ALTERNATIVE SIGNALING PATHWAYS TRIGGERED BY DIFFERENT MECHANISMS OF SERPIN ENDOCYTOSIS

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Abbreviations:

apoER2: apolipoprotein E Receptor2

APP: Amyloid Precursor Protein

CCP: Clathrin-Coated Pit

CCV: Clathrin-Coated Vesicle

CS: Chondroitin Sulfate

DMEM: Dulbecco's Modified Eagle's Medium

ECM: Extracellular Matrix

EGF: Epidermal Growth Factor

ERK: Extracellular signal-Regulated Kinase

FCS: Fetal Calf Serum

GAG: Glycosaminoglycan

GPCR: G-Protein Couples Receptor

GPI: Glycosylphosphatidylionsitol

HS: Heparan Sulfate

HSPG: Heparan Sulfate Proteoglycan

KPI: Kunitz proteinase inhibitor

LDL: Low-Density Lipoprotein

LDLR: LDL receptor

LpL: Lipoprotein Lipase

LRP1: Low-density lipoprotein receptor Related Protein1

MAPK: Mitogen-Activated Protein Kinase

MEF: Mouse Embryonic Fibroblast

MLCK: Myosin Light Chain Kinase

NMDA: N-Methy-D-Aspartate

PAI: Plasminogen Activator Inhibitor

PDGF: Platelet Derived Growth Factor

PN-1: Protease Nexin-1

RAP: Receptor Associating Protein

RTK: Receptor Tyrosine Kinase

SFM: Serum Free Medium

Shh: Sonic hedgehog

SMC: Smooth Muscle Cell

tPA: Tissue-type Plasminogen Activator

uPA: Urokinase-type Plasminogen Activator

uPAR: uPA Receptor

VLDL: Very Low-Density Lipoprotein

VLDLR: VLDL receptor

Summary:

Protease Nexin-1 (PN-1), a 43 KDa glycoprotein, is known as a serpin (**se**rine **p**rotease **in**hibitor) regulating extracellular proteolytic activity. It strongly inhibits the activity of several serine proteases such as thrombin, tissue plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), trypsin and plasmin. Consequently it contributes to tissue homeostasis by inhibiting serine proteases upon formation of high molecular weight complexes that are actively removed from the extracellular space. The internalization of the PN-1 protease complexes is mediated by low-density lipoprotein receptor related protein1 (LRP1) and LRP1's co-receptor heparan sulfate proteoglycan (HSPG).

In this thesis, the mechanism and the consequence of free PN-1 internalization were examined. In cortical primary neuronal cultures prepared from PN-1 reporter mice, endogenous PN-1 was taken up by the neurons that did not expressed PN-1. Internalization of exogenous PN-1 was also studied in both wild type and LRP1-/- mouse embryonic fibroblasts (MEF). It displayed concentration- and time-dependence, and the kinetics of PN-1 uptake in LRP1-/- MEF cells was slower than that of wild type MEF cells. Receptor associated protein (RAP) interfered with PN-1 uptake in wild type but not in LRP1 -/- MEF cells. These data suggested that an alternative receptor mediates PN-1 uptake in the absence of LRP1. We identified syndecan-1, a member of HSPG family to be the receptor mediating PN-1 uptake in LRP1 -/- MEF cells. The following experimental evidences supported this conclusion. First, PN-1 uptake was sensitive to Genistein and β-cyclodextrin, both known to block syndecan-1 mediated endocytosis. Second, PN-1 uptake was increased by over-expression of full-length syndecan-1 and decreased by RNA interference targeting this proteoglycan. Furthermore, over-expression of truncated syndecan-1 lacking its intracellular domain did not influence PN-1 uptake in LRP1 -/- MEF cells. These results demonstrated that syndecan-1 especially the intracellular domain of its core protein was required for syndecan-1-mediated PN-1 internalization in the absence of LRP1.

We also explored the role of PN-1 in signaling transduction and cell migration. PN-1 activated PKA by binding to LRP1. More importantly, in the absence of LRP1, PN-1 stimulated Ras-Raf-MEK-ERK signaling pathway, and enhanced cell migration. The involvement of ERK signaling in PN-1 induced migration was substantiated by the fact that MEK inhibitor U0126 inhibited this migration. As downstream effector of ERK signaling, Rac1 was activated by PN-1, resulting in lamellipodia formation and increased

migration in LRP1-/- MEF cell. We further demonstrated that PN-1's function on cell migration is coupled to syndecan-1, because anti-syndecan-1 antibody inhibited cell migration induced by PN-1. Moreover, an enhanced interaction between PN-1 and syndecan-1, by over-expression of either PN-1 or syndecan-1 in LRP1-/- MEF cells, increased cell migration.

We further identified the upstream of this signaling pathway. We found that both anti-integrin β3 and anti-uPA receptor (uPAR) antibodies inhibited PN-1 enhanced migration in LRP1-/- MEF cell. We were also able to co-immunoprecipitate PN-1 and syndecan-1 with integrin β3. Since it is know that both uPAR and syndecan-1 are the upstream of integrin αvβ3 signaling, taken all these together, we concluded that PN-1 stimulated ERK signaling influencing cell migration went through integrin via interaction either with syndecan-1 or uPAR.

1 Introduction

Mammalian cells are able to take up substances by invaginating the plasma membrane, this process can catch membrane bound and soluble components. Endocytosis takes up large amount of the plasma membrane and is balanced by the recycling of membrane components to the plasma membrane by exocytosis. Endocytosis can be subdivided into the categories of phagocytosis, pinocytosis and receptor-mediated endocytosis. Phagocytosis takes up large particles and bacterial cells; it depends on actin polymerization during particle ingestion. Pinocytosis continuously takes up small amount of extracellular fluids, which requires either clathrin or caveolin. Receptor-mediated endocytosis internalizes cell surface proteins along with extracellular factors, including virus, toxins, nutrients, antigens and antibodies, growth factors and hormones via clathrin-coated pits (CCPs), the clathrin-independent pathway or caveolae.

Endocytosis is a precisely regulated physiological process, which begins with the invagination of small regions of the plasma membrane that ultimately form intracellular vesicles. These internalized vesicles may shuttle back to the plasma membrane to recycle the membrane components or they may be targeted for degradation [O'Bryan et al., 2001]. Endocytosis has long been known to affect receptor density on the cell surface. Recent studies have further demonstrated that it plays a key role in receptor-mediated signal transduction through clathrin- and caveolin-dependent processes. In some cases, blockade of these processes attenuates, or even prevents, signal transduction [Liu et al., 2003]

Cell surface signaling receptors, such as G-protein coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs), are activated upon binding of their ligands. These activated receptors can be internalized by endocytic receptors, a phenomenon to regulate the desensitization of signaling receptors. However, signaling transduction also regulates the endocytic efficiency. For instance, upon epidermal growth factor (EGF) stimulation, EGF receptor signaling causes rapid phosphorylation of the clathrin heavy chain leading to increased recruitment of clathrin to the membrane [Wilde et al., 1999]. It also activates Rab5a, a regulatory GTPase that plays an essential role in endocytosis [Barbieri et al., 2000], thereby promoting the endocytosis of EGF and EGF receptor themselves. Thus RTKs initiate specific signaling cascades, possibly at the plasma membrane, to enhance endocytosis.

1.1 Clathrin-mediated endocytosis and signal transduction

Clathrin-dependent endocytosis begins with the assembly of CCPs, which are composed of the basic building blocks of clathrin and the adaptor-binding protein 2 complex [Smythe et al., 1992]. Once recruited to the plasma membrane, clathrin forms the characteristic lattice network composed of the three-legged triskelia. These triskelia assemble into stable oligomeric complexes that induce curvature in the plasma membrane, and lead to the formation of CCPs [Crowther et al., 1981]. In the presence of accessory factors, the CCPs progress to form clathrin coated vesicles (CCVs), a step that requires the GTPase activity of dynamin to promote the fission of membranes, thereby releasing the CCVs [Herskovits et al., 1993;Sever et al., 1999]. The resulting CCVs then undergo a process of uncoating in which clathrin is removed from the vesicles. The uncoated vesicles are then targeted for several possible fates including fusion with the endosomal compartment, followed by degradation or recycling back to the cell surface.

Clathrin-mediated endocytosis is the internalization mechanism for a wide range of functional ligands, including constitutively recycled receptors such as the low-density lipoprotein related protein1 (LRP1) and the urokinase-type plasminogen activator receptor (uPAR), ion channels, GPCRs and RTKs, cell adhesion molecules, and synaptic vesicle membranes. When signaling occurs on the endocytic pathway, on one hand, endocytosis is the mechanism to desensitize activated signaling receptors. On the other hand, CCPs function as nucleation sites for the organization of signaling complexes on the plasma membrane. Thus the endocytic vesicles provide convenient cellular structures for distribution of signaling protein complexes and for signaling propagation. For example, when RTKs activate extracellular signal-regulated kinase (ERK), activated RTKs recruit Grb2 and mSos to the plasma membrane, which in turn rapidly translocate to CCPs, and activate membrane associated Ras, following by the signaling transduction from Ras through Raf-1, MEK to ERK. Over-expression of mutant dynamin, which specifically blocks endocytic vesicles trafficking, inhibits the ERK activation [Vieira et al., 1996], strongly suggesting that endocytosis is required for signaling propagation from RTKs to activated ERKs.

1.2 Caveolae-mediated endocytosis and signal transduction

Caveolae are flask-shaped invaginations present in the plasma membrane of many cell types, which are insoluble in nonionic detergents at 4°C. However, their composition, appearance and function are cell-type dependent. Caveolae are coated primarily by caveolin [Rothberg et al., 1992], which is essential for the formation and stability of caveolae [Fra et al., 1995]. In addition to caveolin, caveolae are known to contain dynamin, a GTPase localized to the neck of flask-shaped caveolae indentations, and is likely involved in pinching off the caveolae vesicles from plasma membrane [Henley et al., 1998;Oh et al., 1998]. Caveolae are also rich in cholesterol and sphingolipids [Brown et al., 1998;Simons et al., 2000], which are, in fact, important for the formation and stability of caveolae as well [Rothberg et al., 1992]. Recent work has confirmed that caveolae are directly involved in the internalization of numerous ligands including membrane components, toxins, viruses and bacteria [Pelkmans et al., 2002]. The mechanism of internalization via caveolae and the intracellular pathways activated subsequently are just starting to emerge.

One of the major hypothetic functions of caveolae is that they appear to serve as signaling platform by recruiting a wide range of signaling molecules, such as LRP1, growth factor receptors (e.g. platelet-derived growth factor (PDGF) receptor) [Boucher et al., 2002], GPCRs, non-receptor tyrosine kinases (e.g. Src), non-receptor Ser/Thr kinases (e.g. PKA) and the signaling adaptor proteins (e.g. Shc, Grb2) [Williams et al., 2004]. The interactions occur in the scaffolding domain of caveolin [Li et al., 1996;Couet et al., 1997;Williams et al., 2004]. Caveolin seems to inhibit down stream signaling of many of these proteins, the most notable of which is Src tyrosine kinase [Razani et al., 2002]. Furthermore, caveolin has been shown to block signaling from EGF receptor to MAP kinase thereby inhibiting cell proliferation [Engelman et al., 1998], however the inhibitory mechanism still remains unknown.

1.3 Low-density lipoprotein receptor family

Low-density lipoprotein (LDL) receptor (LDLR) gene family represents a group of very important transmembrane receptors, which mediate numerous ligands endocytosis and modulate signaling transduction. It consists of seven core members of cell surface protein, including the LDLR, the very low-density lipoprotein (VLDL) receptor (VLDLR), the apolipoprotein E receptor-2 (apoER2), LRP1, the structurally most similar LRP1b

and megalin (LRP2), and the multiple epidermal growth factor repeat containing protein7 (MEGF7). In addition, LRP3, 4, 5, 6 are also included in this family although they share much less structural similarity with the core members.

1.3.1 Structure of LDLR family

The receptors in this family contain an extracellular domain, which is various in size, but unique in the arrangement of the same structural motifs. The most significant feature of their extracellular domain is that a β-propeller domain is always followed the ligand binding repeats. This domain contains YWTD-motif and is flanked by EGF-like repeats, which are essential for the pH-dependent release of ligands in endosomes [Davis et al., 1987] (Fig. 1a). A single transmembrane domain connects the extracellular domain with the cytoplasmic domain, which displays little sequence similarity between family members. All core members of this family contain one or more arginine-proline-x-tyrosine (NPxY) motifs in the intracellular domain, which serves as an endocytosis signaling. However, they contain a various number of ligand-binding repeats in their extracellular domain, allowing for a large and diverse ligand-binding potential.

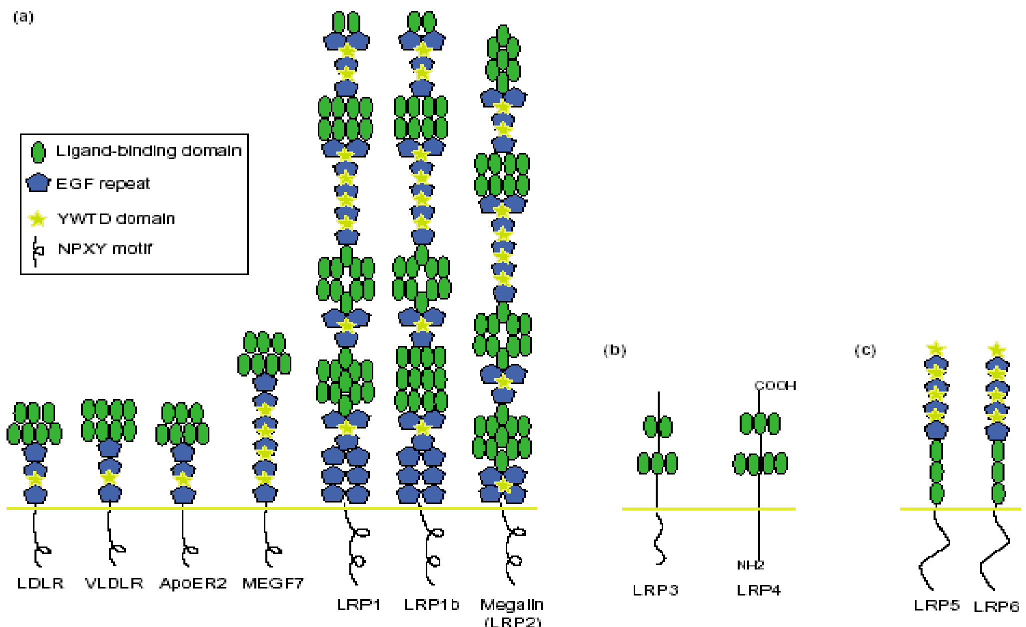


Fig. 1 Gene family of LDL receptor (adapted from Howell and Herz 2001)

Other more distantly related receptors, like LRP3 and LRP4, share little homology with classic receptors apart from the ligand-binding repeats, and both lack the NPxY endocytosis signals (Fig. 1b). Another subgroup of receptors, such as LRP5 and LRP6, has a similar organization of extracellular domain as the core members, except that the YWTD and the EGF repeats are amino-terminal to the ligand-binding repeats, which immediately precede the plasma membrane. Their intracellular domains also lack NPxY motifs (Fig.1c) [Howell and Herz, 2001].

The first family member to be identified was the LDLR, which plays a central role in lipid metabolism and cholesterol homeostasis by mediating the cellular uptake of cholesterol-rich LDL particles [Chen et al., 1990]. Because other family members also bind to lipoproteins, the receptors have been considered to be included in the regulation of cellular and systemic lipoprotein metabolism.

1.3.2 Functions of LDLR family

The dogma that the LDLR family functions predominantly, if not exclusively, in lipid and lipoprotein metabolism has been challenged by recent findings that several members of this family function in signaling processes. They physically or functionally interact with other classes of cell surface proteins or intracellular adaptor proteins, indicating a remarkable functional complexity (Table 1) [May et al., 2003]

Table 1 Mammalian members of LDLR family (adapted from May et al, 2003)

Receptor	Expression	Biological Function	Intracellular interact proteins
LRP1	Expressed by a wild range of cell types and tissues, such as	Endocytosis of a broad range of ligands, including protease/protease inhibitor complexes, signaling	PSD-95, Dab1 JIP1/2, Fe65, Shc
	Hepatocytes	Chylomicron remnant receptor	
	Neurons	Synaptic function?	
	Trophoblasts, embryonic tissues	Embryonic development	
LRP1b	Restricted expression pattern (central nervous system)	Unknown	Unknown
Megalin	Apical plasma membrane of absorptive and secretory epithelia, for example, renal proximal tubule	Vitamin and nutrient supply of tissues in the developing and adult organism: calcium homeostasis, recovery of excreted low molecular weight proteins and vitamin D/vitamin D binding protein complexes	ANKRA, dab1/2, MegBP, JIP1/2, EB-1, Glu1-BP (=semCAP-1), NHE3, CAPON, MAGI-1
	Thyroid and parathyroid gland	Uptake and transcytosis of thyroglobulin: PTH internalization	
	Developmental expression: endometrium during implantation, placental cytotrophoblast, trophoectoderm, visceral yolk sac, neuroectoderm	Supply of nutrients, vitamin and lipid homeostasis, signaling?	
VLDLR	Developing and adult brain, heart and endothelial cells, adipose tissue.	Neuronal migration: synaptic transmission Unknown	Dab1
ApoER2	Developing and adult brain	Neuronal migration: synaptic transmission	Dab1, JIP1/2, PSD-95
	Testis	Male fertility?	
LDLR	Ubiquitous, for example, hepatocytes, macrophages, central nervous system	Cholesterol homeostasis	ARH
MEGF7	Restricted expression pattern: embryogenesis, adult CNS	Unknown	Unknown

1.3.2.1 LDLR family-dependent endocytosis

As indicated in Table 2, LDLR family members internalize broad range of ligands, executing diverse functions, from lipoproteins metabolism to proteases and their inhibitor complexes clearance, vitamin metabolism and signaling transduction as well. The LDLR family-mediated ligand internalization comprises three distinct phases: binding of the ligands to the receptor; followed by internalization via CCPs and releasing of the ligands as a result of a pH drop in the endosomes; recycling of the receptor to the cell surface and degradation of the ligands [Brown et al., 1986]. A unique feature, also shared by all family members, is that the interactions between the ligands and the receptors can be antagonized by a 39-KDa receptor associated protein (RAP), which functions intracellularly as a molecular chaperone, by facilitating receptor folding and by preventing premature ligand interaction with the receptor during their trafficking within

the early secretory pathway [Warshawsky et al., 1994;Bu, 1998]. Since RAP shows rather high affinity to all members of LDLR family, it is commonly used as an antagonist to study LDLRs-mediated endocytosis [Strickland et al., 1995]

Table 2 Examples of ligands for the LDLR-related proteins (adapted from Howell 2001)

Ligand classes and examples	LDLR	VLDLR	ApoE R2	LRP1	Megalin	LRP5	LRP6
Lipoproteins containing							
ApoE	+	+	+	+	+		
ApoB 100	+				+		
lipases		+		+	+		
Carrier Proteins							
DBP					+		
RBP					+		
Proteases and inhibitor complexes							
PA				+	+		
α_2M				+			
PAI-1		+		+	+		
Signaling proteins							
Reln		+	+				
Wnt						(?)	+
TSP-1		+		+			

α_2M,α_2 -macroglobulin; ApoB, apolipoprotein B; PA, plasminogen activator; PAI-1, plasminogen activator inhibitor-1; RBP, retinol-binding protein;TSP-1, Thrombospondin-1 and LRP1b are not listed because their binding properties are unknown

1.3.2.1.1 Lipoprotein and lipid metabolism

Lipoproteins are characterized by an insoluble core of cholesteryl ester and triglyceride surrounded by a shell of amphipathic phospholipids and specialized protein called apolipoprotein (Fig. 2). The main function of lipoproteins is to transport lipid in an aqueous environment [Wasan et al., 1998;Chung et al., 2004]

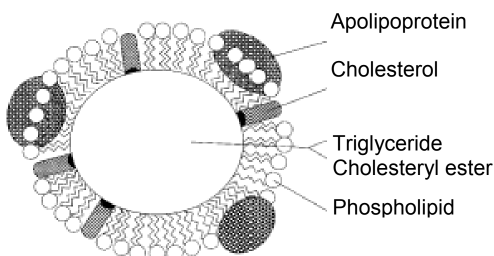


Fig. 2 The lipoprotein particle consists of cholesterol and triglycerides with an apolipoprotein embedded in a phospholipid monolayer (adapted from Chung and Wasan, 2004).

Lipoproteins differ in their content of proteins and lipids, and are classified based on their density into five main categories: chylomicrons, VLDLs, intermediate density lipoproteins, LDLs and high-density lipoproteins (Table 3).

Table 3 Density, size, physical composition and function of human plasma lipoproteins
(adapted from Chung and Wasan, 2004)

Characteristics	Chylomicrons	Very low-density lipoproteins	Intermediate density lipoproteins	Low-density lipoproteins	High-density lipoproteins
Abbreviations		VLDL	IDL	LDL	HDL
Density (g/ml)	< 0.95	0.95 - 1.006	1.006 - 1.019	1.019 - 1.063	1.063 - 1.210
Diameter (nm)	75 - 1200	30 - 80	25 - 35	18 - 25	12-May
Composition (%dry wt.)					
Proteins	1 - 2	8	19	22	47
Triglycerides	86	55	23	6	4
cholesterol	5	19	23	6	4
Phospholipid	7	18	20	22	30
Apoproteins	A1, A2				A1, A2
	B-48	B-100	B-100	B-100	
	C1, C2, C3	C1, C2, C3	C1, C2, C3		C1, C2, C3
	E	E	E		
Main function	Transport of exogenous triglyceride and cholesterol	Transport of endogenous triglyceride	Transport of endogenous cholesterol	Cholesterol transport to all tissues	Reverse cholesterol transport

Chylomicrons contain cholesterol and triacylglycerols from food, which are repacked with apolipoproteins and additional lipids in enterocytes. Subsequently they are secreted into circulation, acquiring cholesteryl ester, apoE and apoC, which facilitate reorganization by lipoprotein lipase (LpL). As a result, chylomicrons become rapidly hydrolyzed, releasing free fatty acid, mono- and diglycerides, and free cholesterol, which are absorbed by neighboring tissues for energy production and storage. The residual particles, known as apoE-rich chylomicron remnants, are taken up by the liver via LRP1 [Salter et al., 1988;Wasan et al., 1998].

VLDLs are the major transporters of endogenous triacylglycerol from the liver to extrahepatic tissue. Both endogenous and exogenous lipids are assembled with lipoproteins, mainly apoB-100, into VLDL particles. In blood, as for chylomicrons, VLDLs acquire cholesteryl ester, apoE and apoC and are then hydrolyzed by LpL, releasing free fatty acids and VLDL remnants. The latter, which are relatively rich in apoB-100, can be removed from circulation by LDLR or LRP1 on hepatocytes [Salter et al., 1988;Wasan et

al., 1998]. The VLDL remnants can also be further catabolized by hepatic lipase to form cholesteryl ester-rich LDL particles [Salter et al., 1988].

LDLs are the main carriers of cholesterol to peripheral tissues for sustaining, for instance steroid production and membrane synthesis. ApoB-100 is essential for recognition of the LDL particles by their receptors and subsequent internalization. They are then degraded in lysosomes, releasing lipids into the cytoplasm for cell use [Brown et al., 1986;Salter et al., 1988].

1.3.2.1.2 LDLR family-mediated lipoprotein and lipid metabolism

Cholesterol homeostasis is maintained by a complex feedback mechanism, in which LDL-derived cholesterol suppresses the intracellular cholesterol and LDLR biosynthesis to prevent further cellular cholesterol overloading. However mammalian cells can synthesize cholesterol in absence of lipoproteins. Thus a constant level of cholesterol is maintained within the cells. When LDL is available, most cells primarily use the LDLR to import LDL cholesterol. Clinically, the most important effect of LDLR deficiency is hypercholesterolemia in the circulation that accelerates development of atherosclerosis, due to a disturbed balance between extracellular and intracellular cholesterol pools [Goldstein et al., 1985].

VLDLR displays high affinity to apoE [Takahashi et al., 1992;Takahashi et al., 1996]. It has been shown to mediate the uptake of chylomicron remnants *in vitro* [Niemeier et al., 1996], and to reverse hypercholesterolemia in LDLR knock-out mice [Kobayashi et al., 1996;Kozarsky et al., 1996]. These results demonstrate that the VLDLR is competent in binding and internalization of apoE-containing lipoproteins, indicating that VLDLR plays a significant role in the metabolism of triglyceride-rich lipoproteins. In VLDLR and LDLR double knock-out mice, a significant increase in serum triglyceride level was detected under high fat diet; and these mice were protected from obesity via a significant reduction in whole-body free fatty acid uptake under a high fat and calorie diet [Goudriaan et al., 2001]. Taken together, VLDLR seems to be a part of machinery transporting triglycerides or free fatty acid to peripheral cells.

LRP1 is produced at high levels in hepatocytes in the liver, where it mediates the uptake of chylomicron remnants, the lipoproteins that shuttle dietary lipids from the intestine to the liver [Willnow et al., 1994c;Gliemann, 1998]. LRP1 also binds the lipoprotein lipases that are directly involved in the generation of the remnant lipoproteins from triglyceride-rich chylomicrons [Beisiegel et al., 1991].

1.3.2.2 LDLR family-independent endocytosis

It was first shown 20 years ago that clearance of remnant lipoproteins by liver was partly mediated through a LDLR-independent pathway [Kita et al., 1982;Rubinsztein et al., 1990;Ishibashi et al., 1994]. In fact the LDLR-independent pathway mediated about one-third of LDL removal from plasma in normal humans and all removal in patients homozygous for receptor-negative familial hypercholesterolemia [Goldstein et al., 1977;Kesaniemi et al., 1983]. As suggested by studies carried out in different laboratories HSPG are the potential receptors for hepatic and arterial catabolism of artherogenic lipoproteins [Williams et al., 1992;Fernandez-Borja et al., 1996;Al Haideri et al., 1997;Seo et al., 1997;Llorente-Cortes et al., 2002;Boyanovsky et al., 2005].

