Platelet-derived Growth Factor Receptor-β (PDGFR-β) Activation Promotes Its Association with the Low Density Lipoprotein Receptor-related Protein (LRP)

EVIDENCE FOR CO-RECEPTOR FUNCTION*

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Activation of the platelet-derived growth factor receptor-β (PDGFR-β) leads to tyrosine phosphorylation of the cytoplasmic domain of LRP and alters its association with adaptor and signaling proteins, such as Shc. The mechanism of the PDGF-induced LRP tyrosine phosphorylation is not well understood, especially since PDGF not only activates PDGF receptor but also binds directly to LRP. To gain insight into this mechanism, we used a chimeric receptor in which the ligand binding domain of the PDGFR-β was replaced with that from the macrophage colony-stimulating factor (M-CSF) receptor, a highly related receptor tyrosine kinase of the same subfamily, but with different ligand specificity. Activation of the chimeric receptor upon the addition of M-CSF readily mediated the tyrosine phosphorylation of LRP. Since M-CSF is not recognized by LRP, these results indicated that growth factor binding to LRP is not necessary for this phosphorylation event. Using a panel of cytoplasmic domain mutants of the chimeric M-CSF/PDGFR-β, we confirmed that the kinase domain of PDGFR-β is absolutely required for LRP tyrosine phosphorylation but that PDGFR-β-mediated activation of phosphatidylinositol 3-kinase, RasGAP, SHP-2, phospholipase C-γ, and Sre are not necessary for LRP tyrosine phosphorylation. To identify the cellular compartment where LRP and the PDGFR-β may interact, we employed immunofluorescence and immunogold electron microscopy. In WI-38 fibroblasts, these two receptors co-localized in coated pits and endosomal compartments following PDGF stimulation. Further, phosphorylated forms of the PDGFR-β co-immunoprecipitated with LRP following PDGF treatment. Together, these studies revealed close association between activated PDGFR-β and LRP, suggesting that LRP functions as a co-receptor capable of modulating the signal transduction pathways initiated by the PDGFR-β receptor from endosomes.

The low density lipoprotein receptor-related protein (LRP)† is a large endocytic receptor composed of a 515-kDa heavy chain to which ligands bind and a non-covalently associated 85-kDa light chain containing a transmembrane and cytoplasmic domain. LRP is a member of the LDL receptor family and is required for embryonic development in mice (1). Although LRP was originally identified as an endocytic receptor for α-2-macroglobulin-protease complexes (1, 2) and apoE-enriched lipoprotein particles (3, 4), this receptor is now known to recognize more than 30 distinct ligands including lipoproteins, proteases, proteinase inhibitor complexes, matrix proteins, bacterial toxins, viruses, intracellular proteins, and growth factors (for a review, see Ref. 5).

In addition to its prominent role in mediating the cellular uptake of a variety of ligands, recent work has implicated LRP in several signal transduction pathways and has expanded its realm to include regulation of cell migration (6–8) and modulation of the integrity of the blood brain barrier (9). In the vasculature, LRP is expressed in smooth muscle cells, and a tissue-specific deletion of the LRP gene in vascular smooth muscle cells causes smooth muscle cell proliferation, aneurysm formation, and increased susceptibility to cholesterol-induced atherosclerosis (10). The mice demonstrated abnormal activation of PDGFR-β, and these effects could be inhibited by treatment of the mice with Gleevec, a known inhibitor of PDGF signaling. These studies indicate that LRP plays a role in protecting the integrity of the vascular wall and preventing atherosclerosis by controlling PDGF activation. The mechanism by which LRP modulates PDGFR function remains unknown and may be related to the ability of the PDGFR-β to mediate tyrosine phosphorylation of the LRP cytoplasmic domain (11, 12). This occurs on a tyrosine residue located within the second NPXY motif in the cytoplasmic domain of LRP (12) and generates a docking site for adaptor molecules such as Shc (12–14), a protein known to be involved in signaling pathways (15–17).

