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Platelet-derived Growth Factor Mediates Tyrosine Phosphorylation of the Cytoplasmic Domain of the Low Density Lipoprotein Receptor-related Protein in Caveolae*

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The low density lipoprotein (LDL) receptor gene family represents a class of multifunctional, endocytic cell surface receptors. Recently, roles in cellular signaling have also emerged. For instance, the very low density lipoprotein receptor (VLDLR) and the apolipoprotein receptor-2 (apoER2) function in a developmental signaling pathway that regulates the lamination of cortical layers in the brain and involves the activation of tyrosine kinases. Furthermore, the cytoplasmic domain of the LDL receptor-related protein (LRP) was found to be a substrate for the non-receptor tyrosine kinase Src, but the physiological significance of this phosphorylation event remained unknown. Here we show that tyrosine phosphorylation of LRP occurs in caveolae and involves the platelet-derived growth factor (PDGF) receptor β and phosphoinositide 3-kinase. Receptor-associated protein, an antagonist of ligand binding to LRP, and apoE-enriched β-VLDL, a ligand for LRP, reduce PDGF-induced tyrosine phosphorylation of the LRP cytoplasmic domain. In the accompanying paper (Loukinova, E., Ranganathan, S., Kuznetsov, S., Gorlatova, N., Migliorini, M., Ulery, P. G., Mikhailenko, I., Lawrence, D. L., and Strickland, D. K. (2002) *J. Biol. Chem.* 277, 15499–15506) Loukinova *et al.* further demonstrate that one form of PDGF, PDGF-BB, binds specifically to LRP and that phosphorylation of LRP requires the activation of Src family kinases. Taken together, these findings provide a biochemical basis for a cellular signaling pathway that involves apoE and LRP.

The low density lipoprotein (LDL)[1] receptor-related protein (LRP) is a member of an ancient and multifunctional gene family that has arisen during the transition from unicellular to multicellular organisms (1). The namesake of this family is the LDL receptor, an endocytic cell surface receptor that controls plasma cholesterol levels by removing cholesterol-rich LDL particles from the circulation via the liver. LRP also participates in the removal of a specific class of lipoprotein particles, the chylomicron remnants, by the liver. However, lipoprotein clearance is only one function of LRP. At present over 30 different ligands are known that interact with this multifunctional receptor (2). Most of the known ligands fall into two classes, depending on whether they function in lipid metabolism or in the regulation of extracellular protease activity.

Much of what we know about the biological functions of LRP has been derived from the study of its role in ligand endocytosis and the routing of the endocytosed ligands toward lysosomal degradation. However, recently increasing evidence has accumulated that suggests that LRP is likely involved in transducing extracellular signals to the cell. First, two other members of the LDL receptor gene family, the very low density lipoprotein receptor (VLDLR) and the apolipoprotein E receptor-2 (apoER2) have been found to be obligate components of a developmental signaling pathway that regulates the lamination of the cortex and of the cerebellum (3). Signaling by the VLDLR and apoER2 ligand Reelin involves the activation of tyrosine kinases and subsequent phosphorylation of the phosphotyrosine binding (PTB) domain containing adaptor protein Disabled-1 in the migrating neurons (4, 5). Second, several ligands for LRP, *e.g.* urokinase-type plasminogen activator, activated α2-macroglobulin, and thrombospondin (TSP), have been shown to activate distinct and different intracellular signaling pathways, including tyrosine kinases (6), mitogen-activated protein kinases (7, 8), and Ca^{2+} currents (9). Third, tissue-type plasminogen activator has been shown to enhance neurotransmission and long term potentiation by a mechanism that is likely LRP-dependent (10). Finally, recent work by Barnes *et al.* (11) have shown that LRP can be phosphorylated on tyrosine residues within the cytoplasmic domain in cells that overexpress the constitutively active tyrosine kinase v-Src. Tyrosine phosphorylation of the LRP tail results in the association with the PTB domain containing adaptor protein Shc. However, the nature of the physiological signaling molecules, the biological significance, and the underlying biochemical mechanisms that are involved in LRP tyrosine phosphorylation remain unknown.

Here, we show that LRP is likely part of a TSP-1-mediated signaling cascade that involves tyrosine phosphorylation of the non-receptor tyrosine kinase Fyn. In as much as receptor and non-receptor tyrosine kinases (12–14) are enriched in caveolae/rafts, we also looked to see if tyrosine phosphorylation of LRP occurs in this membrane domain. Caveolae/rafts compartmen-

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[1] The abbreviations used are: LDL, low density lipoprotein; LRP, low density lipoprotein receptor-related protein; VLDL, very low density lipoprotein; VLDLR, VLDL receptor; apo, apolipoprotein; PTB, phosphotyrosine binding; TSP, thrombospondin; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; DMEM, Dulbecco's modified Eagle's medium; NRK, normal rat kidney; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; PM, plasma membrane; PNS, post-nuclear supernatant; CM, caveolae membrane; NCM, non-caveolae membrane; RAP, receptor-associated protein; PI3K, phosphatidylinositol 3-kinase.

talize a variety of signaling molecules at the cell surface (15), including platelet-derived growth factor receptor β (PDGFR-β) (16). We used cell fractionation to show that a substantial portion of the LRP on the surface is in caveolae/rafts. Incubation of cells in the presence of PDGF-BB stimulated the phosphorylation of the LRP in fractions of caveolae/rafts but not the LRP in non-caveolae fractions. Sequestration of PDGF in the medium by the non-receptor binding competent, native form of α2-macroglobulin or by occupation of the LRP extracellular domain with a large ligand, *i.e.* apoE-enriched β-VLDL, reduced the PDGF-induced tyrosine phosphorylation of LRP.

