LRP: a new adhesion molecule for endothelial and smooth muscle cells

CHUAN HU,1 JUAN A. OLIVER,2 MICHAEL R. GOLDBERG,3 AND QAIS AL-AWQATI1,2

Departments of 2Medicine, 1Physiology and Cellular Biophysics, and 3Pediatrics, College of Physicians and Surgeons of Columbia University, New York, New York 10032

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Hu, Chuan A. Oliver, Michael R. Goldberg, and Qais Al-Awqati. LRP: a new adhesion molecule for endothelial and smooth muscle cells. Am J Physiol Renal Physiol 281: F739–F750, 2001.—We recently generated a monoclonal antibody that disrupted the association of endothelial cells with their target location during kidney development. Here, we purified the antigen of this monoclonal antibody to homogeneity using rat mesangial cell cytosol. Sequence revealed that it is a previously identified protein, termed the “laminin receptor precursor” (LRP). We found that this protein is expressed in most tissues, but immunocytochemistry revealed that it is present largely or entirely in blood vessels where it is located underneath endothelial cells and in between smooth muscle cells of the vascular wall. Vascular smooth muscle cells such as mesangial cells produce and secrete LRP into their extracellular matrix where it is present in several molecular weight forms. Endothelial cells produce very little if any of the protein, but they bind avidly to LRP-coated dishes. Anti-LRP antibodies prevent the binding of smooth muscle cells to uncoated plates, implying that cells that secrete it use it for attachment. In an assay for heterologous cell-to-cell interaction, antibodies to LRP inhibited the binding of smooth muscle cells to endothelial cells. Maturation and differentiation of blood vessels require interaction between endothelial and smooth muscle cells. LRP is a new component of the mesangial matrix, and we propose that it is an adhesion molecule that mediates an interaction between smooth muscle cells and endothelia.

“laminin receptor precursor”; angiogenesis; vascular smooth muscle cells; endothelial smooth muscle adhesion

ANGIOGENESIS, THE SPROUTING of capillaries from preexisting blood vessels, occurs during embryonic development and in brief and regulated periods in the adult, such as during the reproductive cycle in the female genital tract and during wound healing. It also plays a significant role in certain pathological conditions, such as solid-tumor growth, rheumatoid arthritis, and other chronic inflammatory processes. Angiogenesis occurs in multiple steps: the basement membrane must be locally degraded to allow migration of endothelial cells, which need to proliferate and to adhere to each other to form tubes that finally coalesce. Whereas most studies of angiogenesis have focused on endothelial cells, smooth muscle support cells are also necessary to maintain and regulate the blood vessels (6). Little is known about the factors that regulate association between endothelial and smooth muscle cells. During studies of kidney development we identified an antigen that was involved in the targeting of endothelial cells (15, 25). In this study, we demonstrate that this protein is produced by smooth muscle cells and serves as an adhesion molecule for both endothelial and smooth muscle cells.

Kidney development begins when the ureteric bud, an outgrowth of the Wolffian duct, invades the metanephric mesenchyme and induces it to convert to epithelial cells. The ureteric bud is reciprocally induced to grow and branch, and these branches finally form the collecting duct. The converted mesenchyme condenses around the growing tip of the ureteric bud to form a vesicle, which after adhering to the tip of the ureteric bud, undergoes morphogenesis to form a comma-shaped and then an S-shaped body. Endothelial cells migrate from outside the kidney mesenchyme towards the branching ureteric bud, proliferate, and invade the lower slit of the S-shaped body to form the glomerular capillary network (25). It is likely that the vascular structures of the kidney develop by de novo conversion of mesenchyme to endothelial cells, i.e., by vasculogenesis as well as by invasion from the outside (1, 27). The kidney is formed of units, the nephrons, each of which has its own vasculature. Hence, mesenchymal induction and ureteric bud branching must be exquisitely coordinated with the development of the vasculature, in both space and time. It is thus likely that the ureteric bud and/or mesenchymal cells must provide factors that guide endothelial cells to their appropriate locations. The kidney mesenchyme is known to express vascular endothelial growth factor (VEGF), an inducer of angiogenesis, and its receptors, Tie1 (1). The ureteric bud also secretes fibroblast growth factor 2, an inducer of angiogenesis (4). Thrombospondin, an inhibitor of angiogenesis, is also expressed in the developing kidney and may participate in guiding endothelial cells. However, the factors that target endothelial cells to their specific locations are largely unknown.
Given that many of the events in kidney development are mediated by cell-to-cell communication, we generated a library of monoclonal antibodies (MAbs) against extracellular molecules of embryonic day 15 (E15) rat kidney cells (15). The antigen of the MAb 5B6-E4 was distributed in a pattern that was temporally and spatially associated with endothelial cell location and migration during kidney development (25). When E14 kidneys were cultured in vitro, endothelial cells were closely associated with ureteric bud ampullae as in vivo. MAb 5B6-E4 disrupted this association (25). These results suggested that the antigen recognized by 5B6-E4 is involved in endothelial cell targeting during kidney development. In the present study, we purified the antigen to homogeneity, and peptide sequences showed that it was a previously identified protein called the lamin receptor protein or (LRP)/P40 protein (31). This protein was originally identified as a protein that binds to laminin that is expressed in a colon cancer cell line. LRP is a secreted protein and hence, it is not likely to be the receptor for laminin, at least in the traditional use of the term receptor. We show here that in multiple tissues LRP is only present in vascular structures where it is located under endothelial cells and in the extracellular matrix of vascular smooth muscle cells. Vascular smooth muscle cells produce and secrete it, but endothelial cells do not express large quantities of it. Both endothelial and smooth muscle cells bind to dishes coated with purified LRP. Furthermore, the binding of smooth muscle cells to endothelia is blocked by antibodies to LRP. We suggest that LRP may mediate the association between endothelial cells and smooth muscle cells.

