Journal of Cellular Biochemistry 93:1169–1177 (2004)

Effects of Low Density Lipoprotein Receptor-Related Protein-1 on the Expression of Platelet-Derived Growth Factor β-Receptor In Vitro

Lihua Wu, Sanja Arandjelovic, and Steven L. Gonias*

Departments of Pathology and Biochemistry and Molecular Genetics,
University of Virginia School of Medicine, Charlottesville, Virginia, 22908

Abstract The low density lipoprotein receptor related protein-1 (LRP-1) is a cargo transport receptor that undergoes constitutive endocytosis and recycling. Platelet-derived growth factor-BB (PDGF-BB) binds to LRP-1 and may bridge LRP-1 to PDGF receptors. Bridging of LRP-1 to other receptors by bifunctional ligands may represent a general mechanism whereby LRP-1 facilitates internalization of membrane proteins. The goal of this study was to determine whether LRP-1 regulates cell-surface levels of PDGF β-receptor or PDGF β-receptor degradation following treatment with PDGF-BB. Unexpectedly, in both murine embryonic fibroblasts (MEFs) and HT 1080 fibrosarcoma cells, LRP-1 expression was associated with increased levels of PDGF β-receptor. In MEFs, the mechanism involved increased PDGF β-receptor transcription and/or RNA stabilization. LRP-1 expression was not associated with increased levels of PDGF β-receptor in Chinese hamster ovary (CHO) cells, suggesting that cell context is important. The kinetics of PDGF β-receptor phosphorylation, in response to PDGF-BB, and the extent of degradation of PDGF β-receptor were equivalent in LRP-1-expressing and -deficient MEFs. We conclude that PDGF β-receptor expression and cell surface levels may be regulated by LRP-1; however, this activity is cell type-specific. LRP-1 does not directly regulate PDGF β-receptor phosphorylation or degradation in PDGF-BB-treated cells. J. Cell. Biochem. 93: 1169–1177, 2004.

Key words: low density lipoprotein receptor-related protein; platelet-derived growth factor; platelet-derived growth factor β-receptor

Low density lipoprotein receptor related protein-1 (LRP-1/CD91) is a 600-kDa type I membrane protein and a member of the LDL receptor gene family [Strickland et al., 2002]. In its mature form, LRP-1 consists of an N-terminal 515-kDa heavy chain that is entirely extracellular and an 85-kDa light chain, which has a transmembrane domain and cytoplasmic tail. The heavy chain is anchored to the cell surface by non-covalent interactions with the light chain. LRP-1 and other members of this gene family have diverse effects on cell physiology. By binding over 40 distinct ligands, LRP-1 may regulate lipid homeostasis, extracellular proteolysis, growth factor/cytokine activity, the composition of the extracellular matrix, and the immune response [Strickland et al., 2002]. LRP-1 also may regulate the composition of the plasma membrane. A number of plasma membrane proteins, including the urokinase receptor (uPAR), amyloid precursor protein (APP), and tissue factor (TF), undergo endocytosis in complex with LRP-1 [Conese et al., 1995; Knauer et al., 1996; Hamik et al., 1999]. As a result, LRP-1 down-regulates the levels of these proteins at the cell surface. The mechanism by which LRP-1 facilitates endocytosis of other membrane proteins is not completely understood; however, bifunctional ligands or intracellular adaptor proteins may be necessary to bridge LRP-1 to the other membrane proteins [Strickland et al., 2002]. LRP-1 also may regulate the composition of the plasma membrane by regulating membrane protein transport in the secretory pathway [Salicioni et al., 2004].

*Correspondence to: Steven L. Gonias, Department of Pathology, University of California, San Diego, School of Medicine, 9500 Gilman Drive, La Jolla, California 92093.
E-mail: sgonias@ucsd.edu

Received 20 June 2004; Accepted 28 July 2004

DOI 10.1002/jcb.20288

The cytoplasmic tail of the LRP-1 light chain includes two NPxY sequences that may be targets for tyrosine phosphorylation [Barnes et al., 2001]. This is important because NPxY motifs, in LDL receptor family members, function as binding sites for signaling adaptor proteins, such as Shc and Dab1, and thereby regulate cell signaling [Trommsdorff et al., 1998; Gotthardt et al., 2000; Barnes et al., 2001]. Platelet-derived growth factor (PDGF) is unique amongst growth factors in its capacity to induce tyrosine phosphorylation of LRP-1 [Boucher et al., 2002; Loukinova et al., 2002]. When cells are treated with PDGF-BB, the growth factor induces dimerization of either PDGF α- or β-receptors, with subsequent activation of receptor tyrosine kinase activity [Claesson-Welsh, 1994]. Many proteins are tyrosine-phosphorylated downstream of PDGF receptors; however, the response is limited by receptor internalization and degradation in lysosomes [Sorkin et al., 1991; Claesson-Welsh, 1994].

Tissue-specific knock-out of the *LRP-1* gene in mouse vascular smooth muscle cells (VSMCs) increases the level of PDGF β-receptor and its degree of tyrosine-phosphorylation in vivo [Boucher et al., 2003]. Because PDGF-BB binds directly to LRP-1 [Loukinova et al., 2002], the opportunity exists for the formation of bridged PDGF β-receptor–LRP-1 complexes at the cell surface. These complexes may demonstrate altered endocytosis and/or survival compared with PDGF receptor dimers and thus, account for the difference in PDGF β-receptor expression and activation in LRP-1-deficient VSMCs in vivo. Alternatively, regulation of PDGF receptor expression and activity in VSMCs in vivo may reflect release from LRP-1-regulated signaling pathways or the effects of LRP-1 on protein trafficking in the secretory pathway.