In another study in this issue, Loukinova *et al.* (17) show that PDGF binds directly to LRP and that PDGF receptor-dependent phosphorylation of LRP occurs on the tyrosine residue in the second NPXY motif within the cytoplasmic tail of the receptor. Taken together, these data suggest that LRP serves as a coreceptor in conjunction with transmembrane or membrane-associated tyrosine kinases at the cell surface and that LRP ligands may thereby either transduce or modulate cellular signals that involve the activation of tyrosine kinases.

EXPERIMENTAL PROCEDURES

Materials—PDGF-BB and anti-phosphotyrosine antibodies (4G10) were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Rabbit anti-caveolin 1 and anti-rack antibodies were from Transduction Laboratories (Franklin Lakes, KY). ECL Western blotting detection reagents were from Amersham Biosciences, Inc. (Piscataway, NJ). OptiPrep was purchased from Accurate Chemical & Scientific Corp. (Westbury, NY). Protein A-Sepharose CL4B beads and α2-macroglobulin were purchased from Sigma Chemical Co. (St. Louis, MO). Wortmannin and tyrphostin 9 were purchased from BIOMOL (Plymouth Meeting, PA).

Cell Culture—Three cell types were used. Caveolae were isolated from human fibroblasts. Cells were seeded in 150-mm dishes (300,000 cells/dish) and grown to confluency in 20 ml of DMEM supplemented with 10% (v/v) fetal calf serum and antibiotics. We used five dishes for each treatment. Whole cell lysates were prepared from J774 mouse macrophages and normal rat kidney (NRK-SA6) cells seeded in 100-mm dishes and grown to 80–90% confluence in 10 ml of DMEM supplemented with 10% (v/v) fetal calf serum and antibiotics.

Purification of TSP, RAP, and ApoE—Conditioned medium containing recombinant TSP-1 was prepared as follows: HEK293 cells stably transfected with a pCDNA3.1-TSP-1 expression cassette were grown in DMEM containing 10% fetal calf serum and antibiotics. After 48 h, cells were washed three times with PBS and grown for an additional 48 h in DMEM containing 0.2% bovine serum albumin. Media were then collected and stored at −80 °C until used. Control conditioned medium was obtained from mock transfected 293 cells and prepared according to the same protocol. Human GST-RAP and GST fusion proteins were prepared as described previously (18). RAP was prepared by thrombin cleavage of GST-RAP followed by anion-exchange chromatography on a Mono-Q column (Amersham Biosciences, Inc., Piscataway, NJ). Rabbit apoE was isolated from β-VLDL prepared from 20 ml of plasma obtained from cholesterol/coconut oil-fed rabbits (19, 20). For enrichment with apoE, β-VLDL was incubated together with apoE for 1 h at 37 °C in 0.5 ml of DMEM containing 0.2% bovine serum albumin before addition to the culture medium (19, 20).

Caveolae Isolation—Caveolae were isolated using the method of Smart *et al.* (21). Briefly, confluent normal human fibroblasts were collected in hypertonic buffer supplemented with protease and phosphatase inhibitors and Dounce-homogenized 20 times on ice. Plasma membrane (PM) were isolated with a 30% sucrose gradient from postnuclear supernatant (PNS) and then sonicated. The sonicated samples were mixed with OptiPrep (23% final) and a linear 20% to 10% OptiPrep gradient was overlaid on the samples. Samples were then centrifuged at 52,000 × *g* for 90 min at 4 °C. The bottom 1 ml was designated non-caveolae membrane (NCM), and the top 5 ml was collected and mixed with 4 ml of 50% OptiPrep in a second tube. The samples were then overlaid with 1 ml of 5% OptiPrep and centrifuged at 52,000 × *g* for 90 min at 4 °C. An opaque band located between the 15 and 5% interface was collected and designated the caveolae membrane (CM) fraction. Antibodies against caveolin-1, Rack, and LDL receptor were used as control marker of CM and NCM fractions.

Metabolic Labeling—Medium was removed from NRK-SA6 cells and replaced with methionine/cysteine-free medium or with phosphate-free medium for 30 min prior to labeling. 150 μCi of Tran^{35}S-label (PerkinElmer Life Sciences) or 150 μCi of [^{32}P]orthophosphate were added, and incubation was continued for 3 h. Cells were lysed in PBS containing 1% TX-100 and phosphatase inhibitors. Immunoprecipitation with anti-LRP antibody was carried out as described below on the cleared lysates, and labeled proteins were separated by non-reducing gradient SDS-gel electrophoresis. ^{35}S-Labeled gels were treated with Enhance prior to exposure to x-ray film.

Immunoprecipitation of Whole Cell Lysates—Cells were washed with ice-cold PBS and lysed for 20 min on ice in lysis buffer supplemented with protease and phosphatase inhibitors. The lysates were precleared then incubated overnight at 4 °C with the indicated antibody and protein A-Sepharose CL-4B beads (500 μg of protein; 5 μg of IgG; 50 μl of beads). Immunoprecipitates were washed twice with lysis buffer and lysis buffer containing 2 M NaCl, and twice in 10 mM Tris, pH 8, 50 mM NaCl. Proteins were eluted from beads with SDS sample buffer, separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and blotted with the indicated antibody.

Immunoprecipitation of Caveolae-enriched Fractions—Caveolae-enriched fractions were lysed (v/v) in TETN buffer (25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Triton X-100, 0.5 mM benzamidine, 60 mM octylglucoside) containing protease and phosphatase inhibitors for 30 min. Lysates were incubated overnight at 4 °C with the indicated antibody and protein A-Sepharose CL-4B beads as described above.

