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Activation of ERK Signaling Upon Alternative Protease Nexin-1 Internalization Mediated by Syndecan-1

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Abstract Protease nexin-1 (PN-1), an inhibitor of serine proteases, contributes to tissue homeostasis and influences the behavior of some tumor cells. The internalization of PN-1 protease complexes is considered to be mediated by the low-density lipoprotein receptor related protein 1 (LRP1). In this study, both wild-type and LRP1−/− mouse embryonic fibroblasts (MEF) were shown to internalize PN-1. Receptor associated protein (RAP) interfered with PN-1 uptake only in wild-type MEF cells, indicating that another receptor mediates PN-1 uptake in the absence of LRP1. In LRP1−/− MEF cells, inhibitor sensitivity and kinetic values ($t_{1/2}$ at 45 min) of PN-1 uptake showed a similarity to syndecan-1-mediated endocytosis. In these cells, PN-1 uptake was increased by overexpression of full-length syndecan-1 and decreased by RNA interference targeting this proteoglycan. Most important, in contrast to PKA activation known to be triggered by LRP1-mediated internalization, our study shows that syndecan-1-mediated internalization of PN-1 stimulated the Ras-ERK signaling pathway. J. Cell. Biochem. 99: 936–951, 2006. © 2006 Wiley-Liss, Inc.

Key words: ERK signaling; PN-1; syndecan-1; internalization; LRP1

Protease nexin-1 (PN-1) is a secreted 43 KDa protein of the serpin superfamily [Sommer et al., 1987]. It binds and potently inhibits several serine proteases, including thrombin, trypsin, tissue plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), and factor XIa [Baker et al., 1980; Monard, 1993; Knauer et al., 2000]. PN-1 contributes to the tight regulation of extracellular proteolytic activity, which is required for developmental events and maintenance of tissue homeostasis. In addition, higher levels of PN-1 mRNA were detected in pancreatic carcinomas, gastric and colorectal cancer samples, and increased levels of PN-1 protein were measured in prostate cancer cell lines [Buchholz et al., 2003; Chen et al., 2004]. Overexpression of PN-1 enhanced the invasive potential of pancreatic cancer cells in tumor xenografts [Buchholz et al., 2003]. PN-1 has been shown to form inhibitory complexes with serine proteases and such complexes are actively removed from the extracellular environment by internalization via the low density lipoprotein receptor related protein 1 (LRP1) [Knauer et al., 1997b; Crisp et al., 2000; Knauer et al., 2000]. Furthermore, there is evidence for a constant PN-1 secretion and uptake in a free form by cultured cells [Howard and Knauer, 1986], suggesting that PN-1 can be removed from the extracellular matrix (ECM).

LRP1 is a transmembrane receptor belonging to low-density lipoprotein (LDL) receptor family. It plays distinct roles as an endocytosis receptor and as a signal modulator [Herz and Strickland, 2001; van Der, 2002]. LRP1 mediates the catabolism of diverse ligands including lipoproteins, lipoprotein lipases, and serine protease-inhibitor complexes. This function requires cell surface heparan sulfate proteoglycans (HSPGs) as co-receptors to present ligands and facilitate internalization through an unknown mechanism [Kowal et al., 1989; Beisiegel et al., 1991; Knauer et al., 1997b].

HSPGs are transmembrane proteins with one or more heparan sulfate chains covalently attached to their extracellular domains. They

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are ubiquitous on cell surfaces, where they are generally more abundant than most receptors [Bernfield et al., 1999]. HSPGs also mediate LDL receptor- and LRP1-independent internalization of lipoproteins and lipoprotein lipases [Williams et al., 1992; Fernandez-Borja et al., 1996; Al Haideri et al., 1997; Seo and St. Clair, 1997; Llorente-Cortes et al., 2002], among which syndecan-1 and perlecan have been identified as independent endocytic receptor in lipoprotein metabolism [Fuki et al., 1997; Fuki et al., 2000a]. The syndecan-1-mediated endocytosis pathway requires tyrosine kinase activity and involves detergent-insoluble membrane rafts [Fuki et al., 1997; Fuki et al., 2000a,b]. In addition, ligand binding to syndecan-1 is required for the regulation of alphavbeta3 integrin activity in human mammary carcinoma cells [Beauvais and Rapraeger, 2003; Beauvais et al., 2004]. 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].

Endocytosis is coupled with signal transduction. On one hand, ligand binding to cell surface receptors initiates signaling, such as the Ras-ERK pathway, which is mediated by receptor tyrosine kinases. There the endocytic machinery functions as a compartment to recruit signaling molecules. On the other hand, receptor signaling appears to control the endocytic machinery, providing a feedback loop regulating endosomal traffic of cell surface receptors [Teis and Huber, 2003]. For example, LRP1-mediated internalization of apoE has been shown to activate PKA signaling [Zhu and Hui, 2003], and concomitantly the phosphorylation state of LRP1 influences its endocytic function [Li et al., 2001; Ranganathan et al., 2004].

We initiated this study to explore (i) the possibility that free PN-1 is internalized; (ii) whether this internalization solely depends on LRP1; (iii) the type of cell surface receptor involved in case of LRP1-independent internalization; (iv) the consequences of an interaction of PN-1 with distinct cell surface receptors. Using mouse embryonic fibroblasts (MEF), we demonstrate that free PN-1 can be internalized, either through LRP1 or by a different mechanism. We identify syndecan-1 as the receptor responsible for this alternative pathway. Surprisingly we find that the interaction between syndecan-1 and PN-1 activates the Ras-ERK signaling, indicating the occurrence of an alternative signaling pathway linked to the internalization of PN-1.

MATERIALS AND METHODS

Materials

Recombinant PN-1 (rPN-1) was synthesized and purified in our laboratory [Sommer et al., 1989]. Thrombin was purified from human plasma and characterized as described [Stone and Hofsteenge, 1986]. Wild-type and LRP1 deficient MEF have been characterized before [Willnow and Herz, 1994]. 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]. Plasmid (RasN17) expressing dominant negative H-Ras was a kind present from Dr. Yoshikuni Nagamine (Friedrich Miescher Institute, Switzerland) [El Shemerly et al., 1997].

Cell Culture

MEF cells were cultured 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.

