Regulation of the Composition of the Extracellular Matrix by Low Density Lipoprotein-Receptor-related Protein-1

ACTIVITIES BASED ON REGULATION OF mRNA EXPRESSION

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Alban Gaultier‡, Ana Maria Salicioni†, Sanja Aranjelovic§, and Steven L. Gonas†

From the ‡Department of Pathology, University of California San Diego School of Medicine, La Jolla, California 92093 and the §Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, Massachusetts 01003

Low density lipoprotein receptor-related protein-1 (LRP-1) is a catabolic receptor for extracellular matrix (ECM) structural proteins and for proteins that bind to ECM. LRP-1 also is implicated in integrin maturation. In this study, we applied a proteomics strategy to identify novel proteins involved in ECM modeling that are regulated by LRP-1. We show that LRP-1 deficiency in murine embryonic fibroblasts (MEFs) is associated with increased levels of type III collagen and pigment epithelium-derived factor, which accumulate in the substratum surrounding cells. The collagen receptor, uPAR-AP/Endo-180, is also increased in LRP-1-deficient MEFs. Human LRP-1 reversed the changes in protein expression associated with LRP-1 deficiency; however, the endogenic activity of LRP-1 was not involved. Instead, regulation occurred at the mRNA level. Inhibition of c-Jun amino-terminal kinase (JNK) blocked type III collagen accumulation in LRP-1-deficient MEFs, suggesting regulation of JNK activity as a mechanism by which LRP-1 controls mRNA expression. The ability of LRP-1 to regulate expression of the factors identified here suggests a role for LRP-1 in determining blood vessel structure and in angiogenesis.

Low density lipoprotein receptor-related protein-1 (LRP-1)‡ is a single-pass, type I transmembrane receptor composed of two chains, a 515-kDa heavy chain, which is entirely extracellular, and an 85-kDa light chain that includes the transmembrane and cytoplasmic domains. Because LRP-1 binds diverse ligands and undergoes constitutive endocytosis and recycling, one major function of this receptor is to deliver extracellular proteins to lysosomes for degradation (1). Some LRP-1 ligands are multienzyme complexes that include other transmembrane receptors (2, 3). As a result, LRP-1 regulates the protein composition of the plasma membrane. LRP-1 also regulates cell signaling, either by directly binding proteins such as Shc and c-Jun amino-terminal kinase (JNK)-interacting protein (JIP) or by modifying the activity of other signaling receptors such as uPAR (4, 5). It is now clear that LRP-1 is a member of a gene family that includes the low density lipoprotein receptor and other receptors that have partially overlapping or, in some cases, opposing activities (3). In the mouse, LRP-1 gene knock-out is embryonic lethal (6). Conditional inactivation of the LRP-1 gene in vascular smooth muscle cells leads to abnormal proliferation and aneurysm formation (7).

The cytoplasmic tail of LRP-1 includes two NPXY motifs, which may be involved in binding signaling adaptor proteins (4, 8). The second NPXY is also a target for tyrosine phosphorylation by platelet-derived growth factor-BB (9, 10). Phosphorylation of tyrosine, serine, and threonine in the LRP-1 light chain may regulate its function in cell signaling and its effects on various cellular activities, including cell migration and growth (11–13). We have demonstrated that LRP-1 expression is associated with increased platelet-derived growth factor-B receptor mRNA expression in murine embryonic fibroblasts (MEFs) in vitro (14). Thus, LRP-1 may regulate levels of other cellular proteins by diverse mechanisms, in addition to facilitating catabolism.

We are interested in mechanisms by which cells regulate the composition of the extracellular matrix (ECM). This process is important not only in development but also in wound healing, ischemic injury, and cancer (15, 16). Functioning as a catabolic receptor, LRP-1 regulates levels of thrombospondin and fibronectin in pericellular spaces (17, 18). By a yet unclear mechanism, LRP-1 regulates maturation of β1 integrin and thereby alters the amount of integrin available for cell adhesion and ECM modeling (19). Furthermore, by binding and promoting the endocytosis of complexes of proteinases with α2-macroglobulin and members of the Serpin family, LRP-1 cleaves proteinases that otherwise target ECM proteins (1, 3).