RESULTS

RAP Blocks p59^{fyn} Phosphorylation Induced by Thrombospondin-1—TSP-1 can induce cell death signals in vascular endothelial cells by sequential activation of CD36, p59^{fyn}, and stress-activated protein kinase p38 (6). Because TSP-1 can also interact with LRP (22–24), we tested whether LRP might be involved in TSP-1 signaling and p59^{fyn} phosphorylation. The macrophage-like J774 cell line was exposed to conditioned medium from 293 cells that had either been not transfected (Fig. 1*A*, *lanes 1* and *2*) or transfected (*lanes 3–5*) with an expression plasmid encoding TSP-1. Cells were incubated in the absence (*lanes 1–3*) or presence of GST-RAP fusion protein (*lane 4*) or GST control protein (*lane 5*). The 39-kDa receptor-associated protein (RAP) is a universal inhibitor of ligand binding to LDL receptor family members (25) and blocks the binding of TSP-1 to LRP (22–24). Subsequent immunoprecipitation of p59^{fyn} from the cell lysates and immunoblot analysis with anti-phosphotyrosine antibodies revealed TSP-1-dependent tyrosine phosphorylation of p59^{fyn} in the J774 cells (*lane 3*), which was blocked by RAP (*lane 4*), but not by GST (*lane 5*).

RAP Blocks the Tyrosine Phosphorylation of LRP—In Src-transformed cells, LRP can be phosphorylated on tyrosine residues within the cytoplasmic tail (11). However, it is unclear whether and how this tyrosine phosphorylation of the LRP tail occurs under normal conditions. In an early experiment, we had immunoprecipitated LRP from lysates of the NRK-SA6 normal rat kidney cell line after metabolic labeling with [^{35}S]methionine (Fig. 1*B*, *lanes 1–4*) or [^{32}P]orthophosphate (*lanes 5–8*). The results of this experiment demonstrated that the 85-kDa subunit of LRP, which contains the cytoplasmic tail, was phosphorylated and that the phosphate residues could be most effectively removed by incubation with potato acid phosphatase (*lane 7*) but not by alkaline phosphatase (*lane 6*). A protein of ~60 kDa that was heavily phosphorylated but biosynthetically only poorly labeled coprecipitated with LRP. Treatment of the cell lysate with *N*-glycanase revealed that LRP but not the 60-kDa protein carried *N*-linked carbohydrates, suggesting that the coprecipitated protein may be cytoplasmic and thus interact with the LRP tail. In contrast, the ER-chaperone RAP was labeled efficiently with [^{35}S]methionine but remained unphosphorylated. These experiments revealed that LRP can undergo phosphorylation in untransfected cultured cells under steady-state conditions, but they did not determine whether these phosphorylation events include tyro-

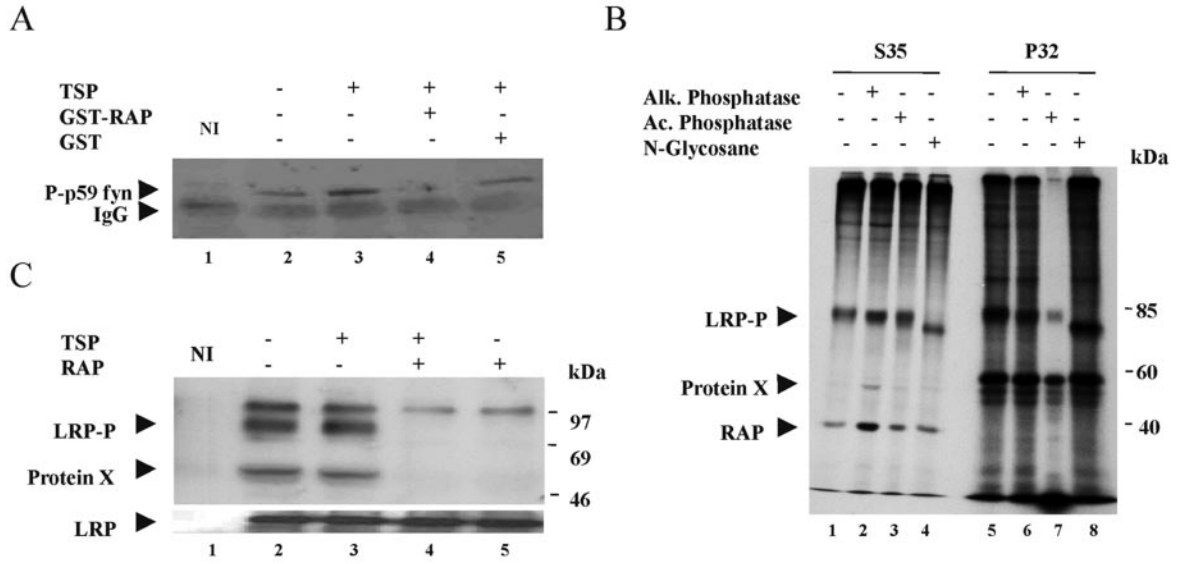

FIG. 1. **RAP blocks TSP-1-induced tyrosine phosphorylation p59^{fyn} and LRP.** *A*, J774 mouse macrophages were treated with control conditioned medium (control), conditioned medium containing thrombospondin-1 (*TSP*), GST-RAP (30 mg/ml), or GST (30 mg/ml). Whole cell lysates were immunoprecipitated with anti-p59^{fyn} IgG and immunoblotted with antibody 4G10 directed against phosphotyrosine. *NI*, non-immune control. *B*, LRP and associated proteins were metabolically labeled in NRK-SA6 cells with [^{35}S]methionine and cysteine or with [^{32}P]orthophosphate, followed by immunoprecipitation with a specific polyclonal antibody directed against LRP. Before separation by SDS-gel electrophoresis, immunoprecipitated samples were either not treated (*lanes 1* and *5*) or treated with calf intestine alkaline phosphatase (*lanes 2* and *6*), potato acid phosphatase (*lanes 3* and *7*) or with neuraminidase (*lanes 4* and *8*). *C*, J774 cells were treated for 30 min as described for *A* in the presence or the absence of TSP-1 or 30 μg/ml of RAP. Cell lysates were immunoprecipitated using polyclonal anti-LRP IgG and immunoblotted with antibodies against phosphotyrosine. The membrane was then stripped and blotted with polyclonal anti-LRP IgG (*lower panel*). *NI*, non-immune IgG was used for immunoprecipitation.