We initiated studies to investigate the mechanism of LRP tyrosine phosphorylation mediated by the PDGFR-β to gain insight into the biochemical link between these two receptors. Using a variety of mutant and chimera PDGFR molecules, we demonstrated that the tyrosine kinase activity of PDGFR is ab-

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‡ The abbreviations used are: LRP, low density lipoprotein receptor-related protein; LDL, low density lipoprotein; M-CSF, macrophage colony-stimulating factor; SYF, src, yes, fyn; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor; PDGFR-β, PDGF receptor-β; PLCγ, phospholipase C-γ; PI3K, phosphatidylinositol 3-kinase; RAF, receptor-associated protein; HRP, horseradish peroxidase; DMEM, Dulbecco's modified Eagle's medium; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; Erk, extracellular signal-regulated kinase; Map, mitogen-activated protein; SFK, Src family kinase; ChiR, chimeric; WT, wild type; KD, kinase-dead.
solutely critical to induce LRP tyrosine phosphorylation, whereas the bridging of LRP and PDGFR via a common ligand PDGF is not necessary for this phosphorylation to take place. We also provided evidence that interaction of LRP and PDGFR occurs within endosomal compartments of the PDGFR-treated cells.

MATERIALS AND METHODS

Proteins and Antibodies—LRP was isolated from human placenta as described previously (1). Human receptor-associated protein (RAP) (19) was expressed in bacteria as fusion proteins with glutathione S-transferase in Escherichia coli as described (19). Recombinant human M-CSF, PDGFR-α, and PDGFR-β were all purchased from R&D Systems. A rabbit polyclonal IgG prepared against purified human LRP (R2629) was affinity-purified over LRP-Sepharose as described (20). Monoclonal anti-LRP 8G1 (2, 21) and monoclonal antibody 11H4 (3) (R2629) was affinity-purified over LRP-Sepharose as described (20).

CSF, PDGF-A, and PDGF-B were all purchased from R&D Systems. Laboratories, whereas HRP-conjugated donkey anti-goat IgG antibody was purchased from Santa Cruz Biotechnology. Anti-actin rabbit polyclonal IgG (BIAcore AB, Uppsala, Sweden). For these studies, a CM5 BIAcore chip surfaces were regenerated as described (12). The binding was measured at 25 °C at a flow rate of 30 μl/min. The fluorescence signal was detected with a SIA 9000 optical biosensor (BIAcore, Sweden). The sensor chip was activated, and LRP was coupled as described (12). An additional flow cell, similarly activated and blocked without immobilization of protein, served as a negative control. A flow cell with immobilized ovalbumin at the level of 500 reactive units was used as a control for nonspecific protein binding. All binding reactions were performed in 10 mM HEPES, 0.15 M NaCl, 0.05% Tween 20, pH 7.4 (BSA-P buffer) (Biacore, Sweden). The binding was measured at a flow rate of 50 μl/min for 2 min followed by 2 min of dissociation. The bulk shift due to changes in refractive index measured on blank surfaces was subtracted from the binding signal at each condition to correct for nonspecific signals. Chip surfaces were regenerated as described (12).

Cell Culture and Growth Factor Treatment—NIH/3T3 cells stably transfected with the M-CSF/PDGFR-β chimeric receptors (Ch1/WT), Ch1/RK/D, or Ch1(F5)) as well as Ph cells were a generous gift of Dr. Andrius Kazlauskas (Boston, MA). NIH/3T3 cells, SYF cells, SYF + c-Src cells, and WI-38 cells were purchased from the ATCC. All cells were cultured at 37 °C in 150-cm² flasks in DMEM containing 10% fetal bovine serum (5% for Ph cells). NIH/3T3 stable transfected cells were supplemented in culture with 1 mg/ml G418. Cells were plated out in 150-cm² plates coated with 0.1% gelatin in 1X PBS. Upon reaching 70% confluence, cells were then incubated for 24 h in serum-free DMEM. Following serum starvation, cells were treated for 15 min with fresh serum-free DMEM supplemented with 40 ng/ml M-CSF, 50 ng/ml PDGFR-α, or 40 ng/ml PDGFB.