1.3.2.3 LDLR family-mediated signal transduction

1.3.2.3.1 VLDLR and ApoER2 function in Reelin signaling pathway

Reelin is a large extracellular protein, which is predominantly synthesized and secreted in the cerebral cortex by the Caja-Retzius cells of the marginal zone, the most outer layer of the developing cortex [D'Arcangelo et al., 1995;Ogawa et al., 1995]. Reelin signaling pathway regulates the cortical layering and positioning of neurons during development [Miyata et al., 1997]. In reeler mice, in which the gene encoding Reelin is defective, the disorganized cortex is approximately inverted. The disordered cortex appears with early-born neurons occupying abnormal superficial positions and later-born neuron adopting abnormal deep positions [Caviness, Jr. et al., 1973].

The cytoplasmic adaptor protein, Disable-1, has been shown to function down-stream of the Reelin signaling pathway [Sheldon et al., 1997;Rice et al., 1998;Howell et al., 1999]. Disable-1 deficient mice develop a phenotype which is identical to that of reeler mice [Howell et al., 1997;Sheldon et al., 1997]. The link between extracellular Reelin and intracellular Disable-1 was not clear until it has been reported that mice lacking both VLDLR and ApoER2 precisely mimic the phenotype of those with Reelin or Disable-1 deficiency [Trommsdorff et al., 1999]. In light with these observations, the NPxY motif of these two receptors has been shown to interact with the phosphotyrosine binding domain of Disable-1 by the yeast two-hybrid system [Gotthardt et al., 2000]. Furthermore, binding of Reelin to its receptors induces phosphorylation of Disable-1, this effect is abolished by RAP or by apoE, which in turn block the binding of Reelin to the receptors [D'Arcangelo et al., 1999;Hiesberger et al., 1999]. These findings demonstrate that

Reelin acts, via VLDLR and apoER2, to induce phosphorylation of Disable-1. The mechanism by which Reelin signaling is influencing neuronal migration is not yet well defined. However it is known that Reelin-receptor interaction activates Src family kinases, such as Src and Fyn, which phosphorylate Dab1 [Arnaud et al., 2003;Bock et al., 2003]. The down stream of Dab1 has just been identified recently as CrkII, an intracellular adaptor protein, which has been shown to regulate cell migration through Rac protein [Chen et al., 2004].

1.3.2.3.2 LRP function in Wnt signaling pathway

The Wnt family of secreted molecules functions in cell-fate determination and morphogenesis during development [Wodarz et al., 1998]. The interaction between canonical Wnts and their receptors, the Frizzled family members, results in dissociation of axin-1, GSK3β and β-catenin complex, thereby reducing the phosphorylation of β-catenin. Unphosphorylated β-catenin is stabilized and forms complexes with the TCF/LEF transcription factors, inducing specific transcriptional activation of target genes. LRP6 null mice have characteristics that closely match the phenotypes of many Wnt mutants, such as Wnt 1, Wnt3a and Wnt7a. For instance, loss of LRP6 results in truncation of the axial skeleton, deletion of the caudal midbrain and limbs patterning defects. However, LRP6 mutants do not display the entire mutations characteristic of Wnt mutants [Hussain et al., 1999], suggesting a requirement for another co-receptor in Wnt signaling.

The genetic experiments conducted in *Xenopus* embryos show that injection of either LRP5 or LRP6, in combination with Wnt5a, activates Wnt-Frizzled signaling, induces Wnt-responsive genes, the dorsal axis duplication, and neural crest cell formation [Tamai et al., 2000]. The LDLR fail to substitute for these LRPs. *In vitro* experiments also show that LRP6 complexes with Frizzled only when Wnt is present, and LRP6 is able to form complex with Wnt as well [Hussain et al., 1999]. These discoveries demonstrate that LRP5 and 6 interact with Frizzled functioning as Wnt co-receptor. However, LRP1 has been shown to sequester Frizzled1 and disrupt the receptor/co-receptor complex formation, leading to the repression of the canonical Wnt signaling. However, this inhibitory effect is not related to its endocytic function [Zilberberg et al., 2004].

1.3.2.3.3 LRP2 (Megalin) function in signaling transduction

Megalin (LRP2) is highly expressed in proximal tubules of the kidney, where it plays an important role in vitamin and iron homeostasis. Megalin mediates the tubular uptake of the complex of vitamin and their carrier proteins such as retinol-binding protein, vitamin D-binding protein, transcobalamin, and transferrin [Moestrup et al., 2001]. This re-absorption mechanism reduces the loss of these vital substances in the urine.

Megalin also regulates calcium homeostasis through parathyroid hormone, which mobilizes calcium and counter-regulates low calcium level. The binding of this hormone to its receptor transduces signal through activation of adenylate cyclase and increases product of cAMP. Megalin competes directly with the receptor of parathyroid hormone for hormone binding and endocytosis, thus down regulates the receptor activity [Nykjaer et al., 2002].

In addition Megalin binds the extracellular molecule Sonic hedgehog (Shh), and regulates Shh signaling. It has been proposed that megalin internalizes a complex of Shh and its receptor Patched, thereby releasing Patched-mediated inhibition of the Shh signaling receptor Smoothened and activating Shh signaling pathway [McCarthy et al., 2002]. Megalin deficiency leads to a subsequent loss of Shh expression in the ventral forebrain, consequently the ventrally derived oligodendroglial and interneuronal cell populations are lost in the forebrain [Spoelgen et al., 2005].

Megalin is also believed to functionally interact with intracellular proteins through adaptors that bind to the receptor tail and regulate its endocytic and signal transducing activities. For example, it has been shown that the intracellular tail of megalin interacts with megalin-binding protein, which is an intracellular adaptor protein. The latter interacts with several transcriptional regulators including SKI-interacting protein. This suggests that megalin directly participates in transcriptional regulation through controlled sequestration or release of transcription factor via megalin-binding protein [Petersen et al., 2003].

Megalin binds to various other intracellular adaptor molecules with role in protein kinase signaling and protein trafficking [May et al., 2003], including c-Jun N-terminal kinase interacting protein 1 and 2 [Gotthardt et al., 2000], and disable-2 [Oleinikov et al., 2000], suggesting that megalin plays a regulatory role in signaling transduction.

1.3.2.4 LRP1-mediated endocytosis and signal transduction

LRP1 is synthesized as a single chain molecule, and processed by furin into a 515 KDa α chain, and an 85 KDa β chain that contains the transmembrane and the

intracellular domains [Herz et al., 1990]. The α and β subunits remain non-covalently associated on the cell surface.

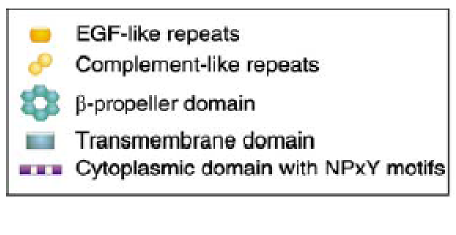
1.3.2.4.1 LRP1-mediated endocytosis: ligand families and their binding sites

LRP1 recognizes at least 30 different ligands that represent several families of proteins (Table 4). These include lipoproteins, proteases, protease-inhibitor complexes, ECM proteins, bacterial viruses and various intracellular proteins [Herz et al., 2001;Strickland et al., 2003].

Table 4 Ligands that bind to the extracellular domain of LRP1 (adapted from Herz and Strickland, 2001;Strickland and Ranganathan, 2003)

Lipoproteins and lipid metabolism	
apolipoprotein E	
Hepatic lipase lipoprotein	Lipid metabolism and cholesterol homeostasis
Lipoprotein lipase	
Sphingolipid activator protein	
Proteases and cofactors	
uPA	Cell migration, wound healing
tPA	Fibrinolysis, signaling function in brain
MMP-9	
MMP-13	Angiogenesis, metastasis
TSP-2/MMP-2	
Factor IXa	
Factor VIII	Blood coagulation
Protease-inhibitor complexes	
α_2M-protease complexes	Pan-protease inhibitors, infection
PZP-protease complexes	
uPA:PAI-1	Regulate uPA/tPA activity
tPA:PAI-1	
Thrombin:PAI-1	
Thrombin:ATIII	
Thrombin:HCII	
Thrombin: PN-1	
Elastase:α_1-AT	Regulate neutrophil elastase
C1s:C1q inhibitor	Regulate C1s activity
TFPI: TFPI-VIIa complex	Regulate blood clotting
APP (KPI isoforms)	Alzheimer disease
Matrix proteins	
Fibronectin	
Thrombospondin-1	TGF-β activation, matrix-cell interactions
Thrombospondin-2	Collagen assembly, matrix-cell interaction
Intracellular proteins	
HSP-96	Chaperon
RAP	Chaperon
HIV Tat protein	transcriptional activation
Calreticulin	
Growth factors	
PDGF	Regulate of signal transduction
Midkine	
Connective tissue growth factor	
Other molecules	
Complement C3	Infection
Lactoferrin	Antibacterial
Rhinovirus	
Pseudomonas exotoxin A	
Circumsporozoite protein	

The ligand recognition sites within LRP1 have been studied by testing the ability to mediate ligand internalization either by each of these single repeats or by fusing various clusters of ligand binding repeats. These studies have yielded some important insights into the ligand recognition properties of LRP1. The major ligand binding sites are located in clusters II and IV; most ligands bind equally to clusters II and IV; no other ligands besides RAP have been shown to bind to clusters III (Fig. 3) [Willnow et al., 1994b;Springer, 1998;Herz et al., 2001].



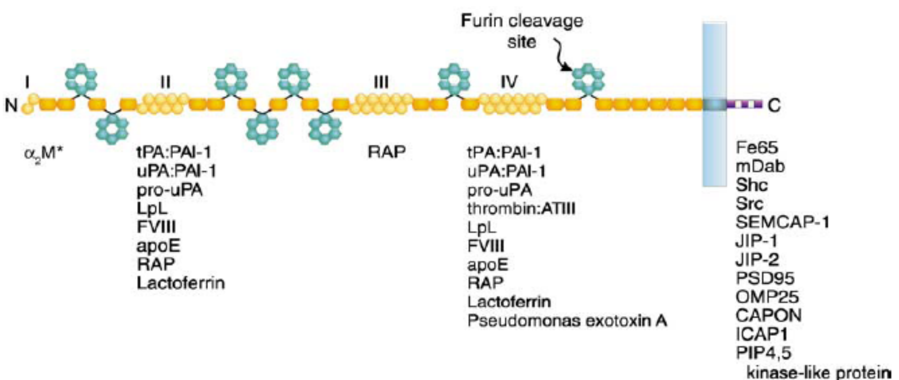


Fig. 3 Domain organization of LRP1. LRP1 contains four ligand-binding clusters with different affinity for distinct ligands as described in the text (adapted from Strickland and Ranganthan, 2003).

1.3.2.4.2 Interactions between LRP1 and intracellular adaptor proteins

Not only does the extracellular domain of LRP1 bind a multitude of biologically diverse ligands, but there is now also an increasing number of cytoplasmic proteins that have been found to interact with the intracellular domain of LRP1 (Table 5) [Herz et al., 2001;Su et al., 2002;Petersen et al., 2004].

Table 5 Adapter and scaffold proteins that bind to the intracellular domain of LRP1 (adapted from Herz and Strickland, 2001, Su et al. 2003, Petersen et al., 2004)

Cytoplasmic ligands	**Implied function**
Diasble-1 (Dab-1)	Activation of Src family kinases, neuronal migration
FE65	App processing, actin remodeling
SEMCAP-1	Axon guidance, vesicular transport
JIP1	Regulation of MAPK and SAPK, including JNK
JIP2	
PSD-95	Scaffolding protein of the postsynaptic density, coupling to NMDA receptors
Talin-like protein	Coupling to actin cytoskeleton
OMP25	Mitochondrial transport
CAPON	Regulation of nitric oxide synthase
PIP4,5 kinase-like protein	Regulation of inositol signaling
ICAP1	Integrin-mediated signaling?
Shc	Ras activation
GULP/CED-6	clearance of apoptotic cells
MafB	hindbrain development

1.3.2.4.3 Potential role of LRP1 in Alzheimer disease

LRP1 also serves as a receptor mediating the clearance of proteins associated with Alzheimer's disease, such as amyloid precursor protein (APP), apoE and α_2M [Beisiegel et al., 1989;Kristensen et al., 1990;Strickland et al., 1990;Hussain et al., 1991;Kounnas et al., 1996]. Single APP gene gives rise to many isoforms due to alternative RNA splicing, among which APP770, 751 and 695 are the major isoforms in the brain. These isoforms are transmembrane proteins; they can be cleaved within their extracellular domain. APP 770 and 751, which contain the Kunitz proteinase inhibitor (KPI) domain, have been shown to be internalized by LRP1 [Kounnas et al., 1995], whereas APP695, lacking this domain, is a poor ligand to LRP1 but believed to be the major source of amyloid β-peptide (Aβ) in brain [Kang et al., 1990;Wertkin et al., 1993]. Once synthesized, APP is processed by two different proteolytic pathways, leading either to the cell surface exposure or to the extracellular space releasing. The amyloidogenic pathway of APP processing, and Aβ production can take place intracellularly in the secretory compartments, or following internalization of cell surface APP by the endocytic pathway [Koo et al., 1994;Cook et al., 1997;Hartmann et al., 1997;Skovronsky et al., 1998]. Most of studies are focused on the interaction between LRP1 and APP 770 or 751 to elucidate the role of LRP1 in Alzheimer's disease.

LRP1 plays dual role in the process of Alzheimer's disease. LRP1 can increase Aβ level, which is generated in endosomal compartments upon internalization of cell surface APP by LRP1 [Ulery et al., 2000]. On the opposite, LRP1 also decreases Aβ level by two

different mechanisms. It mediates the degradation of Aβ in complex with apoE or α_2M [Kang et al., 2000;Shibata et al., 2000]. It also interacts with KPI domain of APP751 therefore favors both APP751 internalization and degradation [Kounnas et al., 1995;Knauer et al., 1996]. However the latter mechanism was challenged by the recent observation by Pietrzik et al. They reported that LRP1 regulated different steps of APP processing, including APP secretion, internalization and Aβ production independent of KPI domain of APP. This function involves the interaction between intracellular adaptor protein FE65 and the cytoplasmic domain of LRP1 [Pietrzik et al., 2002]. Based on these observations, these authors proposed that the interaction between LRP1 and KPI domain of APP might play a role in signaling transduction rather than in endocytosis.

1.3.2.4.4 LRP1 function in neurotransmission

Long term potentiation (LTP) is one of the best models for investigating cellular and molecular mechanisms involved in the strength and stability of synaptic connections, thus approaching issues in the formation and storage of memory [Nicoll et al., 1995]. LTP has two distinct phases: early and late-phase LTP. In contract to the early-phase LTP, the late-phase LTP requires gene transcription, new protein synthesis, activity of cAMP-dependent protein kinase A (PKA) [Nguyen et al., 1994;Schuman, 1997]. It is also known that the induction of LTP requires a calcium influx through NMDA receptor [Malenka, 1991].

Tissue-type plasminogen activator (tPA) has been shown to contribute to activity dependent synaptic plasticity in the hippocampus and cerebellum, and LTP is significantly decreased in mice lacking tPA [Carmeliet et al., 1994;Frey et al., 1996;Huang et al., 1996]. One mechanism has been proposed that tPA may modulate intracellular signaling events such as calcium influx and PKA activation [Zhuo et al., 2000]. As LRP1 is the major receptor binding tPA in hippocampal neurons, the role of LRP1 in LTP was worth exploring. In fact, binding of tPA to LRP1 enhances the activity of PKA, a key player in later-phase LTP [Roberson et al., 1996;Abel et al., 1997]. Moreover, RAP blocks the enhancing synaptic potentiation induced by exogenous tPA in hippocampal slices prepared from tPA knockout mice [Zhuo et al., 2000]. So the interaction between tPA and LRP1 is likely to initiate intracellular signal transduction, including an increase in PKA activity, which in turn regulates late-phase LTP.

In the same context, it is intriguing that LRP1 is implicated in the activation of NMDA receptor and modulation of the calcium influx. tPA cleaves NR1 subunit of NMDA

receptor causing an increase in the sensitivity to NMDA receptor agonists [Nicole et al., 2001]. Second, α_2M, associated with neuronal LRP1, alters NMDA receptor-mediated Ca^{2+} influx [Bacskai et al., 2000;Qiu et al., 2002]. This interaction is likely to occur via the multivalent scaffold protein PSD-95, because PSD-95 associates with NMDA receptor as well as with the cytoplasmic domain of LRP1 [Gotthardt et al., 2000]. The Ca^{2+} influx due to LRP1-mediated activation of NMDA receptor channels may provide a mechanism of altering local synaptic plasticity.

1.3.2.4.5 Role of LRP1 in cell adhesion and migration

LRP1 contributes to the regulation of cell adhesion and migration due to its function on endocytosis and signaling transduction.

LRP1 mediates the internalization of cell surface uPAR [Conese et al., 1995], uPA-plasminogen activator inhibitor (PAI) complex [Nykjaer et al., 1992], and fibronectin [Salicioni et al., 2002], all of which play an important role in cell adhesion and migration. Consequently LRP1 regulates cell surface concentration of these proteins and the cell signaling they are triggering. In mouse embryonic fibroblasts and HT 1080 fibrosarcoma cells, loss of LRP1 expression results in increased uPA accumulation in the medium, increased uPAR on the cell surface, and increased cell migration on vitronectin [Weaver et al., 1997;Webb et al., 2000]. Furthermore, LRP1 deficient mouse embryonic fibroblasts display higher Rac1 activity, shown to be the consequence of accumulated uPAR on the cell surface [Ma et al., 2002]. LRP1 also suppresses cell signaling to ERK by binding free uPA, thus decreasing the free uPA available to interact with uPAR [Webb et al., 2000]. *In vivo* LRP1 is weakly detected in prostates with adenocarcinomas and undifferentiated carcinomas, whereas a significantly higher level of uPAR expression is observed. This indicates an inverse relationship between the expression of LRP1 and the increased activation of plasminogen activators detected in cancers [Gilardoni et al., 2003]. Hence LRP1 provides an indirect mechanism to regulate migration related cell signaling by controlling the concentrations of cell surface ligands and receptors, but not by participating in the actual signaling events.

LRP1 also regulates PDGF signaling and its effect on migration, which requires both endocytic and signaling functions. PGDF-induced vascular smooth muscle cell (SMC) migration and proliferation is a critical step during the formation of atherosclerotic lesions [Ross, 1993]. The role of LRP1 in SMC migration is supported by the observations that RAP and anti-LRP1 antibody inhibit SMC migration, and that apoE, inhibits both PDGF

stimulated proliferation and migration in SMC. The latter effect requires the apoE association with LRP1, as apoE does not affect SMC proliferation and migration when LRP1 is absent, indicating that endocytic function of LPR1 is involved [Swertfeger et al., 2002].

Recently it has been reported that LRP1 mediates cell adhesion by co-operating with other adhesion molecules. It promotes integrin β1 maturation and transport to the cell surface [Salicioni et al., 2004]. It co-localizes with integrin αMβ2, and down-regulation of LRP1 expression abrogates the integrin β2 mediated cells adhesion, indicating cooperation between these two molecules [Spijkers et al., 2005]. LRP1 also functions as co-receptor for membrane-anchored receptor, like calreticulin, which directs focal adhesion disassembly. Blockage of LRP1 activity results in dysfunctional focal adhesion disassembly and cell adhesion [Orr et al., 2003].

1.3.2.4.6 Phosphorylation of LRP1: regulation of endocytosis and signal transduction

As discussed in the previous section, LRP1 executes both endocytic and signaling transducing functions. The interesting question would be: how is this regulated? To answer this question, many studies are focused on the phosphorylation of the intracellular domain of LRP1. LRP1 can be phosphorylated on tyrosine residues [Barnes et al., 2001], which provide a docking site for adapter proteins, such as Shc containing phosphotyrosine binding domain or a carboxyl-terminal Src homology domain. These adapter proteins are involved in signaling cascades induced by protein tyrosine kinases.

In this context LRP1 tyrosine phosphorylation has shown great importance in PDGF-initiated signaling. It is known that PDGFBB induces the tyrosine phosphorylation of LRP1 on the second NPxY motif in the cytoplasmic tail; this effect requires PDGF receptor-β, Src tyrosine kinase and PI3 kinase [Boucher et al., 2002;Loukinova et al., 2002]. Mice lacking LRP1 in vascular SMC under LDLR-/- background display hypersusceptibility to develop atherosclerosis, accompany by hyperactivation of PDGF pathway, over-expression of PDGF, and an increase of phospho-PDGF receptor. It has been proposed that in the absence of LRP1, PDGF binds to its own receptor PDGF receptor-β and activate signaling cascades leading to SMC proliferation and migration (Fig. 4A). In the presence of LRP1 and apoE, PDGF binds to both LRP1 and PDGF receptor-β leading to the tyrosine phosphorylation of NPxY motif in the cytoplasmic tail of LRP1, which is blocked by apoE. Thus really justifies here unphosphorylated LRP1

functions as endocytic receptor to reduce the extracellular PDGFBB by endocytosis and degradation, preventing PDGF-dependent vascular SMC migration and proliferation (Fig. 4B). In the presence of LRP1 but absence of apoE, LRP1 undergoes tyrosine phosphorylation upon PDGFBB stimulation, phosphorylated LRP1 interacts with Shc adaptor protein and favors to the development of atherosclerotic lesion (Fig. 4C) [Boucher et al., 2003].

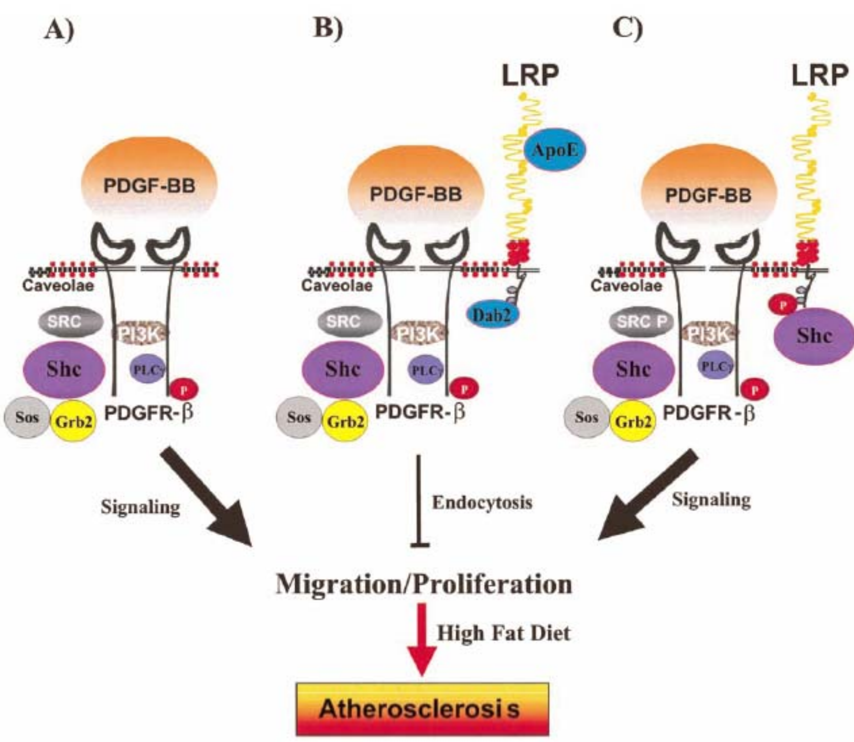


Fig. 4 Model for regulation of PDGF signaling and suppression of atherosclerosis by functional switch of LRP1. (adapted from Boucher 2004).

The example described above indicates that the regulation of LRP1 function involves the amino acid specific phosphorylation of LRP1 in its cytoplasmic domain, especially within the NPxY motifs. LRP1 has also been shown to be phosphorylated in its cytoplasmic domain at serine 73 and serine 76 by cAMP dependent, serine/threonine specific protein kinase PKA. Inhibition of PKA activity leads to a decrease of LRP1 phosphorylation and LRP1-mediated endocytosis, so does the mutation on site serine 76

of LRP1 [Li et al., 2001]. It was thus proposed that serine phosphorylation regulates LRP1-mediated internalization, whereas tyrosine phosphorylation is involved in signal transduction.

However, in a more recent finding, LRP1 was shown to be phosphorylated on serine, threonine and tyrosine residues within its cytoplasmic domain by PKCα. Interestingly mutations of these serine and threonine residues lead to inhibition of phosphorylation of LRP1, and to more rapid internalization rate. This result reveals that phosphorylation reduces the association of LRP1 with adapter proteins of the endocytic machinery, such as Disable-1. Furthermore it has been shown that serine and threonine phosphorylation is necessary for the interaction of LRP1 with Shc, and increases the interaction of LRP1 with other adapter proteins, such as Disable-1 and CED-6/GULP [Ranganathan et al., 2004]. It is likely that the state of LRP1 phosphorylation on serine and threonine residues not only regulates its endocytic function, but also influences the phosphorylation of tyrosine residues, thus as well regulating its impact on signal transduction. Therefore the functional switch of LRP1 seems to be far more complicated than it was originally thought.

1.4 Heparan sulfate proteoglycan

Proteoglycans are proteins substituted with one or more glycosaminoglycans (GAG), which generally either fill the extracellular space or attach to the cell surface. They can be present as membrane-bound or glycosylphosphatidylinositol (GPI)-linked to the cell membrane [Iozzo, 1998;Bernfield et al., 1999]. They act as tissue organizers, influencing cell growth and the maturation of specialized tissues. They play a role as biological filters and modulate growth factor activities, regulate collagen fibrillogenesis and skin tensile strength. They also affect tumor cell growth and invasion, influence corneal transparency and neurite outgrowth [Iozzo, 1998].