sine residues. Phosphorylation of the LRP tail by serine/threonine kinases had previously been reported (26). To address this question and to determine whether the coprecipitated 60-kDa protein may also be phosphorylated on tyrosine residues, we immunoprecipitated LRP from J774 cells that had been treated (Fig. 1*C*, *lanes 3* and *4*) or not treated (*lanes 1*, *2*, *5*) with TSP-1-conditioned medium. The results of this experiment demonstrate that LRP and the coprecipitated 60-kDa protein are phosphorylated on tyrosines under steady-state conditions. This was not affected by the absence or presence of TSP-1 in the incubation medium. Interestingly, however, tyrosine phosphorylation of both LRP and the 60-kDa protein was completely abolished by incubation of the cells with recombinant RAP (*lanes 4* and 5), suggesting that the binding of an extracellular ligand to LRP may be required for the activation of a tyrosine kinase.

LRP Is Present in Caveolae—Tyrosine kinases have been shown to be present in cholesterol-rich microdomains at the cell surface, also known as caveolae and rafts. To test whether LRP phosphorylation might take place in this type of specialized subcellular domain, we first investigated whether LRP was present in caveolae. To achieve this, we used a detergent-free method to purify caveolae from quiescent normal human fibroblasts that had been maintained in the absence of serum for 12 h. We chose human fibroblasts as the model system, because caveolae are abundant in this cell type, and standardized methods have been established for their purification (21). In our experiments, 5 μg of post-nuclear supernatant (PNS), plasma membrane (PM), non-caveolae membranes (NCM), or caveolae membranes (CM) were separated by electrophoresis and analyzed by immunoblotting. When these fractions were detected with anti-LRP antibody (Fig. 2*A*), an ~85-kDa protein corresponding to the smaller subunit was present in the PNS, the PM, and the CM fractions (*lanes 1*, *3*, and *4*). In the NCM fraction, the band was also present, but less intense (*lane 5*), indicating that LRP is primarily present in the caveolae fraction. As expected, when the samples were immunoblotted for the caveolae marker caveolin-1 (Fig. 2*A*), no protein was detectable in the NCM fraction. In another approach, we separated membranes from human fibroblasts on an OptiPrep gradient followed by fractionation (Fig. 2*B*). In this approach, the bulk of the protein was recovered in fractions 8–13 (Fig. 2*C*), termed non-caveolae fraction, separated from the caveolin-1 containing caveolae fractions (fractions 1–7), consistent with the method originally described by Smart *et al.* (21). Each fraction was immunoblotted with antibodies directed against LRP, caveolin-1, the LDL receptor, and the protein kinase C-anchoring protein Rack, the latter two serving as markers for the non-caveolae fraction. LRP was abundant in caveolae (numbers 1–8), but also in the non-caveolae fractions (numbers 9–14) suggesting the receptor is present in both compartments.

PDGF Stimulates Tyrosine Phosphorylation of LRP in Caveolae—The presence of substantial amounts of LRP in caveolae raised the possibility that tyrosine kinases that are abundant in this compartment may be mediating the tyrosine phosphorylation of LRP and the associated 60-kDa protein that we had observed. Because the PDGFβ receptor is a transmembrane tyrosine kinase that is highly expressed in human fibroblasts where it preferentially localizes to caveolae, we decided to test the possibility that the β-receptor-specific ligand PDGF-BB might induce tyrosine phosphorylation of LRP. Fig. 3*A* shows that 15 min of treatment with PDGF-BB-induced tyrosine phosphorylation of LRP in caveolae (*lanes 1–8*) but not in the non-caveolae fraction (*lanes 9–16*). PDGF-induced tyrosine phosphorylation of LRP reached its maximum after 30 min of treatment (Fig. 3*B*, *lanes 1–6*) and was partly decreased by the addition of RAP (Fig. 3*A*, *lane 4*). In the non-caveolae fraction, PDGF had no effect on LRP phosphorylation (Fig. 3*A*, *lanes 9–16*), and only background phosphorylation was observed in NCM fraction compared with CM when equal volumes (500 μl) of CM and NCM fractions were immunoprecipitated (Fig. 3*A*, *lanes 9–16* and Fig. 3*B*, *lanes 7–12*). When comparable amounts of proteins were subjected to immunoprecipitation, no