Immunoprecipitation and Immunoblot Analysis—Immunoprecipitation and immunoblot analysis were performed essentially as described (23). Briefly, cells were washed twice with cold Dulbecco’s phosphate-buffered saline containing 1 mM sodium orthovanadate. Lysates were then prepared in lysis buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, and a protease and phosphatase inhibitor mixture (Calbiochem). After pre-clearing with rabbit IgG (10 μg) and protein G-Sepharose, lysates were immunoprecipitated with R2629 (10 μg) and protein G-Sepharose overnight at 4 °C. Immunoprecipitates were washed, separated by SDS-PAGE on 4–12% Tris-glycine precast gels (Invitrogen), and transferred to nitrocellulose membranes for immunoblot analysis. Following the addition of the detection antibody and horseradish peroxidase-labeled secondary antibody, the membranes were developed with chemiluminescent reagent (Pierce), and bands were visualized using Biomax MR film (Eastman Kodak Co.). For visualizing the immunoprecipitated LRP, the membranes were stripped with Re-blot Western blot recycling kit (Chemicon International), blocked for 30 min in 5% nonfat dry milk in TBS, 0.05% Tween 20, 1 mM CaCl₂, TBS-Tween, and incubated with 11H4 (1 μg/ml) in 5% nonfat dry milk in TBS-Tween buffer overnight at 4 °C. Membranes were then washed 3 × 15 min in TBS-Tween buffer and incubated with goat anti-mouse IgG (H+L)-HRP conjugate secondary reagent (Bio-Rad) (1:3000) in TBS-Tween buffer at room temperature for 1 h. Membranes were then washed 3 × 5 min and developed with chemiluminescent reagent (Pierce).

Results

Growth Factor Binding to LRP Is Not Necessary for Receptor Tyrosine Kinase-mediated Phosphorylation—Recent studies found that PDGF-B mediates the tyrosine phosphorylation of the LRP cytoplasmic domain via PDGFR-β activation (11, 12). LRP tyrosine phosphorylation is specific for PDGF, as other growth factors such as epidermal growth factor, basic fibroblast growth factor, or insulin-like growth factor-1 are unable to promote LRP tyrosine phosphorylation (12). Interestingly,
PDGF directly binds to LRP, whereas epidermal growth factor, basic fibroblast growth factor, and insulin-like growth factor-1 are not recognized by LRP (12). These observations raise the possibility that PDGF-B may facilitate LRP tyrosine phosphorylation by bridging LRP to the activated PDGFR-β. To investigate the significance of the PDGF-B/LRP interaction and to determine whether association of growth factor with LRP is required for its tyrosine phosphorylation, we utilized a chimeric PDGFR-β that has been described by Fambrough et al. (24). This chimeric receptor, termed ChiR(WT), was generated by replacing the ligand binding domain of the PDGFR-β with that from the M-CSF receptor, a highly related receptor tyrosine kinase of the same subfamily but with different ligand specificity. This chimeric receptor functions normally in PDGF receptor signaling pathways but is activated by M-CSF (24).

Initially, we needed to determine whether M-CSF itself is capable of binding directly to LRP and investigated this possibility using surface plasmon resonance. The results (Fig. 1) reveal that even at high concentrations (100 nM), M-CSF was not recognized by LRP. As expected, PDGF-B (50 nM) and RAP (100 nM) were readily recognized by LRP. These results show that M-CSF does not bind to LRP and indicate that ChiR(WT) can be used to test the hypothesis that growth factor binding to LRP is important for the subsequent tyrosine phosphorylation of LRP.

To determine whether LRP tyrosine phosphorylation can be induced by activation of ChiR(WT), we incubated parental NIH/3T3 cells and NIH/3T3 cells stably transfected with ChiR(WT) for 15 min at 37 °C with either PDGF-B or M-CSF. Following incubation, LRP was immunoprecipitated from cell lysates, and the immunoprecipitated proteins were subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phospho-PDGFR-β antibody (LRP). Membranes containing cell lysates were probed with anti-phospho-PDGFR-β, anti-PDGFR-β, anti-phospho-PDGFR-β, anti-PDGFR-β, anti-actin, and anti-human M-CSF receptor (M-CSFR) antibodies. WB, Western blot.