The goal of this study was to determine whether LRP-1 regulates cell-surface levels of PDGF β-receptor in cells in culture. We report the unexpected result, that LRP-1 expression is associated with increased levels of total and cell-surface PDGF β-receptor in fibroblast-like cells, including murine embryonic fibroblasts (MEFs) and in HT 1080 fibrosarcoma cells. The effects of LRP-1 on PDGF β-receptor in MEFs were explained by an increase in RNA transcription and/or stability. In other cell types, including Chinese hamster ovary (CHO) cells and human VSMCs, LRP-1 expression was not associated with increased levels of PDGF β-receptor, suggesting that cell context is important. PDGF β-receptor phosphorylation and subsequent β-receptor degradation were similar in LRP-1-positive and -negative MEFs.

MATERIALS AND METHODS

Reagents

PDGF-BB was purchased from R&D Systems (Minneapolis, MN). Rabbit PDGF β-receptor-specific polyclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phosphotyrosine-specific monoclonal antibody PY20 was from Transduction Laboratories (Lexington, KY). Epidermal growth factor (EGF) receptor-specific antibody was from Upstate (Lake Placid, NY). Mouse monoclonal antibody 8G1, which is specific for the LRP-1 heavy chain, and 11H4, which recognizes the light chain, were purified from hybridoma-conditioned medium. MAP kinase/ERK 1,2-specific antibody was from ZYMED (South San Francisco, CA). Monoclonal β-actin-specific antibody was from Sigma (St. Louis, MO). The biotinylation reagent, sulfo-NHS-LC-biotin, was purchased from Pierce (Rockford, IL). Glutathione-*S*-transferase-receptor associated protein (GST-RAP) was expressed in bacteria and purified as described previously [Webb et al., 1999]. The activity of purified GST-RAP was confirmed by competition for receptor-binding with methylamine-activated α_2-macroglobulin. Activated α_2-macroglobulin binds to LRP-1, but not to any other LDL receptor family members, making this an appropriate reagent for testing the activity of RAP in this study [Strickland et al., 1990; Webb et al., 1995].

Cell Culture

LRP-1-deficient MEFs (MEF-2 cells), LRP-1 +/− MEFs (*Pseudomonas* exotoxin A (PEA)-10 cells), and wild-type MEFs (MEF-1 cells) were obtained from the American Type Culture Collection (ATCC). MEF-2 and PEA-10 cells were cloned from the same culture of LRP-1 +/− cells, following selection with PEA [Willnow and Herz, 1994]. LRP-1 deficiency provides one mechanism by which cells gain resistance to PEA. MEF-1 cells are from the same mouse strain. B6 cells are MEF-2 cells that were transfected for stable expression of full-length human LRP-1. These cells were kindly provided by Dr. Dudley Strickland (Holland Labora-

tories, American Red Cross, Rockville, MD). All MEFs were cultured in DMEM with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2.

HT 1080 fibrosarcoma cells were obtained from the ATCC and cultured in MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2. Rat VSMCs were isolated from rat aortas by enzymatic digestion, as previously described [Geisterfer et al., 1988] and kindly provided by Dr. Gary Owens (University of Virginia). The cells were cultured in DMEM/F12 supplemented with 10% FBS, 0.68 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Human aortic VSMCs were obtained from the ATCC (CRL-1999) and cultured in Ham's F12 medium supplemented with 10% FBS, 2 mM L-glutamine, 0.01 mg/ml insulin, 0.01 mg/ml transferrin, 10 ng/ml sodium selenite, 0.03 mg/ml endothelial cell growth supplement, 0.05 mg/ml ascorbic acid, 10 mM HEPES pH 7.4, 10 mM TES, 100 U/ml penicillin, and 100 μg/ml streptomycin. Wild-type CHO-K1 cells and LRP-1-deficient CHO 13-5-1 cells [FitzGerald et al., 1995] were cultured in Ham's F12 medium, supplemented with 5% FBS optimized for CHO cells (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Preparation of siRNA and Transfection

Duplex siRNA was synthesized by Dharmacom Research (Lafayette, CO). The targeted sequence was determined by analysis of the human LRP-1 cDNA, in the region 50–100 nucleotides downstream of the start codon. We searched for a sequence, AA(N19), with approximately 50% GC-content and identified nucleotides 73–93 (AAGACTTGCAGCCCCAAGCAG) of the coding region. The selected sequence was subjected to a Blast search to optimize specific silencing of LRP-1 expression. A scrambled LRP-1 siRNA was prepared as a control. HT 1080 cells and human VSMCs were seeded into 6-well plates and then transfected with 1.0 μg/well of siRNA for 4 h in serum-free medium, using GeneSilencer transfection reagent (Gene Therapy System, Inc., San Diego, CA). The medium was then replaced with serum-containing MEM or Ham's F12 medium. Cells were assayed to determine LRP-1 expression by immunoblot analysis, using antibody 8G1, 24–120 h after transfection.

Immunoblot Analysis

Cells were washed three times with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS) and then extracted in RIPA buffer, which contains 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and proteinase inhibitors. Equal amounts of cellular protein were subjected to SDS–PAGE, under non-reducing conditions for detection of LRP-1 and under reducing conditions for detection of PDGF β-receptor, EGF receptor, or β-actin. Proteins were electro-transferred to polyvinylidene fluoride membranes. The membranes were probed with specific antibodies followed by peroxidase-conjugated secondary antibodies and developed with Western Lightning Chemiluminescence reagent (Perkin Elmer Life Sciences, Boston, MA). To compare band intensities within a single blot, densitometry was performed and the results analyzed using ImageQuant software.