To identify novel mechanisms by which LRP-1 may be involved in tissue modeling, our laboratory has undertaken a discovery-based proteomics approach. Our experimental system compares LRP-1-deficient MEF-2 cells with LRP-1-positive PEA-10 cells, which were cloned from the same preparation (6). To confirm that identified differences are due to LRP-1, we also examine B4–41 cells (18), which are MEF-2 cells rescued by expression of full-length human LRP-1. In this paper, we report on three proteins with known activities related to tissue formation and remodeling that are regulated by LRP-1. In each case, regulation does not involve LRP-1 endocytic activity but instead reflects the ability of LRP-1 to regulate mRNA expression/stability, probably downstream of LRP-1-dependent cell-signaling pathways. The newly identified targets include: 1) type III collagen, a fibrillar collagen and the second most abundant collagen in the body, linked intimately to blood vessel structure (20); 2) pigment epithelium-derived factor (PEDF), a collagen-associated member of the Serpin family that lacks proteinase inhibitory activity but functions as a potent anti-angiogenesis factor (21); and 3) uPAR-AP/Endo-180, a member of the macrophage mannose receptor family that serves as a catabolic receptor for multiple collagen (22).
Identification of these novel targets suggests new roles for LRP-1 in embryogenesis and angiogenesis.

**EXPERIMENTAL PROCEDURES**

Reagents—Fibronectin, type I collagen, and type III collagen were purchased from Southern Biotech (Birmingham, AL). Type III collagen-specific antibody was from Southern Biotech. Antibodies specific for PEDF and for extracellular signal-regulated kinase (ERK) were purchased from Upstate Biotechnology (Charlottesville, VA). Phosphorylated JNK-specific antibody was purchased from Cell Signaling Technologies (Danvers, MA). uPAR-AP/Endo-180-specific antibody was a generous gift from Dr. Niels Behrendt (Finsen Laboratory, Copenhagen). Receptor-associated protein was expressed as a glutathione S-transferase fusion protein (GST-RAP) in bacteria, as described previously (23). GST-RAP functions as an antagonist of LRP-1 ligand binding activity (24, 25). As a control, we also expressed GST in bacteria transformed with the empty vector, pGEX-2T. Alexa 598-conjugated antibodies and Alexa 488-conjugated phalloidin were from Molecular Probes (Carlsbad, CA). Sulfo-NHS-LC-biotin was purchased from Pierce. Streptavidin-Sepharose, concanavalin A-Sepharose, and suprabodies and Alexa 488-conjugated phalloidin were from Molecular Probes. Receptor-associated protein was expressed as a glutathione S-transferase fusion protein (GST-RAP) in bacteria, as described previously (23). GST-RAP functions as an antagonist of LRP-1 ligand binding activity (24, 25). As a control, we also expressed GST in bacteria transformed with the empty vector, pGEX-2T. Alexa 598-conjugated antibodies and Alexa 488-conjugated phalloidin were from Molecular Probes (Carlsbad, CA). Sulfo-NHS-LC-biotin was purchased from Pierce. Streptavidin-Sepharose, concanavalin A-Sepharose, and suprabodies and Alexa 488-conjugated phalloidin were from Molecular Probes (Carlsbad, CA). Sulfo-NHS-LC-biotin was purchased from Pierce.

PEGF and for extracellular signal-regulated kinase (ERK) were purchased from Southern Biotech (Birmingham, AL). Type III collagen and PEDF were purchased from EMD Biosciences (Darmstadt, Germany). qPCR reagents, including primers and probes for PEDF, type III collagen, hypoxanthine guanine phosphoribosyltransferase, and uPAR-AP/Endo-180 were from Applied Biosystems (Foster City, CA).

**Cell Culture—**MEFs that are genetically deficient in LRP-1 (MEF-2) and LRP-1+/− MEFs (PEA-10) were obtained from the ATCC. PEA-10 and MEF-2 cells were cloned from the same MEF culture, heterozygous for LRP-1 gene disruption, after selection with Pseudomonas exotoxin A (6). B-41 cells are MEF-2 cells that were transfected for stable expression of full-length human LRP-1 (18). uPAR-AP/− MEF-A1 cells and uPAR−/− MEF-C1 cells have been described previously (26). MEFs were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (HyClone Laboratories, Logan, UT). For some experiments, MEFs were cultured in complete medium supplemented with 250 nM GST-RAP or with an equivalent concentration of GST (negative control) for 3 days. The medium, including GST-RAP or GST, was replaced daily. Chinese hamster ovary (CHO-K1) cells were grown in DMEM-low glucose supplemented with 10% fetal bovine serum and non-essential amino acids. Preparation of Conditioned Medium for Two-dimensional PAGE—PEA-10 and MEF-2 cells were cultured in complete medium for 18 h. The cells were washed three times with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS), and cultured for another 24 h in serum-free DMEM supplemented with 10% Prolifex S6. Conditioned medium was recovered and subjected to centrifugation for 5 min at 200 × g to remove non-adherent cells. The media samples were then supplemented with complete proteinase inhibitor mixture (Roche Applied Science, Mannheim, Germany) and 2.5 mM EDTA and concentrated using Centricrons with 10-kDa molecular mass retention filters (Millipore, Billerica, MA). Final samples were dialyzed for 24 h against 0.25 mM NaPO₄, 7 mM NaCl, 0.15 mM KCl, pH 7.5, at 4 °C. Protein concentrations were determined by bichinchoninic acid assay (Sigma). The amount of sample to be analyzed was standardized based on an equivalent amount of cells, as opposed to an equivalent amount of protein, in recognition of the possibility that released proteins may accumulat at a different rate in conditioned medium from the different cell lines. Protein in media samples was precipitated with 4 volumes of cold acetone. After centrifugation, protein pellets w ere resuspended in two-dimensional PAGE first dimension buffer (4% CHAPS, 2 M thiourea, 7 M urea, and 0.5% immobilized pH gradient buffer, pH 4–7).