The GAG chains consist of long, unbranched, highly negatively charged, repeated disaccharides that are modified by sulfation and epimerization during synthesis in the Golgi. They are covalently attached to a core protein through a short polysaccharide linker. GAGs are extremely well conserved structures, indicating specific, essential roles in biology [Williams et al., 1997].

There are four classes of GAGs that attach to proteoglycan core proteins: heparan sulfate (HS)/heparin, chondroitin sulfate (CS), dermatan sulfate and keratan sulfate (Fig. 4) [Prydz et al., 2000]. However, proteoglycans appear primarily attached with HS side

chains, the most ubiquitous cell surface GAG, recognized as heparan sulfate proteoglycan (HSPG).

GAG	Hexuronic or Iduronic acid	Galactose	Hexosamine	Disaccharide composition
Heparan sulphate/ Heparin	D-glucuronic acid **(GlcA)** L-iduronic acid **(IdoA)**	-	D-glucosamine **(GlcNAc)**	**GlcA β(1→4) GlcNAc α((1→4)** **IdoA α(1→4) GlcNAc α(1→4)**
Keratan sulphate	-	Galactose **(Gal)**	D-glucosamine **(GlcNAc)**	**Gal β(1→4) GlcNAc β(1→3)**
Chondroitin sulphate	D-glucuronic acid **(GlcA)**	-	D-galactosamine **(GalNAc)**	**GlcA β(1→3) GalNAc β(1→4)**
Dermatan sulphate	D-glucuronic acid **(GlcA)** L-iduronic acid **(IdoA)**	-	D-galactosamine **(GalNAc)**	**IdoA β(1→3) GalNAc β(1→4)**

Fig. 5 Structure of different GAG chains attached to the HSPG core proteins. The red dotted rectangles marks different sulfation positions in each GAG (adapted from Prydz 2000).

HSPG family comprises primarily two subfamilies of proteins: the transmembrane syndecans and the GPI-linked glypicans. Other cell surface core proteins, including betaglycans and CD44s, have HS chains attached to their extracellular domains, but HS chains attaching to these HSPGs have been thought to have a less important role in the interactions with ligands [Kramer et al., 2003]. There are also matrix HSPGs, like perlecan and agrin, which are secreted and present in the basement membrane [Iozzo, 1998].

Cell surface HSPGs provide highly complex and sophisticated systems to control interactions of ECM components and soluble ligands with the cell surface. They bind to extracellular proteins and form signaling complexes with receptors, therefore regulating their occupancy and response. They also immobilize proteins on the cell surface and mediate protein internalization. The outcome of these interactions depends on whether the ligand is soluble (i.e. growth factor, cytokine) or insoluble (i.e. ECM components), whether it also interacts with a signaling receptor, or binds to the HS chains or the core proteins only [Bernfield et al., 1999].

Binding of cell surface HSPGs to insoluble ligands, such as ECM components, immobilizes HSPGs at the membrane and enables them to interact with actin cytoskeleton, thus mediating the cell-cell and cell-matrix adhesion. For instance, they interact with fibronectin or integrin to mediate focal adhesion formation and cell spreading [Midwood et al., 2004;Beauvais et al., 2004a]. The pattern and timing of HSPGs expression are correlated with the requirement of ECM accumulation or the interaction with ECM at or during specific development stages [Sutherland et al., 1991]. Cell surface HSPGs also act as the co-receptors for soluble ligands like FGF, presenting FGFs at sufficiently high concentration to its receptors, hence regulating FGF signaling and the relevant downstream events such as angiogenesis, wound healing or tissue development [Nugent et al., 2000]. In addition, cell surface HSPGs act as internalization receptor, regulating the concentration of cell surface receptors or ligands and providing a secondary signaling to the cells. Even the shed ectodomain of HSPGs contributes to regulation of ligands activities and the corresponding cell responses [Bernfield et al., 1999].

Most known HSPG functions depend on the interactions between HS chains and their protein ligands. Cell surface HS chains bind to numerous proteins in cellular microenvironment, such as morphogens (e.g. Shh, Wnts), ECM proteins (e.g. fibronectin, vitronectin and laminin), tissue remodeling factors (e.g. uPA PAI-1 and PN-1), growth

factors (e.g. EGF, FGF), cell adhesion molecules (e.g. N-CAM), lipoproteins (e.g. apoB, apoE), lipoprotein lipases, chemokines and cytokines [Bernfield et al., 1999] among other extracellular constituents. The core proteins of HSPGs were considered as much less important for HSPG functions. Nonetheless, Drosophila, Zebra fish, Xenopus and mouse lacking a specific HSPG core protein show a phenotype, even though other core proteins are present, indicating a functional specificity that is required, and this can not be compensated during distinct developmental stages [Kramer et al., 2003].

Cell surface HSPGs can be regulated in at least two different ways. All syndecans can be shed from cell surface [Kim et al., 1994;Spring et al., 1994], an event converting the HSPG from an activator to a potent inhibitor [Lopez-Casillas et al., 1994;Kato et al., 1998]. Localization of HSPG to restricted regions of the cell surface is another mechanism to regulate and focalize HSPG function [Kramer et al., 2003].

1.4.1 Syndecan family

In vertebrates the mammalian syndecan family counts four transmembrane proteins carrying HS and CS chains. Syndecans are expressed in a cell and tissue specific manner during development; their expression is also modified pathologically [Bernfield et al., 1992]. Every mammalian cell expresses at least one type of syndecan. 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, whereas syndecan-4 is found in virtually every cell type [Couchman, 2003].

Each syndecan has an extracellular domain with attachment sites for three to five HS or CS chains, a single-span transmembrane domain, and a short intracellular domain. The extracellular domain bears little primary sequence homology, yet all can be proteolyticly cleaved at a site near the transmembrane domain. The transmembrane and cytoplasmic domains are highly homologous, except for a short variable region in the center of the cytoplasmic domain (Fig. 6) [Rapraeger, 2001]. HS is predominantly attached to syndecans, typically positioned near the amino terminus, allowing interactions with a large number of proteins as described in previous section. The role of CS chains is less clear. A recent study suggested that, in syndecan-1 and syndecan-4, CS chains cooperate with HS chains in the binding of growth factors or laminin [Okamoto et al., 2003;Deepa et al., 2004].

The syndecans modulate the interaction between cells and their environment. They participate in multiple cell behaviors such as growth, adhesion, migration, differentiation, and apoptosis, hence playing essential roles in embryonic development, tumorigenesis and angiogenesis. Their functions depend not only on molecular interactions between syndecan core proteins and cytoskeletal or signaling molecules, but also on binding specificities of their HS chains to extracellular ligands.

1.4.1.1 HS synthesis: generating specific HS binding sites

The fine structures of HS chains begin with the generation of a tetrasaccharide (xylose-galactose-galactose-glucuronic acid) that is covalently attached to serine residues within the core proteins. This tetrasaccharide serves as the connection for all four GAG classes. In the case of HS, N-acetylglucosamine and glucuronic acid are sequentially added in an alternating fashion to generate the disaccharide repeat. The disaccharide chain can be modified in different ways, including sulfation at the N, 3-O or 6-O position of the N-acetylglucosamine, deacetylation of the glucosamine, epimerization of glucuronic acid to iduronic acid, and sulfation at the 2-O position of uronic acid. The combination of these six modifications lead to an extraordinary level of chemical diversity in the pattern of sulfation within HS, which in turn determines the ligand binding specificity.

The specific binding affinity of HS chains is determined by the pattern of sulfotransferases expressed within the Golgi. In the Golgi, domains of HS chains with binding specificity are generated by regulating the transition of GAG substrates through distinct combination of sulfotransferase isoforms [Zako et al., 2003]. It has recently been shown that the amino acid sequence of HS attachment side in the core protein may also play a role in determining HS binding specificity [Nedvetzki et al., 2003]. Consequently when HSPG is transported from the Golgi to the cell surface; the final HS chain has domains of contiguous disaccharides containing N-sulfation, O-sulfation and unmodified domains. In general, specific ligand binding site is either a rare sulfation like 3-O sulfation of glucosamine, like for antithrombin III, or a specific sulfation pattern like 2-O, 6-O and N-sulfation, which are organized into a specific binding domain for FGF-1 and FGFR-1 [Wu et al., 2003].

HS chains serve as a multi-functional regulator of protein activities through different mechanisms. For instance, they serve to simultaneously bind both proteases and cognate inhibitors, such as thrombin and PN-1, thus bringing them together in an

appropriate orientation to accelerate the inhibitory interaction [Scott et al., 1985;Gettins, 2002]. Alternatively they bind to protease inhibitors and induce their conformational change, therefore enhancing the interaction between protease inhibitors and proteases, as for antithrombin III and thrombin or Factor Xa [Olson et al., 1992;Gettins, 2002]. As described before, they also act as co-receptor for FGFR signaling pathway, increasing cell surface localization and concentration of FGF [Turnbull et al., 2001].

1.4.1.2 Syndecan core proteins

Syndecan core proteins contain at least six functional domains (Fig. 6). The extracellular domain is among the most rapidly diverging vertebrate proteins with the exception of their regions for HS attachment. The transmembrane domain is relatively stable, only a few amino acids being different among the vertebrate syndecan sequences. This domain contains the regions for interaction with the other membrane proteins and for localization to distinct membrane compartments. The cytoplasmic domain contains two conserved regions, a membrane proximal common region (C1) containing a serine and a tyrosine, and a C-terminal common region (C2). These two regions are separated by a region (V) with various length and composition. The C1 domain is thought to be involved in syndecan dimerization (all syndecans probably exist as homodimers and high-order oligomers) and in binding of intracellular proteins, such as Src kinase, tubulin and ezrin [Kinnunen et al., 1998;Granes et al., 2000;Granes et al., 2003]. The C2 domain with EFYA motif is the binding site for PDZ containing proteins [Bernfield et al., 1999;Beauvais et al., 2004b].

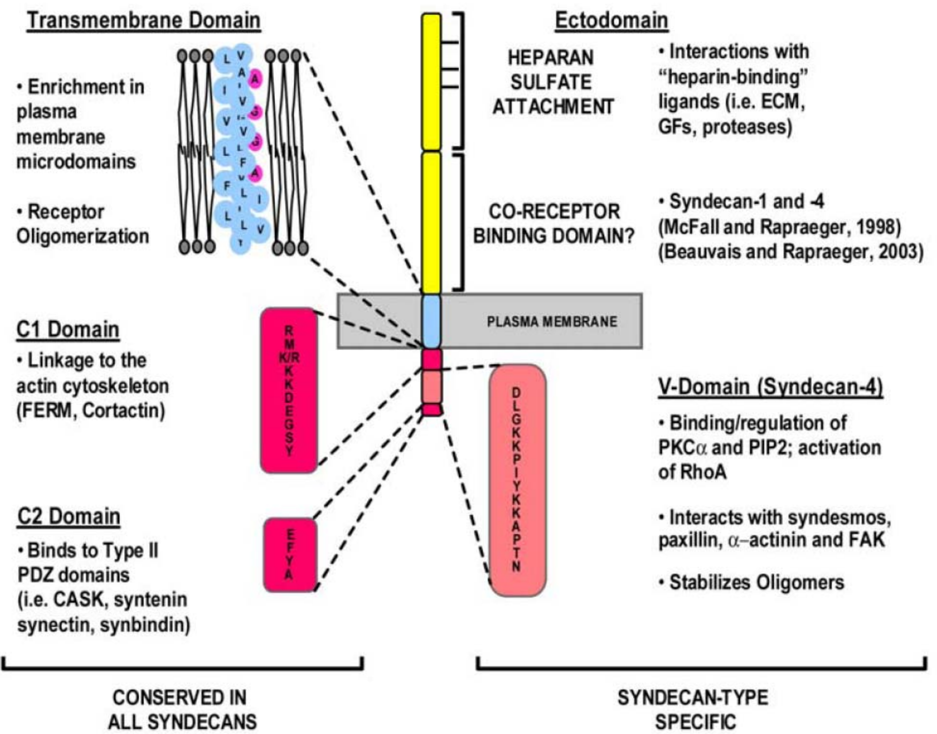


Fig. 6 Functional domains of syndecan core protein, (adapted from Beauvais and Rapraeger, 2004)

1.4.1.3 HSPGs-mediated internalization

1.4.1.3.1 HSPGs function in lipoprotein metabolism as co-receptor

Early work indicated that lipoprotein lipase (LpL) could bridge between lipoproteins and endothelia HSPGs, thereby mediating cell surface attachment of the particles [ROBINSON, 1963]. Later on, this LpL bridge effect was shown to enhance not only the binding but also the internalization and degradation of chylomicrons, VLDL and protein-free emulsions [Eisenberg et al., 1992;Rumsey et al., 1992;Williams et al., 1992;Mulder et al., 1993]. Additional bridge molecules have been found, including apoE and hepatic lipase [Ji et al., 1993;Ji et al., 1994a]. Because lipoproteins enriched in LpL, apoE and

hepatic lipase can bind LDLR family members, it was proposed that HSPG-bound ligands are not internalized directly by the HSPGs, but first required a transfer to the LDLR family members, thus HSPGs function as co-receptor, which facilitate the internalization by their high capacity of ligand binding.

1.4.1.3.2 HSPGs-mediated ligand internalization

HSPGs also mediate LDLR- and LRP1-independent internalization of lipoproteins and lipoprotein lipases [Williams et al., 1992;Fernandez-Borja et al., 1996;Al Haideri et al., 1997;Seo et al., 1997;Llorente-Cortes et al., 2002]. Among those syndecan-1 and perlecan have been identified as independent endocytic receptor in lipoprotein metabolism [Fuki et al., 1997;Fuki et al., 2000a].

Syndecan-1 can directly mediate lipoprotein catabolism. This event is characterized as an endocytic process triggered by syndecan-1 clustering upon ligand binding, involving lipid rafts, requiring tyrosine kinase activity to phosphorylate its cytoplasmic domain, and the association with actin microfilaments. The kinetics of this endocytosis is significantly slower (with $t_{½}$=1h) than that of the LDLR family-mediated internalization [Fuki et al., 1997;Fuki et al., 2000b].

This LDLR-independent pathway of lipoprotein catabolism substantially contributes to the following: 1) chylomicron remnant catabolism in the absence of LDLR [Ishibashi et al., 1994;Mortimer et al., 1995]; 2) LDL clearance, about one-third of which is LDLR-independent in normal human [Goldstein et al., 1977;Kesaniemi et al., 1983]; and 3) arterial lesion development, which is accelerated in the absence of LDLR. Several *in vivo* studies have implicated HSPG-mediated catabolism of lipoproteins. Poor binding of apoE mutants to HS but not to LDLR or LRP1, has been correlated with dominantly inherited forms of type III hyperlipodemia [Ji et al., 1994b;Mann et al., 1995]. Destruction of hepatic HS side chains *in vivo* impairs chylomicron clearance [Ji et al., 1995;Mortimer et al., 1995;Windler et al., 1996]. The clearance of large postprandial remnants appears particularly HS-dependent and LDLR-independent [Windler et al., 1996].

1.4.1.4 Regulation of cytoskeletal organization by syndecans

The extracellular matrix (ECM), in cooperation with growth factors and cytokines, provides cells with a variety of key signals in different physiological and pathological processes, such as proliferation, migration, adhesion, differentiation, and death

[Giancotti et al., 1999]. In particular, the ECM regulates cell morphology through specific plasma membrane receptors triggering signaling events to reorganize cytoskeleton and generate cell polarity. HSPGs have been shown to cooperate with integrins and to regulate cell shape and cytoskeleton assembly [Kusano et al., 2000;Kusano et al., 2004]. The interaction between HSPGs and ECM or growth factors is not only mediated by the HS chains but also by the core proteins [Bernfield et al., 1999].

1.4.1.4.1 Syndecans in signaling transduction and cytoskeleton organization

Several recent studies supported that syndecan-1 is directly involved in signal transduction instead of being just a co-receptor modulating cellular signaling. Syndecans have been shown to interact with syntenin, a PDZ protein binding to the EFYA motif of the cytoplasmic domain of syndecan [Grootjans et al., 1997]. Syntenin is localized at cell adhesion sites and microfilaments, and found to form complex with syndecan-1, E-cadherin, β-catenin at cell-cell contacts. This suggests that it functions as an adaptor to couple syndecan to cytoskeletal proteins or cytosolic downstream signaling effectors [Zimmermann et al., 2001]. Furthermore, it has been shown that integrin αvβ3 and syndecan-1 are functionally coupled. The integrin αvβ3 needs syndecan-1 to become activated and to mediate signals required for cell spreading on vitronectin in human mammary carcinoma. The coupling of syndecan-1 to integrin αvβ3 requires the engagement of syndecan-1 ectodomain [Beauvais et al., 2003;Beauvais et al., 2004a]. This integrin has been associated with ERK signaling in NIH 3T3 fibroblasts [Roberts et al., 2003]. Interestingly syndecan-2 and 4 as well as a truncated syndecan-1 ectodomain have been reported to influence ERK signaling [Viklund et al., 2002;Utani et al., 2003;Chen et al., 2005;Rauch et al., 2005].

Intensive studies have been carried out to further explore the role of HS chains or syndecan core protein in cytoskeleton rearrangement. Ectopic expression of syndecan-1 enables the syndecan-1 deficient Raji-S1 cells to bind and spread on thrombospodin or fibronectin. This effect is not affected by HS removal or by truncation of cytoplasmic domain of syndecan-1 [Lebakken et al., 1996]. Furthermore, the truncation of the syndecan-1 extracellular domain does not affect the initial lamellipodial extension in Raji-S1 cells, but it does inhibit the active membrane ruffling that is necessary for cell polarization. [McQuade et al., 2003]. This suggests that the ectodomain of syndecan-1

has important functions in dynamic cytoskeletal rearrangements, which are independent of, but most likely supplemented by its attached HS chains.

Similar to the observations made in Raji-S1 cells, ectopic expression of syndecan-1 in Schwann cells enhances cell spreading on fibronectin and laminin, reorganization of microfilaments, and focal adhesion formation. Syndecan-1 transiently colocalizes with actin filaments only during cell spreading. Interestingly this effect also requires a 12 amino acid segment within its cytoplasmic domain, especially the tyrosine residue in this region [Carey et al., 1996].

It has been shown that syndecan-1 stimulates Cos-7 cells spreading, fascin spike assembly, and extensive protrusive lateral ruffling on thrombospodin-1. The presence of GAG chains at Ser^{45} or Ser^{47} of the extracellular domain is required for syndecan-1-mediated cell membrane spreading, whereas the V and C2 regions of the cytoplasmic domain are crucial for spreading and fascin structures formation [Adams et al., 2001]. Fascin spike assembly depends on the maintenance of a pool of the non-phosphorylated fascin and the regulation of Rac and Cdc42 small GTPase activities [Adams et al., 2000]. This suggests that syndecan-1 regulates the phosphorylation of fascin, and possible small GTPase activity. Taken together, these data indicate that the role of HS chains and core protein in syndecan-1 mediated-cell spreading is cell-type specific.

The other members of syndecan family also regulate signal transduction and actin cytoskeleton in various cell types. Syndecan-2 is specifically localized on the actin-rich dendritic spines [Halpain, 2000] and promotes the morphological maturation of spine, which requires the PDZ binding motif and the phosphorylation of syndecan-2 by EphB2 receptor tyrosine kinase [Ethell et al., 2001]. Syndecan-2 induces filopodia by a Cdc42-mediated mechanism in fibroblasts [Granes et al., 1999], regulates focal adhesion and stress fiber formation in carcinoma cells [Munesue et al., 2002]. Syndecan-4 induces focal adhesion and stress fiber formation in fibroblasts [Saoncella et al., 1999;Woods et al., 2000]. The molecular mechanism by which syndecan-4 influences both morphology and migration requires its cytoplasmic domain [Longley et al., 1999;Tumova et al., 2000]. In connection to these functions, syndecan-4 is involved in direct transmembrane signaling events, such as the activation of PKCα [Oh et al., 1997] which phosphorylates its cytoplasmic domain [Horowitz et al., 1998a;Horowitz et al., 1998b], the interaction with PDZ protein [Grootjans et al., 1997], the phosphorylation of focal adhesion kinase, and the activation of Rho proteins [Saoncella et al., 1999;Wilcox-Adelman et al., 2002].

1.4.1.4.2 Syndecans in tumor metastasis

Syndecans mediate cell adhesion and control the activities of factors influencing cell growth and motility; they play a critical role in regulating the metastasis behavior of tumor cells by promoting tight cell-cell and cell-ECM adhesion.

In this context syndecan-1 is the most widely studied among all the HSPG family members. Syndecan-1 is highly expressed at the basolateral surface of epithelial cells where it is thought to interact with actin cytoskeleton and to modulate cell adhesion and growth factor signaling [Rapraeger et al., 1986;Sanderson et al., 1988;Kim et al., 1994]. Syndecan-1 expression level at cell surface is correlated with metastasis potential and with survival in a range of epithelia tumors [Inki et al., 1994;Matsumoto et al., 1997;Nackaerts et al., 1997;Kumar-Singh et al., 1998;Stanley et al., 1999]. Loss of syndecan-1 expression induces epithelial-mesenchymal transformation, anchorage-independent growth and increasing motility [Leppa et al., 1991;Leppa et al., 1992;Kato et al., 1995], which is associated with the loss of E-cadherin expression, indicating they are working in concert [Day et al., 1999]. Over-expression of syndecan-1 in transformed S115 cells, in which loss of epithelial morphology is due to syndecan-1 down regulation, restores their epithelial morphology and growth characteristics [Leppa et al., 1992]. Ectopic expression of syndecan-1 in syndecan-1 deficient myeloma cells has the striking effect of reducing invasion; this effect requires its extracellular domain [Liu et al., 1998]. The invasion regulatory domain is further identified as 26 amino acids starting of the transmembrane domain. Importantly, this domain is functionally specific because its mutation does not affect syndecan-1-mediated cell binding to collagen, syndecan-1-mediated cell spreading, or targeting syndecan-1 to specific cell surface domains [Langford et al., 2005]. These data indicate that syndecan-1 is required for maintaining epithelial morphology and behavior, and that the loss of syndecan-1 expression or function may be a prerequisite for tumor cell invasion.

However, in contrast to the general notions that syndecan-1 may be an inhibitor of carcinogenesis; syndecan-1 also demonstrates tumor promoter function. For instance, mammary gland-specific expressed Wnt-1 leads to the tumorigenesis in wild type mice but not in the syndecan-1 deficient mice [Alexander et al., 2000]. Enhanced syndecan-1 expression has been observed in pancreatic, gastric and breast carcinomas, and this over-expression correlates with tumor aggressiveness and poor clinical prognosis [Stanley et al., 1999;Conejo et al., 2000;Wiksten et al., 2001;Burbach et al.,

2003;Barbareschi et al., 2003]. This duality in the role of syndecan-1 in tumorigenesis may reflect tissue or tumor stage specific function.

Both syndecan-2 and syndecan-4 are up regulated in different tumors [Roskams et al., 1998;Park et al., 2002;Gulyas et al., 2003]. Syndecan-2 over-expression in colon carcinoma induces a rounded adhesion phenotype and piling-up of cells in culture [Park et al., 2002;Kim et al., 2003], which also correlates with an invasive phenotype [Contreras et al., 2001]. Syndecan-2 has also been implicated in fibronectin assembly, focal adhesion formation and migration. Reduced syndecan-2 expression leads to failure of focal adhesion and stress fibers formation on fibronectin in a rat Lewis lung carcinoma derived cell line. Increasing the expression of syndecan-2 in this cell line can restore their ability to assemble focal adhesion [Kusano et al., 2000;Munesue et al., 2002], indicating that syndecan-2 plays a role in the signaling that relays adhesion to fibronectin into cytoskeletal events.

1.5 Serine protease inhibitors

1.5.1 Serine protease

Serine proteases comprise almost one third of all proteases found in the nature. This mechanistic class of enzymes is originally identified by the presence of three residues, aspartate, histidine and serine in their catalytic sites. This catalytic site can be found in at least four different structural contexts, thus defining four clans of serine proteases as chymotrypsin, subtilisin, carboxypeptidase Y and the Clp protease.

Serine proteases such as thrombin, tPA, uPA and plasmin belong to the chymotrypsin –like serine protease. Because of their abilities to cleave a wide range of substrates, they are involved in many critical physiological processes including digestion, hemostasis [Neurath, 1984], reproduction [Barros et al., 1996], and immune response [Sim et al., 2000]. The sequential activation cascades of serine proteases appear to be involved in developmental events [LeMosy et al., 1999], tissue remodeling [Van den Steen et al., 2001] and would healing [Li et al., 2003]. Furthermore, they also interact, either dependently or independently of their proteolytic activity, with ECM or transmembrane proteins such as vitronectin or intergrin, thus playing important roles in signal transduction which in turn regulate cell proliferation, differentiation [Selvarajan et al., 2001], apoptosis [Johnson, 2000], adhesion, and migration [Reuning et al., 1998].

1.5.2 Extracellular serine proteases and their receptors in cell migration

Cell migration plays a crucial role in a wide range of biological processes, such as embryogenesis during development, immune response during inflammation, wound healing in adult organism, and metastasis in tumorigenesis. A five-step model of cell migration in three dimensions has been established. First of all, cells undergo polarization and become asymmetric in order to convert intracellularly generated forces into net cell body translocation. In the meantime, extension of cell membrane occurs in the cell front and forms structures identified as lamellipodia or filopodia, which are driven by actin polymerization. Second, stable attachments are formed at the leading edges of lamellipodia and filopodia, which are regulated by cdc42, Rac and Rho. Third, cell surface proteases become concentrated for cleavage of ECM and basement membrane components, allowing cell movement. Fourth, cell contraction generated by myosin enables the cell body translocation. Fifth, the adhesion sites in the rear of the cell are released and the cell moves forward [Lauffenburger et al., 1996;Friedl et al., 2003].