Preparation and Purification of Thrombin-PN-1 Complex

Thrombin and rPN-1 (1:5 ratio) were mixed in assay buffer, which contained 66 mM Tris-HCl, pH 8.0, 133 mM NaCl and 0.13% polyethyleneglycol 6,000, and incubated at 37°C for 1 h. Thrombin-PN-1 complex was purified by FPLC (Superdex 75, 2.0 × 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 24 h before the experiment and grown to 80–90% confluence. Cells were washed twice with pre-warmed PBS

(Ca^{2+} and Mg^{2+} free) and incubated for 3 h at 37°C in serum-free DMEM medium (SFM), supplemented with 0.1 mg/ml BSA delipidated by treatment with activated charcoal and Dextran-T40, 16 μg/ml putresine, 12.5 ng/ml progesterone (Sigma) and 1:5,000 diluted supplement (Sigma, Cat. No.I-1884). After pre-incubation in SFM, cells were washed once with SFM and incubated for 3 h at 37°C in fresh SFM with 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 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), 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 30 min pre-incubation with the last four inhibitors preceded the addition of rPN-1. The subcellular fractions were prepared as described [Ito et al., 1997] with slight modification. Briefly, cells were washed six times with ice-cold PBS and scraped into PBS. Cell suspension was centrifuged (200*g*) at 4°C for 5 min. Cell pellets were solubilized in 100 μl lysis buffer, which contains 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2M sucrose, 2 mM EDTA, 2 mM EGTA and protease inhibitor cocktail (Roche). Cell lysates were centrifuged (10,000*g*) at 4°C for 10 min. An additional centrifugation at 100,000*g* at 4°C for 1 h 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 2 h. The level of PN-1 binding to the cell surface was determined by immunoblotting of plasma membrane fractions.

Immunoblot

Samples from the uptake experiments were separated by 10% SDS–PAGE under reducing conditions. Fifty microgram proteins were 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], 1:2,000 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 wild-type (LRP1+/+).

Immunocytochemistry

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

Syndecan-1 Assay

The 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 15 min 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 5 min. Cell pellets were solubilized in NP-40 buffer on ice for 30 min, and centrifuged (10,000*g*) at 4°C for 10 min. 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 3 h by 10 mU/ml heparinase III and 20 mU/ml chondroitinase ABC (Sigma); fresh enzymes were added after the first 2 h 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 (Invitrogen Life Technologies). 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 kit (AMAXA, Köln, Germany) according to the manufacturers 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 mouse 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. Twenty-four hours 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 (Qiagen). 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 rPN-1 with or without 50 nM RAP was added at 37°C for 10 min. PKA activity was measured with Pep Tag Non-radioactive cAMP-Dependent Protein Kinase Assay Kit (Promega), following the manufacturer's instructions. The results were quantified by Image Master Total Lab (Amersham Pharmacia Biotech).

ERK Signaling Pathway Activation

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 with pre-warmed PBS (Ca^{2+} and Mg^{2+} free) three times, and then switched to SFM for 30 min. 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 with ice-cold PBS three times and solubilized with NP-40 buffer, containing protease inhibitor cocktail (Roche) and 1% phosphatase inhibitor cocktail I and II (Sigma). Cell lysates were kept on ice for 30 min before centrifugation at 10,000*g* at 4°C for 10 min. SDS–PAGE was performed with 20 μg of protein per lane. The samples were blotted against the anti-ERK1/2 phospho-specific antibody (Biosource International, Camarillo, CA). To further identify the upstream of ERK signaling activated by PN-1, we transfected LRP1−/− MEF cells with plasmids expressing dominant negative RasN17, using empty vector as control. Twenty-four hours after transfection, cells were treated in the same way as described above; in this case incubation time with PN-1 was 20 min. Overexpression of H-RasN17 were 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.

RESULTS

PN-1 Interactions With LRP1 Deficient Cells

To exam if active PN-1 could be internalized in the absence of LRP1, we incubated rPN-1 with LRP1−/− and wild-type MEF cells. Upon permeabilization, immunostained PN-1 appeared as punctas in cytoplasm in both wild-type and LRP1−/− MEF cells under normal fluorescence microscopy (Fig. 1A, top panels). We also used confocal microscopy, instead of non-permeabilized immunostaining, to confirm the internalization of PN-1. We observed PN-1 staining throughout the scanned layers (Fig. 1A, bottom panels).

To test the characteristics of free PN-1 binding to the LRP1−/− MEF cells, rPN-1 was added to MEF cells at 4°C in the presence of