Either PDGF-β or ChiR(WT). Since M-CSF does not interact directly with LRP, it appears that growth factor binding to LRP is not a necessity for its subsequent tyrosine phosphorylation by the PDGFR-β.

**PDGFR-β Tyrosine Kinase Activity Is Required for LRP Tyrosine Phosphorylation**—Although our previous studies using synthetic inhibitors revealed the importance of the tyrosine kinase activity of the PDGFR-β for phosphorylating LRP (12), we wanted to confirm the requirement of the tyrosine kinase activity of the PDGFR-β for LRP tyrosine phosphorylation. Fambrough et al. (24) have developed a kinase-dead chimeric receptor, referred to as ChiR(KD), in which lysine 634 is mutated to arginine. Lysine 634 is located in the first tyrosine kinase domain of the cytoplasmic domain of PDGFR-β, and its conversion to arginine results in the complete inhibition of kinase activity of PDGFR-β (24). The results reveal that NIH/3T3 cells stably transfected with ChiR(KD) were unable to mediate tyrosine phosphorylation of LRP in response to M-CSF treatment (Fig. 3, lanes 3 and 4). In addition, ChiR(KD) was also not phosphorylated in response to M-CSF treatment. These data confirmed that tyrosine kinase activity of the PDGFR-β is necessary for LRP tyrosine phosphorylation.

**Activation of PI3K, RasGAP, SHP-2, and PLCγ Does Not Appear to Be Required for Tyrosine Phosphorylation of LRP**—Within the cytoplasmic domain of the PDGFR-β are several tyrosine residues that, upon receptor activation, become phosphorylated and form binding sites for a number of intracellular signaling proteins. Because inhibition of PI3K activity reduced the extent of LRP tyrosine phosphorylation (11), we suspected that binding and activation of PI3K by PDGFR-β might be required for the tyrosine phosphorylation of LRP. To investigate this, we evaluated the ability of a chimeric receptor, termed ChiR(F5), containing tyrosine to phenylalanine mutations at residues 740, 751, 771, 1009, and 1021, to phosphorylate LRP. These mutations block tyrosine phosphorylation at these sites and prevent the receptor from binding PI3K, RasGAP, SHP2, and PLCγ, respectively (24). To determine whether binding of any of these molecules to the PDGFR-β cytoplasmic domain is required for tyrosine phosphorylation of LRP, we investigated the tyrosine phosphorylation of LRP in NIH/3T3 cells stably transfected with the ChiR(F5). The results of this experiment reveal that upon M-CSF treatment, tyrosine phosphorylation of both LRP and ChiR(F5) was induced in the NIH/3T3 cells stably transfected
Slightly increased upon the addition of PDGF-B (Fig. 4, absence of PDGFR-

These data revealed that c-Src readily phosphorylates the LRP (ChiR(F5) and ChiR(KD)) were grown in culture, treated for 15 min at 37 °C with either no growth factor (lanes 1, 3, and 5) or 40 ng/ml M-CSF (lanes 2, 4, and 6), and then lysed and immunoprecipitated with anti-LRP polyclonal R2629. Immunoprecipitates (IP) or cell lysates were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were probed as follows. Immunoprecipitate membranes were probed with anti-phospho-

separated by SDS-PAGE and transferred to nitrocellulose. Membranes with the cytoplasmic domain of the

sites) is absolutely necessary for M-CSF-mediated tyrosine phosphorylation of LRP via ChiR activation.

Src Family Kinases May Not Be Essential for PDGF-B-mediated LRP Tyrosine Phosphorylation—Previously, we showed that the LRP cytoplasmic domain can be phosphorylated by purified Src family kinases (SFKs) in vitro and in cells transfected with LRP mini-receptors (12). Further, we also demonstrated that PDGF-B-mediated tyrosine phosphorylation of LRP is reduced by PP2 (12), an inhibitor of SFKs. To further evaluate the role that SFKs play in the PDGF-B-mediated tyrosine phosphorylation of LRP, we analyzed the PDGF-B-mediated tyrosine phosphorylation of LRP in mouse embryonic fibroblast cells that are deficient in Src, Yes, and Fyn (SYF cells) (25). Src, Yes, and Fyn are prominent members of the SFK family and are ubiquitously expressed. SYF cells respond normally to PDGF but do not express functional Src, Yes, and Fyn kinases (25). Additionally, murine c-Src was added back to the SYF cells via retroviral vector transfection to generate cells (SYF + c-Src cells) that express normal levels of Src but do not express Yes or Fyn (25). Treatment of these cells with PDGF-B resulted in tyrosine phosphorylation of LRP (Fig. 4, lane 2). Expression of c-Src in these cells resulted in constitutive tyrosine phosphorylation of LRP even in the absence of detectable levels of phosphorylated PDGF-B (Fig. 4, lane 3). The extent of LRP tyrosine phosphorylation in SYF + c-Src cells was slightly increased upon the addition of PDGF-B (Fig. 4, lane 4).