Surface-Protein Biotinylation

Confluent cultures were washed three times with PBS and then treated with the membrane-impermeable biotinylation reagent, sulfo-NHS-LC-biotin (0.5 mg/ml), for 30 min at 4°C. After biotin labeling, the cells were washed and then extracted in RIPA buffer. Biotin-labeled proteins in the extracts were affinity-precipitated with Streptavidin–Sepharose (Amersham Biosciences, Uppsla, Sweden), as previously described [Salicioni et al., 2004]. The affinity precipitates were then subjected to immunoblot analysis.

Northern Blot Analysis

The 5.2 kb complete coding sequence of the mouse PDGF β-receptor cDNA was excised with *Bam*HI to generate the 312 base pair probe for Northern blot analysis. Total cellular RNA was isolated from confluent MEF-1 and MEF-2 cells using Trizol reagent (Gibco BRL, Life Technology, Frederick, MD). RNA (10 μg) was subjected to electrophoresis on 1% denaturing formaldehyde/agarose gels and transferred to Nitran nylon membranes. Prehybridization was performed for 20 min at 68°C, using QuikHyb hybridization solution (Stratagene, La Jolla, CA). Membranes were then incubated for 12 h at 68°C with the [^{32}P]-labeled mouse PDGF-β receptor cDNA probe. The membranes were washed twice at 22° C in 2× SSC with 0.1% (w/v) SDS and then once for 30 min at 60°C in 0.1× SSC with 0.1% (w/v) SDS. As a control for load,

membranes were rehybridized with a cDNA probe for β-actin (Ambion, Inc., Austin, TX). PDGF-β receptor and β-actin band intensities were determined by densitometry and the data were analyzed using ImageQuant software. Results were standardized relative to the β-actin signals.

PDGF-BB Stimulation

MEF-1 and MEF-2 cells were seeded in 100-mm^2 plates in DMEM containing 10% FBS and grown to confluence. The cultures were washed three times with PBS and then serum-starved for 18 h. Cells were treated with 0.8 nM PDGF-BB or vehicle at 37°C for different times. After incubation with PDGF-BB, cells were washed with ice-cold PBS and extracted in RIPA buffer. The extracts were subjected to immunoblot analysis to detect tyrosine-phosphorylated proteins and PDGF β-receptor.

Rat VSMCs were serum-starved in DMEM/F12 medium containing 0.5 μM insulin, 5 μg/ml transferrin, 0.2 mM ascorbate, 38 nM selenium, 0.68 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin for 18 h. These VSMCs and serum-starved MEFs were pre-treated with 180 nM GST-RAP or with vehicle (PBS) for 30 min and then stimulated with the indicated concentrations of PDGF-BB (0.01–0.8 nM) for 8 h in the presence of GST-RAP. Surface protein biotinylation and immunoblot analysis were performed to detect cell-surface and total PDGF β-receptor.

RESULTS

LRP-1 Regulates PDGF β-Receptor Expression in MEFs

LRP-1 regulates the cell-surface level of many membrane proteins, including uPAR, APP, TF, and β1 integrin [Conese et al., 1995; Knauer et al., 1996; Hamik et al., 1999; Salicioni et al., 2004]. Given that PDGF-BB binds directly to LRP-1 and therefore may bridge LRP-1 to PDGF receptors [Loukinova et al., 2002], we undertook studies to determine whether LRP-1 regulates the PDGF β-receptor. Initially, we compared total levels of PDGF β-receptor in whole cell extracts from LRP-1-deficient MEF-2 cells, LRP-1(+/−)PEA-10 cells, LRP-1(+/+)MEF-1 cells, and B6 cells, which are MEF-2 cells transfected to express full-length human LRP-1. Extracts were prepared after the cultures became confluent. Figure 1A confirms that