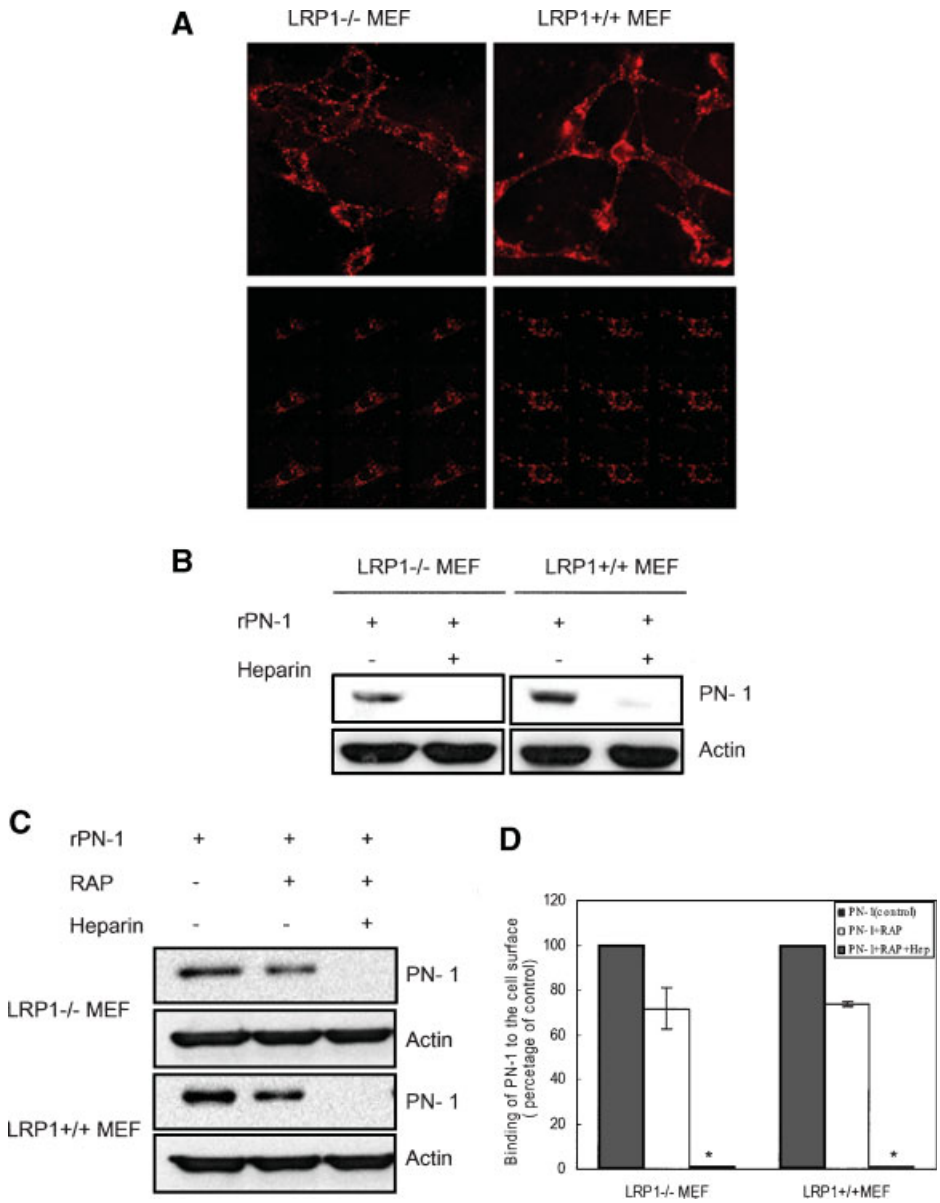

Fig. 1. PN-1 interaction with LRP1 deficient cells. Following the incubation with active PN-1, cells were fixed and immunostained as described in Materials and Methods. PN-1 appeared as punctas in the cytoplasm in both LRP1−/− and LRP1 +/+ MEF cells (**A**, **top panels**). The confocal scanning pictures through the whole cell body confirmed this pattern of PN-1 staining (**A**, **bottom panels**). Cells were incubated with rPN-1 in the absence or presence of heparin (**B**), RAP or combination of RAP and heparin at 4°C (**C**). Immunoblot analysis of plasma membrane fractions showed that soluble heparin completely blocked PN-1 binding to cell surface in both LRP1−/− and LRP1 +/+ cells, whereas RAP reduced PN-1 binding by 30% (**C**, **D**) (data: mean ± SE, white bar: active PN-1 as control, black bar: active PN-1 with RAP, gray bar: active PN-1 with RAP and heparin).

soluble heparin, RAP or both. Heparin strongly reduced most of the PN-1 binding to the cell surface in both MEF cell lines (Fig. 1B). As the interactions with HSPGs have been shown to impede ligand binding to LRP [Wilsie and Orlando, 2003], the effect of RAP was evaluated in presence or absence of heparin. 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. 1C,D). This indicated that HSPG is required as well for the binding of PN-1 to the cell surface of LRP1−/− MEF cells.

LRP1-Independent PN-1 Internalization

We further evaluated PN-1 and purified thrombin-PN-1 complex internalization in MEF cells by immunoblotting following SDS–PAGE of intracellular PN-1 under reducing conditions. Both 43-KDa PN-1 and a PN-1 form with a slightly smaller molecular weight were detected (Fig. 2A). The latter represented the cleaved form of PN-1 following dissociation from the complex [Nick et al., 1990]. Quantification of this immunoblot indicated that after 3 h incubation active PN-1 uptake in LRP1−/−MEF cell could reach 73.4% ± 4.8% of that in wild-type cells, whereas the PN-1-thrombin uptake was 51.3% ± 1.4% of the one evaluated in the presence of LRP1 (Fig. 2B). This data indicated that both free PN-1 and thrombin-PN-1 complex could be internalized by both types of MEF cells. Furthermore, we investigated active PN-1 internalization in the presence of the chemical serine protease inhibitor