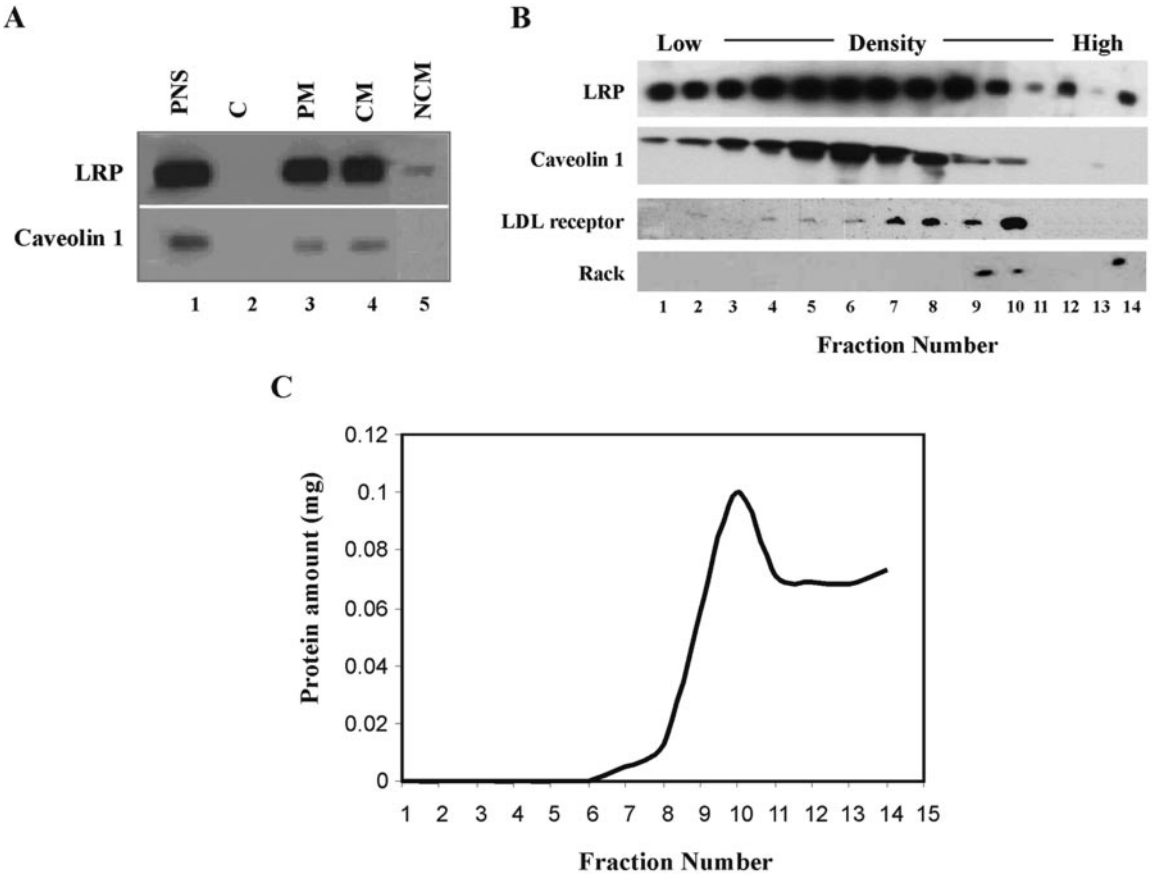

FIG. 2. **LRP is concentrated in caveolae.** *A*, confluent normal human fibroblasts were grown in the absence of serum for 12 h. Caveolae were isolated, and 5 μg of protein from either the post-nuclear supernatant (*PNS*), the cytosol (*C*), the plasma membrane (*PM*), the caveolae membrane (*CM*), or the non-caveolae membrane (*NCM*) fractions were separated by polyacrylamide electrophoresis, transferred to a polyvinylidene difluoride membrane (Millipore), and then blotted with either polyclonal anti-LRP IgG, polyclonal anti-LDL receptor IgG, or monoclonal antibody anti-caveolin IgG. *B*, the OptiPrep gradient was fractionated, and equal volumes of each fraction were loaded on a 4–12% linear gradient SDS-gel, transferred to nitrocellulose, and immunoblotted with the indicated antibodies. *C*, protein concentration profile of the gradient fractions.

LRP phosphorylation was seen in the NCM fraction (Fig. 3*B*, *lanes 13–18*).

PDGF-induced Tyrosine Phosphorylation of LRP in Caveolae Requires PDGF-β Receptor Activity—PDGF-BB is known to activate its receptor, the PDGFR-β in caveolae (16). PDGF binding causes dimerization of the receptor, which results in receptor activation by transphosphorylation in the active loop of the cytoplasmic tyrosine kinase domain (27). To determine, whether PDGFR-β kinase activity is required for PDGF-BB-mediated LRP activation, serum-starved human fibroblasts were preincubated for 30 min with and without tyrphostin 9, a potent reversible inhibitor of intrinsic tyrosine kinase activity of PDGFR-β (28), followed by stimulation for 30 min with 30 ng/ml PDGF-BB. Caveolae and non-caveolae fractions were analyzed by immunoprecipitation with anti-LRP antibodies, and immunoblotted with anti-phosphotyrosine antibodies to detect LRP tyrosine phosphorylation. Tyrphostin 9 prevented PDGF-induced tyrosine phosphorylation of LRP (Fig. 4*A*) in caveolae suggesting that PDGF-BB-mediated LRP activation requires tyrosine phosphorylation and therefore activation of PDGFR-β. Residual tyrosine phosphorylation of LRP in the non-caveolae fractions was not affected (data not shown). Activation of LRP by PDGF could also be largely prevented by pretreatment of the cells with the PI3K inhibitor wortmannin (Fig. 4*B*) suggesting that PDGF-BB-mediated LRP phosphorylation also requires activation of PI3K, and that therefore Src-family kinases that can be activated by PI3K may be involved (29).

Native α2-Macroglobulin Reduces Tyrosine Phosphorylation of LRP in Caveolae—We next sought to determine whether sequestration of PDGF in the medium by binding to the native, receptor-binding incompetent form of α2-macroglobulin (30) would interfere with tyrosine phosphorylation of the LRP tail. Fig. 5*A* shows that tyrosine phosphorylation of the 85-kDa subunit of LRP was indeed markedly reduced in the presence of 5 μg/ml native α2-macroglobulin (*lane 4*). In the absence of PDGF, α2-macroglobulin alone had no effect (*lane 5*).

ApoE-enriched β-VLDL Blocks PDGF-mediated Tyrosine Phosphorylation of LRP—LRP can bind β-migrating very low density lipoproteins (β-VLDL) after they have been enriched by incubation with exogenous apoE (19, 31). ApoE has also been shown to inhibit platelet-derived growth factor-induced vascular smooth muscle cell migration and proliferation (32, 33), and it has been suggested that this effect is mediated by LRP (34, 35). Our results show that purified apoE alone had little effect on the PDGF-induced tyrosine phosphorylation of LRP (Fig. 5*B*, *lanes 4* and *5*). β-VLDL marginally reduced LRP phosphorylation (*lane 6*). However, when β-VLDL was preincubated for 1 h with apoE, PDGF-dependent phosphorylation of LRP was completely prevented (Fig. 5*B*, *lane 7*). These data are consistent with a model, in which tyrosine phosphorylation of the cytoplasmic domain of LRP is required for the transmission of PDGF-mediated migration and proliferation signals that have been shown to be down-regulated by apoE.