**Two-dimensional PAGE—**Samples were loaded on 18-cm immobilized pH gradient strips, pH 4–7, and subjected to isoelectric focusing using an IPGPhor system (Amersham Biosciences) according to the manufacturer’s instructions. In the second dimension, proteins were separated by SDS-PAGE in 10% acrylamide (Protein XL, Bio-Rad). Proteins were visualized by silver staining, as described by Vorum et al. (27). Gels were imaged using a flat-bed scanner (Epson) and analyzed using PDQuest software (Bio-Rad). Spots with at least a three times increase in quantity in LRP-1-deficient cells were targeted for micro-sequencing.

**Mass Spectrometry—**Gel pieces containing spots of interest were transferred to siliconized tubes and washed in 50% methanol overnight. The gel pieces were dehydrated in acetonitrile and then rehydrated for treatment with reductant (10 mM dithiothreitol), followed by 50 mM iodoacetamide, and finally, trypsinization (20 ng/µl) for 12 h at 37 °C. Extracted peptides were evaporated to 25 µl for mass spectrometry analysis.

The liquid chromatography-mass spectrometry system consisted of a Finnigan LCQ ion trap mass spectrometer with a Protein nanospray ion source interfaced to a self-packed 8 cm x 75 µm inner diameter PheromoneX Jupiter 10 µm C18 reversed-phase capillary column. Peptides were eluted from the column in an acetonitrile, 0.1 M acetic acid gradient and injected directly into the mass spectrometry. The nanospray ion source was operated at 2.8 kV. Peptide sequences were analyzed using the Sequest search algorithm. Peptides that were not identified by this algorithm were interpreted manually by searching expressed sequence tag data bases.

**RT-PCR and qPCR—**Total RNA was extracted from confluent MEF cultures that were maintained for 24 h in serum-free medium supplemented with 10% Prolifex S6, using the RNAeasy kit, as directed by the manufacturer (Qiagen). cDNA was synthesized using the Script cDNA synthesis kit (Bio-Rad). Semiquantitative RT-PCR was performed using HotStar Taq polymerase (Qiagen) and specific primers for PEDF, type I collagen, type III collagen, and β-actin. The amplification program consisted of 30 cycles. qPCR was performed using a System 7300 instrument (Applied Biosystems) and a one-step program: 95 °C, 10 min; 95 °C, 30 s, 60 °C, 1 min for 40 cycles. Hypoxanthine guanine phosphoribosyltransferase gene expression was measured as a normalizer for each sample. Results were analyzed by the relative quantity (ΔΔCt) method, as described previously (28). All experiments were performed in triplicate with internal duplicate determinations.