**Experimental Procedures**

**Cells and cell culture.** Rat mesangial cells were cultured in RPMI 1640 media containing 20% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). EJG bovine adrenal medulla endothelial cells, HCT-116 human colon carcinoma cell line, and A7r5 rat vascular smooth muscle cell lines were obtained from the American Type Culture Collection (Manassas, VA). EJG cells were cultured in minimum essential medium supplemented with 10% FBS. HCT-116 cells were cultured in McCoy's 5A medium supplemented with 10% FBS. A7r5 cells were cultured in high-glucose DMEM supplemented with 10% FBS. Human umbilical vein endothelial cells (HUVEC), a gift from Dr. Nancy J. Emnaker and Dr. Samuel C. Silverstein (Columbia University), were cultured in M199 medium supplemented with 20% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mg/ml amphotericin B. All cell media culture were purchased from Gibco BRL (Grand Island, NY).

**Purification of the antigen of monoclonal antibody 5B6E4 and cloning of rat LRP cDNA.** Two hundred 150-cm² tissue culture flasks of mesangial cells were homogenized in lysis buffer containing 250 mM sucrose, 5 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μg/ml pepstatin A by passage through a 22-gauge needle a few times. Western blot with MAb 5B6-E4 was used to assay the 45-kDa antigen during the purification process. The 100,000-g supernatant (800 mg) was concentrated with Centriprep-10 (Amicon, Beverly, MA) and exchanged into 20 mM bis-Tris (pH 7.0; buffer A) with PD-10 columns. The cell extract was loaded onto a 6-ml Resource-Q column (Pharmacia Biotech, Uppsala, Sweden), and the bound material was eluted using a 100-ml linear gradient of 0–0.5 M NaCl in buffer A. The fractions that contained the antigen were pooled, and their media were exchanged to 10 mM sodium phosphate (pH 7.0; buffer B) and loaded on a 5-ml Hitrap heparin column. The bound proteins were eluted with a 36-ml linear gradient of 0–0.6 M NaCl in buffer B. The fraction containing the antigen was then exchanged into 20 mM bis-Tris (pH 7.0), 1.5 M NaCl (buffer C) and loaded on a 1-ml resource phenyl column. The bound proteins were eluted with a 10-ml linear gradient of 1.5–0 M NaCl, followed by a 5 ml buffer C without NaCl. The final two positive fractions containing the antigen (50 mg) were combined, precipitated, and electrophoresed by SDS-PAGE. The 45-kDa protein was isolated from SDS-PAGE and sequenced. The amino acid sequences obtained were compared with protein databases using the Genetics Computer Group software package (Madison, WI). Silver staining was done with Silver Stain Plus (Bio-Rad, Hercules, CA). The protein concentrations were measured with Bio-Rad protein assay kit using BSA as a standard.

To purify LRP from basement membrane matrix (phenol- red-free Matrigel, Becton Dickinson labware, Bedford, MA), Matrigel was extracted twice with 2 M urea, 20 mM bis-Tris (pH 7.0). After centrifugation at 20,000 g for 20 min at 4°C, the supernatant was collected and concentrated with Centriprep-10. The 67-kDa LRP was purified from the supernatant with the same chromatography scheme described above with minor modifications. The 67-kDa LRP was assayed by Western blot using anti-LRP antibodies.