DISCUSSION

The LDL receptor family members VLDLR and apoER2 have recently been recognized to function as coreceptors in a developmental signaling pathway that leads to the activation of

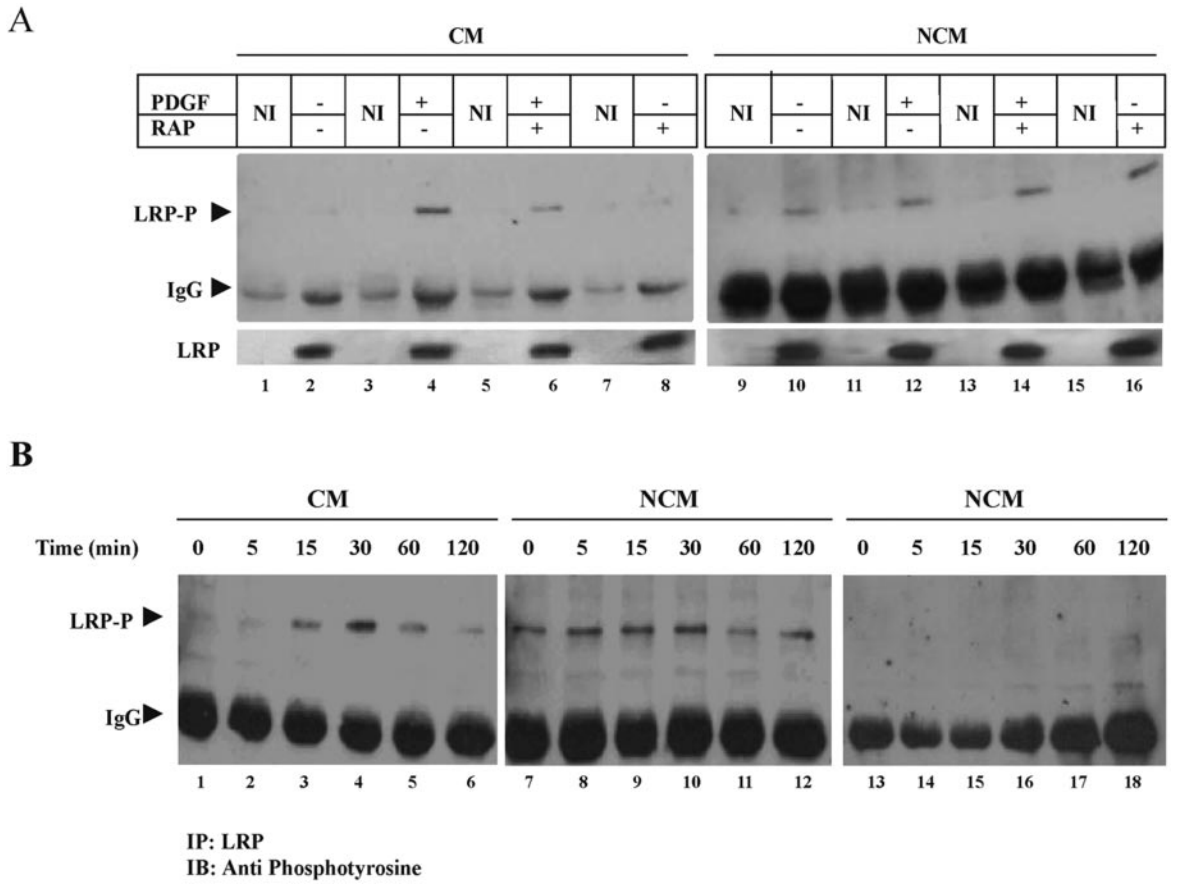

FIG. 3. **PDGF-induced tyrosine phosphorylation of LRP.** *A*, serum-starved confluent human fibroblasts were incubated in the presence or absence of 30 ng/ml PDGF-BB, 30 μg/ml of RAP, or both RAP and PDGF-BB for 15 min. Cells were washed twice with ice-cold PBS, and caveolae were prepared as described by Smart *et al.* (23). Caveolae (*CM*, *left panel*) and non-caveolae fractions (*NCM*, *right panel*) were mixed with an equal volume of TETN buffer and immunoprecipitated by incubation with anti-LRP IgG or preimmune (*NI*) antibody and protein A-Sepharose CL-4B beads. Precipitated proteins were separated by electrophoresis and immunoblotted with antibodies against phosphotyrosine (*upper panels*) or LRP (*lower panels*). *B*, human fibroblasts were incubated for the indicated time in the presence of 30 ng/ml PDGF-BB. 5 μg/lane CM protein (*left panel*), 200 μg/lane NCM protein (*middle panel*), or 20 μg/lane non-caveolae membrane protein (*right panel*) were immunoprecipitated with anti-LRP IgG in TETN buffer, loaded on a 4–12% SDS gradient gel, and analyzed by immunoblotting with antibodies against phosphotyrosine.

tyrosine kinases. Here we have shown that another member of the gene family, the multifunctional receptor LRP, is also involved in tyrosine kinase-mediated signaling events, likely in a manner analogous to that of VLDLR and apoER2 in the Reelin signaling pathway (3). PDGF-BB specifically induced tyrosine phosphorylation of LRP in cultured cells in a PDGFβ receptor-dependent manner. This phosphorylation took place in caveolae, a specialized cholesterol-rich microdomain at the cell surface, and not in the non-caveolar part of the plasma membrane. Another cellular signaling pathway that involves the membrane receptor CD36, the non-receptor tyrosine kinase p59^{fyn}, and TSP-1, a common ligand for LRP and CD36, is blocked by RAP, an antagonist of ligand binding to LRP. This finding suggests that LRP may not only function as a coreceptor in PDGF receptor-dependent signaling pathways but may also participate in a similar role in signaling by TSP-1.