These data revealed that c-Src readily phosphorylates the LRP cytoplasmic domain when expressed in fibroblasts even in the absence of PDGF-B activation. They also suggested that Src, Yes, and Fyn are not essential for PDGF-B-mediated LRP tyrosine phosphorylation.

To more directly determine whether there is a requirement for SFKs in the PDGF-B-mediated tyrosine phosphorylation of LRP, we used a chimeric receptor described by DeMali and Kazlauskas (26). This chimeric receptor represents a fusion of the extracellular, transmembrane, and juxtamembrane domains of the PDGF-α with the cytoplasmic domain of the PDGF-β (26). The chimeric receptor, termed N2WT, is activated by binding to PDGF-A or PDGF-B and maintains normal levels of PDGF-β tyrosine kinase activity (26). A mutant of this chimeric receptor, termed N2F72/74, contains mutations in which tyrosine residues 572 and 574 are converted to phenylalanine. This mutant receptor is also activated by binding to PDGF-A or PDGF-B and has normal receptor tyrosine kinase activity but is not able to bind SFKs. Transfection of the wild type and mutant chimeric receptors into mouse embryonic fibroblast Ph cells, which are homoygous for a deletion that includes the PDGF-α gene, generates cell lines that are useful for investigating the PDGF-mediated activation of Src family kinases (26), as well as the PDGF-mediated tyrosine phosphorylation of LRP via SFKs.

Ph cells stably transfected with the wild type chimeric receptor (N2WT) or with the mutant chimeric receptor (N2F72/74) were stimulated for 15 min at 37 °C with PDGF-A or with PDGF-B. Ph cells stably transfected with an empty control vector were also treated with PDGF-A or PDGF-B. In Ph cells transfected with control vector, LRP tyrosine phosphorylation only occurred upon treatment with PDGF-B (Fig. 5, lane 3). In contrast, tyrosine phosphorylation of LRP occurred in Ph cells transfected with either N2WT or N2F72/74 in response to treatment with both PDGF-A (Fig. 5, lanes 5 and 8, respectively) and PDGF-B (Fig. 5, lanes 6 and 9, respectively). Together, the results in Figs. 4 and 5 suggest that Src, Yes, or Fyn is not required for LRP tyrosine phosphorylation via activation of the PDGF-B. Further, the results revealed that active Src can tyrosine-phosphorylate LRP even in the absence of PDGF-B activation.

Activated PDGF-β and LRP Co-localize in Endosomal Compartments Following PDGF Stimulation—To gain further insight into the mechanism of PDGF-B-mediated LRP tyrosine phosphorylation, we initiated studies to identify the cellular compartments where LRP and the PDGF-β might interact. WI-38 human lung fibroblast cells, which express high levels of both PDGF-β and LRP, were grown to confluence on glass coverslips in complete, serum-containing media. Then, following a 24-h serum starvation period, cells were either fixed and stained with anti-LRP and anti-PDGFR-β IgG (Fig. 6A) or stimulated for 15 min at 37 °C with PDGF-B prior to fixing and staining with the same antibodies (Fig. 6B). In unstimulated cells, LRP and PDGFR-β were not co-localized (Fig. 6A). However, upon treatment with PDGF-B, LRP and PDGFR-β were co-localized in punctate structures that appear to be endosomal compartments.