**In-gel digestion of proteins and sequence analysis.** The SDS-PAGE gel was first stained in 0.05% Coomassie brilliant blue G dissolved in 0.5% acetic acid and 20% methanol for 1 h. After destaining in 30% methanol, the appropriate band was excised in a displaced volume of less than 100 μl. The protein was reduced by submerging the gel in 100 μl of 0.01 M dithiothreitol, 0.1 M Tris, pH 8.5, and by being shaken gently for 1 h at 55°C. The liquid was then removed from the tube and replaced with 100 μl of 0.15 M N-isopropylidioacetamide, 0.1 M Tris, pH 8.5. After reacting for 30 min in the dark, the supernatant containing the alkylating reagent was removed and discarded, and the gel was washed with 500 μl of 0.05 M Tris, pH 8.5, and 50% acetonitrile for 20 min while being shaken. The supernatant was discarded, and the wash was repeated two times. The washed gel pieces were dried completely in a Speed-Vac concentrator. Forty-one microliters of digestion buffer (0.025 M Tris, pH 8.5) containing 0.05 μg trypsin were added to the tube containing the dried gel pieces and incubated for 20 h at 32°C. When the digestion was complete, the gel pieces were extracted three times with 100 μl of 50% acetonitrile, 0.1% trifluoroacetic acid, and shaken for 30 min each time, and the supernatants were transferred to a Hewlett-Packard HPLC injection vial. The combined washes in the injection vial were dried in a Speed-Vac concentrator and redissolved in 200 μl of 0.1% trifluoroacetic acid for injection onto an HPLC column. Of the total digest, 7.5% was reserved for mass spectroscopy analysis. Matrix-assisted laser desorption ionization mass spectroscopic analysis was performed on the digest and on 10% of selected fractions using a PerSeptive Voyager PE- RP mass spectrometer. Sequence analysis was performed on a Perkin-Elmer model 494 sequencer. Proteolysis of LRP and subsequent partial sequence analysis of the isolated peptides yielded two sequences, which are underlined in Fig. 4.

To clone rat LRP cDNA, total RNA was isolated from cultured mesangial cells with RNAazol B (TelTest, Friendswood, TX). RT-PCR was performed using the RNA PCR kit.
(Perkin Elmer, Forster City, CA) with the following primers: 5'-CCGTTTGCTCGGGCCTCGTTTAA3' ; 5'-TCAGGAC-CACCTCGTGTTGGCT-3'.

Northern blot. A mouse multiple tissue blot containing 2
µg of poly (A)' RNA (Clontech, Palo Alto, CA) was hybridized with a 32P-labeled 905-bp fragment of mouse LRP cDNA under high-stringency conditions, and the X-ray film was exposed for 12 h.

Antibodies. Anti-rat CD 31 antibody was purchased from Serotec (Kidlington, UK), anti-von Willebrand factor was from DAKO, monoclonal anti-α-smooth muscle actin was obtained from Sigma, and the p-18 anti-laminin monoclonal antibody was from the Developmental Studies hybridoma bank maintained by the University of Iowa, Iowa City, IA. To generate polyclonal antibodies against rat LRP, the whole open reading frame of rat LRP cDNA was cloned into the pGEX-2TK vector (Pharmacia Biotech, Uppsala, Sweden). Glutathione-S-transferase (GST)-LRP fusion protein was expressed in Escherichia coli BL21 and purified using glutathione-Sepharose 4B. The fusion protein was injected into rabbits to generate two polyclonal antibodies: 13085 and 13068. To remove the portion of the antibodies against the GST part of the fusion protein, the antisera were purified with an immobilized GST column (Pierce, Rockford, IL).

Immunohistochemistry. For immunohistochemistry, 5-µm cryostat sections were made from frozen tissues embedded in Tissue-Tek optimal cutting temperature (OCT) compound (VWR Scientific, Bridgeport, NJ). The sections were fixed with 2% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100, and blocked with 10% fetal bovine serum (FBS) in PBS. For double staining, primary antibodies were mixed and incubated with the sections in 10% FBS for 2 h at room temperature. Purified antisera against LRP were diluted 1:4–1:10, anti-CD 31 antibody was diluted 1:50, and anti-von Willebrand factor was diluted 1:200–1:400. Fluorophore-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) were mixed and used at dilutions of 1:500–1:1,000. Confocal images were collected on a laser scanning confocal microscope (model LSM 410; Carl Zeiss, Jena, Germany). Tissue sections were viewed at ×40, and red and green images were collected as dual-scanned images. The images were analyzed by Zeiss LSM-PC software and processed with Adobe Photoshop software (San Jose, CA).

For immunoelectron microscopy, rat kidneys were isolated and stained with the anti-LRP polyclonal antibody and a 10-nm-size gold-conjugated goat anti-rabbit secondary at 4°C after embedding. They were then fixed with 4.25% glutaraldehyde in 0.1 M sodium phosphate buffer. Tissues were embedded in Epon, and sections were examined under the electron microscope.

Nude mice study. Female 6- to 8-wk-old athymic nude mice (Harlan Sprague Dawley, Indianapolis, IN) were housed in a ventilated facility. HCT-116 colon cancer cells were trypsinized, washed with serum-free McCoy’s 5A medium, and injected subcutaneously into the anterior flank of the nude mice at 5 × 10⁶ cells/mice. After 10 days, the tumors were excised, embedded in OCT compound, and frozen immediately.

Tissue homogenization and Western blot. Fresh rat kidney cortex, liver, placenta, and HCT-116 tumor were homogenized in lysis buffer using a glass Wheaton homogenizer followed by a steel tissue homogenizer. Rat glomeruli were isolated by passing rat kidney cortex through a 150-µm USA standard testing sieve (Fisher Scientific, Springfield, NJ). The glomeruli were lysed in SDS-PAGE sample buffer. The protein samples were separated on 4–15% Tris-HCl Ready gels (Bio-Rad), and Western blot was performed as described (15).