An early observation describing that cancer cells continuously dissolve plasma clots in culture, indicates the connection between increase proteolytic activity and cancer [Pollanen et al., 1991]. In fact, following the evidence that much higher amount of uPA is expressed in transformed cells [Unkeless et al., 1974], and that high levels of uPA and uPAR are relevant to poor prognosis, the proteolytic degradation by uPA or plasmin is thought to facilitate tumor cell migration through the basement membrane and interstitial connecting tissues. Therefore it is a surprise that the high level of PAI-1 is used as a marker for poor prognosis in certain cancers [Schmitt et al., 1997]. The most intensive studies on the mechanism by which serine proteases and their inhibitors regulate cell adhesion and migration have been carried out in the uPA, uPAR and PAI-1 system. It has been shown that uPA-uPAR complex promotes migration in different cell types [Gudewicz et al., 1987;Fibbi et al., 1988;Del Rosso et al., 1990], in a way which is independent of uPA proteolytic activity. However, uPA-uPAR complex-induced signaling requires other transmembrane and scaffold proteins for signal transduction and consequent migration events, because uPAR is only a GPI-anchored protein without intracellular domain.

uPAR functions as receptor for both uPA and vitronectin at distinct binding sites [Ploug et al., 1991;Wei et al., 1994;Kanse et al., 1996]. Association of uPAR to immobilized vitronectin activates Rac 1, a small GTPase, which controls events involved

in cytoskeleton remodeling and migration [Kjoller, 2002;Ma et al., 2002]. uPAR is localized in lipid rafts, focal contacts, and at the leading edge of lamellipodia in migrating cells [Pollanen et al., 1988;Estreicher et al., 1990;Stahl et al., 1995]. It assembles a cascade of extracellular proteases which degrade ECM proteins and facilitate penetration of tissue boundaries [Dano et al., 1985]. Upon binding of paracrine or autocrine uPA, uPAR also triggers multiple signaling pathways, including the Ras-ERK pathway, which control cell growth, apoptosis, and cell migration [Ossowski et al., 2000;Webb et al., 2000;Kjoller, 2002]. ERK and its down stream effectors, such as myosin light chain kinase (MLCK), are responsible for the increase in cell migration [Nguyen et al., 1999;Webb et al., 2000].

1.5.3 Serine protease inhibtor-Serpin family

Serpins form a superfamily of proteins, most of which inhibit serine proteases. However serpins that either inhibit cysteine proteases or even lack any protease inhibitory activity have also been identified [Gettins, 2002]. In fact, the membership of this family is based on the presence of a single domain consisting of three β-sheets and eight to nine α-helices, and on the functional properties that result from the special structure of this single domain (Fig. 7).

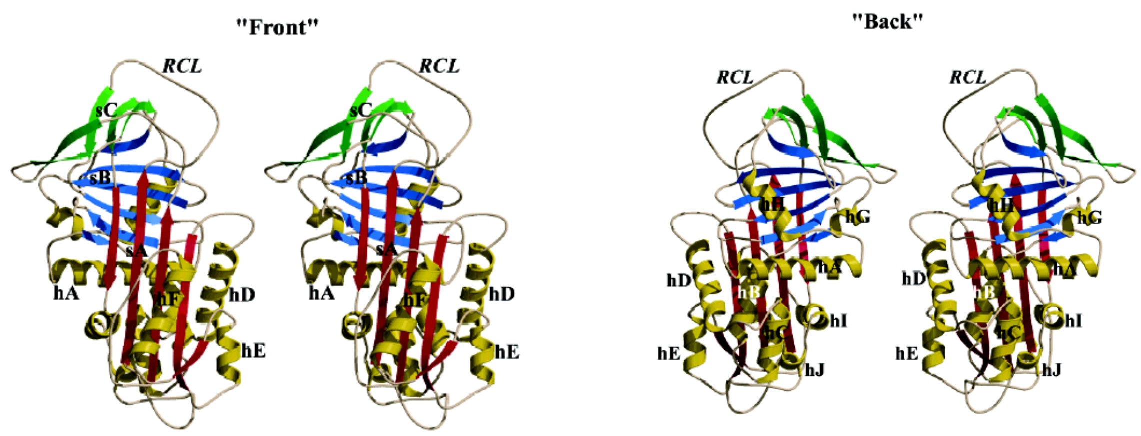


Fig. 7 Front and back stereoviews of a typical serpin. β-sheet A, B, and C are shown in red, blue, and green respectively. The eight α-helices are labeled A through H. RCL: reaction center loop (adapted from Gettins, 2002).

1.5.3.1 Serpin inhibitory mechanism and activity regulation

Serpins serve as suicide substrates to target proteases and form an irreversible and covalent inhibitory complex. The structures of cleaved form of inhibitory serpins have all shown remarkable expansion of β-sheet A, through the insertion of the cleaved reactive center loop as the fifth strand of the sheet, and changing the environment of the reactive center loop from complete solvent-exposed to mostly buried (Fig. 8A,B). The inhibitory mechanism includes the following steps, 1) formation of an initial non-covalent complex, EI, 2) attacking of the active site serine on the peptide bond of the serpin to form intermediate, 3) cleavage of the peptide bond to give a covalent acyl ester intermediate EI' with release of the first product, the free amino group, 4) insertion of the reaction center loop into β-sheet A, translocation of protease and committing the second intermediate to kinetic trapping, E-I*, 5) departure of the second product E+I*. There is an alternative branching pathway at step 4, for the protease to complete the substrate reaction and leave the cleaved serpin behind (Fig. 8C).

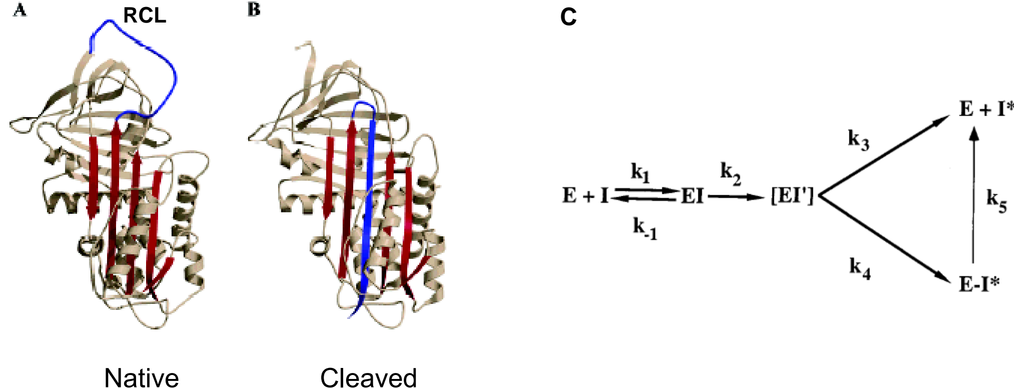


Fig. 8 Comparison of the different conformational states of serpins, the reactive center loop is shown in blue, and the remainder of β-sheet is in red (A, B). Branched pathway mechanism of serpins as suicide substrate inhibitor, as described in the text (C). (adapted from Gettins, 2002)

The activities of serpins can be regulated by different mechanisms. For instance, several serpins found in the blood coagulation and fibrinolysis systems, such as PAI-1 and protease nexin-1 (PN-1) are activated by binding to heparin or other GAGs. The resulting enhancement in the inhibition rate can reach several thousand folds, suggesting an important role for such activation in site-specific regulation. Another example is the regulation of PAI-1 activity by vitronectin. In this case, PAI-vitronectin complex shows much higher inhibitory efficiency and longer half-life. This regulation is physiologically significant since the PAI-1 binding site in vitronectin is close to that of integrin, both tPA and thrombin can spare vitronectin from complex with PAI-1, so that vitronectin is available for integrin interaction, which in turn regulates cell migration [Gettins, 2002].

1.5.3.2 Clearance of serpin-protease complexes

A number of studies have addressed the fate of serpin-protease complexes. Their half-life time in the circulation is shown to be much shorter than that of the native or the cleaved serpins [Ohlsson et al., 1976;Mast et al., 1991], suggesting such rapid removal from circulation is mediated by receptors present on the cell surface. Studies carried out

further revealed that LRP1 is the main receptor responsible for the serpin-protease complexes clearance [Nykjaer et al., 1992;Kounnas et al., 1996]. Nevertheless, in most of the cases, the co-receptor of LRP1, such as HSPG or uPAR, plays an important role in LRP1-mediated internalization [Knauer et al., 1997b;Crisp et al., 2000].

1.5.3.3 Protease Nexin-1

Protease nexin-1 (PN-1) is a 43 KDa glycoprotein of the serpin superfamily [Sommer et al., 1987]. It was first molecularly identified as a protein preventing neurite outgrowth in neuroblastoma cells [Gloor et al., 1986]. It binds and potently inhibits several serine proteases, including thrombin, tPA [Baker et al., 1980], uPA, trypsin [Stone et al., 1987], and Factor XIa [Knauer et al., 2000]. The affinity of PN-1 for thrombin can be dramatically increased upon binding to heparin [Scott et al., 1985].

In vivo PN-1 has a very complex spatial and temporal expression pattern in developing cartilage, lung, skin, urogenital tract, and central and peripheral nervous system, indicating it has tissue and cells type specific functions during development [Mansuy et al., 1993]. *In vitro*, PN-1 is secreted by many different cell types, including fibroblasts, astrocytes, glioma [Guenther et al., 1985], neuroblastoma [Vaughan et al., 1993], astrocytoma [Kasza et al., 2001], and primary Schwann cells [Bleuel et al., 1995]. Following its secretion, PN-1 associates with the ECM by binding to cell surface heparan sulfate [Herndon et al., 1999], vitronectin [Rovelli et al., 1990], and collagen type IV [Donovan et al., 1994].

PN-1 expression can be modulated in response to pathological states. Both *in vitro* and *in vivo* studies have shown that PN-1 expression is either increased [Meier et al., 1989;Bleuel et al., 1995] or decreased [Niclou et al., 1998] following injuries at different never. PN-1 is also up-regulated upon lesion in the substantia nigra [Scotti et al., 1994], and in Alzheimer's diseases [Vaughan et al., 1994;Choi et al., 1995]. Moreover PN-1 has been shown to be over-expressed in metastatic tumor cells, and to improve the transplanted tumor cells migration through an yet unidentified mechanism [Buchholz et al., 2003].

The inhibitory complexes with serine proteases form by PN-1 and its targeted proteases and such complexes are actively removed from the extracellular environment upon internalization via LRP1 [Knauer et al., 1997b;Crisp et al., 2000;Knauer et al., 2000]. In this respect, the level of free PN-1 in fibroblast-conditioned media reaches steady state within 48h due to a constant secretion and uptake in a free form by the cells

[Howard et al., 1986], suggesting that PN-1 can be removed from ECM. Similarly, the active form of neuroserpin, which does not bind to LRP1 directly, has been shown to be internalized by cultured cortical neurons via LRP1 and an unidentified cofactor [Makarova et al., 2003].

The clearance of PN-1-protease complexes starts with their binding to cell surface heparan sulfates. The complexes are concentrated and transferred to LRP1, followed by their internalization and degradation. A lysine-rich heparin-binding site found in PN-1 between residues 71 and 86, is required for heparin-mediated inhibitory acceleration [Stone et al., 1994]. This PN-1 domain is also required in the initial binding of the complex with either thrombin or uPA to the cell surface in the LRP1-mediated internalization [Knauer et al., 1997a;Crisp et al., 2000]. Furthermore the PN1-uPA complex must specifically bind to endosomal heparins at pH 5.5 to be retained and sorted to lysosomes [Crisp et al., 2000]. Another region of PN-1, defined by residues 47-58 close to heparin-binding domain at, functions as LRP1 interacting domain [Knauer et al., 1997a]. An adjacent His-Asp pair within this domain, which is critical for the LRP1-mediated internalization of PN-1 thrombin complexes [Knauer et al., 1999]. Interestingly this LRP1-interacting domain is not required in LRP-mediated clearance of PN-1-uPA complex [Crisp et al., 2000]. It has been proposed that the basic residues of various ligands binding to LDLR family members are involve the interaction with the acidic regions in the ligand binding domain of the receptors [Lalazar et al., 1988;Nielsen et al., 1996;Rodenburg et al., 1998;Stefansson et al., 1998].

1.5.3.4 Serpins in cell adhesion and tumor invasion

The uPA, uPAR and PAI-1 are identified players in cell adhesion [Ciambrone et al., 1990;Nusrat et al., 1991]. PAI-1 disturbs vitronectin-dependent adhesion by complexing with vitronectin while retaining its inhibitory activity [Salonen et al., 1989;Ciambrone et al., 1990;Ciambrone et al., 1992]. PAI-1 has also been proposed to reduce adherence by increasing PAI-1-uPA turnover; an event definitely requires its inhibitory activity [Waltz et al., 1993]. It is further demonstrated that PAI-1 inhibits uPA-vitronectin-dependent cell adhesion by interrupting the interaction between uPA and vitronectin through uPAR [Wei et al., 1994;Kanse et al., 1996]. As the PAI-1 and uPAR binding sites on vitronectin are not identical but overlapping, PAI-1, which has higher affinity to vitronectin, can compete for uPAR binding to vitronectin [Deng et al., 1996]. The picture becomes even more complicated with the finding that the PAI-1 binding site on vitronectin also overlaps with

that for integrin, suggesting that PAI-1 also regulates integrin-mediated cell adhesion and signaling [Waltz et al., 1993;Stefansson et al., 1996]. In summary, PAI-1 detaches cells by disturbing either uPAR-vitronectin or integrin-vitronectin interaction, and the presence of uPA is required in both cases. Finally, PAI-1 is not the only player, which may interact with integrin in this system as uPAR co-localizes with different integrin subunits and consequently modifies integrin functions [Myohanen et al., 1993;Wei et al., 1996]. In addition, the impact of these uPA-PAI-1 [Waltz et al., 1993] and uPAR-uPA-PAI-1-integrin complexes [Czekay et al., 2001;Czekay et al., 2003] in cell-adhesive events can be regulated by LRP1-mediated endocytosis.

Similarly to PAI-1, PN-1 binds to vitronectin with high affinity [Rovelli et al., 1990]. In the presence of active uPA, PN-1 increases the association between vitronectin and uPAR. Through this mechanism PN-1 stimulates uPAR-dependent cell adhesion to vitronectin, when PN-1 and uPAR accumulate and co-localize at the interface between the cells and the matrix. However, in contrast to PAI-1, PN-1 does not influence vitronectin binding to integrins or integrin-mediated cell adhesion [Kanse et al., 2004]. PN-1 has been shown to be up regulated in highly metastatic pancreatic tumors cell lines. PN-1 over-expression greatly enhances the local invasion of the xenograft tumors. It is correlated with a massive increase in ECM production, such as type I collagen, fibronectin and laminin. Moreover, the invasive PN-1-expressing cells tend to adopt a spindle-shaped morphology and strongly express the mesenchymal marker vimentin [Buchholz et al., 2003]. Taken together, these results indicate that serpins can have additional adhesive functions by differentially influencing the impact of some signal transduction pathways mediating cell adhesion, migration, and tumor invasion.

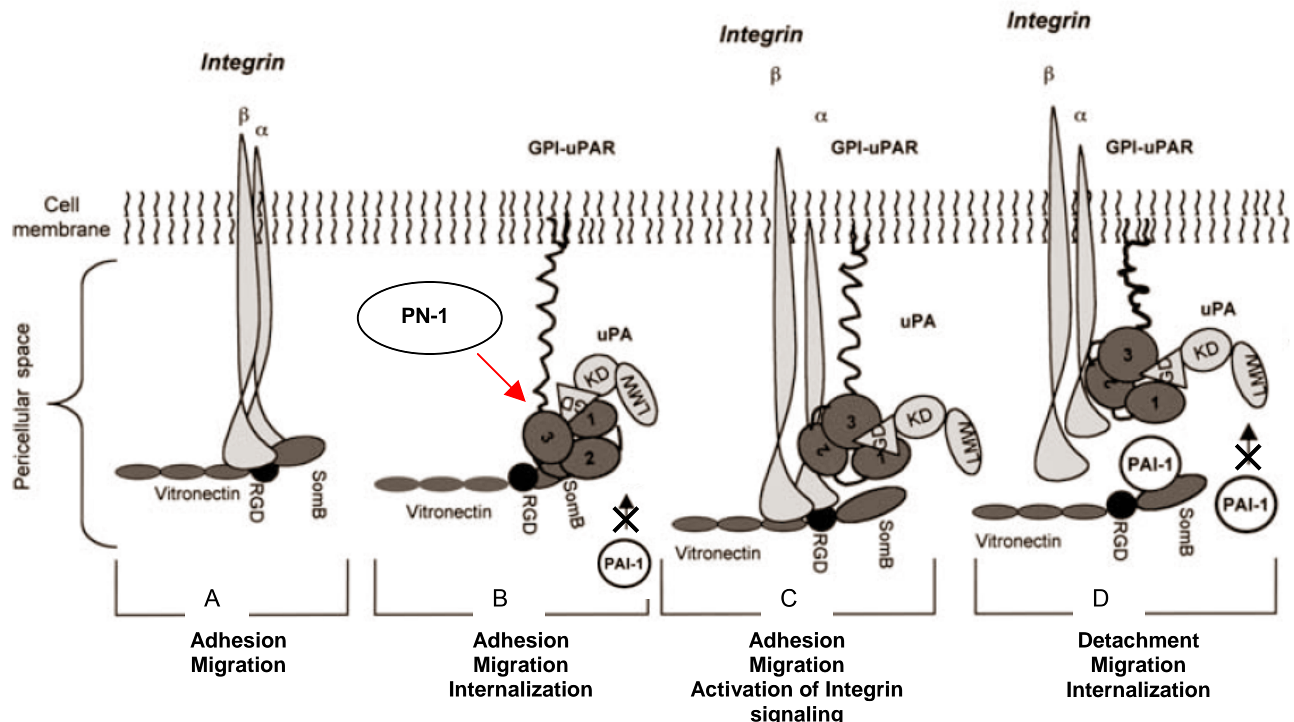


Fig. 9 Schematic model for PA-1 or PN-1 interaction with integrin and vitronectin. A) Integrin binds to the RDG sequence of vitronectin. B) uPAR (-uPA complex) binds to the somatomedin B (SomB) domain of vitronectin. PAI-1 forms complex with uPA triggering the internalization of uPA-uPAR; thereby interrupt the interaction of uPAR and vitronectin. In contrast, PN-1 increases the association between uPAR and vitronectin. C) uPAR (-uPA complex) binds to integrin and promotes cell adhesion and migration. D) PAI-1 also recognizes the SomB domain of vitronectin, therefore PAI-1 can inhibit integrin-vitronectin interaction as well. PAI-1 also forms complex with uPA triggering internalization of uPA-uPAR (adapted from Myöhänen H and Vaheri A, 2004) [Myohanen and Vaheri, 2004].

2 Aim and course of this work

Protease Nexin-1 (PN-1) is known as a serpin regulating extracellular proteolytic activity upon formation of inhibitory complexes with serine proteases. Such complexes are actively removed from the extracellular environment by internalization via LRP1. It has been reported that the level of active PN-1 in fibroblast-conditioned media reaches steady state due to a constant secretion and uptake in a free form by the cell. Removal of active form of PN-1 could be a mechanism to increase the local proteolytic activity that is required for proper biological functions.

In this thesis work, I explored (1) the possibility that active form of PN-1 is internalized; (2) whether this internalization depends on LRP1; (3) the type of cell surface receptor involved in case of an LRP1-independent internalization; (4) the mechanisms and the consequences of an interaction between PN-1 and distinct cell surface receptors.

To address these questions, I first examined whether the cells could take up the endogenous PN-1. For this purpose I prepared primary cortical neuronal culture from previously generated PN-1 reporter mouse (PN-1 KI mouse), with a bi-cistronic construct containing a HA-tagged PN-1 and the β-galactosidase marker gene inserted in the locus of PN-1. Thus I was able to distinguish neurons that synthesize, secret and take up PN-1 from those only internalizing it. I observed that PN-1 was taken up by neurons that did not express PN-1, and that LRP1 ligand RAP did not block this uptake. I further investigated whether PN-1 was taken up in an active form or in a complexed form, and whether this PN-1 internalization was LRP1 dependent or not. I used LRP1-/- and wild type MEF cells to show that both forms of exogenous PN-1 are taken up. The internalization of PN-1 was mediated predominantly by LRP1 in wild type MEF cells, because it was blocked by RAP or an inhibitory peptide known to interfere LRP1-PN-1 interaction. In contrast in MEF cells lacking LRP1, PN-1 internalization was not influenced by any of these inhibitors, indicating an alternative pathway of PN-1 endocytosis.

I then identified the receptor responsible for PN-1 internalization in the absence of LRP1. By comparing the kinetics and inhibitor sensitivity of PN-1 internalization in wild type and LRP1-/- MEF cells, I found out that the properties of PN-1 endocytosis in LRP1-

/- MEF were similar to those described for syndecan-1-mediated endocytosis. I then asked whether change of syndecan-1 expression level would influence PN-1 uptake? And how it would be influenced under different genetic background of LPR1? Therefore I tried to increase or reduce syndecan-1 expression level in MEF cells by over-expressing full-length syndecan-1 or by using siRNA. I observed, only in LRP1-/- MEF cells, that PN-1 uptake was strongly influenced by the changes of syndecan-1 expression. I also provided evidence that the intracellular domain of syndecan-1 was required for active PN-1 internalization.

To characterize the consequences of PN-1 interaction with distinct cell surface receptors, I investigated its effect on signal transduction and on cell behavior. In wild type MEF cells, PN-1 activated PKA upon binding to LRP1, whereas it activated ERK signaling in LRP1-/- MEF cells upon interaction with syndecan-1. These findings triggered my interest to explore the potent role of PN-1 in a signal transduction related event such as cell migration. In LRP1-/- MEF cells, PN-1 activated Rac1 and induced lamellipodia formation, thereby increasing cell migration. This finding also raised the question how PN-1 signaling was propagated? To further investigate this issue, I evaluated whether functional blocking antibodies against uPAR, syndecan-1, and integrin $\beta3$, which are possible upstream effectors of ERK signaling, could inhibit PN-1-induced cell migration. Each of these three antibodies could antagonize PN-1 stimulation at cell migration. I was also able to co-immunoprecipitate integrin $\beta3$ with PN-1 and syndecan-1. These results suggested that PN-1 interacted with either uPAR or syndecan-1 or both to activate integrin $\alpha v\beta3$ signaling and its down stream effectors, such as ERK and Rac1 to enhance cell migration.

3 Materials and Methods

3.1 Materials

Recombinant PN-1 was synthesized and purified in our laboratory [Sommer et al., 1989]. Thrombin was purified from human plasma and characterized as described [Stone et al., 1986]. Wild type and LRP1 deficient mouse embryonic fibroblasts (MEF) have been characterized before [Willnow et al., 1994a]. GST- receptor associated protein (RAP) [Herz et al., 1991] was a kind gift from Dr. Michael Etzerodt (Department of Molecular and Structural Biology, University of Aarhus, Denmark). Peptide 960 (P960), corresponding to the residues Pro^{47}-Ile^{58} in the domain of PN-1 considered to interact with LRP1, and the scrambled control peptide 965 (P965) were synthesized as described [Knauer et al., 1997a]. Plasmids expressing dominant negative H-RasN17 were a kind present from Dr. Yoshikuni Nagamine (Friedrich Miescher Institute, Basel, Switzerland) [El Shemerly et al., 1997].

3.2 Methods

Cell culture: Both MEF cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) in a humidified atmosphere of 5% CO_2 at 37°C.

Primary cortical neuronal culture: Primary neurons were cultured on glass cover slips. Cover slips were cleaned by HNO_3 (1:6 diluted) for 10min, washed with lots of water followed by rinsing with methanol once, and finally sterilized at 160°C for 2h. Sterile cover slips were coated with 1 mg/ml poly-L-lysine (in borate buffer) at 37°C overnight and rinsed with sterile water. Method for preparing cortical neuronal culture has been described previously [Bartlett et al., 1984]. In brief, cortexes were dissected from the mice embryonic brains at E16-18 in HBSS buffer (10 ml 10x Hank’s BSS, 1 ml 1M HEPES PH 7.3 (GIBCO), 89 ml H_2O), followed by trypsin treatment (1:10 diluted trypsin-EDTA, (Sigma Cat No. 25300-054)) at 37°C for 15min. Cell suspensions were prepared in HBSS buffer by tituation using a fire-polished Pasteur pipette. Dissociated cells were plated on cover slips in HC-MEM (500 ml 1x MEM (GIBCO), supplemented with 15 ml 20% glucose and 10% horse serum). After allowing cells to adhere for 1.5-2h, neurons attached to cover slips were transferred to a new dish with B-27 medium (50 ml

Neurobasal medium, 1 ml B-27 supplement (GIBCO), 0.125 ml 25 mM L-Glutamine). The culture was maintained by changing fresh B-27 medium every five days.

β-galactosidase cytochemistry: Primary neurons cultured on poly-L-lysine coated cover slips were fixed in 4% paraformaldehyde (PFA) in PBS (Ca^{2+}, Mg^{2+} free) at room temperature for 15min then washed with PBS (Ca^{2+}, Mg^{2+} free) 3 x 10min. Neurons were incubated in staining solution containing 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) (Roche), 2 mM $MgCl_2$, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide in PBS (Ca^{2+}, Mg^{2+} free) at 37°C overnight.

Immunocytochemistry of HA tagged PN-1: Primary neurons prepared from PN-1 knock-in mice were fixed in 4% PFA plus 15% picric acid in PBS (Ca^{2+}, Mg^{2+} free) at room temperature for 15 min and washed with PBS (Ca^{2+}, Mg^{2+} free) three times for 10min each. Fixed cells were permeabilized in the working buffer (PBS (Ca^{2+}, Mg^{2+} free) plus 0.2% triton X-100) at room temperature for 15min followed by blocking in the blocking buffer (3% BSA in the working buffer) at room temperature for 30min. Cells were incubated with anti-HA antibody (Roche, clone 12CA5), diluted 1:200 in blocking buffer at room temperature for 2 h, washed in the working buffer and followed by secondary antibody incubation.