Cellular localization of LRP and the PDGFR-β was further
examined by immunogold electron microscopy. In resting cells, we rarely noted co-localization of PDGFR-β with LRP (Fig. 7A). However, following stimulation for 15 min at 37 °C with PDGF-B, we noted extensive co-localization of the PDGFR-β with LRP in clathrin-coated pits (Fig. 7B). Together with the immunofluorescence studies, these results revealed that PDGFR-β and LRP co-localize following activation of PDGFR-β.

We next sought to determine whether the PDGFR-β and LRP interact with one another in cells using co-immunoprecipitation analysis. WI-38 cells were stimulated for 15 min at 37 °C with PDGF-B, and cell lysates were subjected to immunoprecipitation and immunoblot analysis. The results revealed that phosphorylated forms of PDGFR-β co-precipitate with LRP in WI-38 cells following treatment with PDGF-B (Fig. 8).

**DISCUSSION**

Recent studies indicate that LRP is a physiological regulator of the PDGF signaling pathway (10), although the precise role played by LRP in this process is unknown. Activation of the PDGFR-β upon binding of its ligand leads to a transient tyrosine phosphorylation of the LRP cytoplasmic domain (11, 12), suggesting that LRP may function as a co-receptor with the PDGFR-β. In the present study, we have addressed the mechanism by which the PDGFR-β mediates the tyrosine phosphorylation of the cytoplasmic domain of LRP and investigated whether or not these two receptors interact with one another in cells.

Our earlier observation that LRP binds to PDGF-B (12) raised the possibility that this growth factor may bridge LRP to the PDGFR-β, thereby facilitating tyrosine phosphorylation of the LRP cytoplasmic domain. To test this hypothesis, we employed a chimeric receptor that has been previously used to study PDGFR-β function (24). This receptor, constructed by fusing the ectodomain of the M-CSF receptor to the transmembrane and cytoplasmic domain of the PDGFR-β, retains the signaling properties of the PDGFR-β but is only activated upon binding of M-CSF. Our results showed that the chimeric receptor readily phosphorylates the LRP cytoplasmic domain upon stimulation with M-CSF. Since M-CSF, unlike native PDGF-B, does not bind directly to LRP, the results of these experiments revealed that growth factor binding to the LRP ectodomain is not necessary in order for LRP to undergo tyrosine phosphorylation by the PDGFR-β.

Stimulation of the PDGFR initiates a variety of signaling pathways. Next, we wished to determine whether activation of particular signaling molecules such as Src family kinases or...
PI3K is required for LRP tyrosine phosphorylation. To address this, we employed several mutant chimeric PDGFR receptors. These included a kinase-inactive receptor, a receptor defective in binding and activating PI3K, RasGAP, SH2P2, and PLCγ (24), and a receptor defective in binding and activating Src (26). The results from this work showed that only the kinase-inactive receptor failed to mediate the tyrosine phosphorylation of LRP, indicating that PDGFR-β kinase activity is essential for this process. Both of the other two mutant receptors readily mediated LRP tyrosine phosphorylation when activated, suggesting that activation of PI3K, RasGAP, SH2P2, PLCγ, or Src may not be required for LRP tyrosine phosphorylation. However, these results are in apparent contradiction of previous work using specific inhibitors of Src family kinase members (12) and PI3 kinase (11), which revealed that these kinases contribute to the tyrosine phosphorylation of the LDL receptor. It could be that the PDGFR chimeric receptor used in this study may transmodulate endogenous PDGFR-β either indirectly or directly through heterodimerization, which in turn could activate PI3 kinase or Src. Alternatively, it is well known that the PDGFR signaling can transmodulate and activate other receptor tyrosine kinases, such as the epidermal growth factor receptor (27, 28), which in turn would activate PI3 kinase and/or Src. Further, SYF cells likely express other Src family kinase members that could phosphorylate LRP. Thus, the data from the current study need to be interpreted with caution. Interestingly, in SYF cells transfected with c-Src, we found LRP to be constitutively tyrosine-phosphorylated even in the absence of PDGFR-β activation. Thus, Src and PI3K seem to modulate the extent of LRP tyrosine phosphorylation, but their activity may not be required for this process. These data did not discount the possibility that the PDGFR-β may be capable of directly phosphorylating LRP. However, this will need to be determined.