Biotinylation of extracellular LRP. Subconfluent mesangial cells were washed three times with ice-cold PBS and incubated with 0.5 mg/ml of sulfo-NHS-LC-biotin (Pierce) for 1 h at 4°C. The cells were then washed three times with ice-cold PBS and lysed with a lysis buffer containing 50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 1 mM EDTA, and 150 mM NaCl. The lysate was centrifuged at 12,000 g for 10 min, and the supernatant was precleared with preimmune serum and protein A beads. The lysate was then incubated with anti-LRP serum for 1 h at 4°C and precipitated with protein A beads. The beads were then washed sequentially with lysis buffer twice, and once each with lysis buffer containing 0.1% or no Triton X-100. The beads were then boiled in SDS-PAGE sample buffer, and the biotinylated proteins were identified by blotting with horseradish peroxidase-conjugated avidin (Pierce).

Cell adhesion assay. Various amounts of purified LRP or laminin were coated on polystyrene 48-well cell culture cluster plates (Costar, Corning, NY) and air-dried overnight. Immediately before use, the plates were washed once with PBS and blocked with 2% BSA in PBS, pH 7.4. Cells were harvested with trypsin/EDTA, washed, and suspended in MEM (GIBCO BRL) supplemented with 0.5% BSA (MEM-BSA). Cells were added to appropriate wells at a concentration of 100,000 cells/0.2 ml MEM-BSA and allowed to adhere for various times at 37°C in 5% CO₂. The wells were then gently washed three times with MEM-BSA, and cell attachment was determined with CellTiter 96 AQueous One solution cell proliferation assay (Promega, Madison, WI) or by a hemocytometer. For antibody-blocking experiments, total IgG was purified from anti-LRP sera or control sera with Hitrap protein A column (Pharmacia Biotech, Uppsala, Sweden). The cells were incubated with antibodies for 30 min at room temperature before being added to the appropriate wells. Each data point was calculated from triplicate wells.

Cell-cell adhesion assay. Confluent EJG or HUVEC endothelial cells were cultured in 48-well cell culture cluster plates. Immediately before the experiment, the endothelial cells were washed and incubated with MEMaa-BSA. Mesangial cells and A7r5 smooth muscle cells were labeled overnight with [35S]methionine/cysteine (Express label; NEN Life Science Products, Boston, MA) at 0.4 mCi/ml in methionine/cysteine-free medium with dialyzed FBS. The cells were harvested with trypsin/EDTA and washed and suspended in MEMaa-BSA. The cells were incubated with total IgG purified from the sera for 30 min before being added to the appropriate wells at a concentration of 100,000 cells/0.2ml. The cells were allowed to adhere to the endothelial cell layer for 15 min at 37°C in 5% CO₂. The wells were then gently washed three times with MEMaa-BSA, and 0.3 ml 1% SDS in PBS, pH 7.4, was added to lysis the cells. The radioactivity was counted by liquid scintillation.

RESULTS

LRP/P40 is the antigen of monoclonal antibody 5B6-E4. In adult kidney, the monoclonal antibody 5B6-E4 stained glomeruli and blood vessels, (Fig. 1). In the glomeruli, its staining pattern suggested that the protein was located in the glomerular basement membrane and the mesangium with the staining lying beneath endothelial cells. In renal blood vessels, the antibody stained the vascular smooth muscle layer (Fig. 1, top left corner). Using immunoblot analysis and cell fractionation of cultured mesangial cells, we found that the antibody recognized a 45-kDa protein most abundant in the cytosol (15). To purify the antigen of
5B6-E4, a chromatographic scheme was designed, using immunoblots as the detection assay. Soluble proteins from cultured mesangial cells were separated sequentially with Q anion exchange, heparin, and phenyl Sepharose columns (Fig. 2). The 45-kDa protein was purified to homogeneity; Fig. 3A shows silver-stained SDS-PAGE. Microsequencing of two peptides showed that they matched amino acids 18–37 and 53–57 of the rat LRP/P40 protein (Fig. 4, underlined peptides) (30).

To confirm that LRP is the antigen of MAb 5B6-E4, the cDNA of rat LRP was first cloned by RT-PCR using mRNA isolated from cultured mesangial cells. The sequence of the cloned cDNA was identical to the published rat LRP cDNA (30). A full-length fusion protein was expressed in E. coli, and the MAb 5B6-E4 specifically recognized the GST-LRP fusion protein (Fig. 3B), confirming that LRP/P40 is the antigen of MAb 5B6-E4. Polyclonal antibodies were generated in rabbits against the fusion protein, and they recognized mesangial cell proteins that were identical in molecular weight to those recognized by 5B6-E4 (Fig. 3C). The polyclonal antibodies stained rat glomeruli in a pattern identical to that of the 5B6-E4, Fig. 1, right panel. These studies in the aggregate demonstrate that the antigen to the MAb 5B6-E4 is LRP.