Jimenez *et al.* (6) have shown a CD36-dependent role of TSP-1 in the regulation of apoptosis during angiogenesis. Signaling by TSP-1 involves tyrosine phosphorylation of p59^{fyn}, which interacts with the cytoplasmic domain of CD36 (36). Because CD36 and LRP both bind TSP-1 at different sites on the molecule (24), we hypothesized that both receptors might be obligate components of a signaling complex that is assembled by TSP-1 at the cell surface. This hypothesis was supported by our finding that RAP, which blocks the binding of TSP-1 to LRP (22–24) also prevented the TSP-1-mediated phosphorylation of p59^{fyn} (Fig. 1*A*). When the 85-kDa subunit of LRP, which harbors the cytoplasmic domain of the receptor, is phosphorylated, a phosphoprotein of ~60 kDa coprecipitates with LRP (Fig. 1*B*), and the LRP tail had recently been shown to be phosphorylated on tyrosine residues, allowing it to associate with the PTB-domain containing adaptor protein Shc (11). Taken together, these findings raised the possibility that TSP-1 signaling might not only induce tyrosine phosphorylation of p59^{fyn}, but also of the LRP cytoplasmic domain. Immunoblot analysis of immunoprecipitated LRP with an anti-phosphotyrosine antibody (Fig. 1*C*) revealed substantial phosphorylation of LRP and the coprecipitated phosphoprotein under steady-state conditions in J774 cells. However, phosphorylation levels were not induced by TSP-1, yet, RAP completely abolished phosphorylation of LRP and of the 60-kDa protein, indicating that tyrosine phosphorylation of these proteins occurred by an LRP-dependent signaling pathway that did not involve TSP-1. The nature of the coprecipitating phosphoprotein was thus not investigated further, and we instead decided to first explore the potential mechanisms that might mediate tyrosine phosphorylation of LRP.

Caveolae and rafts are cholesterol-rich microdomains of the plasma membrane that are selectively enriched in a variety of signaling molecules, including kinases and phosphatases (15, 16). Signaling molecules that are concentrated in these subcellular domains include non-receptor tyrosine kinases of the Src family but also receptor tyrosine kinases such as the EGF receptor and the PDGF receptor. The role of the PDGF receptor

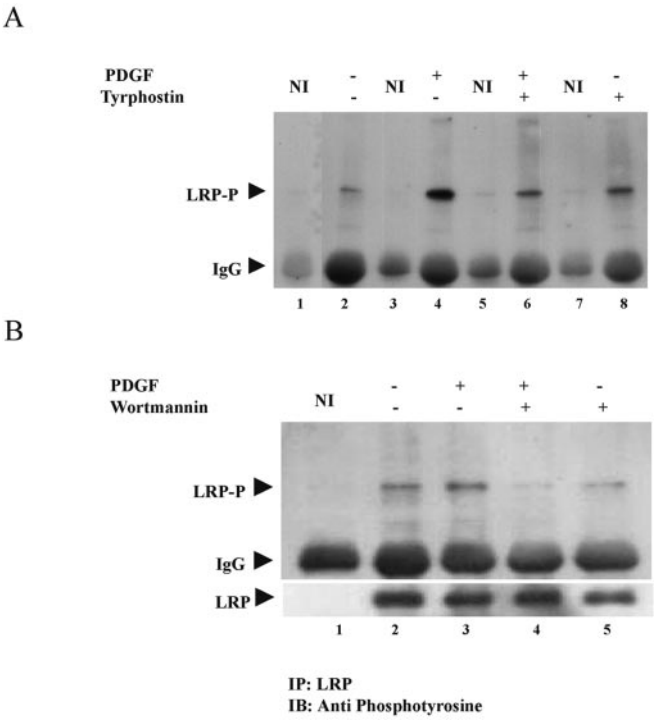

FIG. 4. **Tyrphostin 9 and wortmannin reduce the PDGF-induced tyrosine phosphorylation of LRP in caveolae.** *A*, human fibroblasts were grown in the absence of serum for 12 h and were then incubated for 30 min in the presence or absence of 30 ng/ml PDGF-BB and/or 2 μM tyrphostin 9. Caveolae were isolated, and 5 μg of protein from caveolae-enriched fractions was immunoprecipitated in TETN buffer using anti-LRP (*even-numbered lanes*) or non-immune (*NI*) IgG (*odd-numbered lanes*). Immunoprecipitated proteins were separated by electrophoresis and immunoblotted with antibodies directed against phosphotyrosine. *B*, cells were preincubated for 30 min in the presence of 500 nM wortmannin followed by incubation for 30 min in the presence or absence of 30 ng/ml PDGF-BB and/or 500 nM wortmannin. Caveolae were isolated treated as described above.