Using membrane fractionation techniques, prior work located LRP in caveolae (11), lipid raft-enriched membrane domains where PDGFR-β is also known to reside (29). LRP is a constitutively active endocytic receptor and is rapidly internalized from the cell surface independent of ligand binding, whereas in the case of the PDGFR-β, ligand binding is accompanied by clustering of the receptors, and the receptor-ligand complexes are internalized and ultimately delivered to lysosomes for degradation (30–32). In the current study, immunofluorescence microscopy as well as immunogold electron microscopy demonstrated that PDGFR-β and LRP co-localize within endosomal compartments, which is especially evident following stimulation of the cells with PDGF. In resting cells, we did not detect significant co-localization of these two receptors; however, rigorous quantitation and distance measurements are required before conclusions regarding the distribution of LRP and PDGFR-β in resting cells can be reached. Recent studies have found that caveolar vesicles can be targeted to early endosomes in a Rab5-dependent pathway to form distinct and stable membrane domains (33), and it is conceivable that the PDGFR-β could be delivered to endosomes via this mechanism. Internalization of signaling receptors was initially thought to result in termination of the signaling pathway and/or in down-regulation of the receptor itself via degradation. However, accumulating evidence suggests that signaling can take place not only from the cell surface but also from endosomes and that compartmentalized signaling could be physiologically important (34, 35). Various proteins may act as scaffolds that recruit other factors either to the plasma membrane or to the endosomes, which mediate alternative pathways downstream of PDGF signaling. 15 minutes following PDGF stimulation, both PDGFR-β and LRP seem to be confined to endosomal compartments, a region where PDGFR-β signaling events can occur. Our observation that phosphorylated, i.e. activated, forms of PDGFR-β co-immunoprecipitate with LRP following PDGF stimulation was consistent with the notion that these two receptors interact within endocytic compartments upon receptor internalization. However, it should be pointed out that at present, the specific cellular compartment where LRP is tyrosine-phosphorylated remains to be determined. We propose that LRP forms a signaling complex with the PDGFR-β in endosomes that may involve adaptor proteins such as Shc, which binds to tyrosine-phosphorylated forms of LRP. It is clear from in vivo studies that PDGF influences the levels as well as functional activity of the PDGFR-β signaling pathways, as Boucher et al. (10) demonstrated that deletion of the LRP gene in vascular smooth muscle cells of mice also deficient in the LDL receptor results in increased levels and activity of PDGFR-β. These in vivo studies indicate that LRP plays an important role in controlling PDGFR activation, thereby protecting the vessel wall from insult and minimizing the development of atherosclerosis. The mechanism of this effect, however, remains unclear. Surprisingly, in murine fibroblasts, LRP expression has been correlated with an increased expression of the PDGFR-β (36, 37), an observation that is contrary to that observed in vivo. Increased expression of the PDGFR-β in LRP(+/-) murine fibroblasts when compared with LRP(-/-) fibroblasts has been attributed to increased PDGFR-β message levels (36) or a decreased half-life of the PDGFR-β protein resulting from a decrease in ubiquitination and endocytosis (37). It is apparent that the ability of LRP to modulate PDGFR-β levels needs to be examined in more detail, especially in vascular smooth muscle cells, which are the primary target for PDGF-B.

In summary, our data have shown that LRP associates with activated forms of the PDGFR-β and that growth factor binding by LRP is not necessary for this event to occur. In resting WI-38 fibroblasts, we noted very little, if any, co-localization between the PDGFR-β and LRP. However, 15 min following stimulation, these two receptors co-localize within endosomal compartments. This association leads to tyrosine phosphorylation of LRP cytoplasmic domain, which enhances the association of adaptor proteins such as Shc, a molecule that plays an important role in mitogen-activated protein kinase activation as well as other signaling pathways (38). The significance of Shc asso-
cation with the LRP cytoplasmic domain is not yet completely understood, but this interaction may recruit Shc to the specific intracellular membrane compartments where it appears to be phosphorylated by Src or other Src family kinases and may then initiate Ras activation.

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