LRP is present in vascular structures. To further study the function of LRP, we first investigated the expression of LRP in other tissues. Using a multiple-tissue Northern blot (Fig. 3D), we found that all tissues expressed a 1.2-kb LRP mRNA. LRP expression was highest in skeletal muscle, liver, kidney, and heart, whereas brain and testis expressed it at a lower level.

Immunohistochemical studies showed that LRP was largely localized to blood vessels. In large blood vessels of the kidney, LRP was present in the subendothelial space and among smooth muscle cells (Fig. 5A). Simultaneous staining of LRP with an endothelial cell marker such as CD31 confirmed the location of LRP in other tissues. In skeletal muscle (Fig. 5B), LRP was
only present in the continuous capillaries. In the heart (Fig. 5C), LRP staining was also underneath CD31-staining cells in the capillary beds surrounding cardiac muscle cells. In the liver, LRP was located in the central vein, whereas the sinusoidal capillaries were negative for LRP (Fig. 5D). The endothelial cells of sinusoidal capillaries of liver usually adhere to the neighboring epithelial cells, and the absence of smooth muscle cells may be the reason that the capillaries in the liver were negative for LRP staining.

Although LRP was restricted to vascular structures, it did not stain endothelial cells [note the absence of colocalization (yellow images) in Figs. 1 and 5]. Because immunofluorescence studies are not of sufficiently high sensitivity to exclude the expression of LRP by other cell types, we can only conclude that LRP-positive cells may support the invasion of endothelial cells.

**LRP** is an extracellular matrix protein that is present in many forms. Using immunoblots of mesangial cells we detected three forms of LRP: 45, 67, and 200 kDa. Whole liver and HCT-116 tumor homogenates had two forms: 45 and 67 kDa (Fig. 6B). In kidney cortex, the 45-kDa LRP was present only in the low-speed soluble fraction, whereas the 67-kDa form was in both the soluble and pellet fraction. A 200-kDa form was only present in the hard pellet and was highly enriched in isolated glomeruli. In addition to the 45- and 67-kDa forms, placenta also expressed a weak 200-kDa band.

To determine the subcellular location of the LRP proteins, we fractionated mesangial cells cultured in serum-free media. LRP proteins could be detected in the medium (Fig. 7A, M) but at a much lower level than that present in the cell. Solubilization of cells by 2% Triton X-100 revealed that LRP was present in mesangial cells mostly as the 45-kDa form (Fig. 7A, C). The
67-kDa form was the least abundant in these cells, comprising <10% of the total immunoreactive LRP. All three LRP forms were preserved in the material that was extracted by 4 M guanidine, 0.5% CHAPS, and 5 mM EDTA [Fig. 7A, extracellular matrix (ECM)]. Because this fractionation procedure is the one usually used for ECM proteins, it suggested that some of the forms may be present in the ECM. To provide additional evidence for this, we treated mesangial cells with an impermeant biotinylation reagent (sulfo-NHS-biotin). The cells were then solubilized with 2% Triton and immunoprecipitated, and the immunoprecipitate was probed with streptavidin. Figure 7C shows that only the 67- and 200-kDa forms of LRP were exposed to the biotinylation reagent. As a control, we labeled cells with [35S]methionine followed by solubilization and immunoprecipitation. The 35S-labeled immunoprecipitate contained the three forms of LRP, 46, 67 (very faint), and 200 kDa with the 46-kDa being the most abundant. This is to be compared with the biotinylated forms, which were only the 67- and 200-kDa species. These studies suggest that only the higher molecular mass forms are extracellular.

To document the localization of LRP further, we stained mesangial cells without permeabilization and found that LRP was present outside the cells in a...
distribution similar to that of an authentic ECM protein, laminin (Fig. 7B). Interestingly, LRP was only accessible to the antibody in a location beneath the cells, similar to the location of laminin even though the cells secreted LRP into the medium. In permeabilized mesangial cells, LRP and laminin colocalized (i.e., yellow staining in Fig. 7B) only when their pattern of staining was fibrillar, a pattern only seen in the unpermeabilized cell study. These studies suggest that LRP is secreted to the site of adhesion of the cell, a theme that is common in other ECM proteins. Mesangial cells permeabilized by Triton showed that there was abundant intracellular LRP (and laminin), further documenting the fact that only a fraction of total cell-associated LRP is located in the ECM. The most definitive method for evaluation of the location of LRP in the ECM is immunoelectron microscopy. We used the immunogold labeling method on rat glomeruli (Fig. 8). The glomerular basement membrane and mesangium are produced by three cell types: podocytes, endothelial cells, and mesangial cells. Using a postembedding method, we found that LRP is present in the glomerular basement membrane (G in Fig. 8) and in the mesangium (M in Fig. 8, top and bottom). We performed a quantitative analysis (13) of the abundance of LRP in glomerular structures by first estimating the volume fraction of the following elements: Bowman’s space (B in Fig. 8), capillary lumen (C), endothelial cell (E), podocyte (P), and mesangial cell (MC). The number of gold particles in each element was then counted. Five independent images were thus tabulated (Table 1). We found that the “concentration” of LRP was enriched by a factor of 3.12 ± 0.7 in the glomerular basement membrane (P < 0.01). None of the other structures showed enrichment above what could be expected from random distribution (i.e., a concentration of 1.0). These results demonstrate that LRP is a new glomerular basement membrane protein.