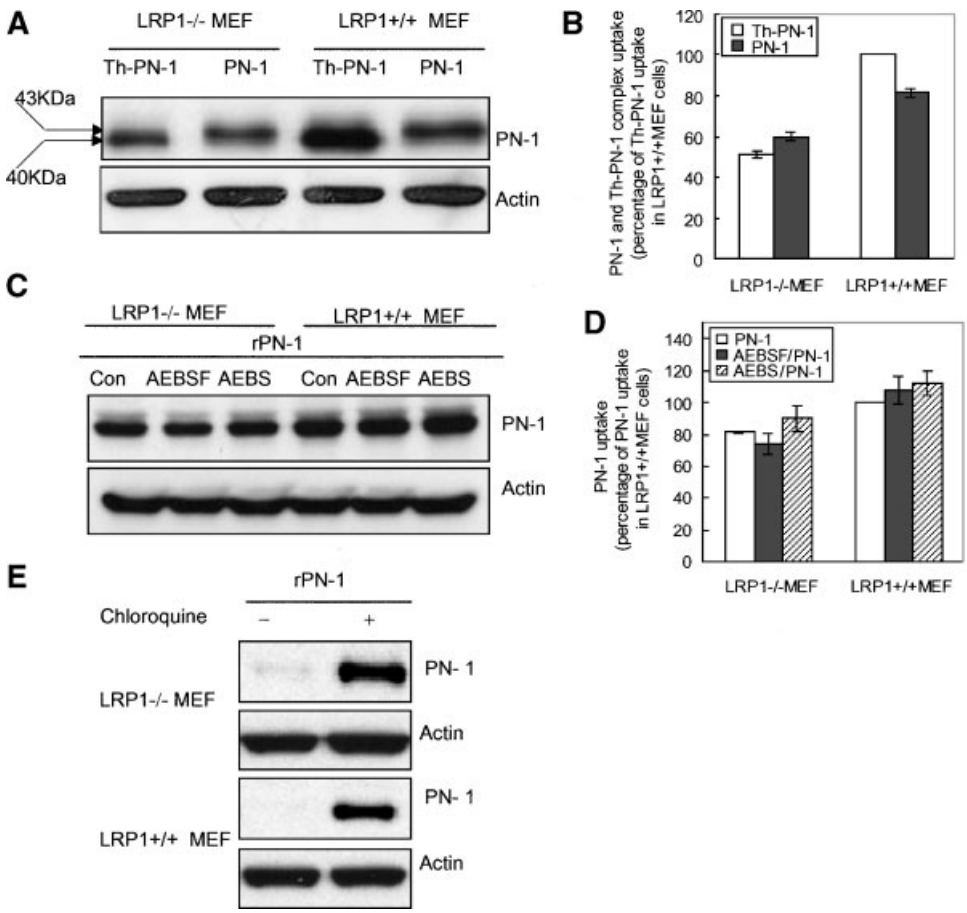

Fig. 2. LRP1-independent PN-1 internalization. Immunoblot analysis of intracellular PN-1 under reducing conditions detected internalization of both active PN-1 and PN-1 complexed with thrombin after 3-h incubation in both types of cells; active PN-1 migrated at a slightly higher molecular weight than PN-1 dissociated from the complex (**A**). 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) (**B**). Immunoblot analysis and quantification showed that the serine protease inhibitor AEBSF and its inactive form AEBS did not affect active PN-1 uptake (**C**, **D**) (data: mean ± SE, white bar: active PN-1, black bar: active PN-1 with AEBSF, gray bar: active PN-1 with AEBS). Overnight incubation with PN-1 in presence of chloroquine caused accumulation of intracellular PN-1 (**E**).

AEBSF. AEBSF did not affect active PN-1 uptake in either of MEF cell lines (Fig. 2C,D), suggesting that the PN-1 uptake detected here was independent of any serine protease activity. As a control for this experiment, AEBS, which is structurally related but without inhibitory activity, was also shown to have no effect on PN-1 internalization. The fate of internalized PN-1 was further analyzed. In both types of cells, overnight incubation of rPN-1 with 200 μM chloroquine, a weak base that inhibits lysosomal proteolysis, led to an increased intracellular accumulation of 43-KDa PN-1, indicating that free PN-1 is degraded in lysosomes (Fig. 2E). The PN-1 uptake in LRP1−/− MEF cells demonstrated by immunocytostaining and Western blot analysis suggested that an LRP1-independent endocytic pathway could trigger PN-1 internalization.

Characteristics of PN-1 Uptake in LRP1−/− MEF Cells

To further investigate the difference of PN-1 processing in the presence or absence of LRP1, we compared the kinetics of PN-1 uptake in both MEF cell lines. In the cells lacking LRP1, PN-1 uptake displayed a much slower kinetic rate ($t_{1/2}$ around 45 min) than in wild-type MEF cells ($t_{1/2}$ around 15 min) (Fig. 3A,B), further

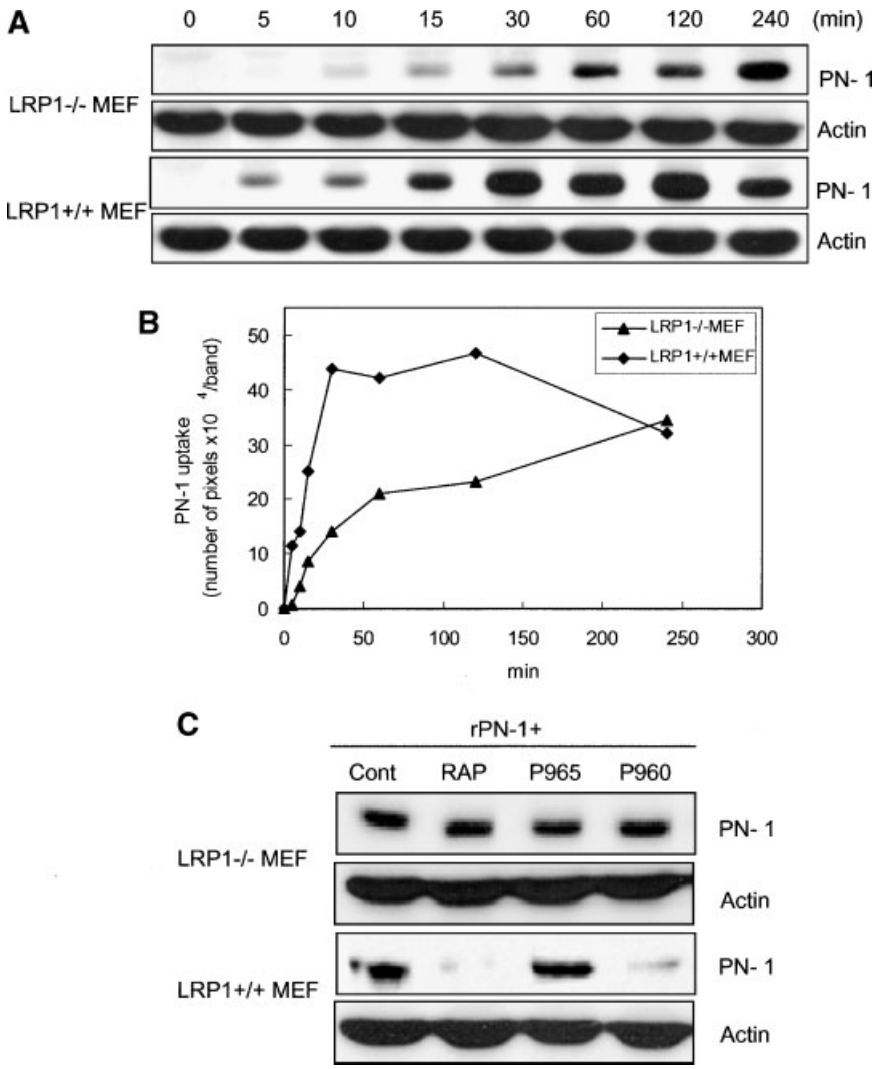

Fig. 3. Characteristics of PN-1 uptake in LRP1−/− and LRP1+/+ MEF cells. Active PN-1 was incubated with both types of MEF cells for the indicated times. Internalized PN-1 levels were detected by immunoblot (**A**) and semi-quantified by densitometry as plotted in (**B**). LRP1−/− MEF cells (triangles) showed uptake kinetics ($t_{1/2}$ 45 min) different from LRP1+/+ MEF cells (diamonds) ($t_{1/2}$ 15 min) (data represents one out of three independent experiments) (**B**). MEF cells were incubated with active PN-1 in the presence or absence of RAP, control peptide (P965) and antagonist peptide 960 (P960), respectively. PN-1 uptake was inhibited by RAP and p960 in LRP1+/+ but not in LRP1−/− MEF cells (**C**).

supporting our observation that the receptor involved is different from LRP1. Finally, RAP and P960, two putative inhibitors which interfere with the LRP1 endocytic pathway by preventing the PN-1-thrombin complex binding to LRP1 were tested [Knauer et al., 1997a]. The scrambled peptide P965, composed of the same amino acids as in 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 internalization in LRP1−/− MEF cells (Fig. 3C). This data also confirmed an LRP1-independent uptake of PN-1 in LRP1−/− MEF cells.