**Surface-Protein Labeling and Affinity Precipitation—**Cell-surface proteins were biotinylated using the membrane-impermeable reagent, sulfo-NHS-LC-biotin (1 mg/ml), as described previously (29). Labeled cells were washed with 100 mM glycine in PBS. Cells extracts were prepared in 1% Triton X-100, 0.125% Tween 20, 0.5% deoxycholate, 50 mM HEPES, pH 7.5, 0.5 M NaCl, and complete proteinase inhibitors (Roche Applied Science). Cellular debris was removed by centrifugation at 13,000 × g at 4 °C for 15 min. Equal amounts of cellular protein were incubated with streptavidin–agarose for 4 h at room temperature, and in some cases, the unbound fraction was then incubated with concanavalin A-Sepharose overnight at 4 °C. Streptavidin beads and concanavalin A beads were washed two times with 20 mM Tris-HCl, 150 mM NaCl, pH 7.4 (TBS), 1% Triton X-100 and again with TBS. Proteins were eluted with SDS sample buffer for analysis.
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SDS-PAGE and Immunoblotting—Unless otherwise specified, cells were extracted in 1.0% Triton X-100, 0.125% Tween 20, 0.5% deoxycholate, 50 mM HEPES, 0.5 mM NaCl, pH 7.5, and complete proteinase inhibitor mixture (Roche Applied Science). Equal amounts of cellular protein were subjected to one- or two-dimensional SDS-PAGE, using the Laemmli buffer system (30), and electrotransferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad). Proteins were visualized using 0.2% Ponceau Red in 3% trichloroacetic acid prior to immunoblotting. Membranes were blocked with 5% nonfat dry milk in TBS, 0.1% Tween 20. Purified primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) were diluted in the same buffer. Detection was performed using Immunostar-horseradish peroxidase chemiluminescence (Bio-Rad) and Kodak Biomax films.

Immunofluorescence—PEA-10 and MEF-2 cells were plated on coverslips coated with poly-d-lysine or type I collagen (100 μg/ml), maintained for 72 h in serum free medium supplemented with 10% FBS, fixed with 4% formaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS, and incubated in PBS containing 1% BSA and primary antibody. The cells were then washed. Secondary anti-rabbit IgG coupled to Alexa 598 (10 μg/ml) and phalloidin-coupled to Alexa 488 (1 unit/ml) were added to the cells after dilution in PBS, 1% BSA. Cells were imaged with a Leica DMRE inverted microscope.

Cloning, Expression, and Purification of PEDF—Specific primers corresponding to the 5’ and 3’ ends of the mouse PEDF open reading frame were designed for cloning PEDF into pCDNA3.1His-myc (Invitrogen). The primers included HindIII and KpnI restriction sites. PCR was performed with total cDNA obtained from MEF-2 cells, and the resulting amplicon was cloned into the vector. The sequence of the construct was verified. CHO-K1 cells were transfected with the expression construct using FuGENE 6 (Roche Applied Science) according to manufacturer’s instructions. After 3 weeks of selection with G418 (0.5 mg/ml), PEDF-expressing cells were single cell-cloned. PEDF protein was purified by affinity chromatography, using the His tag in the construct (29).

PEDF Ligand Blot—Purified PEDF (50 μg) was labeled with Na125I using IODO-BEADS (Pierce), according to manufacturer’s instructions. BSA, fibronectin, type I collagen, and type III collagen (5 μg) were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked for 1 h in PBS, 0.1% Tween 20, 5% dry milk and then incubated with 5 nm 125I-PEDF in the same buffer for 12 h at 4 °C. Membranes were washed extensively with PBS, 0.1% Tween 20 and imaged using a phosphorimager (Bio-Rad).

RAP Activity—To confirm that GST-RAP is active, we studied inhibition of binding of activated a3M to LRP-1, as described previously (31). Briefly, purified activated a3M was labeled with Na125I using IODO-BEADS and incubated with confluent cultures of PEA-10 or MEF-2 cells for 4 h at 4 °C. The concentration of 125I-labeled a3M was 1 nm. Unlabeled a3M (0.2 μM) or 0.25 μM GST-RAP was added to some wells. After washing the cultures, cell-associated radioactivity was recovered and determined in a γ counter. Cellular protein was determined by bicinechonic acid assay. Specific binding was determined as the fraction of total binding inhibited by unlabeled a3M.

Type III Collagen Internalization—Type III collagen was labeled with Na125I using IODO-BEADS. Cells were seeded in 24-well plates and cultured until confluent. To begin an experiment, the cells were washed once and then incubated for 1 h in binding buffer, which consisted of DMEM, 20 mM HEPES, pH 7.4, 15 mg/ml BSA, and insulin-transferrin-selenium complement (Invitrogen). The medium was replaced by fresh binding buffer containing 5 nm 125I-labeled type III collagen. Incubations were allowed to proceed for 3 h at 37 °C. In some wells, GST-RAP (0.5 μM) was added. To determine cellular uptake, the cultures were washed twice with cold binding buffer and then treated for 15 min with 0.25% Pronase in DMEM at 4 °C. The detached cells were pelleted by centrifugation at 3000 × rpm for 3 min. Radioactivity associated with the cell pellet was measured in a γ counter.