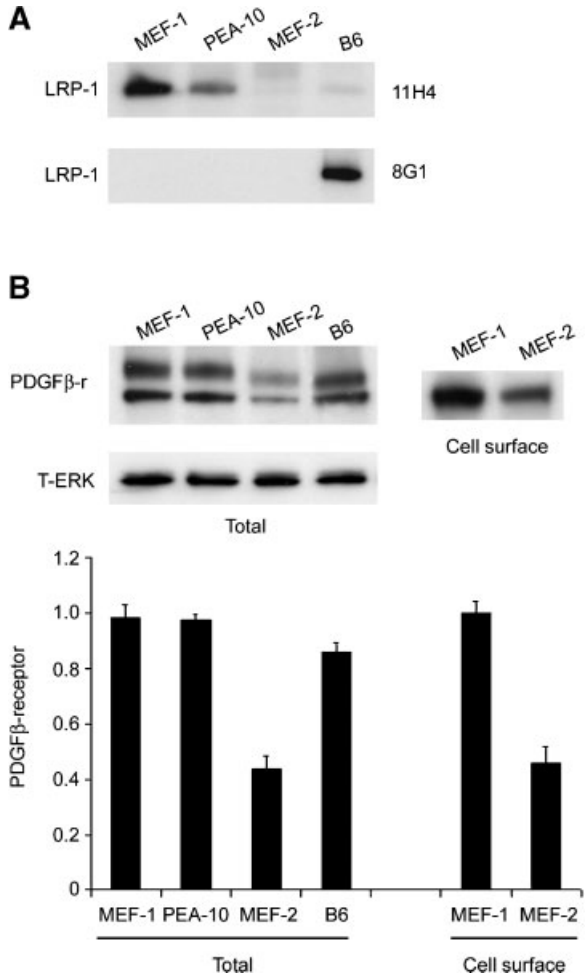

Fig. 1. Platelet-derived growth factor (PDGF) β-receptor expression in murine embryonic fibroblasts (MEFs). MEF-1, *Pseudomonas* exotoxin A (PEA)-10, MEF-2, and B6 cells were cultured until confluent in serum-containing medium. Cell extracts were then prepared and equal amounts of cellular protein were subjected to immunoblot analysis. **A**: LRP-1 content was determined using antibodies 11H4 and 8G1. Antibody 11H4 recognizes the light chain whereas 8G1 recognizes the heavy chain and is specific for human LRP-1. **B**: The same cell extracts were examined by immunoblot analysis for PDGF β-receptor. These lanes are labeled ''total.'' Membranes were also probed for total ERK (T-ERK), as a control for load. In the lanes labeled ''cell surface,'' cells were surface-labeled with biotin and Streptavidin affinity precipitates were prepared. These were probed by immunoblot analysis for PDGF β-receptor. The bar graph compares the total level of PDGF β-receptor (total) or the level of cell-surface PDGF β-receptor (cell surface) to that present in MEF-1 cells. The levels were determined by densitometry and analyzed using ImageQuant software (mean ± SEM, n = 3).

MEF-2 cells lack immunoreactivity for the LRP-1 light chain. The level of LRP-1 in PEA-10 cells was decreased compared with MEF-1 cells, as previously described [Willnow and Herz, 1994]. B6 cells demonstrated low levels of LRP-1, as

determined with antibody 11H4; however, this antibody may preferentially detect murine LRP-1, compared with human LRP-1. A more robust signal was observed when we probed the same membranes with antibody 8G1, which recognizes the human LRP-1 heavy chain.

MEF whole cell extracts were then probed to detect total cellular PDGF β-receptor. As shown in Figure 1B, the level of PDGF β-receptor was decreased by greater than 50% in the LRP-1-deficient MEF-2 cells, compared with either MEF-1 cells or PEA-10 cells, both of which express LRP-1. Importantly, both the mature form of PDGF β-receptor (190-kDa) and the 170-kDa species, which is an intracellular PDGF β-receptor precursor [Claesson-Welsh et al., 1988], were affected similarly, suggesting that the difference in PDGF β-receptor levels did not reflect altered maturation of newly synthesized protein. To confirm that the decrease in PDGF β-receptor in MEF-2 cells was due to LRP-1 deficiency, we examined B6 cells, in which LRP-1 is re-expressed. In these cells, increased amounts of PDGF β-receptor were observed, suggesting phenotypic rescue.

To compare cell-surface PDGF β-receptor in MEFs, we used a membrane-impermeable biotinylation reagent. Streptavidin affinity precipitates were prepared and subjected to immunoblot analysis for PDGF β-receptor. Figure 1B shows that the level of cell-surface PDGF β-receptor was decreased in MEF-2 cells and the extent of the decrease was in proportion to that observed in whole cell extracts.

To further explore the relationship between LRP-1 expression and expression of PDGF β-receptor, we compared cells that were maintained under different conditions of culture confluency and serum-supplementation. In LRP-1-expressing MEF-1 cells, high confluency and serum deprivation favored PDGF β-receptor expression (Fig. 2A), as has been observed in other cells [Barrett et al., 1996]. The equivalent pattern of PDGF β-receptor expression was observed in MEF-2 cells. As a result, the relationship between LRP-1 and PDGF β-receptor expression was maintained under all culture conditions. LRP-1 levels were also increased in MEF-1 cells, when the cells were cultured in serum-free medium (n = 4); however, the effect was modest.

To determine the mechanism by which LRP-1 expression is associated with increased PDGF β-receptor levels in MEFs, we performed Northern blot analysis to detect PDGF β-receptor mRNA. The level of PDGF β-receptor mRNA was decreased by about 40% in MEF-2 cells, compared with LRP-1-expressing MEF-1 cells (Fig. 2B), suggesting that LRP-1 regulates PDGF β-receptor expression in MEFs by altering RNA transcription and/or stability.