Preparation and purification of thrombin-PN-1 complex: Thrombin and recombinant PN-1 (rPN-1) (1:5 ratio) were mixed in assay buffer, which contained 66 mM Tris-HCl, pH8.0, 133 mM NaCl and 0.13% polyethyleneglycol 6000, and incubated at 37°C for 1h. Thrombin-PN-1 complex was purified by FPLC (Superdex 75, 2.0 x 25 cm, flow rate 0.5 ml per minute) (Amersham Pharmacia). Eluant fractions were collected by indicated molecular weight.

Uptake experiments: MEF cells were plated 24h before the experiment and grown to 80-90% confluence. Cells were washed twice with pre-warmed PBS (Ca^{2+}, Mg^{2+} free) and incubated for 3h at 37°C in serum-free DMEM medium (SFM), supplemented with 0.1 mg/ml stripped BSA, 16 μg/ml putresine, 12.5 ng/ml progesterone (Sigma) and 1:5000 diluted supplement (Sigma, Cat. No.1-1884). After pre-incubation in SFM, cells were washed once with SFM followed by incubation at 37°C for 3h in fresh SFM with either 300 ng/ml active rPN-1 or purified thrombin-PN-1 complex (thrombin 300 ng/ml). Cells

were also incubated with rPN-1 in the presence of different inhibitors at different concentrations described as following: 50 nM RAP, 25 μg/ml P965 and P960, 200 μM chloroquine [Takayama et al., 2005]; 100 μM serine protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), and its analogous 4-(2-aminoethyl)-benzenesulfonamide (AEBS) (Sigma) [Makarova et al., 2003]; 300 μM Genistein, 10 mM β-cyclodextrin, 25 μM N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89) or 1 μM phenylarsine oxide (PAO) (Sigma). A 30min pre-incubation with the last four inhibitors preceded the addition of rPN-1. The sub-cellular fractions were prepared as described [Ito et al., 1997] with slight modification. Briefly, cells were washed six times with ice-cold PBS and scraped in PBS. Cell suspension was centrifuged (200 × g) at 4°C for 5min. Cell pellets were solubilized in 100 μl lysis buffer, which contains 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2 M sucrose, 2 mM EDTA, 2 mM EGTA and protease inhibitor cocktail (Roche). Cell lysates were centrifuged (10,000 × g) at 4°C for 10min. An additional centrifugation at 100,000 × g at 4°C for 1h was performed to safely remove the plasma membrane fraction. The resulting supernatant containing low-density microsomals (LDM) and cytosolic fractions was used to monitor internalized PN-1 [Shisheva et al., 1994;Shisheva et al., 2001]. The pellets, resuspended in the same buffer, contained the plasma membrane bound PN-1.

Binding experiments: MEF cells were cultured as for the uptake experiments except that the plates were coated with 1% gelatin (Sigma). After pre-incubation in SFM, cells were incubated with 300 ng/ml active rPN-1 in fresh SFM in the presence or absence of 1 mg/ml heparin (Sigma), 250 nM RAP, or combination of both at 4°C for 2h. The level of PN-1 binding to the cell surface was determined by immunoblotting of plasma membrane fractions.

Immunoblotting: Samples from the uptake experiments were separated by 10% SDS-PAGE under either reducing or non-reducing conditions [Laemmli, 1970]. For the latter, the samples were incubated overnight at 4°C in buffer containing only 0.4% SDS and no β-mercaptoethanol. Certain amount of protein was loaded per lane, proteins were transferred to PVDF membrane (Millipore) and probed overnight at 4°C by monoclonal antibody against rPN-1 (4B3) [Meier et al., 1989], diluted 1:2000 in blocking buffer containing 3% skim milk powder in PBS with 0.2% tween 20. The results were quantified by Image Master Total Lab (Amersham Pharmacia Biotech). The amount of PN-1 or PN-

1 complex internalized under different conditions was normalized to the percentage of that in LRP1+/+MEF cells.

Immunocytochemistry for PN-1: After the PN-1 uptake experiments, cells were fixed and immunostained with 4B3 antibody as described [Bleuel et al., 1995].

Semi-quantitative PCR: Total RNA from MEF cells was extracted by RNeasy Kit (Qiagen), followed by single step transcription and amplification by SuperScript one-step RT-PCR kit (Invitrogen Life Technologies). Primers were designed to amplify 300-400 bp fragments of syndecans. Syndecan-1: sense 5'CTC CCG CAA ATT GTG GCT G3', antisense 5'TGG GCT GTG GTG ACT CTG A3'. Syndecan-2: sense 5'TGG ATC CTG CTC ACC TTG G3', antisense 5'TTT TAT AGC AGG GCC CAG CT3'. Syndecan-3: sense 5'CAA TGA GAA CTT CGA GAG GC3', antisense 5'CAG GTG CTG TGG CCA TAG T3'. Syndecan-4: sense 5'CGG AGA GTC GAT TCG AGA G3', antisense 5'TGC CAA GAC CTC AGT TCT CT3'. The PCR was done for 25,30 and 35 cycles and products were tested on 2% agarose gel. The relative mRNA level of actin was tested as a loading control.

Syndecan-1 assay: Cells were washed twice with ice-cold 0.5 mM EDTA-PBS and incubated with 1.5 ml of 20 µg/ml TPCK-treated trypsin (Sigma) in the same buffer for 15min on ice. Soybean trypsin inhibitor (Sigma) was then added to 100 µg/ml. After scraping, cell suspensions were centrifuged (200 × g) at 4°C for 5min. Cell pellets were solubilized in NP-40 buffer on ice for 30min, and centrifuged (10,000 × g) at 4°C for 10min. The protein content of the lysates was determined using the DC-protein assay kit (Bio-Rad). After normalization to the same amount of protein, the supernatants containing the ectodomain of syndecan-1 were digested at 37°C for 3h by 10 mU/ml heparinase III and 20 mU/ml chondroitinase ABC (Sigma); fresh enzymes were added after the first 2h of incubation. Following digestion, the samples were applied on SDS-PAGE (4-15% gradient gel, Bio-Rad) under reducing conditions and transferred to PVDF membrane (Millipore). The membrane was probed by anti-syndecan-1 antibody (BD Biosciences Pharmingen) as described [Park et al., 2000].

Expression plasmids of syndecan-1 and transfection: The cDNA of full-length murine syndecan-1 (Genebank accession NM_011519) was amplified by SuperScript one-step

RT-PCR kit. The PCR product was subcloned into the Eco RI and Xba I sites of pcDNA3.1 (+) (Invitrogen Life Technologies). To generate a syndecan-1 mutant containing only nine amino acids in the cytoplasmic domain, the 34-base new C-terminal anti-sense primer 5'GCT CTA GAG CTC AGC TGC CTT CGT CCT TCT TCT T 3' was used with the regular N-terminal sense primer. The PCR product was subcloned in the same vector. Both expression constructs were confirmed by sequencing. The expression plasmids were linearized by Bgl II and transfected into MEF cells by Nucleofector MEF1 kit combined with program T20 (AMAXA, Köln, Germany) following the manufacturer's instructions. After transfection cells were cultured in presence of G418 sulfate (GIBCO) to select stable transfected clones for further experiments.

siRNAs design, synthesis and transfection: Two different siRNAs were chosen within the syndecan-1 gene (GenBank accession NM_011519), targeting nucleotides 566-586 and 755-775 of syndecan-1 mRNA sequence. Both siRNA sequences were BLAST searched against all mouse sequences in GenBank, no significant homology (>15 contiguous nucleotides of identity) was found. siRNAs were synthesized and annealed by Qiagen and delivered to MEF cells by Nucleofector kit. siRNAs of syndecan-1 were transfected either separately or together. 24h after transfection, cells were trypsinized as described above to collect cell surface syndecan-1, total RNA was extracted from the cell pellets by RNeasy kit. Only the siRNA targeting nucleotides 566-586 coming from the coding region of syndecan-1 ectodomain reduced syndecan-1 level in both MEF cells, consequently the siRNA targeting nucleotide 755-775 coming from the coding region of the transmembrane domain was used as negative control.

Quantitative RT-PCR: Total RNA extracted following siRNA transfection was reversely transcribed using AMV rev. transcriptase kit (Promega). Quantitative PCR was performed on ABI Prism7000 by using SYRB green I master mix (Applied Biosystems). Several pairs of primers for syndecan-1 were tested by comparing the dissociation curves and those that did not produce primer dimer peak were chosen (forward 5' CCA CTT CTC TGG CTC TGG CAC A 3', reverse 5' AAC AGC CAC ACG TCC TTC CAA 3'). The primers for β-actin were used as described [Giulietti et al., 2001]. The level of mRNA encoding syndecan-1 was normalized relative to β-actin mRNA level.

PKA activity measurement: MEF cells were plated and cultured as described for uptake experiments. After overnight incubation in SFM, fresh SFM containing 300 ng/ml active rPN1 with or without 50 nM RAP was added at 37°C for 10min. PKA activity was measured with Pep Tag Non-radioactive cAMP-Dependent Protein Kinase Assay Kit (Promega), following the manufacture's instructions. The results were quantified by Image Master Total Lab (Amersham Pharmacia Biotech).

Activation of ERK signaling pathway: MEF cells were plated in 6 well plates and kept in DMEM supplemented with 10% FCS until confluency. Before the experiment, the cells were washed three times with pre-warmed PBS (Ca^{2+}, Mg^{2+} free), and then switched to SFM for 30min. After this pre-incubation, the medium was changed to fresh SFM containing 300 ng/ml active rPN-1 alone or in presence of 10 mM β-cyclodextrin. After different incubation periods, cells were washed three times with ice-cold PBS and solubilized with NP-40 buffer, containing proteases inhibitor cocktail (Roche) and 1% phosphatase inhibitor cocktail I and II (Sigma). Cell lysates were kept on ice for 30min before centrifugation at 10,000 × g at 4°C for 10min. SDS PAGE was performed with 20µg of total protein per lane. The samples were probed against the anti-ERK1/2 phospho-specific antibody (Biosource International, Camarillo, CA). To further identify the upstream effector of ERK signaling activated by PN-1, we transfected LRP1-/- MEF cells with plasmids expressing dominant negative RasN17. Empty vector was used as control. 24h after transfection, cells were treated in the same way as described above; in this case incubation time with PN-1 was 20min. Over-expression of H-RasN17 was detected on immunoblot by anti-c-H-Ras antibody (Merk Bioscience Ltd., Nottinham, UK). For the immunoblot quantification, the phosphorylation level of ERK was normalized to the percentage of that in empty vector transfected LRP1-/-MEF cells without PN-1 stimulation.

In vitro wound healing assay: MEFs cells were plated at $15x10^4$ cells per well in 6 well plates, and kept in DMEM supplemented with 10% FCS until confluency. Monolayer of cells was wounded by scratching with a 200 µl plastic pipette tip; detached cells were washed away with pre-warmed PBS (Ca^{2+}, Mg^{2+} free). Cells were incubated in SFM for 30min, and then switched to fresh SFM containing 300 ng/ml rPN-1 alone or in the presence of MEK inhibitor U0126 at 10 µM or anti-uPAR antibody at 10 µg/ml. At various time points, cell migration was visualized by a reverse microscope and photographed for

migration into denuded space. In each photograph, the distance between two opposing lead edges of cellular migration was measured at three evenly spaced intervals manually [Weaver et al., 1997].

Migration assay: MEF cells were cultured in DMEM supplemented with 10% FCS for 24h before starting the experiments. Migration assay was performed in modified Boyden chambers with 8 μm polycarbonate filters (Costar Cooperation) coated with vitronectin 10 μg/ml (Sigma) at 37°C for 2h on the bottom side and then blocked with 2 mg/ml BSA at 37°C for 1h. Cells were plated at a density of 5.0 x 10^4 per chamber in SFM, in the presence or absence of 300 ng/ml active rPN-1 in the top chamber. Plated cells were allowed to migration for 4h with SFM in the lower chamber. Non-migrating cells on the upper side of the filter were removed by cotton swab, the migrating cells adhering to the bottom side of the filter were fixed in 4% PFA at room temperature for 15min and stained with 0.1% crystal violet (in water) for 30min. The number of stained cells in each well was counted under inverted microscope. In the migration inhibitory experiments, LRP1-/- MEF cells were pre-incubated with blocking antibodies against integrin β3 (Biolegend), syndecan-1 (BD Biosciences Pharmingen) and uPAR (Research and Diagnose system) at 10 μg/ml in SFM, 37°C for 30min before plating in the top chambers.

Rac1 activation: Cells were cultured and incubated with rPN-1 as described for *in vitro* wound healing assay. Rac/ Cdc42 assay kit contains fragments corresponding to residues 67-150 of p21-activated kinase fused to GST and coupled to glutathione-agarose, which binds activated Rac1 specifically (Upstate Biotechnology). Following incubation with rPN-1, cell lysates were prepared and analyzed following the manufacturer's instructions.

Cell morphology: MEF cells were maintained in DMEM supplemented with 10% FCS for 24h. Cells were suspended and plated in 4-well cell culture chamber (Nalge Nunc International) at 2.0 x 10^4 cells per well in SFM with or without 300 ng/ml rPN-1 at 37°C for 1h. At the end of incubation, cells were washed, fixed and blocked according to the protocol described above for immunocytostaining. For actin staining, Alexa Fluro488 phalloidin (Molecular Probes), diluted 1:60 in the blocking buffer was incubated with cells at room temperature for 20min, cells were washed with PBS (Ca^{2+}, Mg^{2+} free) to remove excess phalloidin.

Adhesion assay: 96 well plate was coated with vitronectin 10 µg/ml (Sigma) at 37°C for 2h, and rinsed with PBS (Ca^{2+}, Mg^{2+} free) 3 x 10min. Unspecific binding sites were blocked by 1% BAS in DMEM at 37°C for 1h. Cells were trypsinized at room temperature for 1min, followed by incubation with 0.1 mg/ml soybean trypsin inhibitor in PBS (Ca^{2+}, Mg^{2+} free) at 37°C for 1min to quench trypsin activity. 5000 cells per well were plated in SFM, and allowed to attach at 37°C for 45min. Non-attached cells were removed by washing with pre-warm DMEM 2 x 5min. The attached cells were fixed in 2% glutaraldehyde in PBS (Ca^{2+}, Mg^{2+} free) at room temperature for 15min, washed with PBS (Ca^{2+}, Mg^{2+} free) 3 x 10min. After washing, cells were stained with 0.5% crystal violet in 20% ethanol at room temperature for 30min. To quantify the result, the staining was dissolved by 500 µl of 10% acitic acid per well for 5min and measured the optical density at 590nm.

Immunoprecipitation: LRP1-/- MEF cells were plated and incubated with 300 ng/ml active rPN-1 as described for PN-1 uptake experiment. Afterwards, cell lysates were prepared and immunoprecipitated by using anti-integrin β1 (MB1.2, CHEMICON) or anti-integrin β3 (clone 2C9.G2, Biolegend) antibodies, as described previously [Roberts et al., 2003]. PN-1 was detected by immunoblotting as described for uptake experiments. Alternatively, cells were transfected with plasmid encoding the full-length syndecan-1. 24h later, these cells were incubated with rPN-1 and processed as described above. Syndecan-1 was detected by immunoblotting following the same protocol described in syndecan-1 assay. After stripping the immunoblot, 4B3 antibody was applied to detect PN-1.

4 Results

4.1 PN-1 uptake in cortical primary neuronal culture

To investigate PN-1 uptake, we prepared cortical neuronal primary culture from PN-1 knock-in mice (Fig. 10A), in which a construct of HA-tagged PN-1-IRES-LacZ was inserted in the PN-1 locus, allowing us to monitor PN-1 transcription following the expression of β-galactosidase; and to detect PN-1 protein level by HA immunocytostaining [Kvajo et al., 2004]. In this cortical primary neuronal culture we observed that only a small population of cortical neurons were expressing PN-1, indicated by both β-galactosidase (X-gal) and HA positive immunocytostaining (Fig. 10B left panels). However PN-1 non-expressing neurons could take it up from adjacent neurons expressing PN-1, indicated by β-galactosidase negative and HA positive immunocytostaining (Fig. 10B left panels). These observations suggested both the secretion and the uptake of PN-1 was secreted and taken up in this culture model. This result matched with earlier observations made in our lab, that exogenous GFP-PN1 was taken up in different cell types, including hippocampal neurons and HepG2 cells (Seiler F. and Albrecht H., unpublished data). The uptake of endogenous PN-1 was not blocked upon incubation with RAP; a well-known antagonistic ligand for LRP1 mediated endocytosis, at a concentration up to 500 nM for 72h (Fig. 10B, right panels). These data suggested that endogenous PN-1 could be taken up in an LRP1-independent manner, although it was not clear yet, whether PN-1 was taken up in an active or/and complexed form.

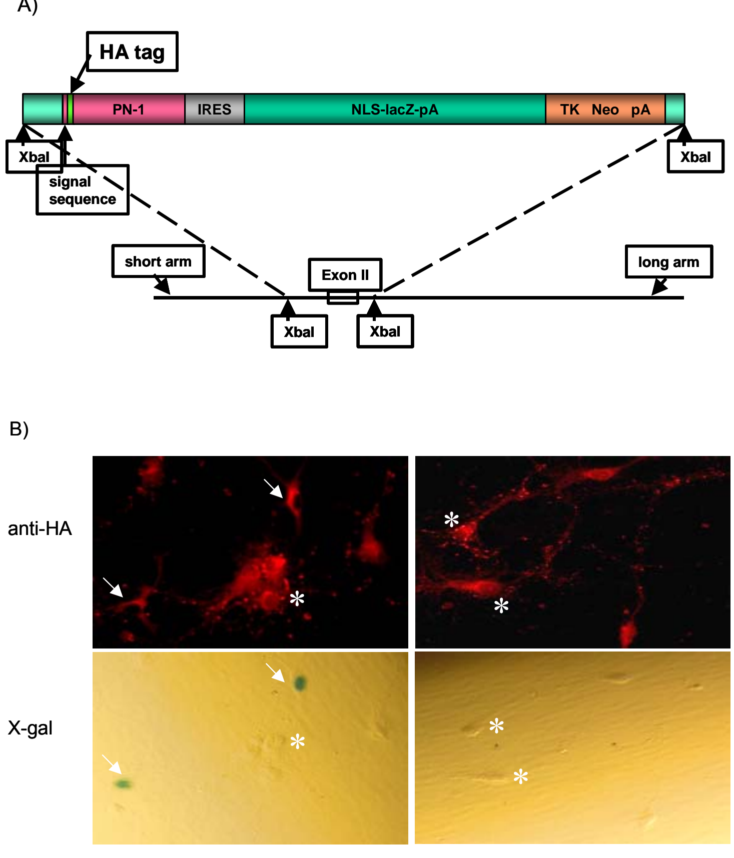


Fig 10. Endogenous PN-1 uptake in cortical neurons from PN-1 KI mice. (A) Schematic representation of the HA-PN-1 genomic locus with relevant restriction sites. (B) In cortical neuronal primary culture, HA-PN-1 was taken up by the neurons that did not express HA-PN-1, showing HA positive (left, top panel, asterisk) but X-gal negative (left panels, asterisk) staining. In primary neurons incubated with RAP 500 nM, 37°C for 72h, the uptake of HA-PN-1 (right panels, asterisk) was maintained the same as in the control neurons (left panels, arrow head). Pictures were taken at 40x magnificence (arrow head: neurons expressing PN-1, asterisk: neurons internalizing PN-1).

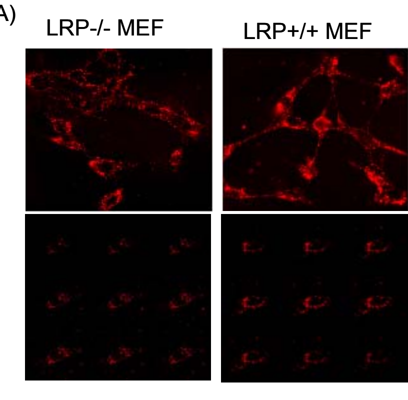
4.2 Both active and complexed PN-1 is internalized in both LRP1-dependent and independent pathways

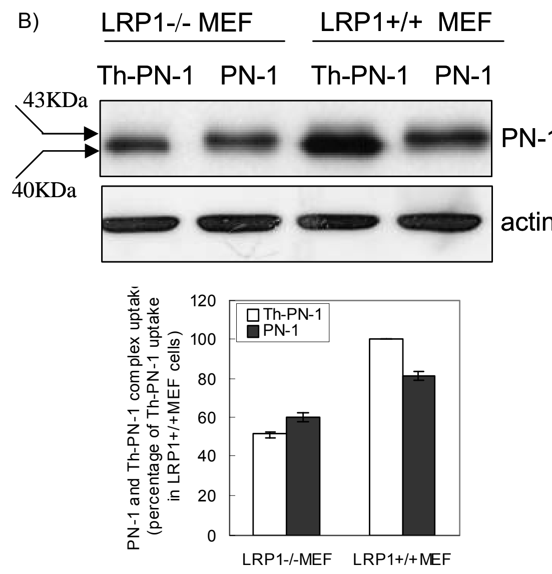
To further investigate the mechanism of PN-1 internalization, we performed PN-1 uptake experiments in LRP1-/- and wild type MEF cells examining the intracellular levels of PN-1 by immunocytochemistry and immunoblotting. Upon permeabilization, immunostained PN-1 appeared as punctas in cytoplasm in both LRP1-/- and wild type MEF cells under normal fluorescence microscope (Fig. 11A, top panels). We also used confocal microscopy, instead of non-permeabilized immunostaining, to confirm the internalization of PN-1. PN-1 staining was observed throughout the scanned layers (Fig. 11A, bottom panels), suggesting that PN-1 could be taken up in both LRP1-dependent and independent pathways. To confirm this result and to better quantify the PN-1 uptake in MEF cells, we evaluated the internalization of active PN-1 and purified thrombin-PN-1 complex by immunoblotting following SDS-PAGE of intracellular PN-1 under reducing conditions. Upon incubation with active PN-1, the internalized PN-1 appeared as single 43 KDa band, otherwise as single band with slightly smaller molecular weight at around 40 KDa upon incubation with rPN-1-thrombin complex (Fig. 11B). This 40 KDa form of PN-1 on immunoblot represents the cleaved form of PN-1 following dissociation from the complex with serine protease [Nick et al., 1990]. There was no significant difference between the internalization of active PN-1 and PN-1-thrombin complex observed in either LRP1-/- or wild type MEF cells. These data indicated that not only complexed but also active PN-1 could be internalized in both LRP1-dependent and independent pathways.

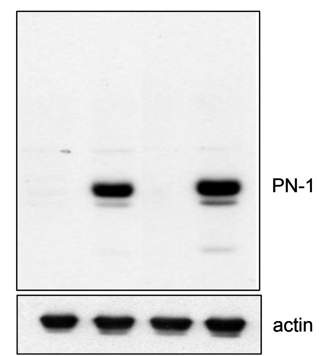
Since this is the first time that active PN-1 internalization was observed, we carried out three additional experiments to exclude any possible artifact. The first one was to confirm that the active PN-1 detected on immunoblot is not dissociated from a serine protease complex. It has been reported that PN-1-serine protease complex is SDS-resistant [Baker et al., 1980]. Therefore PN-1 complex can be detected on immunoblot under non-reducing conditions, if rPN-1 is only taken up in a complex form, we should not be able to detect 43 KDa form of PN-1 under non-reducing conditions. Thus PN-1 uptake experiments were performed with both types of MEF cells and the internalized PN-1 was examined on immunoblot under non-reducing conditions. We could only detect the 43-KDa PN-1 band from cells incubated with free PN-1. Upon exposure to thrombin-PN-1 complex, the 40 KDa form of PN-1 was not detected anymore, as the

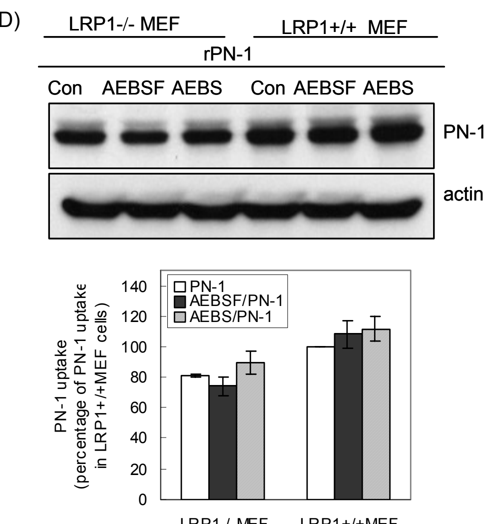
complex did not dissociate under the non-reducing conditions [Travis et al., 1983] (Fig. 11C). Since the available antibody poorly recognizes PN-1 complex, PN-1 complex was not detected neither. Thus, we concluded that the PN-1 detected at 43 KDa was taken up in the active form. In the second experiment, serine proteases activity was blocked by AEBSF to prevent PN-1 to form a complex with any serine protease. Under such conditions uptake of active PN-1 in both MEF cells was still detected, suggesting that PN-1 uptake is independent of any serine protease activity (Fig. 11D). A third experiment was performed to exclude the possible mixing of plasma membrane bound PN-1 and internalized PN-1 during ultra-centrifugation (see Methods). We incubated rPN-1 with both MEF cell lines at 4°C and active PN-1 was detected exclusively in the plasma membrane fractions by immunoblotting, this ruling out a cross contamination (Fig. 11E).

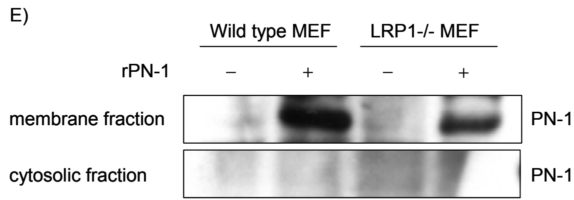
We further analyzed the fate of internalized PN-1. In both types of MEF cells, overnight incubation of rPN-1 with 200 μM leupeptin combined with pepstatin A, or chloroquine, a weak base that inhibits lysosomal proteolysis, led to an increased intracellular accumulation of 43-KDa PN-1, indicating that active PN-1 was degraded in lysosomes (Fig. 11F,G). The PN-1 uptake in LRP1-/- MEF cells demonstrated by immunocytostaining and immunoblot analysis suggested that in the absence of LRP1 another endocytic receptor mediates the internalization of PN-1.