Matrigel is a basement membrane extract from the Engelbreth-Holm-Swarm mouse sarcoma that has multiple biological activities, including the differentiation of epithelial cells, tumor cell invasion, and angiogenesis. We found that both the 45- and the 67-kDa forms of LRP are components of Matrigel (Fig. 7D, polyclonal). The presence of LRP proteins in Matrigel made it possible to purify large quantities of LRP proteins to study their functions. Using the same chromatography scheme, the 67-kDa protein was purified to homogeneity from Matrigel, and a silver-stained fraction is shown in Fig. 7C. The attempt to purify the

![Fig. 7. LRP is an extracellular matrix (ECM) protein. A: fractionation of mesangial cells into supernates (M). Then the cells were solubilized with Triton X-100 (C), and the ECM was extracted with 4 M guanidine HCl (ECM). B: kidney cortex was fractionated into a soluble (S) and a low speed pellet (P). Glomeruli were isolated as described in EXPERIMENTAL PROCEDURES. Placenta and HCT-116 tumors in nude mice and liver were homogenized, and the total homogenate was placed in each lane.](image1)

![Fig. 7. LRP is an extracellular matrix (ECM) protein. A: fractionation of mesangial cells into supernates (M). Then the cells were solubilized with Triton X-100 (C), and the ECM was extracted with 4 M guanidine HCl (ECM). B: kidney cortex was fractionated into a soluble (S) and a low speed pellet (P). Glomeruli were isolated as described in EXPERIMENTAL PROCEDURES. Placenta and HCT-116 tumors in nude mice and liver were homogenized, and the total homogenate was placed in each lane.](image2)
45-kDa LRP from the Matrigel failed because it was degraded by protease activity from the Matrigel during purification, activity that was resistant to a cocktail that includes EDTA, PMSF, leupeptin, aprotinin, pepstatin A, and E64.

**Endothelial cells bind to LRP.** The secretion of LRP into the ECM by vascular smooth muscle cells suggested that the target of LRP function was endothelial cells. Using the purified 67-kDa LRP from Matrigel, we found that there was no effect on migration or proliferation of endothelial cells in culture (data not shown). In adhesion assays, we coated cell culture dishes with the 67-kDa LRP and found that it promoted adhesion of EJG endothelial cells (Fig. 9A, ). At 20 min, very few EJG cells adhered to a BSA-coated dish, but 10% of the added endothelial cells bound to the LRP-coated dish, increasing to nearly 25% of added cells by 60 min, nearly sevenfold greater than those bound to BSA-coated dishes (Fig. 9A, ). Another endothelial cell, HUVEC, had much faster kinetics of binding to tissue

![Fig. 8. LRP is located in the glomerular basement membrane (GBM). Immunoelectron microscopy of rat kidney glomeruli is shown. Sections were incubated with the antibody after embedding followed by secondary antibodies coupled to 10-nm gold particles, and random images were photographed. Using stereological principles, the fraction of the surface area of the following elements were estimated: Bowman’s Space (B), capillary lumen (C), endothelial cell (E), podocyte epithelial cell (P), GBM (G), mesangial cell (MC), and mesangium (M). The number of gold particles in each of these elements was measured in 5 randomly chosen samples that had easy-to-identify structures. Three representative images are shown here. Only the G had a significantly enhanced level of accumulation of gold particles (P < 0.005). Bar = 200 nm.](image)

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<th>Table 1. Quantitative analysis of immunogold staining of glomerular structures</th>
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<tr>
<td>Bowman’s</td>
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<td>No. of gold particles</td>
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<td>Fraction of total gold particles</td>
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Data are from analysis of 5 independent images. RBC, red blood cells; GBM, glomerular basement membrane. Each image was analyzed separately for all the parameters listed. The total number of gold particles counted was 775. Statistics were performed on the 5 individually calculated “concentration” values, i.e., gold particles (as a fraction of total)/fractional area. *P < 0.01.
culture plastic coated with BSA, such that within 20 min, 11% of the cells were bound to BSA-coated plates. However, significantly more HUVEC bound to LRP-coated plates at every time point between 0 and 20 min. The effect of LRP on endothelial cell adhesion was dose dependent (data not shown). Mesangial cells also bound to LRP-coated dishes (data not shown). These results suggest that the 67-kDa LRP is sufficient to promote adhesion of endothelial cells.

To test whether cells that highly express and secrete LRP use it for adhesion, we plated mesangial or A7r5 on tissue culture plastic with or without anti-LRP antibodies (Fig. 9B). Anti-LRP polyclonal antibodies blocked mesangial cell adhesion to culture dishes (Fig. 9B). However, when the dishes were first coated with laminin, mesangial cell adhesion was not affected by the anti-LRP antibodies, indicating that the ability of mesangial cells to bind to LRP is specific (Fig. 9C).