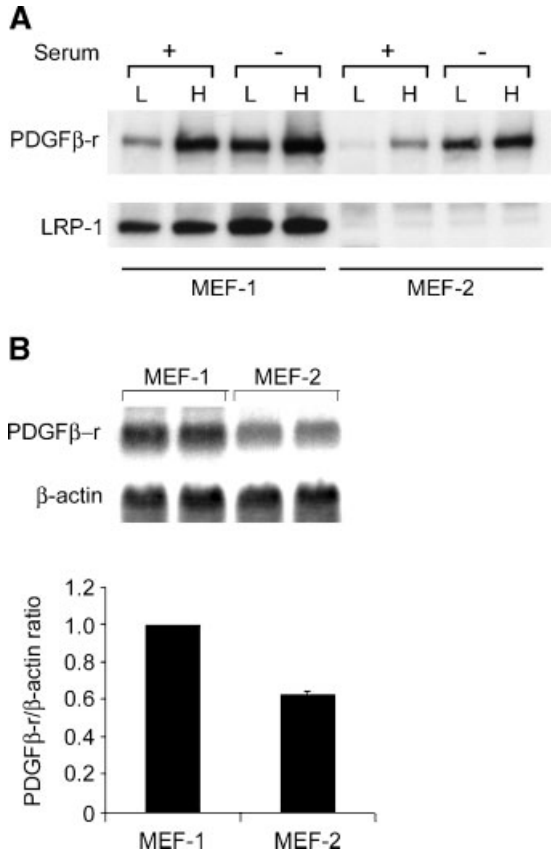

Fig. 2. Regulation of PDGF β-receptor expression. **A**: MEF-1 and MEF-2 cells were plated in 100 mm^2 culture dishes at low density ($3.5 \times 10^3/mm^2$) (L) or at high-density ($3.5 \times 10^4/mm^2$) (H). Cultures were maintained for 24 h in DMEM with (+) or without (−) 10% fetal bovine serum (FBS). Equal amounts of cellular protein were subjected to immunoblot analysis for PDGF β-receptor and for LRP-1, using antibody 11H4. **B**: Total cellular RNA was isolated from confluent cultures of MEF-1 and MEF-2 cells. Northern blots were probed for PDGF β-receptor mRNA and for β-actin mRNA, as a control for load. Densitometry was performed and analyzed using ImageQuant software. The bar graph compares PDGF β-receptor mRNA in MEF-1 and MEF-2 cells (mean ± SEM, n = 4).

Regulation of PDGF β-Receptor in Other Cell Lines

To assess the effects of LRP-1 expression on PDGF β-receptor levels in a second model system, we utilized an siRNA approach. In HT 1080 cells, synthetic duplex siRNA induced

maximum knock-down of LRP-1 at about 72 h (Fig. 3A). An 80% decrease in LRP-1 was observed at that time (Fig. 3B). Concurrently, we observed a 50% decrease in PDGF β-receptor, compared with control cultures that were treated with transfection reagent alone. Specificity was demonstrated because LRP-1 knock-down did not affect cellular levels of EGF receptor or β-actin. Furthermore, a scrambled LRP-1 siRNA duplex had no effect on LRP-1 or PDGF β-receptor (results not shown). Thus, in HT 1080 cells, as in MEFs, LRP-1 expression is associated with increased levels of PDGF β-receptor. However, the relationship between LRP-1 expression and PDGF β-receptor was cell type-specific. We were not able to demonstrate a decrease in PDGF β-receptor in LRP-1-deficient CHO cells [FitzGerald et al., 1995] or when LRP-1 was knocked down by 70% with siRNA in human VSMCs (results not shown).

Response to PDGF-BB in LRP-1-Deficient Cells

Because PDGF-BB binds to both PDGF receptors and LRP-1 [Loukinova et al., 2002], we tested whether LRP-1 influences the kinetics of PDGF β-receptor phosphorylation or survival following exposure to PDGF-BB. Confluent cultures of MEF-1 and MEF-2 cells were serum-deprived for 18 h and then treated with PDGF-BB (0.8 nM). In both cell types, we observed tyrosine phosphorylation of a 190-kDa band, corresponding to the mature form of the PDGF β-receptor (Fig. 4A). Phosphorylation persisted for 1–30 min. This was followed by disappearance of the 190 kDa band, probably due to degradation, as was anticipated [Sorkin et al., 1991]. When the total amount of 190-kDa PDGF β-receptor was analyzed by densitometry, a modest survival advantage was observed from 1 to 30 min in LRP-1-expressing cells (Fig. 4B); however, by 2 h, PDGF β-receptor declined by approximately 80% in both cell types. Thus, LRP-1 did not alter the ultimate extent of PDGF β-receptor degradation after exposure to a high concentration of PDGF-BB.

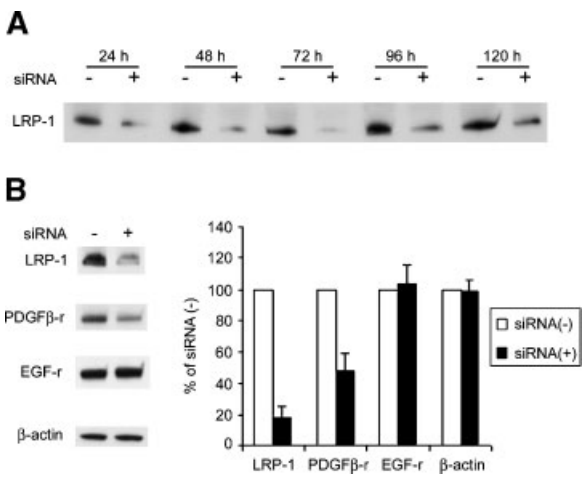

Fig. 3. Effects of LRP-1 knock-down on PDGF β-receptor levels in HT 1080 cells. **A**: HT 1080 cells were transfected with synthetic duplex siRNA targeting human LRP-1. Immunoblot analysis was performed to detect LRP-1 in cultures treated with siRNA (+) or with transfection reagent only (−), at the indicated times after initiating transfection. LRP-1 was detected with antibody 8G1. **B**: HT 1080 cells that were treated with siRNA (+) or with transfection reagent alone (−) were extracted 72 h later and subjected to immunoblot analysis to detect the indicated proteins. Immunoblots were subjected to densitometry and the level of each protein was compared in siRNA-treated and control cultures (mean ± SEM, n = 3).