Heparan Sulfate Proteoglycans are Involved in PN-1 Uptake in LRP1−/− MEF Cells

Cell surface heparan sulfate is mainly associated with HSPGs of two families, the syndecans and the glypicans [Bernfield et al., 1999], which have been shown to mediate LRP1-independent lipoprotein metabolism [Fernandez-Borja et al., 1996; Al Haideri et al., 1997; Seo and St Clair, 1997; Llorente-Cortes et al., 2002]. As the kinetic experiment indicated a similarity to syndecan-1-mediated internalization, we focused our attention on this HSPG [Fuki et al., 1997]. In the syndecan-1-mediated endocytic pathway, the phosphorylation of the intracellular domain of syndecan-1 by a tyrosine kinase activity is required for its endocytic function [Fuki et al., 1997], which involves detergent-insoluble membrane rafts instead of clathrin-coated pits [Fuki et al., 2000b]. Therefore, we tested whether PN-1 uptake in LRP1−/− MEF cells could still be mediated by syndecan-1 using chemicals known to interfere with syndecan-1-mediated endocytosis. Two sets of inhibitors were used: (1) Genistein, a general tyrosine kinase inhibitor and β-cyclodextrin, which depletes cholesterol and disrupts membrane rafts [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 and Mueller, 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. 4A). 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.

Syndecan-1 Plays a Predominant Role in PN-1 Uptake in LRP1−/− MEF Cells

To evaluate the contribution of syndecan-1 to the uptake of free PN-1 in LRP1−/− MEF cells, the full-length syndecan-1 was overexpressed in both MEF cell lines (Fig. 4B). This led to two times more PN-1 uptake (202.5% ± 12.0%) in LRP1−/− but not in wild-type MEF cells (Fig. 4C,D). The association of the cytoplasmic domain with the cytoskeleton upon ligand-triggered clustering represents an important step in the endocytosis mediated by syndecan-1 [Carey et al., 1996]. In addition, tyrosine kinase activity is required for endocytosis, suggesting that the phosphorylation of the highly conserved tyrosine residues within the cytoplasmic domain of syndecan-1 could be of importance. Furthermore, either deletion of the C-terminal 23 amino acids or point mutation of tyrosine residues within this domain are known to abolish syndecan-1 and microfilament association [Carey et al., 1996]. Truncated syndecan-1 (DNsyn1), which retains only nine amino acids of the cytoplasmic domain, was therefore overexpressed (Fig. 4B). This did not alter PN-1 uptake in either MEF cell line (Fig. 4C,D). These results suggested that the intracellular domain of syndecan-1 is also required for the PN-1 uptake in LRP1−/− MEF cells. To better address the functional contribution of syndecan-1, siRNA was used to transiently knock down its level of expression (Fig. 5A,B). Decrease of syndecan-1 levels resulted in a significant reduction of PN-1 uptake (47% ± 9.2%) in LRP1−/− MEF cells, whereas it remained without effect in wild-type MEF cells (Fig. 5C,D). Altogether the data demonstrated that syndecan-1 plays a predominant role in PN-1 internalization in LRP1−/− MEF cells.

Syndecan-1-Mediated PN-1 Internalization Triggers a Switch to the Ras-ERK Signaling Pathway in LRP1−/− MEF Cells

As PKA is activated upon apoE interaction with LRP1 [Zhu and Hui, 2003], we tested whether PN-1 could also activate PKA in MEF cells. This was the case in wild-type MEF cells and this effect was abolished by RAP. Upon ligand interaction with LRP1−/− MEF cells, PKA activity was reduced (Fig. 6A). Cross talk