Based on the results that purified LRP increased cell adhesion of both endothelial cells and mesangial cells, and anti-LRP antibodies blocked smooth muscle cell adhesion to cell culture dishes, we reasoned that LRP may be important for the adhesion between endothelial cells and smooth muscle cells. To test this, a cell-cell adhesion assay was set up, in which 35S-labeled mesangial or A7r5 smooth muscle cells were added to confluent HUVEC endothelial cell layers in the presence or absence of anti-LRP antibodies. At 15 min, ~50% of the added mesangial or A7r5 cells adhered to the endothelial cells. Anti-LRP antibodies significantly decreased the number of mesangial cells or A7r5 cells adhering to the endothelial cell layer, while the normal rabbit IgG had no effect (Fig. 10). Microscopic examination after 2 days of coculture showed that smooth muscle cells had burrowed underneath the endothelial cells (data not shown). In this, the smooth muscle cells resembled the migration of leukocytes through an endothelial monolayer. Remarkably, when mesangial cells were allowed to adhere to plates until they formed a monolayer, endothelial cells added to this layer failed to adhere in an LRP-dependent manner (Fig. 10B). This is likely due to the fact that all adhesion molecules secreted by mesangial cells are located underneath the mesangial cells rather than on the surface that faces the medium (see Fig. 7B). This result suggests that LRP can mediate adhesion between endothelial cells and smooth muscle cells.

**DISCUSSION**

LRP cDNA was first cloned as a 67-kDa protein that binds to laminin 1 (31) and is highly expressed in a variety of human tumors such as colon and breast cancer (23, 32). The human LRP gene was localized to 3p21.3, a “hot spot” for genetic alterations in a variety...
of cancers, especially in small-cell lung carcinoma (19). Interestingly, two monoclonal antibodies against LRP labeled a dorsoventral gradient in developing retina (26), suggesting that it may be important in retinal development. Genetic studies in Drosophila demonstrated that LRP was required during oogenesis and imaginal disc development (21). Despite these extensive studies, little is known about the specific function of LRP. Several lines of evidence are incompatible with the hypothesis that LRP is a laminin receptor. First, LRP cDNA does not have a typical stretch of hydrophobic amino acids long enough to serve as either a signal peptide or a transmembrane domain. Second, during our purification of functional 67-kDa LRP it became clear that it was a soluble protein, which argues against its being a transmembrane protein. Furthermore, it was not clearly demonstrated that either recombinant LRP or the 67-kDa LRP bound to laminin in a physiologically specific manner. It seems that the evidence for LRP being a receptor was based on the finding that it was retained by a laminin column. Adding to the confusion in the field was the finding that a 40-kDa cytoplasmic protein (P40) was encoded by the same cDNA sequence but was associated with ribosomes in sucrose density gradients (3, 12). However, these studies did not exclude the possibility that the association of P40 with ribosomes occurred after the cells were homogenized or that P40 was present in some cellular structures, for example, fragments of extracellular matrix, that could migrate at the same position as ribosome subunits in sucrose gradient. Thus LRP might be a multifunctional protein; alternatively these findings might be explained by its spurious binding to other proteins. Clearly, more work needs to be done to resolve this question.

Using a functional assay, we found that LRP was important for endothelial cell targeting during kidney development (25). Although there were reports about LRP's distribution in tissues and tumors, the precise location of LRP protein was unclear. Using double immunohistochemical staining with anti-LRP antibodies and antibodies against endothelial cell markers (CD31 and von Willebrand factor), we demonstrate here that LRP was only present in vascular structures. LRP staining was localized in the glomerular basement membrane underneath the endothelial cells and among vascular smooth muscle cells. Primary cultures of endothelial cells (HUVEC) did not contain any significant amount of LRP, whereas all smooth muscle cells tested expressed large amounts of it. These data suggest that LRP was synthesized by smooth muscle cells or pericytes and was secreted into the vascular ECM. The distribution in developing kidney and adult tissues suggests that LRP may be important for blood vessel formation and maintenance, which was supported by the observation that LRP is highly expressed by cells underneath the invading endothelial cells in tumors induced in nude mice. Using LRP purified from Matrigel, we found that both vascular cell types strongly bound to it. Furthermore, anti-LRP antibodies blocked adhesion of smooth muscle cells to cell culture surface and to an endothelial cell layer. Whereas binding of endothelial (or other cells) in vitro to a protein need not be construed as decisive evidence for its role in situ, we point out that we had previously demonstrated that a monoclonal antibody to LRP prevented the association of LRP with ureteric bud branches during kidney development. These in vitro studies suggest that LRP mediates the adhesion between endothelial cells and other cell types such as epithelial cells of the ureteric bud and vascular smooth muscle cells.

In the glomerulus, the basement membrane is synthesized by endothelial, epithelial, and mesangial cells. Each protein component, such as fibronectin, collagen IV, and laminin, can be shown to facilitate adhesion of each of these cell types to tissue culture plates. Similarly, we show here that LRP is present in the glomerular basement membrane and facilitates adhesion of endothelial and mesangial cells to plates in vitro. Like other components of the basement membrane, LRP need not bridge the whole distance between endothelial and mesangial cells; rather, it is likely that it is secreted where it participates in the major function of the basement membrane, which is to provide an adhesive surface for endothelial and other cell types.