Cell-signaling Pathways That Regulate Type III Collagen Expression—Confluent cultures of PEA-10 and MEF-2 cells were treated with JNK inhibitor (10 μM), LY294002 (10 μM), PD98059 (40 μM), DAPT (10 μM), or vehicle (Me2SO) for 18 h. mRNA was recovered and qPCR analyses were performed.

In separate experiments, MEF-2 and PEA-10 cells were cultured for 18 h in JNK inhibitor or Me2SO. Cell extracts were subjected to immunoblot analysis for phosphorylated JNK, type III collagen, and total ERK.

RESULTS

Proteomics Analysis of Conditioned Medium from LRP-1-deficient and LRP-1-expressing MEFs—To identify novel proteins that may accumulate differentially in the cellular microenvironment depending on whether LRP-1 is expressed, we analyzed conditioned medium from PEA-10 and MEF-2 cells by two-dimensional PAGE. Differences in silver staining intensity were assessed by PDQuest software. Representative gels are shown in Fig. 1. The insets show two spots that were consistently (n = 4) and substantially increased in LRP-1-deficient MEF-2 cells. These proteins were subjected to microsequence analysis. For the spot migrating with an apparent mass of 52 kDa, 23 peptides were identified, all corresponding in sequence to PEDF. The identified peptides covered 31% of the sequence of PEDF. The apparent mass of PEDF in our two-dimensional PAGE system was consistent with the reported mass of PEDF monomer (32).

The second spot migrated with an apparent mass of 60 kDa. Fifteen peptides isolated by proteolysis of the spot corresponded exactly in sequence to type III collagen. The 15 peptides included 11% of the protein length and were all restricted to the C-terminal portion of the type III collagen subunit. The absolute C terminus of type III collagen includes the 25-kDa propeptide, which is released by proteolysis after formation of the triple helix (33). A 60-kDa fragment has been reported previously to be released from the C terminus by mammalian collagenases (33).
As a first step to validate our results, the conditioned medium samples shown in Fig. 1 were subjected to two-dimensional PAGE and immunoblot analysis. The immunoblot for type III collagen is shown in Fig. 2A. Only the section of the blot with immunoreactivity is shown. In MEF-2 cell conditioned medium, a broad band of immunoreactivity is observed, probably reflecting heterogeneity in glycosylation and thus differential migration in the isoelectric focusing first dimension. By contrast, almost no immunoreactivity was detected in PEA-10 cell-conditioned medium. The apparent mass of the immunoreactive region in the MEF-2 cell blot is most consistent with the known mass of the N-terminal region of type III collagen monomer after collagenase processing (33). The absence of larger monomer fragments in the two-dimensional PAGE system probably reflects the specimen processing procedure (see Fig. 4). As shown in Fig. 2B, the immunoblot for PEDF showed a pattern that was very similar to that observed by silver staining.

**PEDF Is Increased at the Protein and mRNA Levels in LRP-1-deficient MEFs**—To further explore the relationship between LRP-1 and PEDF, we compared cell extracts of PEA-10 and MEF-2 cells with extracts of B-41 cells, which are MEF-2 cells that express full-length human LRP-1. The level of human LRP-1 in B-41 cells is similar to the level of murine LRP-1 in PEA-10 cells (18). Immunoblot analysis, performed this time using 1D-PAGE, showed increased PEDF in MEF-2 cell extracts, compared with extracts of PEA-10 cells (Fig. 3A). In B-41 cell extracts, the level of PEDF reverted to that observed in PEA-10 cells.

PEDF is known to associate with type I collagen (34). Thus, immunofluorescence microscopy experiments were performed to compare PEDF in the immobilized phase of cultures of PEA-10 and MEF-2 cells plated on type I collagen. Immunoreactivity was observed in a fibrillar pattern surrounding MEF-2 cells (Fig. 3B). Almost no extracellular PEDF was observed in PEA-10 cell cultures. Ligand blot analysis with purified [125]I-PEDF confirmed binding to type I collagen (Fig. 3C). By contrast, no [125]I-PEDF-binding was detected with an equivalent load (5 μg) of type III collagen or fibronectin. Thus, the increase in PEDF association with the substratum is not related to type III collagen deposition, which may be increased in MEF-2 cell cultures.

RAP binds directly to LRP-1 and blocks the binding of other known ligands (24, 25). To test whether LRP-1 acts as a catabolic receptor for PEDF, we cultured PEA-10 cells in the presence of 250 nM GST-RAP for...
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24 h. Control cells were treated with 250 nM GST. PEDF accumulation in conditioned medium was unchanged by RAP (data not shown), indicating that the endocytic function of LRP-1 is not responsible for regulating PEDF.