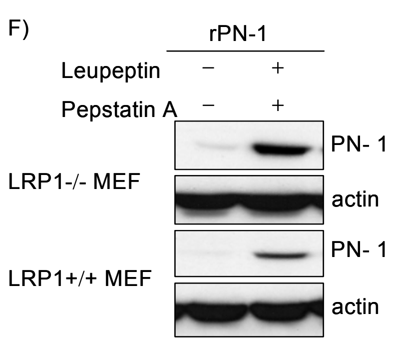

A)
LRP-/- MEF
LRP+/+ MEF


B)
LRP1-/- MEF
LRP1+/+ MEF
Th-PN-1
PN-1
Th-PN-1
PN-1
43KDa
40KDa
PN-1
actin
PN-1 and Th-PN-1 complex uptake
(percentage of Th-PN-1 uptake
in LRP1+/+MEF cells)
Th-PN-1
PN-1
120
100
80
60
40
20
0
LRP1-/-MEF
LRP1+/+MEF


C)
LRP1-/- MEF
LRP1+/+ MEF
Th-PN-1 PN-1 Th-PN-1 PN-1
PN-1
actin


D)
LRP1-/- MEF
LRP1+/+ MEF
rPN-1
Con AEBSF AEBS
Con AEBSF AEBS
PN-1
actin
PN-1 uptake
(percentage of PN-1 uptake
in LRP1+/+MEF cells)
PN-1
AEBSF/PN-1
AEBS/PN-1
140
120
100
80
60
40
20
0
LRP1-/-MEF
LRP1+/+MEF


E)
Wild type MEF
LRP1-/- MEF
rPN-1
–
+
–
+
membrane fraction
cytosolic fraction
PN-1
PN-1


F)
rPN-1
Leupeptin – +
Pepstatin A – +
LRP1-/- MEF
LRP1+/+ MEF
PN- 1
actin
PN- 1
actin

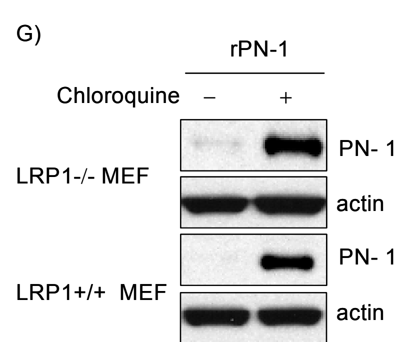

G)
rPN-1
Chloroquine – +
LRP1-/- MEF
LRP1+/+ MEF
PN- 1
actin
PN- 1
actin

Fig. 11 PN-1 internalization in LRP-/- and wild type mouse embryonic fibroblasts. (A) MEF cells were incubated with 300 ng/ml rPN-1 at 37°C for 3h. Cells were fixed and immunostained as described in Materials and Methods. PN-1 appeared as punctas in the cytoplasm in both LRP1-/- and wild type MEF cells (A, top panels). The confocal scanning pictures through the whole cell body confirmed this pattern of PN-1 staining (A, bottom panels). Pictures were taken at 40x magnificence. (B) Internalization of 300 ng/ml rPN-1 or thrombin-rPN-1 complex (thrombin 300 ng/ml) in MEF cells was analyzed by immunoblotting under reducing conditions. Both active and complexed PN-1 were taken up in LRP1-/- and wild type MEF cells, with active PN-1 migrating at a slightly higher molecular weight than PN-1 dissociated from the complex. Quantification of these results indicated lower level of either PN-1 or PN-1-thrombin complex internalized in the cells devoid of LRP1 (data: mean ± SE, white bar: PN-1-thrombin complex, black bar: active PN-1). (C) Under non-reducing conditions, active PN-1 uptake was still detectable, but not in the case of complexed PN-1. (D) Serine protease inhibitor did not interfere with PN-1 uptake, indicating that active PN-1 uptake was not requiring any serine protease activity (data: mean ± SE, white bar: active PN-1, black bar: active PN-1 with AEBSF, grey bar: active PN-1 with AEBS). (E) Immunoblotting on PN-1 showed that PN-1 could only be detected in the plasma membrane fraction when the cells were incubated with rPN-1 at 4°C. (F) Overnight incubation with rPN-1 in presence of the lysosomal inhibitors leupeptin combined with pepstatin A lead to intracellular PN-1 accumulation. (G) Chloroquine showed the same effect as leupeptin and pepstatin A on PN-1 uptake.

4.3 Properties of PN-1 uptake in LRP1-/- and wild type MEF cells

In the next experiment, we compared the efficiency of PN-1 uptake in both types of MEF cells to reveal the differences between the endocytic pathways involved. The PN-1 internalization showed a time-dependent fashion in both types of MEF cells. It is noticeable that in LRP1-/- MEF cells the PN-1 uptake was less prominent and showed a slower kinetic ($t_{1/2}$ around 45min) than in wild type MEF cells ($t_{1/2}$ around 15min) (Fig. 12A,B). The PN-1 internalization also displayed a concentration-dependent fashion in both types of MEF cells (Fig. 12C). We observed that in LRP1-/- MEF cells PN-1 uptake was less efficient at given concentrations than in wild type MEF cell. Taken together, these results suggested that the receptor contributing to PN-1 uptake in LRP1-/- MEF cells has different properties.

In this context we tested whether RAP and peptide 960 (P960) could interfere PN-1 uptake in LRP1-/- MEF cells. RAP, among all the ligands, has the highest affinity to members of LDLR family including LRP1, whereas P960 represents the PN-1 domain binding to LRP1. Both are putative inhibitors which interfere with LRP1 endocytic pathway by preventing the PN-1-thrombin complex binding to LRP1 [Knauer et al., 1997a]. The scrambled peptide P965 with the same amino acids content as P960 was used as control. Both RAP and P960 reduced PN-1 uptake in wild type MEF cells up to about 90%, but did not affect PN-1 uptake in LRP1-/- MEF cells (Fig. 12D). These data not only further confirmed an LRP1-independent uptake of PN-1, but also indicated that in the absence of LRP1 the receptor mediating PN-1 internalization was not from the LDLR family.

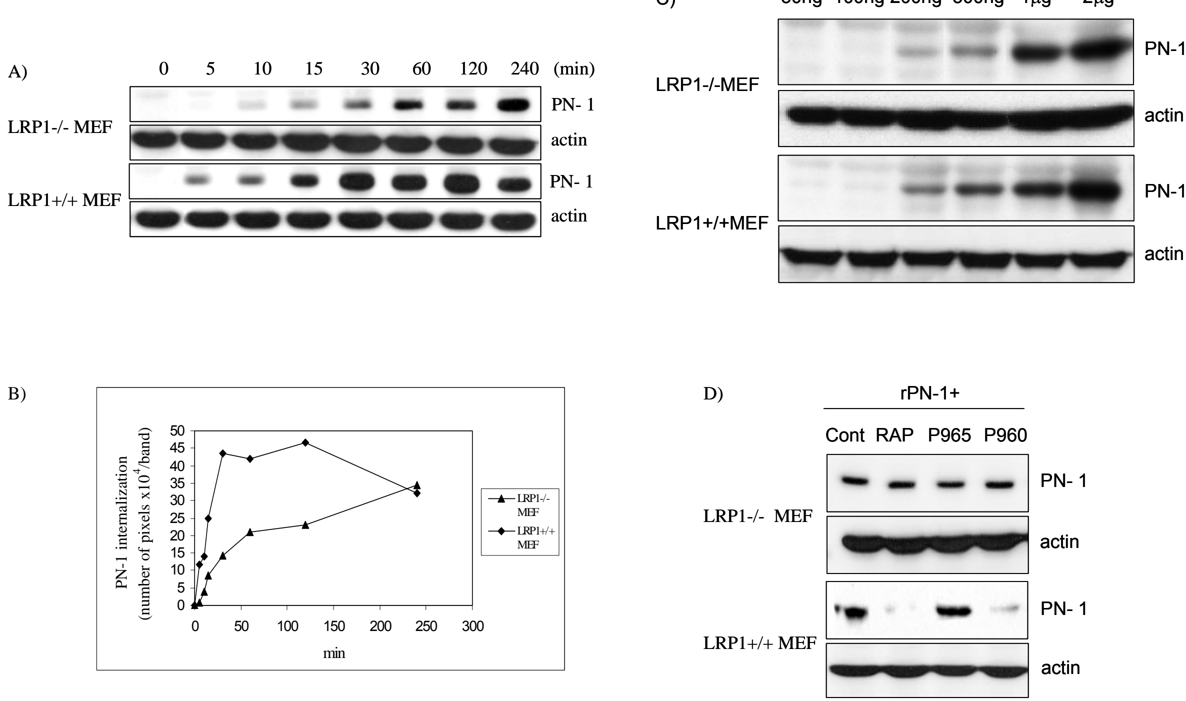


Fig. 12 Properties of PN-1 uptake in LRP1-/- and wild type MEF cells. (A) Both types of MEF cells were incubated with 300 ng/ml rPN-1 at different time points. The intracellular PN-1 levels were detected by immunoblotting. (B) Semi-quantification and densitometry plotting of PN-1 internalization. In LRP1-/- MEF cells (triangle) showed kinetic parameter ($t_{1/2}$ 45min) different from wild type MEF cells (square) ($t_{1/2}$ 15min) (Data represents one experiment out of three individual experiments). (C) MEF cells were incubated at 37°C for 3h with rPN-1 at different concentrations as indicated and the intracellular PN-1 levels were detected by immunoblotting. (D) MEF cells were incubated with 300 ng/ml rPN-1 at 37°C for 3h in presence or absence of 50 µM RAP, 25 µg/ml control peptide (P965), or antagonist peptide 960 (P960) respectively. PN-1 uptake was inhibited in wild type but not in LRP1-/- MEF cells.

4.4 HSPGs are involved in PN-1 uptake in LRP1-/- MEF cells

The next step was to identify the endocytic receptor, which mediated both active PN-1 and PN-1 complex internalization in LRP1-/- MEF cells. It is well known that binding of PN-1-protease complex to cell surface heparan sulfate is the initial step of its internalization. Thus free heparin antagonizes ligand binding to the cell surface [Howard et al., 1987]. To test whether this was also the case for active PN-1 uptake, rPN-1 was added to MEF cells at 4°C in the presence of soluble heparin. Membrane-bound PN-1 levels were determined in the plasma membrane fractions by immunoblot analysis. Heparin strongly reduced most of the PN-1 binding to the cell surface in both MEF cell lines (Fig. 13A). In addition, the interactions with HSPGs has been shown to impede ligand binding to LRP1 [Wilsie et al., 2003], the effect of RAP was evaluated in presence or absence of heparin. In this case rPN-1 was added to MEF cells at 4°C in the presence of soluble heparin, RAP or both. Membrane-bound PN-1 levels were determined in the plasma membrane fraction. In presence of RAP, PN-1 binding was reduced by about 30%, and abolished by the combination of RAP and heparin in both wild type and LRP1-/- MEF cells (Fig. 13B,C). This suggested that the initial binding step of active PN-1 also required heparan sulfate on the cell surface of LRP1-/- MEF cells.

Cell surface heparan sulfate is mainly associated with HSPGs of two families, syndecans and glypicans [Bernfield et al., 1999]. HSPG has been shown to mediate LDLR or LRP1-independent lipoprotein metabolism [Williams et al., 1992;Fernandez-Borja et al., 1996;Al Haideri et al., 1997;Seo et al., 1997;Llorente-Cortes et al., 2002], among which syndecan-1 and perlecan have been identified as independent endocytic receptors [Fuki et al., 1997;Fuki et al., 2000a]. Being different from LRP1-mediated endocytosis, syndecan-1-mediated endocytosis is triggered by ligand binding and clustering. It involves detergent-insoluble membrane rafts instead of clathrin–coated pits [Fuki et al., 2000b]. It requires intact actin microfilaments and tyrosine kinase activity, which in turn phosphorylates the intracellular domain of syndecan-1 [Fuki et al., 1997], thus facilitating the endocytosis. Ligands are processed with $t_{1/2}$ of approximately 1 hour by this pathway [Fuki et al., 1997;Fuki et al., 2000b], which is obviously slower than in the endocytosis mediated by LDLR family members. Consequently, we examined whether PN-1 uptake in LRP1-/- MEF cells was mediated by syndecan-1. We used chemicals, known to interfere with syndecan-1-mediated endocytosis to test whether

they could block PN-1 uptake in MEF cells. Two sets of inhibitors were used: (1) Genistein, a general tyrosine kinase inhibitor, and β-cyclodextrin, which depletes cholesterol and disrupts membrane rafts, have been shown to inhibit syndecan-1-mediated endocytosis [Fuki et al., 1997;Fuki et al., 2000b], (2) H89, an inhibitor of PKA, and PAO, a tyrosine phosphatase inhibitor, which interfere with LRP1-mediated internalization via clathrin-coated pits [Goretzki et al., 1997]. Both Genistein and β-cyclodextrin nearly abolished PN-1 uptake only in LRP1-/- MEF cells. In contrast, H89 and PAO reduced PN-1 uptake only in wild type MEF cells, with H89 showing a much stronger inhibitory effect. In LRP1-/- MEF cells, H89 increased PN-1 uptake whereas PAO showed no effect (Fig. 13D). These data suggested that syndecan-1, possibly together with an alternative carrier, could function as a receptor mediating PN-1 uptake in LRP1-/- MEF cells.

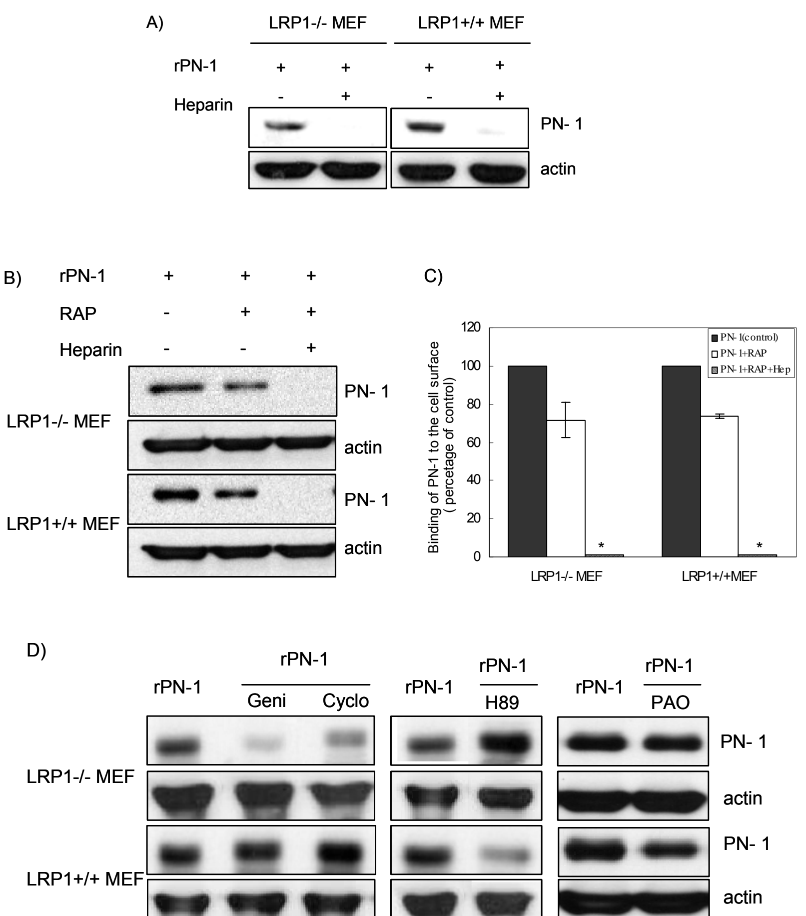


Fig. 13 Heparan sulfate proteoglycans are involved in PN-1 binding and internalization in LRP1-/- MEF cells. (A) Cells were incubated with 300 ng/ml rPN-1 in absence or presence of 1 mg/ml heparin at 4°C for 2h. Immunoblot analysis of the plasma membrane fraction showed that soluble heparin inhibited PN-1 binding to cell surface in both LRP1-/- and wild type MEF cells indicating that the heparin binding site of PN-1 is also required for PN-1 processing in MEF cells. (B, C) Cells were incubated with rPN-1 in the absence or presence of 250 nM RAP or combination of RAP and 1 mg/ml heparin at 4°C for 2h. Immunoblot analysis of the plasma membrane fraction showed that soluble heparin combined with RAP completely blocked PN-1 binding to cell surface in both LRP1-/- and wild type cells, whereas RAP reduced only 30% of PN-1 binding (data: mean ± SE, black bar: active PN-1 as control, white bar: active PN-1 with RAP, grey bar: active PN-1 with RAP and heparin). (D) Both types of cells were also incubated with 300 ng/ml rPN-1 alone or in presence of 300 μM Genistein (Geni), 10 mM β-cyclodextrin (Cyclo), 25 μM H89 and 1 μM PAO respectively at 37°C for 3h. Immunoblot analysis of cell lysates showed that Genistein and β-cyclodextrin inhibited PN-1 uptake only in LRP1-/- MEF cells. In contrast, H89 and PAO reduced PN-1 uptake only in wild type MEF cells, with H89 showing much stronger inhibitory effect. In LRP1-/- MEF cells, H89 increased PN-1 uptake whereas PAO show no effect.

4.5 Syndecan-1 plays a predominant role in PN-1 uptake in LRP1-/- MEF cells

As syndecan-1 was indicated to be important in PN-1 uptake in the absence of LRP1, we further evaluated its contribution to this event in MEF cells. Syndecan-1 is the only cell surface HSPG known to be expressed during early mouse embryonic development [Bernfield et al., 1999]. Our results from semi-quantitative PCR also showed that syndecan-1 and –4 were the most abundant syndecans expressed in MEF cells (Fig. 14A,B). Nevertheless, there is no indication that syndecan-4 is involved in any endocytic pathway; we therefore mainly focused on exploring the role of syndecan-1 in PN-1 internalization. The idea was to exam whether modifying syndecan-1 expression level could influence PN-1 internalization. Therefore the full-length syndecan-1 was over-expressed in both types of MEF cells (Fig. 14C). This led to more PN-1 uptake only in LRP1-/- MEF cells (Fig. 14D). On the other hand, over-expression of full-length syndecan-1 could not facilitate PN-1 uptake in wild type MEF cells (Fig. 14D), suggesting that in this case LRP1 was the predominant receptor.

It is known that the association of the cytoplasmic domain of syndecan-1 to the cytoskeleton upon ligand-triggered clustering represents an important step in syndecan-1-mediated endocytosis. In addition, tyrosine kinase activity is required for endocytosis, suggesting that the phosphorylation of the very conserved tyrosine residues within cytoplasmic domain of syndecan-1 can be important for endocytosis [Fuki et al., 1997]. It is also known that either deletion of the C-terminal 23 amino acids or the point mutation of tyrosine residues within this domain abolishes syndecan-1 association with microfilaments [Carey et al., 1996]. The truncated syndecan-1, which retains only nine amino acids of the cytoplasmic domain (DNsyn1), was therefore over-expressed (Fig. 14C). This did not alter PN-1 uptake in either MEF cell lines (Fig. 14D). These results suggested that the intracellular domain of syndecan-1 was also required for the PN-1 uptake in LRP1-/- MEF cells. Therefore we considered that syndecan-1 not only provided its heparin sulfate side chains as a docking site for PN-1 binding to the cell surface but also, actually more importantly, interacted with PN-1 via its core protein, especially its intracellular domain, for PN-1 internalization. To better address the functional contribution of syndecan-1 to PN-1 internalization, siRNA was applied to transiently knock down its expression level (Fig. 14F,G). Decrease of syndecan-1 levels resulted in a significant reduction of PN-1 uptake in LRP1-/- MEF cells, whereas it

remained without effect in wild type MEF cells (Fig. 14H). Taken together, these data demonstrated that syndecan-1 played a predominant role in PN-1 uptake in LRP1-/- MEF cells.

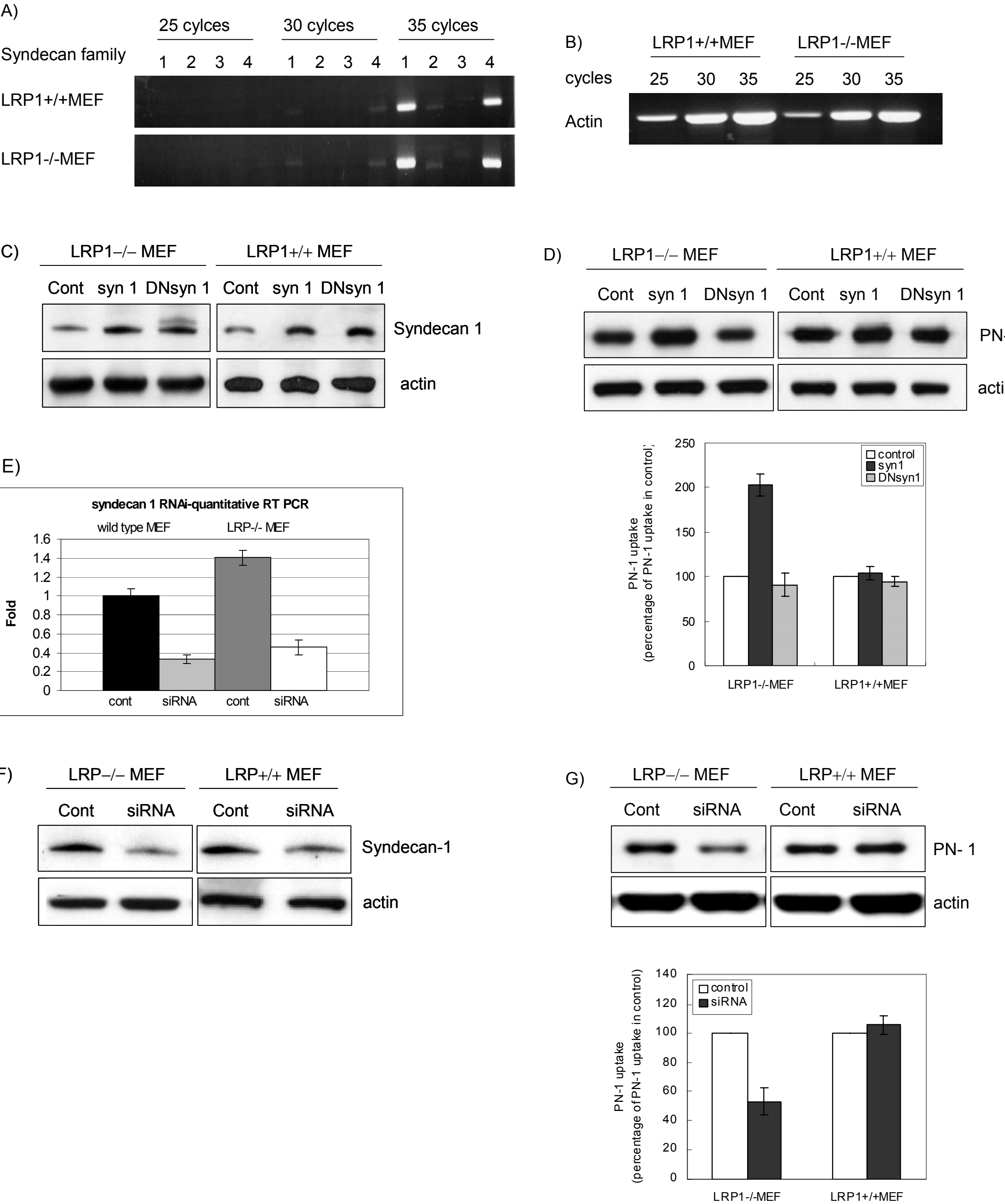


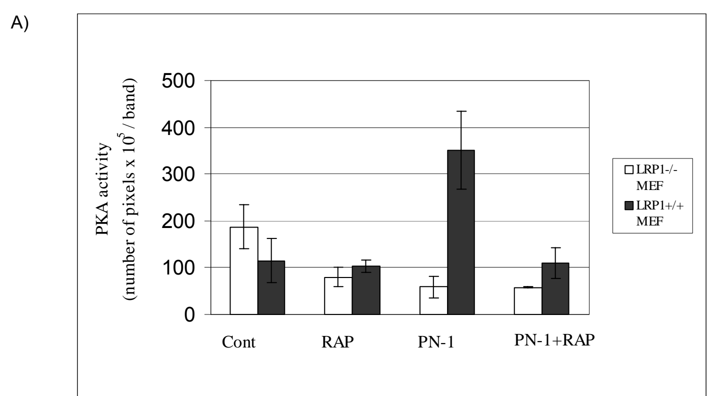
Fig. 14 Syndecan-1 mediated PN-1 internalization in LRP1-/- MEF cells. (A, B) The mRNA levels of members of syndecan family were measured in MEF cells by semi-quantitative RT-PCR, taking actin as control. (C) Both types of MEF cells were transfected with plasmids encoding either full-length (Syn1) or truncated syndecan-1 lacking 23-amino acids of its C-terminal (DNsyn1). Stable clones were selected and the levels of syndecan-1 in the derived cell lines confirmed by immunoblotting. (D) Uptake of rPN-1 was increased significantly in LRP1-/- MEF cells over-expressing full-length syndecan-1, but was only barely changed in LRP1-/- MEF cells over-expressing truncated syndecan-1. The over-expressed syndecans did not significantly affect PN-1 internalization in wild type cells (data: mean ± SE). (E, F) Both types of MEF cells were also transiently transfected with siRNA targeting syndecan-1. 24h later changes in syndecan-1 mRNA and protein levels were confirmed by real time PCR (data: mean ± SE) and immunoblotting. (G) After siRNA transfection, lower level of syndecan-1 led to a significant decrease in rPN-1 uptake only in LRP1-/- MEF cells (data: mean ± SE).