Endothelial cell layers in vein and artery are covered by smooth muscle cells, whereas most capillaries are covered by pericytes. Recruitment of smooth muscle support cells to the endothelial tube is an important component of angiogenesis. Recent studies had shown that angiopoietin 1 might act as a signal between smooth muscle and endothelia. Mice lacking angiopoietin 1 or its Tie2 receptor demonstrate a defect in recruiting smooth muscle cells to early endothelial tubes, leading to embryonic lethality (29). Angiopoietin 1 is produced by smooth muscle support cells, and the Tie2 receptor is located on endothelial cells, resulting in a signaling loop between these two cell types (14). A number of studies suggest that the association between endothelial cells and smooth muscle cells mediates stabilization or maturation of blood vessels (11). For instance, proliferative retinopathy in diabetic patients is preceded by loss of pericytes (28). During development, immature vessels differ from mature ones in their dependence on VEGF for survival (2), and the association of developing vessels with the support cells marks the end of VEGF dependence (5). Furthermore, studies reveal that contact between endothelial and smooth muscle cells leads to the induction of transforming growth factor (TGF)-β, which induces maturation of blood vessels by inhibiting endothelial cell proliferation and migration, and by induction of smooth cell differentiation (17). TGF-β also stimulates production of a number of ECM proteins (16), including LRP (Hu C, unpublished observations). We propose that LRP is a critical molecule that plays a role in blood vessel maturation by enhancing the adhesion of endothelial cells to smooth muscle. During angiogenesis, the support cells highly express LRP, which may be used to initiate contact with endothelial cells. We spec-
ulate that this contact may trigger the activation of TGF-β, which induces the maturation of the developing blood vessels. Interestingly, LRP expression is preserved in adult tissues, suggesting that it may be important for the maintenance of vascular structures. The unique role of LRP in angiogenesis makes it a candidate target for cancer therapy.

Although the cDNA of LRP does not encode a signal peptide, it is secreted into the ECM. LRP was accessible to antibodies and impermeant biotinylation reagents in unpermeabilized mesangial cells, demonstrating that it is indeed an extracellularly secreted protein. Finally, our quantitative immunoelectron microscopy definitively demonstrates that LRP accumulates in the glomerular basement membrane. There is now increasing evidence for the presence of a secretory pathway for proteins that do not possess hydrophobic signal peptides (reviewed in Refs. 9 and 24). More than 10 such secreted proteins had been identified, including interleukin 1, basic fibroblast growth factor (FGF), acidic FGF, platelet-derived growth factor, galectin-1 and galectin-3, and all are secreted without traversing the endoplasmic reticulum and Golgi apparatus. These proteins are present in the cytosol and are not glycosylated, and similarly we and others found that LRP is not glycosylated (Hu C, unpublished observations and Ref. 8). It is likely that LRP is synthesized and secreted through the nonclassic pathway.

The sequence of LRP cDNA has been cloned in more than 10 species and shows striking conservation. At the protein level, the murine LRP sequence is different from the human one by only two amino acids. Only one active gene was found in the human and chicken genomes (19). We, like others, detected a single mRNA species of around 1.2 kb, which encodes a polypeptide with a calculated molecular mass of 32 kDa. The different species of 67 and 200 kDa might represent posttranslational modification, although treatment of the 67-kDa LRP with neuraminidase, O-glycanase, or N-glycosidase F had no effect on the apparent molecular mass of the protein, indicating that it is not glycosylated (Hu C, unpublished observations). Using mass spectrometry, the 67-kDa protein was found to be covalently bound by palmitate, stearate, and oleate, and determination of its amino acid composition indicated that it was a homodimer (20). Treatment with cerulein and hydroxylamine confirmed that fatty acid acylation was involved in the processing of the 67-kDa protein, which by increasing its hydrophobicity may lead to very strong intermolecular interactions that are even resistant to SDS-PAGE conditions. It is also possible that LRP multimers might be composed of cross-linked subunits, a finding that is known to occur in other ECM proteins. In pulse-chase experiments, the metabolic label was present first in the 40-kDa protein and gradually chased into the 67-kDa one, suggesting a precursor-product relationship (7), and we have confirmed this finding (18). Our results that the higher molecular mass species are extracellular (exposed to biotinylation reagents) suggest that the homo- (or hetero-)polymerization occurs in the ECM by still-to-be-defined mechanisms. The 200-kDa form we detected here was also detected by others using mass spectrometry (6). It may represent a higher molecular weight aggregate form of LRP, and interestingly we found that it only exists in some tissues.

In summary, we describe here a protein that is localized to blood vessels and is likely to mediate adhesive interactions between endothelial and smooth muscle cells. Its location and function suggest that it plays a role in the maturation and maintenance of blood vessels.

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REFERENCES