Next, we performed studies to determine whether LRP-1 regulates PEDF mRNA expression. Transcriptional regulation may occur downstream of the various signaling pathways that are controlled by LRP-1. Conventional RT-PCR and qPCR were performed, comparing PEDF mRNA levels in MEF-2 cells, compared with PEA-10 cells, providing an explanation for the increase in PEDF protein. In B-41 cells, PEDF mRNA was decreased to below the level observed in PEA-10 cells. Thus, LRP-1 regulates PEDF expression at the mRNA level.

Type III Collagen Is Increased in LRP-1-deficient MEFs—In MEF-2 cell extracts, type III collagen monomer (150-kDa) was abundantly present (Fig. 4A). No evidence of proteolytic processing was detected, as anticipated. By contrast, type III collagen was almost undetectable in extracts of LRP-1-expressing PEA-10 cells and B-41 cells. Equivalent results were obtained when conditioned medium was collected from MEF-2, PEA-10, and B-41 cells and subjected to immunoblot analysis. Type III collagen expression was detected only in the medium of MEF-2 cells and not in PEA-10 or B-41 cell medium. Evidence of incomplete type III collagen processing was observed in the one-dimensional gel system. Immunofluorescence microscopy showed type III collagen fibrils accumulating in the extracellular immobilized phase in association with MEF-2 cells but not PEA-10 cells (Fig. 4B). Thus, type III collagen that is expressed by LRP-1-deficient MEFs accumulates both in conditioned medium and in the substratum.

As was the case with PEDF, RAP (250 nM) failed to increase accumulation of type III collagen in PEA-10 cell conditioned medium (results not shown). Thus we examined type III collagen expression, at the mRNA level, by RT-PCR and by qPCR. Fig. 4C shows that there was no detectable difference in type I collagen mRNA expression in PEA-10, MEF-2, and B-41 cells. By contrast, type III collagen mRNA was detected almost exclusively in MEF-2 cells. Thus, human and murine LRP-1 shut down type III collagen expression at the mRNA level.

uPAR-AP/Endo-180 Is a Type III Collagen Receptor Regulated by LRP-1—The inability of RAP to promote type III collagen accumulation in PEA-10 cell conditioned medium suggested that LRP-1 is not an endocytic receptor for type III collagen. To confirm this finding, we incubated radioliodinated type III collagen with cultures of PEA-10 and MEF-2 cells at 37 °C (Fig. 5A). Radioligand internalization was measured. Unexpectedly, LRP-1-deficient MEF-2 cells internalized greatly increased amounts of type III collagen, compared with PEA-10 cells. In PEA-10 cells, type III collagen internalization was not inhibited by RAP. RAP also was ineffective with MEF-2 cells, suggesting that another RAP-sensitive low density lipoprotein receptor homologue is not responsible for type III collagen endocytosis in this cell type.

In control experiments, GST-RAP completely inhibited specific binding of activated α₂M to PEA-10 cells (Fig. 5B), indicating that our GST-RAP preparation is active. Specific binding of α₂M was not observed in MEF-2 cells, as anticipated, since LRP-1 is the major receptor for α₂M (35).

Further studies were conducted to determine why type III collagen endocytosis is increased in MEF-2 cells. To compare cell-surface pro-
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To confirm that uPAR-AP/Endo-180 is differentially expressed in LRP-1-negative and -positive cells, PEA-10, MEF-2, and B-41 cells were labeled with biotin. Streptavidin-affinity precipitates were prepared and subjected to SDS-PAGE, electrotransferred to PVDF membranes, and probed with peroxidase-conjugated streptavidin. Eight equal amounts of cellular protein from detergent-soluble cell extracts of PEA-10, MEF-2, and B-41 were subjected to immunoblot analysis for uPAR-AP/Endo-180. C, cell-surface proteins in PEA-10, MEF-2, and B-41 cells were labeled with biotin and isolated by affinity precipitation. Remaining glycoproteins, presumed to be primarily intracellular in origin, were purified by affinity for concanavalin A-Sepharose. These samples were subjected to immunoblot analysis for uPAR-AP/Endo-180.

MEF-2 cells, compared with PEA-10 cells. In B-41 cells, uPAR-AP/Endo-180 mRNA was decreased to undetectable levels. Thus, uPAR-AP/Endo-180 is regulated by LRP-1 at the mRNA level.