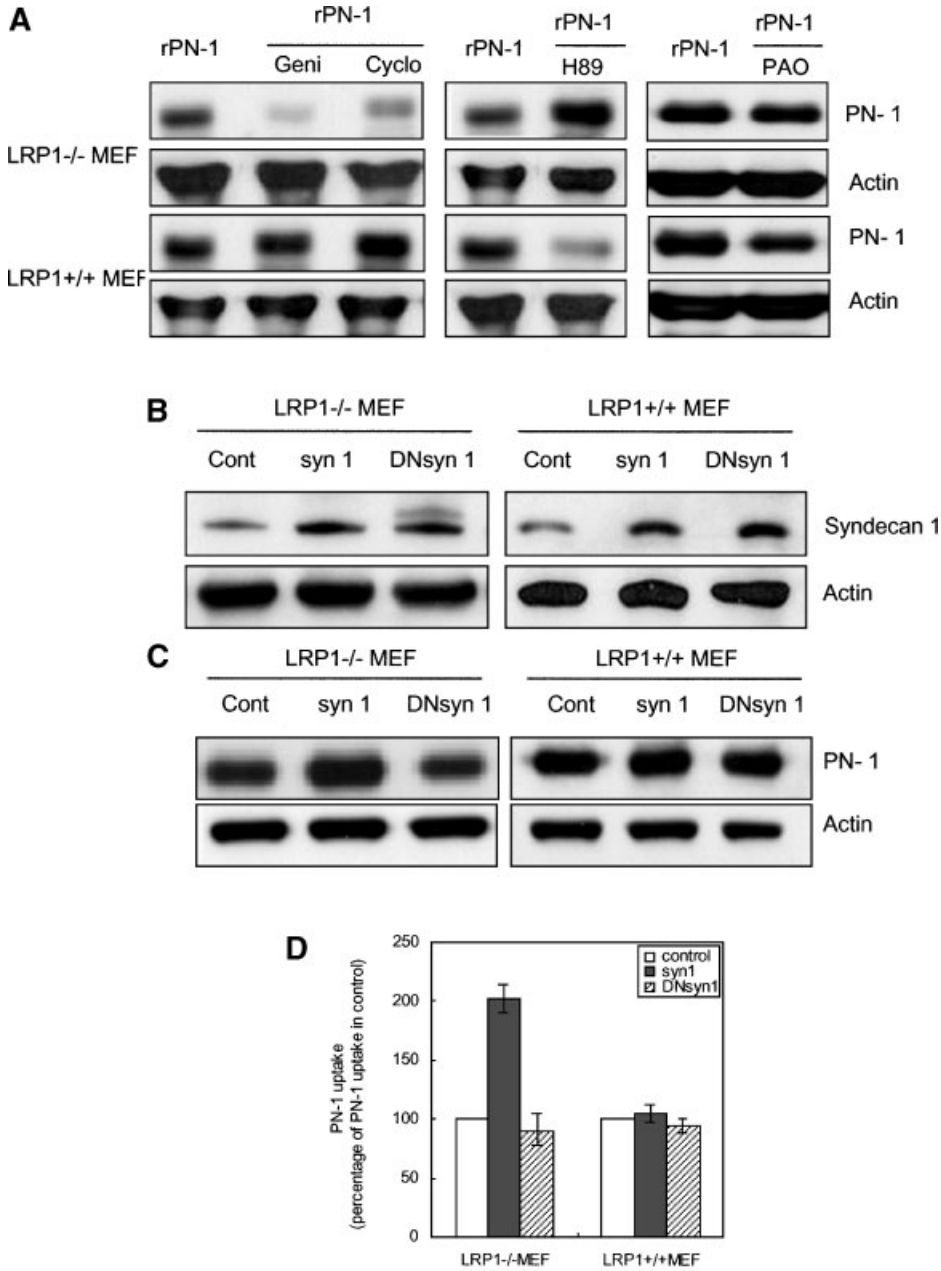

Fig. 4. PN-1 uptake in LRP1−/− MEF cells requires the intracellular domain of syndecan-1. Both types of MEF cells were incubated with active PN-1 alone or in the presence of genistein (Geni), β-cyclodextrin (Cyclo), H89 and PAO respectively. Immunoblot analysis of cell lysates showed that genistein and 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 a much stronger inhibitory effect. In LRP1−/− MEF cells, H89 increased PN-1 uptake whereas PAO showed no effect (**A**). 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 level of syndecan-1 in the derived cell lines confirmed by immunoblot (**B**). Uptake of PN-1 was detected by immunoblot (**C**), and normalized to the PN-1 level in each control condition (**D**). PN-1 internalization was increased 2 times more in LRP1−/− MEF cells overexpressing full-length syndecan-1, but barely changed by overexpressing truncated syndecan-1. The overexpressed syndecans did not significantly affect PN-1 internalization in LRP1+/+ MEF cells (**C**, **D**).

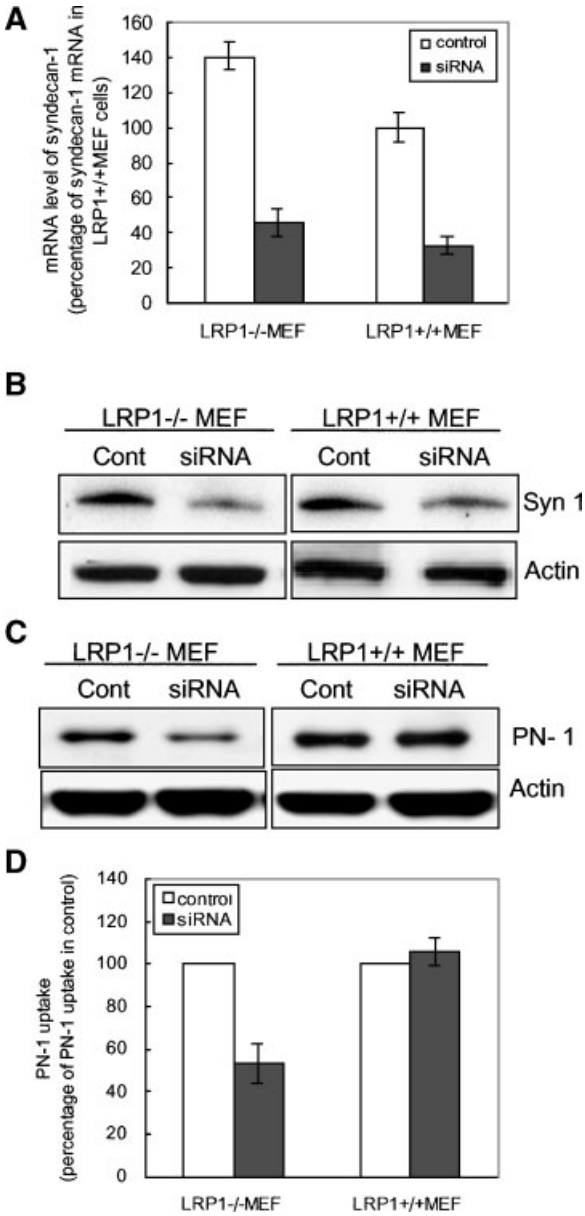

Fig. 5. Reducing the expression level of syndecan-1 decreased PN-1 uptake only in LRP1−/− MEF cells. Both types of MEF cells were transiently transfected with siRNA targeting syndecan-1. Twenty-four hours later the change in syndecan-1 mRNA and protein level was confirmed by real time PCR (**A**) and immunoblot (**B**). Uptake of PN-1 was detected by immunoblot (**C**), and normalized to the PN-1 level in each control condition (**D**). After siRNA transfection, the lower level of syndecan-1 led to a significant decrease (47% ± 9.2%) in PN-1 uptake only in LRP1−/− MEF cells.

between PKA and MAP kinase signaling, which regulates cell proliferation and migration, is well established [Bornfeldt and Krebs, 1999; Stork and Schmitt, 2002]. The presence of phospho-ERK was, therefore, monitored to further evaluate downstream effects of PN-1 uptake in the absence of LRP1. As phosphorylation of ERK increases with time after serum deprivation in LRP−/− 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. 6B). Co-incubation of rPN-1 with β-cyclodextrin, which blocked PN-1 internalization through syndecan-1, abolished ERK activation (Fig. 6C). To identify the upstream effector of ERK signaling, the dominant negative H-Ras (RasN17) was overexpressed in LRP1−/− MEF cells. PN-1 significantly increased phospho-ERK in LRP1−/− MEF cells transfected with empty vectors, but this increased phosphorylation of ERK was abolished by overexpression of dominant negative Ras (Fig. 6D,E). These results indicated that syndecan-1-mediated PN-1 internalization activates the ERK signaling through Ras-ERK pathway in the absence of LRP1.