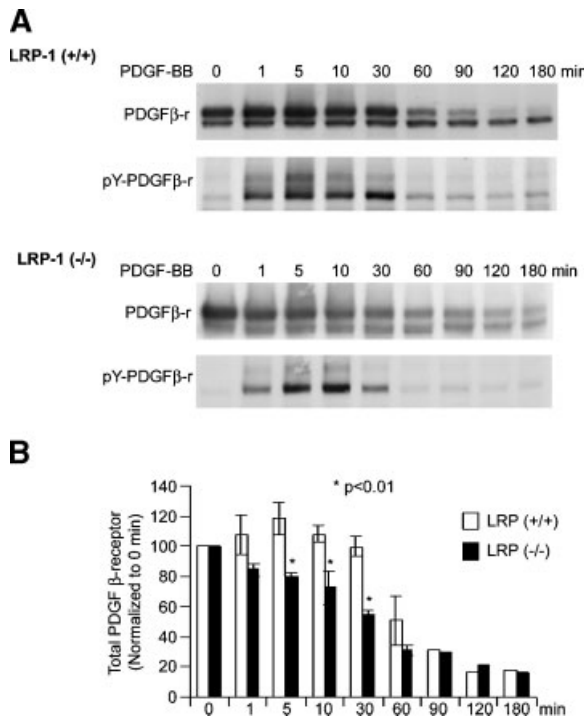

Fig. 4. Treatment of MEFs with PDGF-BB. **A**: Confluent cultures of MEF-1 (LRP-1+/+) and MEF-2 (LRP-1−/−) cells were serum-starved for 18 h. The cells were then treated with PDGF-BB (0.8 nM) for the indicated times. Cell extracts were prepared. Equal amounts of cellular protein were subjected to immunoblot analysis for PDGF β-receptor and phosphotyrosine. The major band that was detected with phosphotyrosine-specific antibody corresponded in mobility exactly to the 190-kDa mature form of the PDGF β-receptor, as anticipated, and is thus labeled ''pY-PDGF β-r.'' **B**: Densitometry was performed and the level of mature PDGF β-receptor (190-kDa band from **upper** immunoblot in each series) was plotted as a function of time (mean ± SEM, n = 3).

We also tested whether LRP-1 affects PDGF β-receptor survival in cells treated with lower concentrations of PDGF-BB over an extended period of time (8 h). Our strategy was to pre-treat MEF-1 cells for 30 min with RAP, which binds to LRP-1 and blocks the binding of other LRP-1 ligands [Herz et al., 1991]. Association of PDGF-BB with LRP-1 is at least partially blocked by RAP [Loukinova et al., 2002]. Thus, we hypothesized that RAP may block the formation of bridged receptor complexes in which PDGF-BB binds simultaneously to the β-receptor and LRP-1. The long incubation time was chosen to potentially reveal subtle changes in the recycling efficiency of PDGF β-receptor, which may be due to LRP-1.

Figure 5A shows a control experiment, in which 180 nM RAP entirely blocked specific-binding of activated α_2-macroglobulin to LRP-1, proving that our RAP preparation is active. However, the same concentration of RAP failed to affect the extent of PDGF β-receptor degradation or the change in cell-surface PDGF β-receptor, after exposure to PDGF-BB (Fig. 5B). In both MEF-1 cells and rat VSMCs, PDGF-BB at concentrations of 0.2 nM or higher induced significant loss of cell-surface and total PDGF β-receptor. PDGF-BB at 0.01–0.04 nM had little or no effect on these levels. These results argue against a model in which LRP-1 regulates PDGF β-receptor by forming bridged receptor complexes that undergo endocytosis as a unit, as has been demonstrated for LRP-1 and other receptors, such as uPAR and TF [Conese et al., 1995; Knauer et al., 1996; Hamik et al., 1999].

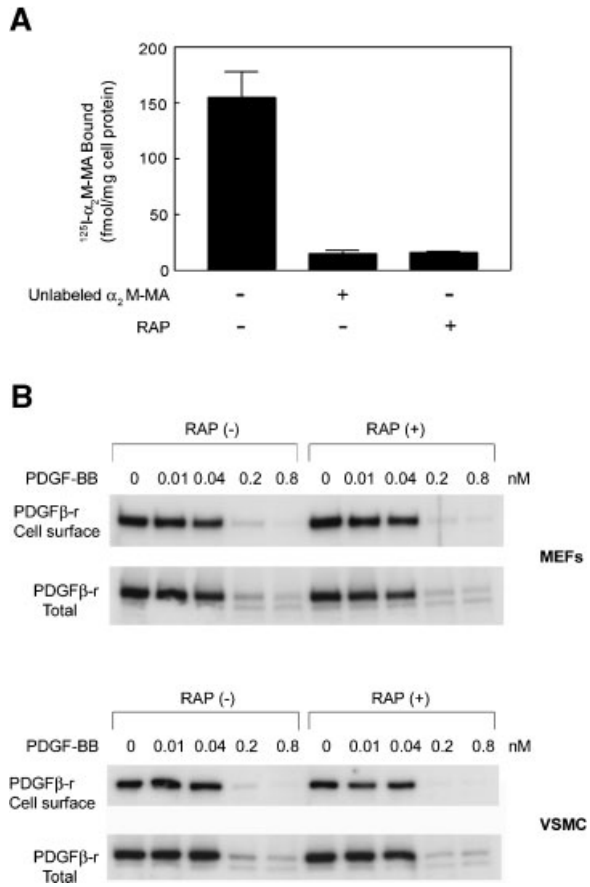

Fig. 5. Effects of the receptor associated protein (RAP) on PDGF-BB-induced PDGF β-receptor degradation in MEF-1 cells and rat vascular smooth muscle cells (VSMCs). **A**: ^{125}I-labeled activated α_2-macroglobulin (1.0 nM) was incubated with RAW 264.7 cells, which are a rich source of LRP-1, in the presence of 180 nM glutathione-*S*-transferase (GST)-RAP or 200 nM unlabeled activated α_2-macroglobulin. By convention, specific ^{125}I-α_2-macroglobulin binding represents that which is blocked by excess unlabeled α_2-macroglobulin. GST-RAP completely blocked specific binding of ^{125}I-α_2-macroglobulin. **B**: Confluent cultures of MEF-1 cells and rat VSMCs were serum-starved for 18 h. The cells were pre-treated with 180 nM GST-RAP for 30 min and then treated with the indicated concentrations of PDGF-BB for 8 h, in the presence or absence of RAP. The cells were then chilled to 4°C. Surface proteins were biotinylated and Streptavidin affinity-precipitates were prepared. These were subjected to immunoblot analysis for PDGF β-receptor (PDGF β-r cell surface). Alternatively, equal amount of cellular protein from whole cell extracts were subjected to immunoblot analysis for total PDGF β-receptor (PDGF β-r total).