LRP-1 deficiency in MEFs is associated with increased levels of cell-surface uPAR and activation of uPAR-dependent cell-signaling pathways (26, 37). To test whether uPAR-AP/Endo-180 expression may be regulated indirectly by LRP-1, due to its control of uPAR-dependent signaling, we examined uPAR-AP/Endo-180 expression in uPAR+/+ and uPAR−/− MEFs, which are described previously (26). Immunoblot analysis of whole cell extracts showed equivalent level of uPAR-AP/Endo-180 in both cell lines (Fig. 7B). These results are consistent with a model in which regulation of uPAR-AP/Endo-180 mRNA by LRP-1 occurs independently of uPAR signaling.

LRP-1-dependent Cell-signaling Pathways Regulate Type III Collagen Expression—To identify signaling pathways that may control type III collagen mRNA expression in MEF-2 and PEA-10 cells, we cultured these cells in the presence of inhibitors of signaling proteins previously shown to be regulated by LRP-1. These included: JNK inhibitor, selected because JIP binds to LRP-1 and this interaction regulates JNK activity (4, 38); the Class I phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, selected because PI3K activation is regulated by Shc and Dab1, which bind to LRP-1 (8, 39); and the γ-secretase inhibitor DAPT, selected because the LRP-1 cytoplasmic tail may be cleaved by a γ-secretase-like activity and translocated into the nucleus for gene regulation (40). We also assessed the activity of the MEK-antagonist, PD98059.

As shown in Fig. 8A, PD098059 and DAPT did not significantly affect type III collagen mRNA expression in MEF-2 cells. LY294004 decreased type III collagen mRNA by 60% in both cell types. JNK inhibitor decreased type III collagen mRNA by 60% in both cell types. JNK inhibitor decreased type III collagen mRNA by 65% in MEF-2 cells and to a lesser extent (~20%) in PEA-10 cells.

The mechanisms by which LRP-1 may regulate JNK activity are complex. Binding of JIP to LRP-1 may promote JNK activation; however, JNK sequestration by the LRP-1–JIP complex may prevent JNK translocation to the nucleus, which is necessary for regulation of transcription (38). Fig. 8B shows that the basal level of phosphorylated JNK was slightly increased in MEF-2 cells, compared with PEA-10 cells, despite

FIGURE 6. LRP-1 regulates expression of uPAR-AP/Endo-180. A, cell-surface proteins in PEA-10 and MEF-2 cells were labeled with biotin. Streptavidin-affinity precipitates were prepared and subjected to SDS-PAGE, electrotransferred to PVDF membranes, and probed with peroxidase-conjugated streptavidin. B, equal amounts of cellular protein from detergent-soluble cell extracts of PEA-10, MEF-2, and B-41 were subjected to immunoblot analysis for uPAR-AP/Endo-180. C, cell-surface proteins in PEA-10, MEF-2, and B-41 cells were labeled with biotin and isolated by affinity precipitation. Remaining glycoproteins, presumed to be primarily intracellular in origin, were purified by affinity for concanavalin A-Sepharose. These samples were subjected to immunoblot analysis for uPAR-AP/Endo-180.

FIGURE 7. Mechanistic studies regarding uPAR-AP/Endo-180 expression by LRP-1. A, total RNA was isolated from PEA-10, MEF-2, and B-41 cells. uPAR-AP/Endo-180 mRNA was quantitated by qPCR. B, equal amounts of cellular protein from detergent-soluble extracts of uPAR+/+ and uPAR−/− MEFs were subjected to immunoblot analysis for uPAR-AP/Endo-180.
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In this study, our goal was to identify novel LRP-1-regulated proteins that may be involved in ECM modeling. We anticipated that regulation may result from the endocytic activity of LRP-1; however, our results demonstrate that LRP-1 also regulates ECM modeling based on its ability to control mRNA expression/stability. Using a proteomics-based strategy, we determined that PEDF, type III collagen, and uPAR-AP/Endo-180 are present at increased levels in LRP-1-deficient MEFs. In each case, human LRP-1 reversed the change associated with murine LRP-1 deficiency. To test whether LRP-1 endocytic activity is involved in regulation of the identified targets, PEA-10 cells were cultured in the presence of RAP. We used protocols that were equivalent to those used previously by us and others to demonstrate the importance of LRP-1 endocytic activity in regulating extracellular proteins such as fibronectin (18) and MMP-9 (42) and plasma membrane proteins such as uPAR (43) and amyloid precursor protein (44); however, RAP was ineffective in promoting accumulation of the LRP-1 target proteins identified here. We then demonstrated that regulation of PEDF, type III collagen, and uPAR-AP/Endo-180 occurs at the level of mRNA. Thus, these proteins do not appear to be classic LRP-1 ligands but, instead, are regulated downstream of LRP-1. The lack of LRP-1 activity as an endocytic receptor for uPAR-AP/Endo-180 is consistent with the work of Engelholm et al. (22), who demonstrated that uPAR-AP/Endo-180 mediates collagen endocytosis in an LRP-1-independent manner. Our results suggest that transcriptional regulation may be a major activity of LRP-1 related to its overall function.