DISCUSSION

This study shows that not only complexed but also free PN-1 is internalized by MEF cells, and that this event is mediated by both LRP1-dependent and LRP1-independent pathways. We identify syndecan-1, a member of the HSPG family, as the receptor mediating internalization of free PN-1 in LRP1-deficient MEF cells. Most importantly, our results provide evidence that, in contrast to LRP1-mediated internalization, which triggers the PKA pathway, PN-1 interaction with syndecan-1 activates Ras-ERK signaling. This effect is abolished by β-cyclodextrin, which blocked free PN-1 internalization only in LRP1 deficient MEF cells.

Endocytosis is an important mechanism by which cells interpret or respond to their environment, receiving extracellular information and translating it into a specific biological function. This mechanism also initiates the clearance of serine proteases complexed to their inhibitors. It is known that PN-1 binds to its target proteases such as thrombin, uPA, and tPA, forming complexes which are internalized through LRP1 to be degraded [Knauer et al., 1997b; Crisp et al., 2000]. Our observation that free PN-1 is also internalized indicates that the active inhibitor as well can be removed from the extracellular

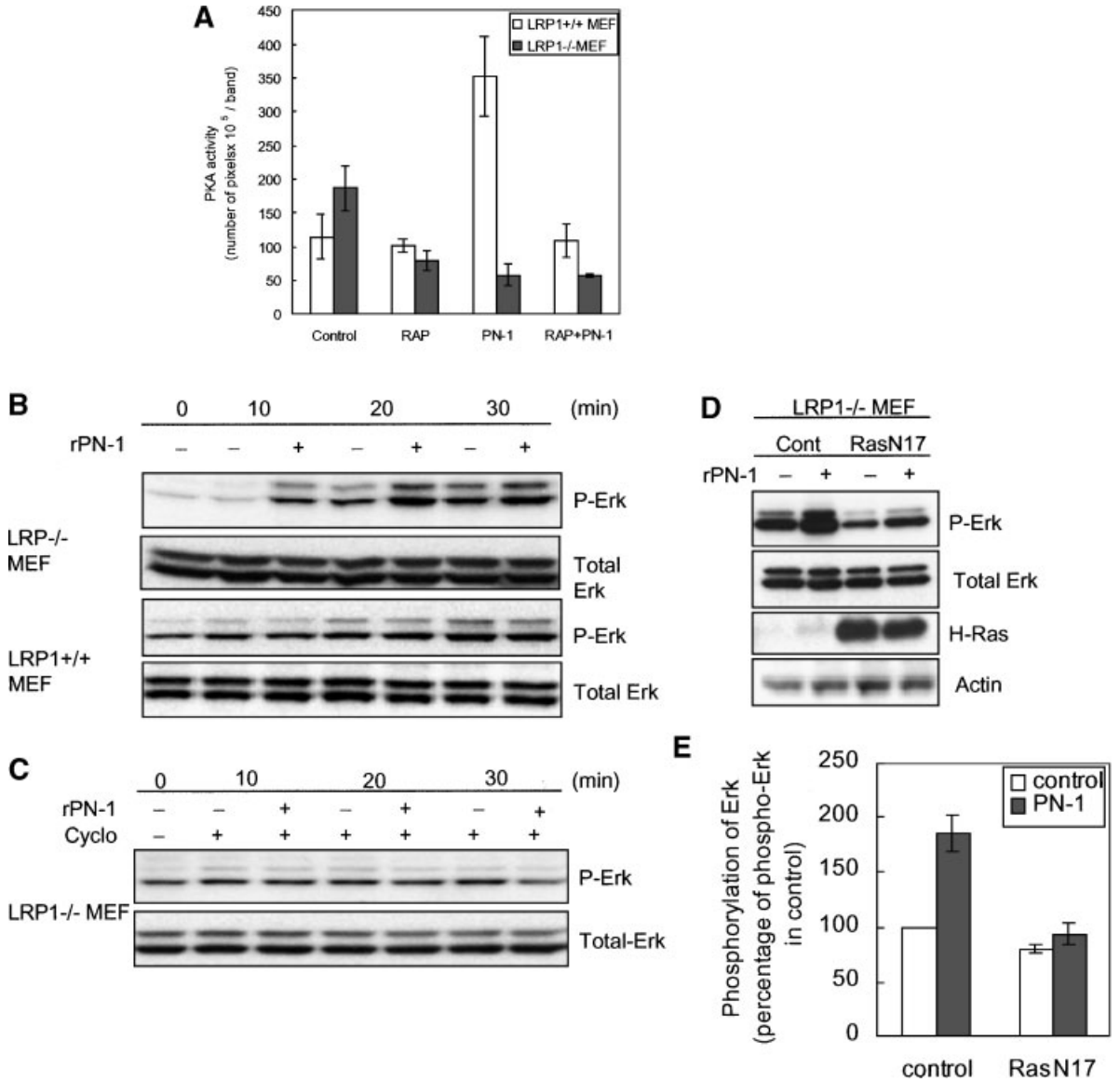

Fig. 6. PN-1 internalization triggered a switch to the Ras-ERK signaling in absence of LRP1. Both MEF cells were plated at the same density as for uptake experiments and pre-incubated overnight in SFM before incubation with active PN-1 in presence or absence of RAP. A threefold increase of PKA activity was detected and effectively blocked by RAP in LRP1+/+ MEF cells (white bars). The level of PKA activity in LRP1−/− MEF cells (black bars), was reduced by PN-1 or RAP alone, or in combination (**A**). After incubation with active PN-1, a transient increase of phospho-ERK was detected in LRP1−/− but not in LRP1+/+ MEF cells (**B**). In the presence of β-cyclodextrin, which interfered with syndecan-1-mediated internalization, the effect of PN-1 was abolished (**C**). Overexpression of dominant negative Ras (RasN17) in LRP1−/− MEF cells also abolished phosphorylation of ERK which was induced by PN-1, as showed by immunoblot (**D**) and quantification thereof (**E**) (data: mean ± SE, white bar: control, black bar: active PN-1).

environment (Fig. 1A). This phenomenon could alter the regulation of extracellular proteolytic activity and consequently the turnover of ECM proteins. The relevance of this mechanism is emphasized by the fact that PN-1 inhibits the activity of the invasion suppressor prostasin, a membrane-bound serine protease detected in prostate cancer [Chen et al., 2001], thus removal of PN-1 from ECM could contribute to inhibition of tumor invasion. Obviously, other mechanisms influencing expression and secretion of PN-1 may also be important for determining the local level of the inhibitor [Bouton et al., 2003; Richard et al., 2004].