DISCUSSION

VSMC-specific knock-out of the *LRP-1* gene in the mouse up-regulates PDGF β-receptor levels in these cells and increases the degree of PDGF β-receptor tyrosine-phosphorylation [Boucher et al., 2003]. There are multiple possible explanations for this result. A unique activity of LRP-1 and related receptors of the LDL receptor family is the ability to facilitate the endocytosis of other membrane proteins [Conese et al., 1995; Knauer et al., 1996; Hamik et al., 1999]. Because PDGF-BB is a bifunctional dimer, which binds to both PDGF receptors and LRP-1, we hypothesized that bridged receptor complexes may form and that these complexes may alter the signaling response to PDGF-BB or survival of PDGF receptors. We found no evidence that this is the case. PDGF-BB-induced phosphorylation of β-receptors was comparable in LRP-1-expressing and deficient cells. The extent of β-receptor degradation, following PDGF-BB treatment, was comparable in LRP-1-expressing and -deficient cells. Furthermore, RAP had no effect on PDGF β-receptor survival

in MEFs or VSMCs that were exposed to low concentrations of PDGF-BB over a prolonged period of time.

An unanticipated finding was the relationship between LRP-1 expression and PDGF β-receptor expression in fibroblasts and fibrosarcoma cells. MEF-2 cells, which are LRP-1 deficient, demonstrated more than a 50% decrease in total and cell-surface PDGF β-receptor. A number of results support the hypothesis that this decrease is directly related to LRP-1 deficiency. First, we compared MEF-2 cells to two separate LRP-1-expressing MEF lines, one that had been selected with PEA and one that had not [Willnow and Herz, 1994]. The two LRP-1-expressing cell lines had equivalent levels of PDGF β-receptor. Second, we examined MEF-2 cells that were transfected for re-expression of LRP-1. The amount of PDGF β-receptor was increased in these cells. Thus, LRP-1 re-expression rescued the phenotype associated with LRP-1 deficiency in MEF-2 cells.

The equivalent relationship between LRP-1 expression and PDGF β-receptor levels was demonstrated in HT 1080 cells. These fibrosarcoma cells express approximately 5,000 copies of cell-surface LRP-1/cell [Webb et al., 2000]. With synthetic siRNA, we knocked down LRP-1 by 80% in HT 1080 cells and observed a concomitant decrease in PDGF β-receptor. This was a specific effect because the level of EGF receptor was unchanged. In MEFs, the effects of LRP-1 on PDGF β-receptor were explained by differences in the amount of PDGF β-receptor mRNA, suggesting altered gene transcription or mRNA stability. These processes may be controlled downstream of cell-signaling pathways that are regulated by LRP-1 or by other cell-signaling receptors such as uPAR, which are activated when the function or expression of LRP-1 is neutralized [Ma et al., 2002]. Another possible mechanism for the regulation of PDGF β-receptor mRNA is suggested by recent studies demonstrating that the intracytoplasmic tail of LRP-1 may be cleaved, yielding a fragment that enters the nucleus and functions as a transcription regulator [May et al., 2002; Kinoshita et al., 2003].

The effects of LRP-1 on PDGF β-receptor were cell type-specific. LRP-1 expression was not associated with increased levels of PDGF β-receptor in CHO cells or in human VSMCs. Incomplete LRP-1 knock-down (70%) with siRNA may explain the lack of an effect in the VSMCs. Another possible contributing factor is the very high level of LRP-1 in VSMCs. Rat VSMCs express over 20,000 copies of LRP-1/cell [Weaver et al., 1996].

Our in vitro experiments do not provide an explanation for the results observed when LRP-1 is neutralized in VSMCs in vivo [Boucher et al., 2003]; however, we can argue against specific models, as described above. The results presented here are most consistent with a model in which the effects of LRP-1 on PDGF β-receptor expression and activity in vivo are indirect, reflecting the cell signaling pathways that are downstream of LRP-1 and/or its co-receptors, such as uPAR. Furthermore, in the intact blood vessel, the opportunity exists for paracrine regulatory pathways to have a substantial impact. These cannot be readily modeled or duplicated in vitro.

REFERENCES

Barnes H, Larsen B, Tyers M, van Der Geer P. 2001. Tyrosine-phosphorylated low density lipoprotein receptor-related protein 1 (LRP1) associates with the adaptor protein SHC in Src-transformed cells. J Biol Chem 276: 19119–19125.

Barrett TB, Seifert RA, Bowen-Pope DF. 1996. Regulation of PDGF receptor expression by cell context—Overrides regulation by cytokines. J Cell Physiol 169:126–138.

Boucher P, Liu P, Gotthardt M, Hiesberger T, Anderson RG, Herz J. 2002. Platelet-derived growth factor mediates tyrosine phosphorylation of the cytoplasmic domain of the low density lipoprotein receptor-related protein in caveolae. J Biol Chem 277:15507–15513.

Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J. 2003. LRP: Role in vascular wall integrity and protection from atherosclerosis. Science 300:329–332.

Claesson-Welsh L. 1994. Platelet-derived growth factor receptor signals. J Biol Chem 269:32023–32026.