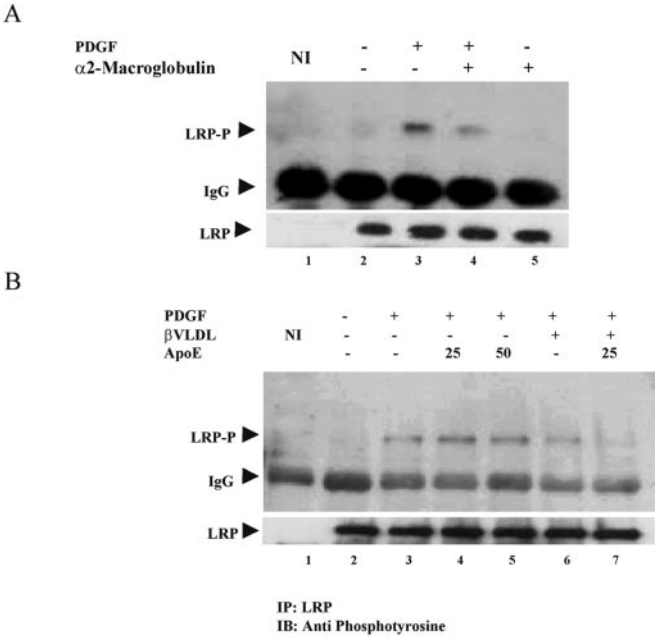

FIG. 5. **Effects of α2-macroglobulin, β-VLDL, and apoE on the PDGF-induced tyrosine phosphorylation of LRP in caveolae.** *A*, serum-starved human fibroblasts were preincubated for 30 min in the presence of 5 μg/ml native α2-macroglobulin followed by incubation for 30 min in the presence or absence of 30 ng/ml PDGF-BB and/or 5 μg/ml α2-macroglobulin. Caveolae were isolated, and 5 μg of protein from caveolae-enriched fractions was immunoprecipitated in TETN buffer with anti-LRP IgG or non-immune (*NI*) antibodies. Immunoprecipitated proteins were separated by SDS-gel electrophoresis and analyzed by immunoblotting with antibodies directed against phosphotyrosine. *B*, cells were incubated for 30 min in the presence or absence of 30 ng/ml PDGF-BB and/or 25 μg/ml or 50 μg/ml purified apoE, 30 μg/ml β-VLDL, or 30 μg/ml β-VLDL enriched with 25 μg/ml rabbit apoE. 5 μg of protein from caveolae fractions was immunoprecipitated and analyzed by immunoblotting as described above.

in caveolar signaling in particular has been extensively investigated (16, 37). To determine, whether growth factor tyrosine kinases such as the PDGF receptor might be involved in tyrosine phosphorylation of LRP, we first sought to investigate whether a fraction of LRP also resides in caveolae. Our findings demonstrate that a substantial portion of cellular LRP is indeed concentrated by an estimated 40-fold in caveolae in human fibroblasts and thus separated from the LDL receptor, another member of the LDL receptor gene family that mediates the endocytosis of LDL and has no known role in cellular signaling (Fig. 2). The finding that a fraction of cellular LRP localizes to a different compartment than the LDL receptor suggested that LRP was indeed engaged in functions that were different from mere ligand endocytosis.

Only LRP that was localized to caveolae but not to the non-caveolar part of the plasma membrane was specifically phosphorylated on tyrosine residues by stimulation of the cells with PDGF prior to isolation of the caveolae (Fig. 3). PDGF-mediated phosphorylation of LRP could be competed for by RAP, which interferes with ligand binding to LRP, suggesting that LRP phosphorylation was not a mere bystander effect, but required ligand association. Loukinova *et al.* (17) show that PDGF itself binds to LRP, suggesting a model in which PDGF might induce the formation of a transient complex between the PDGF receptor and LRP, thereby bringing the LRP tail in close contact with the signaling complex that assembles on the cytoplasmic domain of activated PDGF receptors (16, 29, 38).

Tyrosine kinase activity of the PDGF receptor was required for LRP tail phosphorylation, because the specific PDGF receptor kinase inhibitor tyrphostin 9 abolished PDGF-BB-induced LRP phosphorylation (Fig. 4*A*). However, the PDGF receptor kinase itself does not mediate LRP tail phosphorylation directly, because wortmannin, an inhibitor of PI3K, which is activated by the PDGF receptor, also completely blocked LRP phosphorylation (Fig. 4*B*). This finding indicates that a caveolar tyrosine kinase that requires the prior activation of the PDGF receptor and of PI3K mediates the phosphorylation of LRP. Src family members can be activated by PDGF receptor/PI3K-dependent signals (29, 39), and LRP is a substrate for the constitutively active v-Src (11). Furthermore, Loukinova *et al.* (17) show that the Src family kinase inhibitor PP2 almost completely blocked LRP tyrosine phosphorylation. Taken together, these findings make it likely that PDGF-induced activation of Src family tyrosine kinase in caveolae/rafts is responsible for tyrosine phosphorylation of the LRP tail.

PDGF binding to the cells and specifically to LRP, as shown by Loukinova *et al.*, is necessary for LRP phosphorylation. Sequestration of PDGF in the medium by binding to native α2-macroglobulin (30) (Fig. 5*A*), which is incapable of binding to LRP (2), blocking LRP ligand binding by RAP (Fig. 3*A*), and occupation of the LRP extracellular domain by a large competing ligand, apoE enriched β-VLDL (Fig. 5*B*), all abolished or greatly reduced the PDGF-dependent phosphorylation of LRP.

As Loukinova *et al.* also show, tyrosine phosphorylation occurs on the second NPXY motif in the cytoplasmic tail of LRP. Two different specific PDGF receptor inhibitors, tyrphostin 9 (this study) and tyrphostin AG1296 (17), prevented LRP tyrosine phosphorylation, indicating that PDGF receptor tyrosine kinase activity is absolutely required.

In summary, the present studies have revealed an LRP-dependent branch of PDGF receptor signaling that takes place in caveolae/rafts at the plasma membrane. This pathway may be important for the regulation of smooth muscle cell migration and proliferation during the atherosclerotic transformation of the vascular wall where apoE locally has a protective role (40). Pioneering work by David Hui and his group (32, 33) has demonstrated that apoE counteracts the migration-promoting effect of PDGF in vascular smooth muscle cells. Moreover, a knockdown of LRP by an antisense approach significantly reduced the apoE-mediated inhibition of PDGF-induced cell migration (35). Alteration of cellular responses to mitogens by apoE has also been reported (41). Together, these findings suggest that apoE binding to LRP may play a protective role during the atherosclerotic transformation of the vascular wall by modulating PDGF-mediated signaling through a complex that contains PDGF receptors, PI3K, Src family tyrosine kinases, and LRP.

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