In both wild-type and LRP1−/− MEF cells, cell surface binding is blocked by heparin and

reduced by RAP (Fig. 1B–D). These results confirm the requirement of the heparin-binding site of PN-1 for the interaction with the cell surface [Howard and Knauer, 1987; Herndon et al., 1999]. They are, however, in contrast to the report that RAP increases VLDL binding to HSPG in LDL receptor deficient cells [Wilsie and Orlando, 2003]. This implies that RAP does not solely interact 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 and Stanley, 1994], thus altering the conformation of the heparan sulfate side chains, which in turn change the affinity to different ligands.

PN-1 internalization is inhibited in the wild-type but not in the LRP1−/− cells by both RAP and a peptide corresponding to the domain of PN-1 considered to interact with LRP1 (Fig. 3C). 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 3 h 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 was reduced after 5.5 h while an increased HSPG-dependent internalization was detected by 16 h [Wilsie and Orlando, 2003]. In line with this proposal, activation of ERK pathway was not detected upon 15-min 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.

Cell surface receptors play important roles in the mediation of endocytosis. Such receptors can share the same ligand, or one receptor can mediate the internalization of distinct ligands [Nykjaer and Willnow, 2002]. We showed here that, in addition to the described internalization of complexed-PN-1 [Knauer et al., 1997b], free PN-1 could be endocytosed as well by LRP1-dependent mechanism (Fig. 2). In addition, we have identified syndecan-1 as an alternative receptor for the internalization of free PN-1 in LRP1−/− cells (Figs. 4 and 5). The contribution of other HSPG family members cannot be excluded since this study was not carried out under syndecan-1 knockout conditions.

LRP1 is known to mediate the internalization of many different ligands, including PN-1 and its complexes [Nykjaer and Willnow, 2002]. However, the level of LRP1 is not necessarily the same in different cell types in vivo [Field and Gibbons, 2000; Kang et al., 2000; Gilardoni et al., 2003], not that of syndecan-1 [Conejo et al., 2000; Barbareschi et al., 2003; Matsuzaki et al., 2005]. Thus, the relevance of our findings resides in the identification of an alternative function of syndecan-1 as a receptor becoming important in the absence of LRP1. Such an alternative would also imply a different mode of internalization. Incidentally, up to one-third of plasma LDL removal is due to LDL receptor-independent pathways in normal humans, and all removal in patients with homozygous deficiency for this receptor [Goldstein and Brown, 1977; Kesaniemi et al., 1983]. As yet to be identified HSPG is hypothesized to partially take over lipoprotein internalization [Williams et al., 1992; Ji et al., 1993; Al Haideri et al., 1997]. The nature of the cross talk between these two types of receptors remains unknown. Furthermore, it has been reported that the synthesis of syndecan-1 and perlecan is increased in cells with impaired clathrin-dependent endocytosis [Llorente et al., 2001], suggesting that compensatory mechanisms exist.

As internalization is usually coupled with signal transduction, such alternative endocytic pathways can also be of importance for associated signaling functions [McPherson et al., 2001]. The signaling complexes are recruited to the vicinity of the endocytic machinery and delivered to specific subcellular compartments, thus regulating cell surface receptor activity. As has been shown for the binding of apoE to LRP1 [Zhu and Hui, 2003], we observed that interaction between PN-1 and LRP1 causes PKA activation (Fig. 6A). As the PKA-dependent phosphorylation state of LRP1 influences its endocytosis [Li et al., 2001], this activation of this kinase is likely to increase internalization efficiency. Surprisingly, we found that PN-1 activates Ras-ERK signaling pathway in LRP1−/− MEF cells (Fig. 6B,D). Blockade of PN-1 uptake by β-cyclodextrin-mediated disruption of intact lipid rafts abolished this ERK activation (Fig. 6C). In wild-type MEF cells, PKA activation has been shown to prevent Raf-1 activation, thus inhibiting Ras-ERK signaling [Stork and Schmitt, 2002]. Our results indicate that this interference could be abolished at low

level or in the absence of LRP1. Consequently, the impact of Ras-ERK signaling caused by the PN-1 and syndecan-1 interaction would be enhanced and trigger distinct signal transduction, and thus different cellular responses. The selective or balanced activation of these two signaling pathways depends on the respective levels of LRP1 and syndecan-1 available at the cell surface at any given time [Conejo et al., 2000; Field and Gibbons, 2000; Kang et al., 2000; Barbareschi et al., 2003; Gilardoni et al., 2003; Matsuzaki et al., 2005]. Such a mechanism may alter cellular responses over a wide range depending on cell types. In this context, it was recently reported that down-regulation of MEK-ERK signaling by E-Cadherin-dependent PI3K/PKB pathway initiates cell cycle arrest and induces differentiation processes in intestinal epithelial cells [Laprise et al., 2004].

Although the mechanism behind the coupling of syndecan-1-mediated endocytosis and signal transduction has to be further investigated, our results show that syndecan-1 is indeed an important and direct mediator of cellular signaling. Syndecan-1 was reported to regulate the activity of alphavbeta3 integrin, thereby mediating cell spreading and migration [Beauvais and Rapraeger, 2003; Beauvais et al., 2004]. PN-1 was also shown to promote local invasion of pancreatic tumor cells [Buchholz et al., 2003]. In light of our data, it is especially interesting that syndecan-1 is upregulated in these tumors [Conejo et al., 2000]. It will thus be of interest to further study how PN-1 influences the migration of certain types of cells and whether the stimulation of the Ras-ERK signaling pathway upon its interaction with syndecan-1 is involved.

In conclusion, our findings are contributing to the idea that serpins do not exert their biological functions only by inhibiting serine proteases. To our knowledge, they are the first to provide evidence that the mode of internalization of active serpins can trigger distinct signaling pathways. Such switches could have important consequences for the tuning of tissue homeostasis and tumor cells invasiveness.

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