Collagens are the most abundant proteins in the ECM. The 19 members of this gene family demonstrate specific tissue distributions and differences in function (45). Type III collagen is the second most abundant collagen, after type I collagen, and is present in the skin, blood vessels, and numerous internal organs (46). Like type I, II, V, and XI collagen, type III collagen is fibrillar and forms homotrimeric composed of three α chains, each containing about 1000 amino acids (47). Inactivation of the Col3a1 gene in mice generates animals with a reduced lifespan. The cause of death is related to instability of the major blood vessels (20). This phenotype is in good agreement with pathology observed in humans because mutations in the Col3a1 gene have been implicated in type IV Ehlers-Danlos syndrome. This disease can lead to sudden death from rupture of large arteries (48, 49).

Type III collagen co-localizes with type I collagen in the same fibrils and may control the diameter of the structure (50). Importantly, type III collagen expression is regulated during development. The proportion of type III collagen, relative to type I collagen, decreases with age, suggesting important changes in fibril ultrastructure (51). Processes that may regulate the switch from type III collagen to type I collagen are not understood. Our studies suggest a possible role for LRP-1 in the regulation of type III collagen expression and, thus, in determining the ultrastructure of collagen fibrils in the ECM.

The second identified LRP-1 target, PEDF, is a member of the Serpin superfamily, but unlike many other Serpins, PEDF does not express proteinase inhibitory activity (32). PEDF is a multifunctional protein. In cell culture, PEDF induces neuronal differentiation of Y79 retinoblastoma cells (33). PEDF also expresses anti-angiogenic activity, inhibiting capillary morphogenesis by endothelial cell (53). In vivo, PEDF inhibits neovascularization in the eye and tumor angiogenesis (21). Thus, PEDF and collagen III are both LRP-1-regulated gene products involved in blood vessel formation and stabilization.

The third LRP-1-regulated protein described here is the plasma membrane protein, uPAR-AP/Endo-180, an endocytic receptor and member of the macrophage mannose receptor family. uPAR-AP/Endo-180 associates with uPAR in the plasma membrane (54) and functions as a catabolic receptor for MMP-13 and for multiple collagens (22, 36). uPAR-AP/Endo-180 is expressed by fibroblasts, macrophages, and endothelial cells (55). In PEDF-deficient mice, breast car-
cinoma growth is retarded due to increased collagen accumulation in the spaces surrounding the cells (56). Because our results suggest that uPAR-AP/Endo-180 expression may be substantially increased when LRP-1 is absent, LRP-1 may indirectly control collagen catabolism. In this regard, it is interesting to mention that degradation of collagenase-3 by uPAR-AP/Endo-180 has been reported to be a two-step process that also requires LRP-1 (33). This may represent another mechanism by which LRP-1 regulates collagen metabolism.

The relationship of the LRP-1 targets identified in this study to collagen structure and function is observed at multiple levels. The antiangiogenic activity of PEDF depends on its interaction with type I collagen (57). Our results demonstrate that the PEDF-collagen interaction is specific for type I collagen; type III collagen does not substitute. LRP-1 expression has not been formally linked to angiogenesis; however, the ability of LRP-1 to regulate type III collagen and PEDF may implicate LRP-1 in blood vessel formation and stability. During angiogenesis, endothelial cells come into direct contact with fibrillar collagens. It has been shown that bovine aortic endothelial cells express type I collagen just before angiogenesis and that expression is limited to the vicinity of newly forming blood vessels (58, 59). By inhibiting expression of type III collagen and uPAR-AP/Endo-180, LRP-1 may increase the proportion of type I collagen within fibrils to which angiogenic endothelial cells are exposed and stabilize ECM boundaries that eventually limit further endothelial cell tissue invasion. At the same time, by suppressing PEDF expression has not been formally linked to angiogenesis; however, the antiangiogenic activity of PEDF depends on its interaction with type I collagen within fibrils to which angiogenic endothelial cells are exposed.

The relationship of the LRP-1 targets identified in this study to collagen metabolism is not always based on endocytic activity. Instead, LRP-1 may also require LRP-1 (33). This may represent another mechanism by which LRP-1 regulates collagen metabolism.

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