Claesson-Welsh L, Eriksson A, Moren A, Severinsson L, Ek B, Ostman A, Betsholtz C, Heldin CH. 1988. cDNA cloning and expression of a human platelet-derived growth factor (PDGF) receptor specific for B-chain-containing PDGF molecules. Mol Cell Biol 8:3476–3486.

Conese M, Nykjaer A, Petersen CM, Cremona O, Pardi R, Andreasen PA, Gliemann J, Christensen EI, Blasi F. 1995. Alpha-2 macroglobulin receptor/LDL receptor-related protein (LRP)-dependent internalization of the urokinase receptor. J Cell Biol 131:1609–1622.

FitzGerald DJ, Fryling CM, Zdanovsky A, Saelinger CB, Kounnas M, Winkles JA, Strickland D, Leppla S. 1995. *Pseudomonas* exotoxin-mediated selection yields cells with altered expression of low-density lipoprotein receptor-related protein. J Cell Biol 129:1533–1541.

Geisterfer AA, Peach MJ, Owens GK. 1988. Angeotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. Circ Res 62:749–756.

Gotthardt M, Trommsdorff M, Nevitt MF, Shelton J, Richardson JA, Stockinger W, Nimpf J, Herz J. 2000.

Interactions of the low density lipoprotein receptor gene family with cytosolic adaptor and scaffold proteins suggest diverse biological functions in cellular communication and signal transduction. J Biol Chem 275: 25616–25624.

Hamik A, Setiadi H, Bu G, McEver RP, Morrissey JH. 1999. Down-regulation of monocyte tissue factor mediated by tissue factor pathway inhibitor and the low density lipoprotein receptor-related protein. J Biol Chem 274: 4962–4969.

Herz J, Goldstein JL, Strickland DK, Ho YK, Brown MS. 1991. 39-kDa protein modulates binding of ligands to low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. J Biol Chem 266:21232–21238.

Kinoshita A, Shah T, Tangredi MM, Strickland DK, Hyman BT. 2003. The intracellular domain of the low density lipoprotein receptor-related protein modulates transactivation mediated by amyloid precursor protein and Fe65. J Biol Chem 278:41182–41188.

Knauer MF, Orlando RA, Glabe CG. 1996. Cell surface APP751 forms complexes with protease nexin 2 ligands and is internalized via the low density lipoprotein receptor-related protein (LRP). Brain Res 740:6–14.

Loukinova E, Ranganathan S, Kuznetsov S, Gorlatova N, Migliorini MM, Loukinov D, Ulery PG, Mikhailenko I, Lawrence DA, Strickland DK. 2002. Platelet-derived growth factor-induced tyrosine phosphorylation of the low density lipoprotein receptor-related protein. Evidence for integrated co-receptor function between LRP and the PDGF. J Biol Chem 277:15499–15506.

Ma Z, Thomas KS, Webb DJ, Moravec R, Salicioni AM, Mars WM, Gonias SL. 2002. Regulation of Rac1 activation by the low density lipoprotein receptor-related protein. J Cell Biol 159:1061–1070.

May P, Reddy YK. Herz J. 2002. Proteolytic processing of low density lipoprotein receptor-related protein mediates regulated release of its intracellular domain. J Biol Chem 277:18736–18743.

Salicioni AM, Gaultier A, Brownlee C, Cheezum MK, Gonias SL. 2004. Low density lipoprotein receptor-related protein-1 promotes beta 1 integrin maturation and transport to the cell surface. J Biol Chem 279:10005–10012.

Sorkin A, Westermark B, Heldin CH, Claesson-Welsh L. 1991. Effect of receptor kinase inactivation on the rate of internalization and degradation of PDGF and the PDGF beta-receptor. J Cell Biol 112:469–478.

Strickland DK, Ashcom JD, Williams S, Burgess WH, Migliorini M, Argraves WS. 1990. Sequence identity between the α_2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that the molecule is a multifunctional receptor. J Biol Chem 265:17401–17404.

Strickland DK, Gonias SL, Argraves WS. 2002. Diverse roles for the LDL receptor family. Trends Endocrinol Metab 13:66–74.

Trommsdorff M, Borg JP, Margolis B, Herz J. 1998. Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein. J Biol Chem 273:33556–33560.

Weaver AM, McCabe M, Kim I, Allietta M, Gonias SL. 1996. Epidermal growth factor and platelet-derived growth factor-BB induce a stable increase in the activity of low density lipoprotein receptor-related protein in vascular smooth muscle cells by altering receptor distribution and recycling. J Biol Chem 271:24894–24900.

Webb DJ, Hussaini IM, Weaver AM, Atkins TL, Chu CT, Pizzo SV, Owens GK, Gonias SL. 1995. Activated α_2-macroglobulin promotes mitogenesis in rat vascular smooth muscle cells by a mechanism that is independent of growth factor-carrier activity. Eur J Biochem 234: 714–722.

Webb DJ, Nguyen DHD, Sankovic M, Gonias SL. 1999. The very low density lipoprotein receptor regulates urokinase receptor catabolism and breast cancer cell motility in vitro. J Biol Chem 274:7412–7420.

Webb DJ, Nguyen DHD, Gonias SL. 2000. Extracellular signal-regulated kinase functions in the urokinase receptor-dependent pathway by which neutralization of low density lipoprotein receptor-related protein promotes fibrosarcoma cell migration and Matrigel invasion. J Cell Science 113:123–134.

Willnow TE, Herz J. 1994. Genetic deficiency in low density lipoprotein receptor-related protein confers cellular resistance to *Pseudomonas* exotoxin A. Evidence that this protein is required for uptake and degradation of multiple ligands. J Cell Sci 107:719–726.