het PIP₂ bindingsdomein of het PDZ-bindingsdomein resulteerde in een FGF2-specifiek

Figuur 1. Effect van 'dominant-negatieve' syndecan-4 mutaties.

Overexpressie van een gemuteerd syndecan 4 gen met een verstoorde sequentie voor de PIP₂ bindingsplaats (*links*) veroorzaakt accumulatie van syndecan 4 in het Golgi gebied en leidt tot sequestratie van de PDZ bindende eiwitten van de plasma membraan. In tegenstelling tot dit effect vormt syndecan-4 met een gemuteerd PDZ-bindings motief (*rechts*) oligomeren met endogeen eiwit waardoor het niet kan binden met de PDZ bevattende eiwitten.

dominant negatief fenotype in endotheelcellen dat gekenmerkt werd door een afname van hun proliferatie en migratie capaciteit. Verder toonden deze cellen verstoring in buis-formatie in matrigel-assays. Dit effect werd veroorzaakt door een afname in de syndecan-4 geïnduceerde activatie van PKC α .

Dus, PDZ-bindende partners van syndecan-4 spelen een belangrijke rol in de modulatie van FGF-2 signaaltransductie. Een syndecan-4 mutant met een deficiënte PIP₂- bindingsplaats werd niet tot expressie gebracht aan het oppervlak, maar accumuleerde in het golgi-gebied. We hebben daarom de hypothese opgesteld dat depletie van de PDZ-bindingspartner door competitieve binding aan het in het golgi-gebied gelegen syndecan-4, verantwoordelijk is voor het beschreven fenotype (Figuur 1). In tegenstelling tot enkelvoudige mutaties, resulteerden mutaties met verlaagde affiniteit van FGF2 voor zowel de PIP₂ bindingsplaats als het PDZ domein in een fenotype met een normale respons op FGF2 stimulatie. De rol van het PDZ bindingsmotief alsmede de identificatie van het kritische interactie-eiwit wordt verder beschreven in hoofdstuk 6.

In hoofdstuk 4 werd de membraandistributie van het syndecan-4 transmembraan en C-terminale gedeelte onderzocht door gebruik te maken van een Fc receptor (FcR)-syndecan-4 chimera vóór en na FGF-2 of antilichaam geïnduceerde clustering. De voorheen beschreven cholesterol-rijke compartimenten, lipid rafts

Figuur 2. Mechanisme van syndecan-4 geïnduceerde endocytose.

Binding van FGF2 aan syndecan-4 resulteert in een verplaatsing van het FGF2/syndecan-4 complex naar 'lipid rafts' en het complex wordt dan geïnternaliseerd via constitutieve, CDC-42 afhankelijke macropinocytose.

genoemd, bevatten een variatie aan signaaltransductiemoleculen waaronder de syndecan-4 bindingspartners Src en PKC α . Terwijl ongeclusterd syndecan-4 vooral aanwezig was in de non-raft compartimenten, zorgde clustering voor redistributie van syndecan-4 naar non-caveolae lipid rafts.

Clustering van syndecan-4 resulteerde eveneens in endocytose. Syndecan-4 positieve vesikels migreerden in tandem migreren met caveoline-vesikels. De rol van deze interactie blijft onbekend. FGF-2 geïnduceerde syndecan-4 opname speelt hoogstwaarschijnlijk een belangrijke rol in de modulatie van de signaaltransductie van groeifactoren.

In hoofdstuk 5 werden de initiële stappen van syndecan-4 endocytose, alsmede zijn rol in FGF2 internalisatie gekarakteriseerd. We zagen dat syndecan-4 opname na FGF2 of antilichaam geïnduceerde clustering, de integriteit van de plasmamembraan en de lipid rafts vereist voor de initiatie van de internalisatie, en dat de internalisatie onafhankelijk is van clathrin en dynamin. Onze resultaten tonen dat FGF2 endocytose afhankelijk is van clustering van syndecan-4 waarna rac1 activatie optreedt. Verder is een intacte CDC42-afhankelijke macropinocytose pathway noodzakelijk. Er zijn dus 2 stappen tijdens syndecan-4 opname. Eerst wordt syndecan-4, na clustering, verplaatst naar de lipid rafts. Vervolgens assembleert syndecan-4 een signaaltransductie-complex dat leidt tot de activatie van rac1. Vervolgens ondergaan de syndecan-4 bevattende lipid rafts endocytose en maken ze hierbij gebruik van CDC42 geïnduceerde macropinocytose (Figuur 2).

Activatie van rac1 is een kritische stap in de polarisatie en migratie van cellen. In hoofdstuk 6 hebben we de migratie van endotheelcellen na clustering van syndecan-4 onderzocht. We hebben aangetoond dat syndecan-4,

Figuur 3. Mechanisme van syndecan-4 geïnduceerde cell migratie.

Het syndecan-4 geïnduceerde signaal transductie complex is in staat om Rac1 te activeren. Interactie van syndecan-4 met PDZ-domein bevattend eiwit remt deze activatie. Clustering van syndecan-4 leidt tot activering van Rac1 door selectieve onderbreking van dit remmend effect, hetgeen leidt tot een toename van cel migratie (*links*). Mutatie van het PDZ-bindingsmotief leidt er toe dat het PDZ-domein bevattende eiwit activatie van Rac1 niet kan onderbreken en resulteert in een hoog basaal niveau van Rac1 activiteit (*rechts*).

na ligand binding, cel migratie versterkt door activatie van rac1, dat vervolgens aan de rand van de cel lokaliseert. Cellen die syndecan-4 met een gemuteerd PDZ-bindingsdomein tot expressie brengen, en cellen deficient in syntenin, een eiwit dat een PDZ-domein bevat dat een interactie aangaat met syndecan-4, migreren niet na syndecan-4 clustering (Figuur 3). Hieruit kunnen we concluderen dat syndecan-4/syntenin interacties cruciaal zijn voor syndecan-4 clustering geïnduceerde migratie.