4.6 PN-1 activates the ERK signaling pathway in LRP1-/- MEF cells

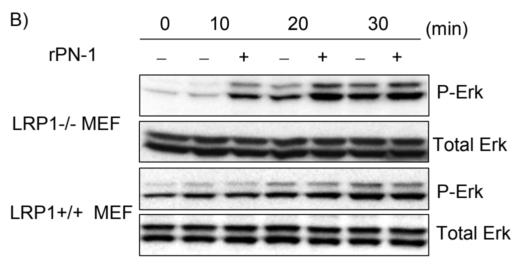
It has been shown that PKA is activated upon apoE binding to LRP1 [Zhu et al., 2003]. Consequently it is worth investigating the signaling pathway activated by the interactions between PN-1 and distinct cell surface receptors, such as LRP1 and syndecan-1. As expected, in wild type MEF cells, PN-1 activated PKA and this effect was abolished by RAP (Fig. 15A). In LRP1-/- MEF cells, PKA activity was reduced upon incubation with either RAP or PN-1; and in the presence of both ligands PKA level was not changed (Fig. 15A). The cross talk between PKA and MAP kinase signaling, which regulates cell proliferation and migration, is well established [Bornfeldt et al., 1999;Stork et al., 2002]. The presence of phospho-ERK was therefore monitored to further evaluate downstream effects of PN-1 uptake in the absence of LRP1. As described, phosphorylation of ERK increased with time after serum deprivation in LRP1-/- MEF cells [Ma et al., 2002], we set up controls for each time point when testing for PN-1's effect on activation of ERK signaling. Upon incubation with rPN-1, increased levels of phosphorylated ERK were detected in LRP1-/-, but not in wild type MEF cells (Fig. 15B). Co-incubation of rPN-1 with β-cyclodextrin, which blocked PN-1 internalization through syndecan-1, abolished ERK activation triggered by PN-1 (Fig. 15C). These results indicated that PN-1 activated the ERK signaling pathway in the absence of LRP1, an effect that may be mediated by interaction with syndecan-1.

To identify the upstream of ERK signaling, we over expressed the dominant negative H-Ras (RasN17) [El Shemerly et al., 1997] in LRP1-/- MEF cells. PN-1 significantly increased phospho-MEK and phospho-ERK in LRP1-/- MEF cells transfected with empty vectors, but this increased phosphorylation of MEK and ERK was largely reduced by over-expression of dominant negative Ras (Fig. 15D). These data indicated that PN-1 actually activated ERK signaling through Ras-Raf-MEK pathway.

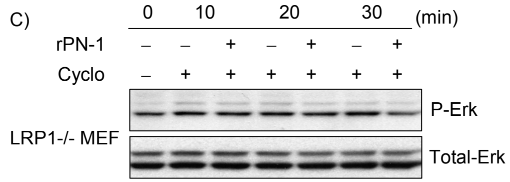
A)



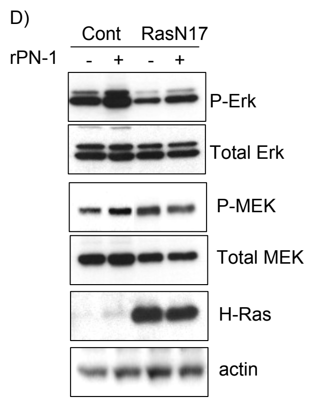
B)



C)



D)



E)

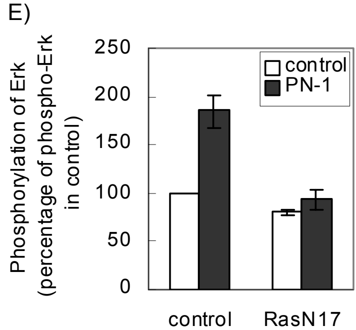
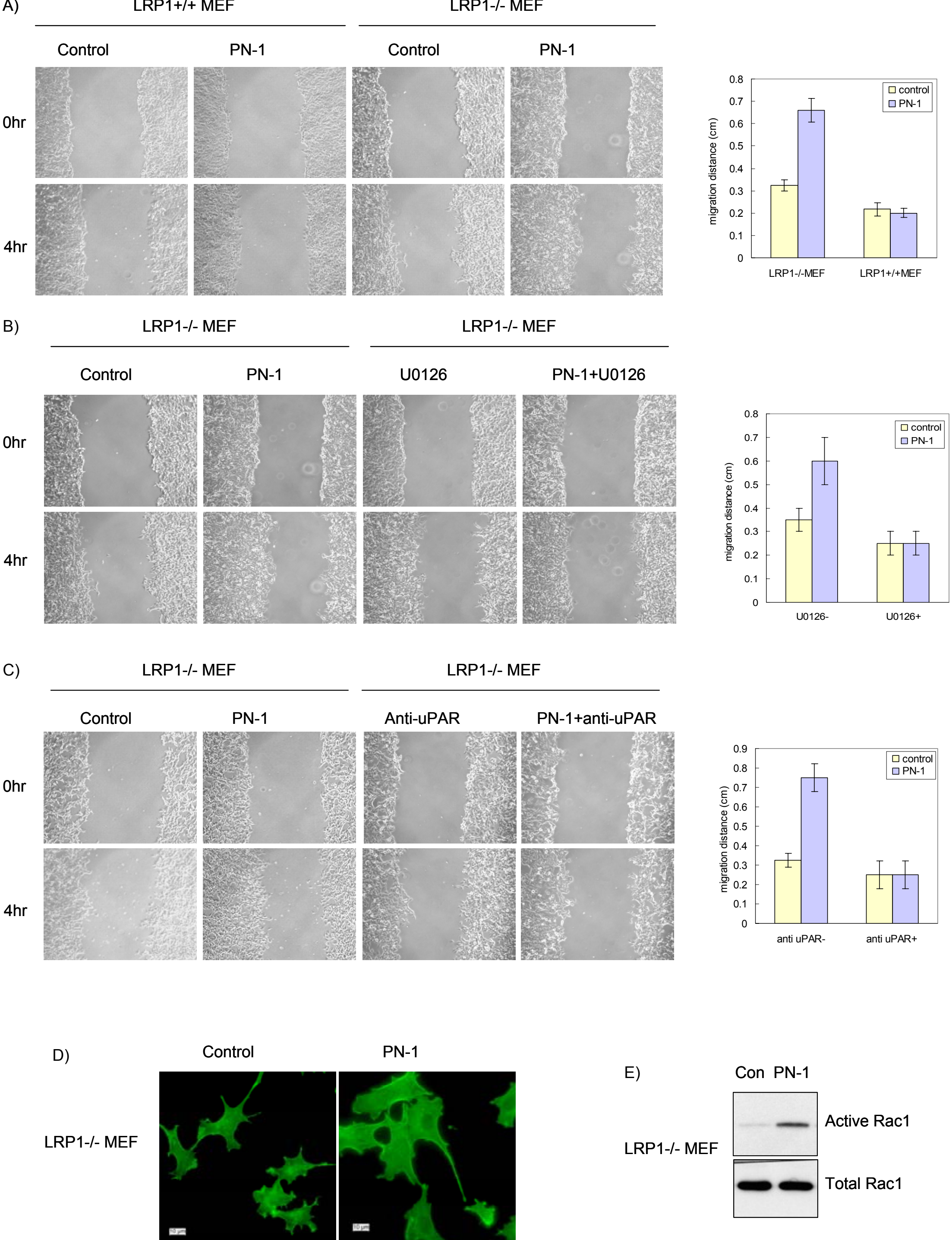


Fig. 15 PN-1 activates different cell signaling pathways upon interaction with distinct cell surface receptors. (A) Both type of MEF cells were plated at the same density as for uptake experiments and pre-incubated overnight in SFM before incubation with 300 ng/ml active PN1 in presence or absence of 50 nM RAP, at 37°C for 10min. A three-fold increase of PKA activity was detected and effectively blocked by RAP in LRP1+/+ MEF cells (black bars). The level of PKA activity in LRP1-/- MEF cells was reduced by incubation with PN-1 or RAP alone, also by PN-1 combined with RAP, but it remained unchanged between PN-1 and PN-1 combined with RAP (white bars). (B) MEF cells were cultured until confluence and switched to SFM fro 30min before applying PN-1. After incubation with 300 ng/ml active PN1 at 37°C for 10, 20 and 30min, a transient increase of phospho-ERK was detected in LRP1-/- but not in LRP1+/+ MEF cells. (C) In the presence of 10 mM β-cyclodextrin that interfered with syndecan-1-mediated internalization, the effect of PN-1 was abolished. (D, E) Over-expression of dominant negative Ras (RasN17) in LRP1-/- MEF cells also abolished ERK phosphorylation induced by PN-1, as showed by immunoblotting and quantification thereof (data: mean ± SE, white bar: control, black bar: active PN-1).

4.7 *PN-1 increases LRP1-/- MEF cell migration by activating ERK and its downstream effector Rac1*

The migration behavior of LRP1-/- MEF cells has been well studied. Loss of LRP1 expression in MEF cells leads to uPA accumulation in the medium, uPAR accretion on the cell surface, and to Rac1 activation, thus stimulating cell migration [Weaver et al., 1997]. The underlying mechanisms were partially elucidated. It is known that uPA-uPAR interaction activates ERK and its downstream MLCK [Nguyen et al., 1999]. LRP1 suppresses uPA-uPAR-mediated signaling to ERK by binding free uPA, thus decreasing the free uPA available to interact with uPAR [Weaver et al., 1997]. Consequently lose LRP1 expression promotes MEF cell migration. Two major reasons justify exploring the function of PN-1 on cell migration. First, PN-1 has been reported to be up regulated in metastatic tumors and to increase local tumor invasion [Buchholz et al., 2003]. Second, in our working model, PN-1 activated ERK signaling, which is obviously important for MEF cell migration. In the *in vitro* wound healing experiments, PN-1 increased LRP1-/- MEF cell migration by nearly 2 fold, but it had no effect on wild type MEF cells (Fig. 16A). To confirm that PN-1’s function on migration is due to its ability to activate ERK signaling, we examined the effect of a MEK inhibitor on PN-1-induced cell migration. U0126, which inhibits both active and inactive MEK, antagonized the PN-1-stimulated migration in LRP1-/- MEF cells (Fig.15B). To identify the upstream effector of signaling pathway activated by PN-1, we coincubated LRP1-/- MEF cells with PN-1 and anti-uPAR antibody, which is functionally blocking ligand binding to uPAR. This antibody abolished PN-1 induced migration as well (Fig 15C). We then further studied the downstream effectors of ERK signaling activated by PN-1, which consequently promotes cell migration. Upon PN-1 incubation in LRP1-/- MEF cells, we observed promoted lamellipodia formation; and this was due to the activation of small GTPase Rac1, already known to regulate such morphological changes in migrating cells (Fig. 16D,E).



A)
LRP1+/+ MEF
LRP1-/- MEF
Control
PN-1
Control
PN-1
0hr
4hr
migration distance (cm)
control
PN-1
LRP1-/-MEF
LRP1+/+MEF
B)
LRP1-/- MEF
LRP1-/- MEF
Control
PN-1
U0126
PN-1+U0126
0hr
4hr
migration distance (cm)
control
PN-1
U0126-
U0126+
C)
LRP1-/- MEF
LRP1-/- MEF
Control
PN-1
Anti-uPAR
PN-1+anti-uPAR
0hr
4hr
migration distance (cm)
control
PN-1
anti uPAR-
anti uPAR+
D)
Control
PN-1
LRP1-/- MEF
E)
Con PN-1
LRP1-/- MEF
Active Rac1
Total Rac1

Fig. 16 PN-1 increases LRP-/- MEF cell migration by activation of Rac1. (A) MEFs cells were cultured in presence of 10% FCS until confluency. Monolayer was wounded and incubated in SFM containing 300 ng/ml rPN-1 37°C for 4h. Cell migration into denuded space was recorded and plotted. PN-1 increases LRP1-/- but not the wild type MEF cell migration. (B, C) LRP1-/- MEF cells were culture under the same conditions as describe above, and incubated with 300 ng/ml rPN-1 in the presence or the absence of either 10 µM U0126 or 10 µg/ml anti-uPAR antibody. Cell migration into denuded space was recorded and plotted. Both U0126 and anti-uPAR abolished PN-1 induced migration of LRP1-/- MEF cells. (D) LRP1-/- MEF cells were plated in 4-well cell culture chamber at 2.0 x 10^4 cells per well in SFM with or without 300 ng/ml rPN-1 at 37°C for 1h and stained by phalloidin for cytoskeleton. PN-1 induced lamellipodia formation in LRP1-/- MEF cells. (E) LRP1-/- MEF cells were cultured and wounded under the same conditions as described above. Afterwards cells were incubated with or without 300 ng/ml PN-1 at 37°C for 1h; activated Rac1 was pull down by GST-fused p21-activated kinase. PN-1 activated Rac1 in LRP1-/- MEF cells.

4.8 Enhanced interaction between PN-1 and syndecan-1 promotes LRP1-/- MEF cell migration.

Previous results showed that 1) β-cyclodextrin inhibited PN-1 internalization mediated by syndecan-1. 2) it also blocked the activation of phospho-ERK induced by PN-1. Based on these observations, we hypothesized that the interaction between PN-1 and syndecan-1 was involved in the activation of ERK signaling in LRP1-/- MEF cells. Thus enhanced interaction between PN-1 and syndecan-1 would activate ERK signaling and increase cell migration. To test this hypothesis, we designed two experiments to enhance PN-1 and syndecan-1 interaction. The first one was to over-express syndecan-1, the transmembrane receptor for PN-1 in LRP1-/- MEF cells, and to incubate such cells with rPN-1 during the migration process. The second one was to over-express PN-1, and to test ERK phosphorylation level and the corresponding migration behavior of such cells. Upon PN-1 incubation, LRP1-/- MEF cells over-expressing syndecan-1 (Fig. 17A) showed a significant 3-fold increase in cell migration on vitronectin (Fig. 17C). Compared to the control cells, MEF cells over-expressing PN-1 showed a constitutively higher level of phospho-ERK (Fig. 17B) and a nearly 2-fold enhancement in cell migration on vitronectin (Fig. 17C).

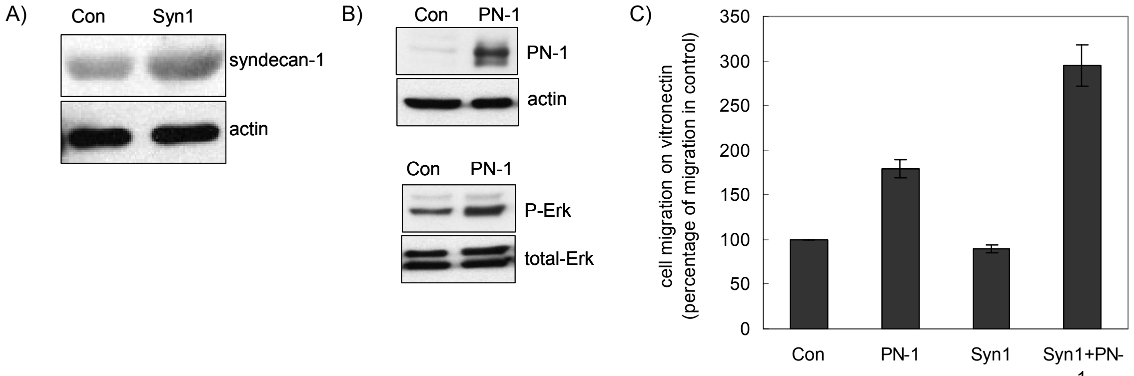
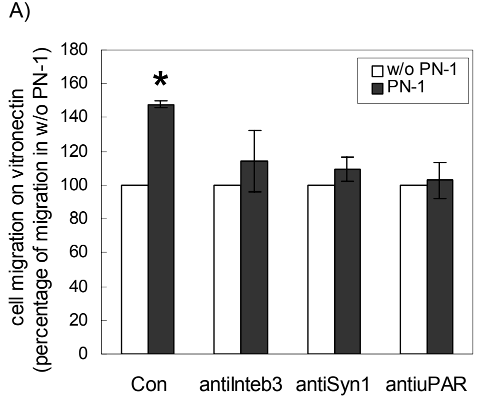
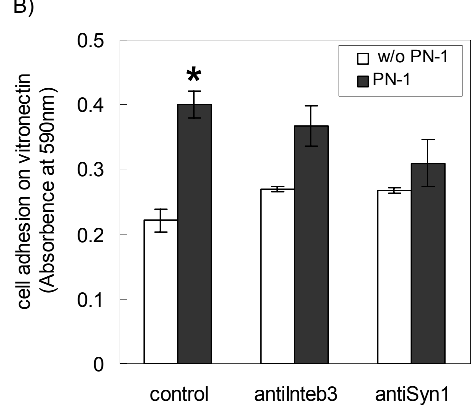


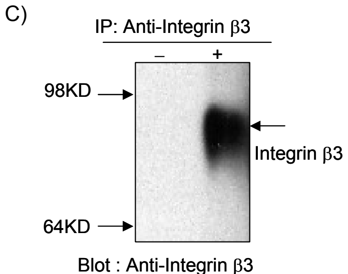
Fig. 17 Enhanced interaction between PN-1 and syndecan-1 activates ERK signaling and increases LRP1-/- MEF cell migration (A) Over-expression of syndecan-1 in LRP1-/-MEF cells was confirmed by immunoblotting (B) Over-expression of PN-1 in LRP1-/-MEF cells (top panels) constitutively activated ERK signaling (bottom panels). (C) Cell migration was tested in modified Boyden chamber with vitronectin-coated filter and with SFM in both top and bottom chambers. LRP1-/- MEF cells were allowed to migrate at 37°C for 4h. Cell migration was increased either by over-expression of PN-1 or by over-expression of syndecan-1 and incubation with rPN-1.

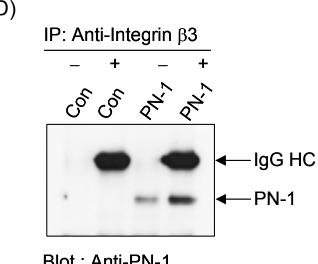
4.9 PN-1 and syndecan-1 are coimmunoprecipitated with integrin β3

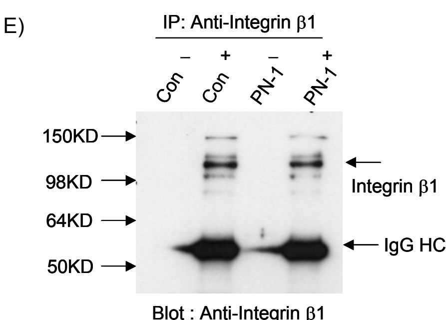
Given the previous results, one could conclude that in LRP1-/- MEF cells, the interaction between PN-1 and syndecan-1 activated ERK signaling and its downstream effector Rac1 promoting cell migration on vitronectin. It was then justified to explore the molecular mechanisms underlying this signaling transduction. Syndecan-1 is known to regulate the activity of integrin αvβ3 and to mediate cell spreading and migration requiring signaling by integrin αvβ3 in human carcinoma cells [Beauvais et al., 2003;Beauvais et al., 2004a]. Furthermore, ERK has been shown to be downstream of integrin signaling [Wary et al., 1996;Roberts et al., 2003]. So one can consider that PN-1 participates syndecan-1-integrin machinery and activates ERK through integrin signaling, thus mediating cell migration. To validate this hypothesis, we first tested whether functional blocking antibodies against syndecan-1, integrin β3 or uPAR, which is known to be involved in integrin αvβ3 signaling [Adachi et al., 2001], would inhibit the migration triggered by PN-1 in LRP1-/- MEF cells. Cells were pre-treated with antibodies before incubation with rPN-1 in the migration or adhesion assays. Each of these three antibodies showed inhibitory effects on both migration and adhesion mediated by PN-1 (Fig. 18A,B). Secondly we tested whether PN-1 and integrin β3 formed complexes in LRP1-/- MEF cells. Following incubation with PN-1 immunoprecipitation was performed using specific antibody against integrin β3. The detection of integrin β3 on immunoblot of the immunoprecipitates demonstrated the specificity of the antibody (Fig. 18C). PN-1 was also detected in the same immunoprecipitates (Fig. 18D). As control, we used the same samples to perform immunoprecipitation with anti-integrin β1 antibody. Only integrin β1 but not PN-1 was detected after such immunoprecipitation (Fig. 18E, F). To better support our working hypothesis, syndecan-1 was over-expressed in LRP1-/- MEF cells, which were then incubated with PN-1. The analysis of the immunoprecipitates using specific antibody against integrin β3 revealed syndecan-1 and PN-1 in the same blot (Fig. 18G, H). Taken together, we concluded that PN-1 specifically interacts with syndecan-1 and integrin β3.

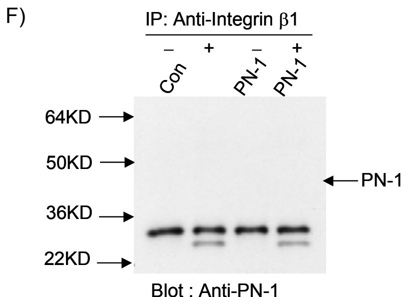

A)
cell migration on vitronectin
(percentage of migration in w/o PN-1)
180
160
140
120
100
80
60
40
20
0
w/o PN-1
PN-1
*
Con
antiInteb3
antiSyn1
antiuPAR


B)
cell adhesion on vitronectin
(Absorbence at 590nm)
0.5
0.4
0.3
0.2
0.1
0
w/o PN-1
PN-1
*
control
antiInteb3
antiSyn1


C)
IP: Anti-Integrin β3
–
+
98KD
64KD
Integrin β3
Blot : Anti-Integrin β3


D)
IP: Anti-Integrin β3
–
+
–
+
Con
Con
PN-1
PN-1
IgG HC
PN-1
Blot : Anti-PN-1


E)
IP: Anti-Integrin β1
–
+
–
+
Con
Con
PN-1
PN-1
150KD
98KD
64KD
50KD
Integrin β1
IgG HC
Blot : Anti-Integrin β1


F)
IP: Anti-Integrin β1
–
+
–
+
Con
PN-1
PN-1
64KD
50KD
36KD
22KD
PN-1
Blot : Anti-PN-1

IP: Anti-integrin β3

G) control Syndecan-1 Syndecan-1 PN-1

antibodies - + - + - +

64 KDa

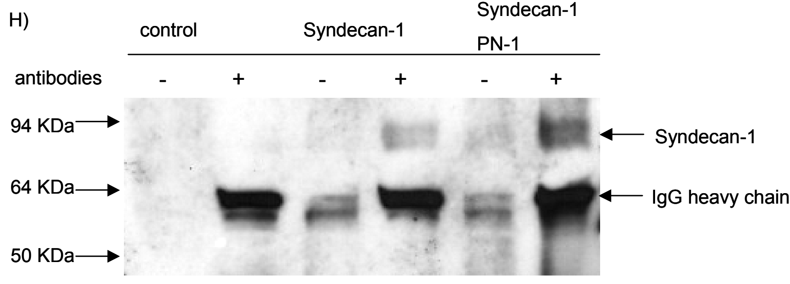
50 KDa

IgG heavy chain

PN-1

Blot: Anti-PN-1

IP: Anti-integrin β3



Blot: Anti-Syndecan-1

Fig. 18 PN-1 and syndecan-1 are coimmunoprecipitated with integrin β3 in LRP1-/- MEF cells. (A, B) Antibodies against integrin β3, syndecan-1 or uPAR inhibited PN-1-stimulated LRP1-/- MEF cell migration and adhesion on vitronectin (asterisk indicates the statistical significance, $p<0.05$). (C) Integrin β3 was detected in the samples, which were immunoprecipitated using anti-integrin β3 antibodies. (D) LRP1-/- MEF cells were incubated with 300 ng/ml active PN-1 at 37°C, for 1h. Upon immunoprecipitation using anti-integrin β3 antibodies, PN-1 was detected only in the samples from the cells, which had been incubated with PN-1. (E, F) In the control experiments, samples were immunoprecipitated using anti-integrin β1 antibodies. Integrin β1 was detected after immunoprecipitation, but PN-1 was not detected. (G, H) Full-length syndecan-1 was over-expressed in LRP1-/- MEF cells, and such cells were incubated with 300 ng/ml active PN-1 at 37°C for 1h. Both syndecan-1 and PN-1 were detected on the same blot after immunoprecipitation using anti-integrin β3 antibodies.

5 Discussion and Outlook

Extracellular proteolysis plays an important role in cell-cell contacts and cell-ECM interactions. Serine proteases form an important subclass of extracellular proteases, and their proteolytic activity is delicately controlled by the serine protease inhibitors. Our experimental results show that active, free PN-1 is also internalized by cells. In addition, we provide evidence that depending on the receptor involved, different signaling pathways are activated, leading to distinct cellular responses to an environmental challenge. Serine protease inhibitors are vital then not only for the control of extracellular proteolysis, but also for sensing and providing information about the extracellular space to the cell via its receptors.

It is commonly accepted that PN-1 binds to its target proteases such as thrombin, uPA, and tPA forming complexes, and that these complexes bind to HSPG, are subsequently internalized through LRP1 and degraded [Knauer et al., 1997b;Crisp et al., 2000]. In this established model of PN-1 complexes interaction with LRP1, it is assumed that active PN-1 cannot bind to the receptor although the receptor recognizes a specific PN-1 moiety within PN-1-protease complexes [Low et al., 1981]. The formation of PN-1-protease complexes leads to a conformational change of PN-1 and this is a prerequisite for binding to the endocytic receptor. However, active PN-1 internalization has been observed in fibroblast cell culture. Howard et al have reported that the levels of endogenous free PN-1 in fibroblast-conditioned media reach steady state within 48 h due to a constant secretion and removal in a free form by cells [Howard et al., 1986]. Unpublished observations made in our lab also showed that PN-1 could be internalized in different cell types, including HepG2 cells and primary hippocampal neurons (Seiler F. and Albrecht H., unpublished data). In our experimental results, active PN-1 is taken up via either LRP1-dependent or -independent endocytic pathway. Nevertheless, in the presence of LRP1, active PN-1 uptake was less efficient than that of the PN-1-thrombin complex (Fig. 11B). This result accords with the above hypothesis that formation of PN-1 complexes leads to the conformational change of PN-1, which facilitates the recognition or binding to the endocytic receptor, such as LRP1. Interestingly, in the absence of LRP1, uptake of active PN-1 is the same as that of PN-1-thrombin complex (Fig. 11B),

indicating that the conformational change is not always necessary for internalization of PN-1 or PN-1 complexes.