Sammenvattend beschrijft dit proefschrift de regulatie van cellulaire en orgaanfuncties door syndecan-4. Dit varieert van de eenvoudige toename van heparan sulfaat massa tot complexe intracellulaire interacties. De beschreven 'trafficking' en signaaltransductie eigenschappen van syndecan-4 dragen bij aan het begrijpen van groeifactor en cel-matrix geïnduceerde angiogenese.

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Professional Positions

- 2001-pres Research Associate, Angiogenesis Research Center, Dartmouth Medical School (Hanover, NH)
- 1998-2001 Research Assistant, Angiogenesis Research Center, Beth Israel Deaconess Medical Center (Boston, MA)
- 1997-1998 Research Assistant, Department of Pediatric Oncology, Dana-Farber Cancer Institute (Boston, MA)
- 1993-1997 Researcher, Department of Biochemistry, Irkutsk Medical Institute (Irkutsk, Russia)

Awards and Professional Activities

- 2005 Junior Investigator Award. Annual North American Vascular Biology Organization meeting in Chicago, IL.
- 2003 Finalist for the Scientific Sessions Poster Competition in Basic Science. American Heart Association, Orlando, FL.
- 2002 North American Vascular Biology Organization travel award. XII International Vascular Biology meeting in Karuizawa, Japan.

Publications

- Tkachenko E, Elfenbein A, Tirziu DC, Simons M. Syndecan-4 clustering induces migration in synectin dependent manner.(*submitted*)
- Tkachenko E, Rhodes JM, Simons M. Syndecans. Endothelial Biomedicine. W. C. Aird. Cambridge, MA, Cambridge U Press. (*submitted*)
- Tkachenko E, Rhodes JM, Simons M. Syndecans- new kids on the signaling block. Review. Circ Res 96(5):488-500.18 March 2005
- Stan RV, Tkachenko E, Niesman I. PV1 Is a Key Structural Component for the Formation of the Stomatal and Fenestral Diaphragms. Mol Biol Cell 2004 15: 3615-3630, August 2004
- Tkachenko E, Lutgens E, Stan RV, Simons M. Fibroblast growth factor 2 endocytosis in endothelial cells proceeds via syndecan-4-dependent activation of rac1 and a cdc42-dependent macropinocytic pathway. Journal of Cell Science 117: 3189-3199, 1 July 2004
- Tkachenko E, Simons M. Clustering induces redistribution of syndecan-4 core protein into raft membrane domains. Journal of Biological Chemistry. 277(22): 19946-51, 31 May 2002

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- Li J, Partovian C, Li J, Hampton TG, Metais C, Tkachenko E, Sellke FW, Simons M. Modulation of microvascular signaling by heparan sulfate matrix: studies in syndecan-4 transgenic mice. *Microvascular Res*. 64(1): 38-46., July 2002

Oral Presentations

- 2005 Eugene Tkachenko, Arye Elfenbein, Daniela Tirziu, Michael Simons. Syndecan-4 clustering induces migration in synectin-dependent manner. North America Vascular Biology Organization, Chicago, IL.
- 2002 Eugene Tkachenko, Michael Simons. Synectin is an essential PDZ protein involved in FGF-2 signaling mediated by syndecan-4. American Society of Cell Biology, San Francisco, CA.
- 2002 Eugene Tkachenko, Michael Simons. Role of Syndecan-4 in modulation of FGF2 signaling. University of Washington, Seattle, WA.

Poster Presentations

- Radu V. Stan, Eugene Tkachenko. (2004) PV1 is a necessary and sufficient component for the formation of the stomatal and fenestral diaphragms. European Life Science Organization, Nice, France.
- Eugene Tkachenko, Arye Elfenbein, Daniela Tirzui, Ty Lanahan, Michael Simons. (2004). Syndecan-4 clustering results in endothelial cell migration. International Society of Vascular Biology, Toronto, Ontario, Canada.
- Eugene Tkachenko, Esther Lutgens, Radu-Virgil Stan, Michael Simons.(2003). Fibroblast growth factor 2 endocytosis in endothelial cells proceeds via syndecan-4-dependent activation of rac1 and a cdc42-dependent macropinocytic pathway. American Society of Cell Biology, San Francisco, CA.
- Esther Lutgens, Eugene Tkachenko, Michael Simons.(2003).Internalization of Syndecan-4 is clathrin, caveolin and dynamin independent and requires activation of Rac1. American Heart Association, Orlando, FL.
- Eugene Tkachenko, Esther Lutgens, Radu-Virgil Stan, Michael Simons. (2003). Syndecan-4 undergoes internalization via a clathrin- and caveolin-independent pathway. EuroConference on Lipid Rafts, Tomar, Portugal.
- Eugene Tkachenko, Arie Horowitz, Michael Simons. (2002).Synectin-dependent syndecan-4 translocation from lateral to apical plasma cell membrane specifically regulates FGF2 signaling. American Heart Association, Chicago, IL.
- Eugene Tkachenko, Michael Simons. (2002). Clustering of the Syndecan-4 induces PDZ-dependent apical targeting and rapid internalization. International Society of Vascular Biology, Karuizawa, Japan.
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- Eugene Tkachenko, Nicholas W. Shworak, Arie Horowitz, Michael Simons. (2001) Syndecan-4, FGF2-signaling receptor, is present in lipid rafts in endothelial cells. American Society of Cell Biology, Washington, DC.