In both wild type and LRP1-/- MEF cells, cell surface binding is blocked by heparin and reduced by RAP (Fig. 13 A,B,C). These results confirm the requirement of the heparin binding site of PN-1 for the interaction with the cell surface [Howard et al., 1987;Herndon et al., 1999]. They are however in contrast to the report that RAP increases VLDL binding to HSPG in LDLR deficient cells [Wilsie et al., 2003]. This implies that RAP does not interact solely with ligand binding to LRPs. Consequently, our data is rather in line with the proposal that RAP may also interact with HSPG core proteins [Vassiliou et al., 1994], thus altering the conformation of the heparan sulfate side chains, which in turn changes the affinity to different ligands.

PN-1 internalization is inhibited in the wild type but not in LRP1-/- cells by both RAP and a peptide corresponding to the domain of PN-1 considered to interact with LRP1 (Fig. 12D). These results indicate that HSPG could take over, at least partially, the endocytic function of LRP1. However the potent RAP inhibition detected in wild type cells after 3h of incubation seems to indicate that HSPG does not substitute for LRP1 function in such cells. A possible explanation for this phenomenon could be that wild type cells would need time to switch to an HSPG–dependent mechanism. Such a delay has been reported for VLDL internalization. In the presence of RAP, LRP1-dependent internalization is reduced after 5.5h while an increased HSPG-dependent internalization is detected by 16h [Wilsie et al., 2003]. In line with this proposal, activation of ERK pathway is not detected upon 15min incubation of wild type cells in presence of rPN-1, RAP or both (results not shown). The mechanism, by which cells switch from LRP1 to HSPG to mediate endocytosis requires further study.

Endocytosis is an important mechanism by which cells interpret or respond to their environment, receiving the extracellular information and translating it into a specific biological function. This mechanism also initiates the clearance of serine proteases complexed to their inhibitors, thus providing the accurate cell surface proteolysis required by cells. Cell surface proteolysis is an important mechanism for generating biologically active proteins that mediate a range of cellular functions and contribute to biological processes such as migration and invasion. Our observation that active PN-1 is

also internalized indicates that the active inhibitor can be removed from the extracellular environment. This mechanism could function to regulate local extracellular proteolytic activity. Similarly the active form of neuroserpin has been reported to be internalized, but through an LRP1-dependent mechanism that requires an additional co-factor [Makarova et al., 2003]. Obviously, other mechanisms influencing PN-1 expression and availability may also be important for determining the local level of the inhibitor. This may represent a mechanism of short-term proteolytic regulation, as opposed to long-term, resulting from protein expression changes in response to prolonged challenges. Examples of this include the down regulation of PN-1 expression by incubation with thrombin for more than 18h in aortic smooth muscle cells, or the up regulation of PN-1 expression in pathological situations such as hypertension [Bouton et al., 2003;Richard et al., 2004]. Furthermore, active PN-1 interaction with cell surface receptors could also be important for its function in signaling transduction. We observed that in cortical or cerebellar primary neuronal culture, there was only a small population of neurons expressing PN-1, but some non-expressing neurons took it up from the vicinity (Fig. 10B). In cerebellar primary neuronal culture, PN-1 was shown to compete with Shh for LRP1 binding, thereby regulating Shh-mediated cell proliferation during cerebellum development (Vaillant C., et al, 2006, submitted). In LRP1-/- MEF cells, exogenous PN-1 activates Ras-MEK-ERK signaling by interaction with syndecan-1 and promotes lamellipodia formation and cell migration (Fig. 15,16).

Endocytosis is mediated by different cell surface receptors. These receptors can share the same ligand, or one receptor can mediate the internalization of distinct ligands, such as members of LDLR family [Nykjaer et al., 2002]. We have showed here, that in addition to the described internalization of complexed PN-1 [Knauer et al., 1997b], active PN-1 can be endocytosed as well by LRP1-dependent mechanism (Fig. 11A,B). We also have identified syndecan-1 as an alternative receptor for the internalization of active PN-1 in LRP1-/- cells (Fig. 13,14). The contribution of other HSPG family members cannot be excluded since this study was not carried out under syndecan-1 knockout conditions.

It has been known for more than 20 years that there is clearance of remnant lipoproteins by liver, which is mediated partly through an LDLR-independent pathway [Kita et al., 1982;Rubinsztein et al., 1990;Ishibashi et al., 1994]. The LDLR-independent pathway mediates about one-third of LDL removal from plasma in normal humans and

all removal in patients homozygous for receptor-negative familial hypercholesterolemia [Goldstein et al., 1977;Kesaniemi et al., 1983]. As shown by studies carried out in different laboratories, LRP1 and HSPG are the probable receptors for hepatic and arterial catabolism of atherogenic lipoprotein [Fernandez-Borja et al., 1996;Al Haideri et al., 1997;Seo et al., 1997;Llorente-Cortes et al., 2002]. All these observations indicate a functional correlation and/or overlapping between LRP1 and HSPG *in vivo*. Furthermore, there is also difference between these two classes of receptors in term of endocytic function. In LRP1-mediated endocytosis, HSPG or another co-receptor must present their ligands to cell surface LRP1 because LRP1 cannot capture a ligand by itself [Knauer et al., 1997b;Crisp et al., 2000]. It is proposed that HSPG-induced changes in conformation lead to a higher affinity of these ligands to LRP1. In contrast, HSPG is sufficient to bind, internalize, and deliver ligands to lysosomes [Williams et al., 1997]. HSPG shows better affinity to and higher capacity for the ligands as well; because HSPG is more abundant than most cell receptors and heparan sulfate (HS) side chains can bind more than one ligand at one time. However, the kinetics of HSPG-mediated internalization is lower than for LRP1. In part it is because LRP1 has a much fast turnover than HSPG. Hence it is likely that the relative roles of these two mechanisms will depend on the ligand affinity, and on the specific expression pattern of LRP1 and HSPG in different cell types. In the latter case, it also depends on the HS side chain structure and specific HSPG core protein. In this study, LRP1 shows a predominant role over syndecan-1 in endocytic function, because changes of syndecan-1 expression level cannot interfere with PN-1 internalization in wild type MEF cells (Fig. 14D,G). Down-regulation of syndecan-1 could be compensated by other cell surface HSPGs, whereas up-regulation of syndecan-1 does not benefit PN-1 uptake either. This indicates that LRP1 is the rate-limiting factor in LRP1-dependent internalization. It also implicates that in the presence of LRP1 other unidentified factors may be required to activate syndecan-1-mediated endocytosis. However, in wild type MEF cells as already discussed, syndecan-1 did not respond even when LRP1 pathway was blocked by RAP or peptide 960 (Fig. 12D). The decision made by the cells seems to be more complicated than simply switching from one to the other pathway, because different modes of endocytosis would also lead to regulation of signaling transduction, to which endocytosis is coupled.

It is known that the level of LRP1 is not necessarily the same *in vivo*, especially under pathological situation or during the progressing of different diseases. Low levels of LRP1

expression have been correlated with aging, prostate cancer and Alzheimer's disease [Field et al., 2000;Kang et al., 2000;Gilardoni et al., 2003]. Thus, the importance of our findings resides in the identification of the alternative function of syndecan-1 as a receptor that could compensate for low level or absence of LRP1 and that may trigger different cell responses by the same ligand. Although the nature of the cross talk between these two types of receptors remains unknown, it has been reported that syndecan-1 synthesis is increased in cells with impaired clathrin-dependent endocytosis [Llorente et al., 2001], implying that compensatory mechanisms exist for low LRP1 level.

Besides their endocytic function, such alternative endocytic pathways could also be important for associated signaling functions [McPherson et al., 2001]. The signaling complexes are recruited to the vicinity of endocytic machinery and delivered to specific subcellular compartments, thus regulating cell surface receptor activity. As has been shown for the binding of apo E to LRP1 [Zhu et al., 2003], we observed that interaction between PN-1 and LRP1 caused PKA activation (Fig. 15A). Li et al reported that a PKA-mediated phosphorylation of the intracellular domain of LRP1 leaded to an increase of LRP1-mediated internalization [Li et al., 2001]. Therefore we consider that PKA activation induced by PN-1 is a kind of positive feedback mechanism to increase internalization efficiency. This could also explain the higher efficiency on the uptake of PN-1 or PN-1 complex in the presence of LRP1 (Fig. 11A,B). Surprisingly, we have found that PN-1 activates ERK signaling pathway only in LRP1-/- MEF cells (Fig. 15B). Blockade of PN-1 uptake, by β-cyclodextrin-mediated disruption of intact lipid rafts, abolished this ERK activation (Fig. 15C). This implies that PN-1 and syndecan-1 interaction achieves a different signal transduction outcome depending on the presence of LRP1. Although the mechanism behind the coupling of syndecan-1-mediated endocytosis and signal transduction is still unknown, the observation that PN-1 activates ERK signaling in LRP1 -/- MEF cells can be of crucial importance. The cross talk between PKA and ERK signaling is extremely complicated. Briefly, PKA can either positively or negatively regulate ERK signaling through several specific mechanisms, which all involve the regulation of phosphorylation of Raf. More importantly, the outcome of this cross talk is highly cell-type specific [Stork et al., 2002]. It has been reported that in MEF cells, PKA activates Src and its downstream effecter Rap1, which in turn blocks Ras activation of Raf-1 [Schmitt et al., 2002]. Our results indicate that this interference can be abolished in the absence of LRP1, therefore the impact on ERK signaling caused

by the interaction of PN-1 and syndecan-1 is enhanced and triggers distinct and different cellular responses. The selective or balanced activation of these two signaling pathways depends on the respective levels of LRP1 and syndecan-1 available on the cell surface at any given physiological or pathological situation [Day et al., 1999;Hsueh et al., 1999]. Such a mechanism may alter cellular responses in different cell types over a wide range.

The finding that PN-1 activates Ras-MEK-ERK signaling pathway in the absence of LRP1 is very interesting, although PN-1 is not the first member of serpin family that is reported to be involved in signal transduction. It is well known that serine protease including uPA, their receptor such as uPAR, and their inhibitor such as PAI-1 are involved in signal transduction, which in turn regulate tumor growth, invasion, and angiogenesis [Durand et al., 2004]. tPA has recently reported as a potent activator of PDGF-CC [Fredriksson et al., 2004]. Taken together, serine proteases and their cognate inhibitors play an important role in signal transduction, which is rather different from their classic role in proteolysis regulation. This may lead us to a new understanding of PN-1 function on cell behavior, for example cell migration, which is mediated by such signaling transduction. We observed that in LRP1-/- MEF cells, PN-1-induced ERK activation was blocked by β-cyclodextrin, a reagent that inhibits syndecan-1 mediated PN-1 internalization (Fig. 15). This observation actually raises the question, whether syndecan-1 would play a role in ERK signaling activated by PN-1? If so, what the underlying mechanism would be? It has been reported recently that syndecan-1 regulates integrin αvβ3 activity and mediates cell spreading and migration, which require integrin αvβ3 signaling in human carcinoma cells [Beauvais et al., 2003;Beauvais et al., 2004a]. Coincidentally, both PN-1 and syndecan-1 are up-regulated in pancreatic tumor, and PN-1 promotes local invasion of pancreatic tumor cells [Conejo et al., 2000;Buchholz et al., 2003]. It will be interesting to identify other co-factors of PN-1 in this signaling pathway, and investigate how the interactions between PN-1 and co-factors would possibly influence the migration of certain types of cells.

It was even more exciting when we observed that, in the absence of LRP1, PN-1 promoted cell migration by activation of ERK signaling (Fig. 16B). We also showed that pre-incubation of cells with antibodies against uPAR, syndecan-1, and integrin β3 can block PN-1-enhanced migration (Fig. 16C,18A). Moreover, we coimmunoprecipitated PN-1, syndecan-1, and integrin β3 (Fig. 18 G,H). Taken together, we propose a working

model of the mechanism by which PN-1 promotes cell migration (Fig. 19). In this model Integrin $\alpha v\beta 3$ is the central player in this pathway, PN-1 may activate ERK signaling either via interaction with uPA-uPAR system or via with syndecan-1. This could further activate the downstream effectors of integrin Rac1 and MLCK in this case, both of which have been shown play an important role in cell migration.

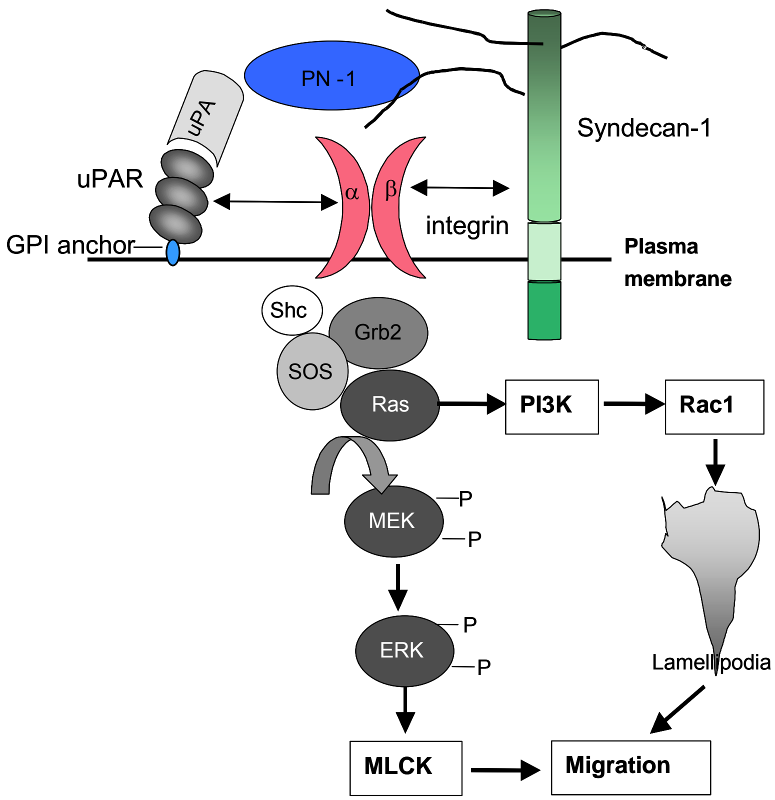


Fig. 19 Model for PN-1 involvement in both uPA-uPAR-initiated and syndecan-1-dependent activation of integrin. Active PN-1 interacts with uPA, leading to uPAR conformation change, and activation of integrin αvβ3 and its downstream Ras-ERK signaling. Alternatively, PN-1 ligation with HS chains (or the ectodomain) of syndecan-1 promotes syndecan-1 coupling to integrin and activates integrin-mediated signaling. Activation of both pathways results in cell migration either through MLCK or through Rac1.

There are many published data suggesting that serine proteases/receptors such as uPA/uPAR, together with their inhibitors (serpins), are organizers of cell-ECM contacts. They cover the full range of activities required to promote and disrupt cell attachment sites, therefore influencing cell migration and invasion. They play significant roles in these events by modulating proteolysis, destroying the ECM, or by mediating cell-ECM interaction and activating cell signaling. Moreover, they interact with ECM molecules, like vitronectin, and cell surface adhesion receptor, like integrin to execute these functions. Thus the coordinated expression and activation of adhesion receptors and of cell-associated serine proteases are required to enable the cells to adhere, to migrate, and to invade surrounding tissues.

The uPA-uPAR system is central to a spectrum of biological processes including fibrinoloysis, inflammation, atherosclerotic plaque formation, and matrix remodeling during wound healing, tumor invasion, angiogenesis, and metastasis. Binding of uPA to uPAR initiates a proteolytic cascade that results in the conversion of plasminogen to plasmin. Plasmin, through its own proteolytic function, degrades a range of extracellular basement membrane components and activates others proteases such as the metalloproteinases, which also degrade ECM proteins. Independent of its catalytic activity, uPA is involved in cell signaling through uPAR. The interactions between uPAR and cell surface/ECM molecules modulate cell-ECM adhesion, an indispensable requirement to establish the ‘grip’, which is necessary for the invading cell to progress within a tissue. Over-expression of uPA or uPAR is a feature of tumor malignancy, and is correlated with tumor progression and metastasis. In contrast, inhibition of expression of these components leads to a reduction in the invasive and metastatic capacity of many tumors. Unexpectedly, not only uPA but also PAI-1 is up-regulated in various tumors and considered to be a strong negative prognostic marker in different cancers [Rakic et al., 2003;Noel et al., 2004].

Degradation of connective tissues is thought to be a necessary step to allow malignant cells to invade locally, enter the lymphatic or blood circulation, and metastasize. The serine protease system (mainly uPA, uPAR) is one of the major systems that are believed to degrade pericellular ECM. They provide the cell surface with necessary enzymatic activity, which can activate the pro-enzyme form of the ECM degradation proteases. This induces directly or indirectly the formation of a provisional

matrix, which scaffolds the leading edge of the invading cells. In this sense we would expect that PN-1, by inhibiting serine proteases activities, would inhibit cell migration.

However, it is well known that PN-1 inhibits catalytic activity of uPA in the presence of ECM molecule such as collagen type IV [Crisp et al., 2002], therefore it may contribute to determine a stable ECM and favor the maintenance of the adhesion sites required for cell adhesion and spreading. It also increases association between uPAR and vitronectin in the presence of active uPA, stimulates uPAR-dependent cell adhesion but does not influence vitronectin binding to integrin or intergrin-mediated cell adhesion. This effect of PN-1 is concentration-dependent [Kanse et al., 2004]. PN-1-uPA complex can be internalized via uPAR and LRP1, during which process uPAR is recycled to the cell surface [Conese et al., 1995]. Thus PN-1 could also disrupt cell adhesion by interfering with the cell surface uPAR pool, which in turn will change the ratio between engaged and free vitronectin and/or integrin. Changes in these ratio could influence the effect of cell detachment mediated by serpin [Czekay et al., 2003]. It is likely that PN-1 plays dual roles namely promoting or destabilizing adhere in cell adhesion, which may depend on the local availabilities of different factors, including PN-1 itself.

Cell migration is intrinsically linked to adhesiveness. Cells require attachment sites in ECM to assemble their cytoskeleton and to initiate membrane protrusions important to migration. However, cell-ECM contact sites cannot be too avid, otherwise the cells would be unable to detach and move.

Apart from their abilities to degrade ECM, uPA and uPAR are involved in signal transduction from the extracellular environment to the intracellular compartments, which influences the cell responsiveness to the extracellular stimuli. In some cell types uPAR localizes in caveolae, which contain clusters of signaling molecules and scaffolding proteins. Alternatively, uPAR can also be found at the leading edge of cells advancing toward a chemotactic stimulus, or at focal contacts of invasive cells, usually in association with molecules of the ECM, integrins, signaling factors, and cytoskeletal elements. These types of co-localizations situate uPAR to play a central role in the activation of integrins and signaling cascades, which in turn induce cytoskeleton reorganization and benefit cell migration.

The uPAR-integrin signaling pathway has been established. uPAR interacts with integrin αvβ3, activates integrin-mediated Ras-ERK signaling pathway by the adaptor protein Shc [Wary et al., 1996;Adachi et al., 2001]. It has been proposed that one of the downstream effectors of Ras-ERK is MLCK, which upon activation phosphorylates the myosin light chain of actin, thereby initiating cytoskeleton contraction and cell migration in an integrin- and matrix-dependent fashion [Adachi et al., 2001]. Another downstream effector of ERK signaling is Rac1. Upon activation (probably through PI3K) it enables changes of the cell cytoskeleton, induces lamellipodia formation and promotes cell migration as well [Adachi et al., 2001]. The activation of uPAR-integrin signaling may or may not need uPA or uPA complex binding to the receptor. Nevertheless, serine proteases and serpins such as uPA and PN-1 play dual roles in this signaling cascade, either by binding and changing the conformation of uPAR, which is required to initiate uPAR signaling [Ossowski et al., 2000] , or by initiating uPAR internalization via LRP1 to reduce uPAR on the cell surface. The outcome of this regulation may be cell type specific. In the case of LRP1-/- MEF cells, where uPAR accumulates on the cell surface [Weaver et al., 1997], we would expect PN-1 to facilitate signal transduction. Indeed this is what we observed, namely the activation of Ras-ERK signaling, activation of Rac1 and lamellipodia formation, and consequently increase of cell migration (Fig. 16). Furthermore we observed that a pre-incubation LRP1-/- MEF cells with anti-uPAR antibody abolishes PN-1 induced-cell migration on vitronectin (Fig. 16C, 18A). Taken together, we conclude that PN-1 activates the Ras-ERK signaling pathway and promotes cell migration via uPAR-mediated signaling in LRP1-/- MEF cells.

However, this may not be the only way that PN-1 is able to promote cell migration. PN-1 interacts with syndecan-1, which is involved in regulation of integrin activity, integrin-mediated cell spreading, and migration. It has been reported that the ectodomain of syndecan-1 and integrin αvβ3 are functionally coupled, and that the integrin is dependent on syndecan-1 to become activated and to mediate signals required for carcinoma cell spreading and migration [Beauvais et al., 2003;Beauvais et al., 2004a]. It is not yet clear how this functional coupling happens, whether it is via a signaling pathway or via a direct interaction between these two receptors. Nevertheless, we have evidence that PN-1 could play a role in the syndecan-1-integrin αvβ3 machinery. First of all, we have shown that in LRP1-/- MEF cells, migration is increased either by over-expression of PN-1 or by over-expression of syndecan-1 followed by incubation

with PN-1 (Fig. 17). This suggests that an enhancement of PN-1 and syndecan-1 interaction increases cell migration. Second, the pretreatment of MEF cells with either anti-integrin β3 or anti-syndecan-1 antibodies blocks PN-1-enhanced cell migration. Third, syndecan-1 and PN-1 are co-immunoprecipitated specifically with integrin β3, but not with integrin β1 (Fig. 18D,F). Beauvais et al. have reported an important feature of syndecan-1 and integrin coupling required to regulate integrin activity. It is that syndecan-1 has to be engaged to a ligand, such as vitronectin, and this engagement requires the HS side chains of syndecan-1. In the light of this observation, active PN-1 interaction with syndecan-1 via HS side chains may provide the prerequisite ligation of syndecan-1-dependent integrin activation, and the subsequent signaling transduction.

In addition, the interaction of PN-1 and HS side chains of syndecan-1 may have further consequences. It is well known heparin increases the affinity between PN-1 and serine proteases. Therefore, the PN-1-uPA complex, facilitated by the presence of syndecan-1, especially its HS side chains, could actively promote uPAR-mediated signaling as well. In summary, PN-1 may be involved in regulation of integrin-mediated signaling through either uPAR or syndecan-1, or both. This would position PN-1 (-uPA complex) a cross-talking point for these two pathways. In either case, PN-1 may or may not need to be in complex with a serine protease, such as uPA to exert its functions.

In conclusion, the data described here provide the first experimental evidence that not only complexed but also active PN-1 is internalized by MEF cells. This event is mediated by both LRP1-dependent and LRP1-independent pathways. We have identified syndecan-1, a member of the heparan sulfate proteoglycan family as the receptor mediating internalization of free PN-1 in LRP1-deficient MEF cells. We have also shown that, in contrast to LRP1-mediated internalization that triggers the PKA pathway, PN-1 interaction with syndecan-1 activates Ras-Raf-MEK-ERK signaling cascades. This effect is abolished by β-cyclodextrin, which blocks active PN-1 internalization only in LRP1 deficient MEF cells. As a consequence of the activation of Ras-ERK signaling, PN-1 activates downstream effectors of ERK signaling such as Rac1 and induces cytoskeleton reorganization, therefore promoting LRP1-/- MEF cell migration on vitronectin. Interestingly, this effect is blocked by different antibodies against integrin β3, syndecan-1, and uPAR. In addition, syndecan-1 and PN-1 are specifically co-immunoprecipitated with integrin β3 indicating that integrin could be upstream of PN-1-

activated ERK signaling, which is activated by PN-1. PN-1 interacts with either uPAR or syndecan-1, or both of them to activate integrin β3 and its downstream signaling including Rac1 and/or MLCK. Taken together, these findings show that serpins serve additional physiological functions, besides their roles as protease inhibitors, by differentially modulating specific cellular signaling pathways, and consequently regulating cell migration, which could be important in cancer development.

As shown in Fig.19, PN-1 interacts with cell surface proteins and receptors, such as uPA-uPAR and syndecan-1 thereby activating signal transduction pathways, which have been implicated in regulating tumor growth, invasion and angiogenesis [Durand et al., 2004]. Clearly it is important to further investigate the role of PN-1 in tumorigenesis. We would like to continue *in vitro* experiments to provide further evidence for the interaction between PN-1 and uPAR and on the regulation of integrin αvβ3 activity, and *in vivo* experiments to investigate functions of PN-1 in tumor formation and metastasis. We would like to inject breast tumorigenetic cells, showing significant difference in PN-1 expression and invasive behavior, into PN-1 deficient mice. This may provide us insight into the importance of a PN-1 expressing environment for different steps of tumorigenesis. Additionally, we will modify PN-1 expression level in these cells, and inject them as well into a PN-1 deficient background. We will be able to find out, for instance, if PN-1 over-expression changes the metastatic feature of PN-1 negative cells, and at which specific steps in metastasis it happens. We also hope to answer the question if over-expression of PN-1 leads to cellular invasion into blood or lung, to the microproliferation, and to changes in the sensitivity of these tumor cells to oxidative stress or resistance to apoptosis.

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