Endothelial Localization of Receptor Tyrosine Phosphatase, ECRTP/DEP-1, in Developing and Mature Renal Vasculature

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Abstract. Developmental assembly of the renal microvasculature requires spatially and temporally coordinated migration, assembly, differentiation, and maturation of endothelial cells in the context of adjacent epithelial and mesangial cells. In this study, endothelial expression and distribution of the receptor tyrosine phosphatase ECRTP/DEP-1 were evaluated during and after developmental assembly of the renal microvasculature. Monoclonal antibodies against ECRTP/DEP-1 ectodomain epitopes localize its expression to membrane surfaces of endothelial cells in glomerular, peritubular capillary, and arterial renal sites of mature human and murine kidney. During kidney development, ECRTP/DEP-1 immunostaining is evident on a subpopulation of metanephric mesenchymal cells and on putative progenitors of glomerular capillary endothelial cells early in their recruitment to developing glomeruli. ECRTP/DEP-1 is prominently displayed on luminal membrane surfaces with punctate accumulations at inter-endothelial contacts that overlap with vascular endothelial-cadherin staining. ECRTP/DEP-1 is recruited to inter-endothelial contacts in confluent cultured human renal and dermal microvascular endothelial cells, yet experimental dissociation of vascular endothelial-cadherin from endothelial junctional complexes fails to redistribute ECRTP/DEP-1. These findings indicate that ECRTP/DEP-1 is expressed in anticipation of glomerular capillary endothelial recruitment during development, and suggest that ECRTP/DEP-1 ectodomain interacts with endothelial surface ligands that are engaged by cell-cell contact.

Development of renal glomerular capillaries is anatomically segregated and temporally staged in a multistep process that involves recruitment of endothelial progenitors from adjacent mesenchyme, assembly of an arborized branching network, and maturation and specialization of endothelial cells adjacent to mesangial and visceral epithelial cells (1,2). Endothelial cell surface receptors are important mediators of this assembly process, because they interact with ligands secreted by adjacent cells, with extracellular matrix, and with surface molecules on cells they contact.

Among growth factors, vascular endothelial growth factor (VEGF) is an important participant. VEGF is induced in S stage glomerular epithelial cells, and endothelial progenitors that are recruited to glomerular capillaries from the adjacent metanephric mesenchyme express the VEGF receptor flk-1 (3,4). Neutralizing VEGF antibodies interrupt postnatal murine glomerular capillary development (5). Homozygous deletion of either platelet-derived growth factor-β (PDGF-β) receptor or PDGF B/c-sis genes in mice causes defective recruitment of mesangial cell precursors with failure of glomerular development (6,7). Type II transforming growth factor-β receptors mediate in vitro capillary morphogenesis of endothelial cells derived from bovine glomeruli (8). Receptor tyrosine kinases of the Eph family and their membrane-bound ephrin ligands are expressed in isolated mesenchymal cells in a pattern similar to that of flk-1 (9), and oligomerized forms of ephrin-B1 stimulate in vitro assembly of human renal microvascular endothelial cells (RMEC) into capillary-like structures (10).

Although roles for receptor tyrosine phosphatases (RTP) in glomerular capillary assembly have yet to be assigned, targeting roles are anticipated, based on biologic precedent. One member of a subclass of RTP, Drosophila receptor tyrosine phosphatase DPTP10D, functions to direct axonal migration and neural network assembly (11). A second DPTP10D subclass member, DEP-1, has been identified in arterial sites in mammalian kidney (12). DEP-1 (high cell density enhanced protein tyrosine phosphatase-1) cDNA was initially cloned by Ostman et al. from HeLa cells (13). Independently, our laboratory cloned DEP-1 sequences from human RMEC cDNA and designated the high identity product ECRTP (endothelial cell receptor tyrosine phosphatase). Structural orthologues of ECRTP/DEP-1 (also called HPTPb, Byp-1, PTPβ2, and CD148) have been identified in neonatal smooth muscle cells, in breast and thyroid cancer cell lines, and in all hematopoietic lineages (14,15). Although ECRTP/DEP-1 expression was identified in arterial endothelial cells of the kidney, in situ...
hybridization experiments failed to detect glomerular capillary localization of ECRTP/DEP-1 mRNA (12).

Like other members of the class III receptor tyrosine phosphatase family, including glomerular epithelial protein tyrosine phosphatase-1 (GLEPP-1), stomach cancer-associated PTP (SAP-1), and DPTP 10D, ECRTP/DEP-1 is a type I membrane protein comprised of a large extracellular domain containing eight or more fibronectin type III repeats and a single cytoplasmic domain phosphatase catalytic domain (13). GLEPP-1 is structurally similar to ECRTP/DEP-1, yet shows renal expression limited to glomerular visceral epithelial cells, where it is structurally similar to ECRTP/DEP-1, yet shows renal expression limited to glomerular visceral epithelial cells, where it has been implicated in podocyte integrity (16). Unlike the MAM (neprin-XenopusA2-mu) domain containing receptors PTPμ and PTPκ, which interact through homophilic binding, class III RTP appear to bind yet unidentified ligands.

Monoclonal antibodies against ECRTP/DEP-1 ectodomain epitopes were used to define its distribution in the renal circulation of mature and developing kidney. ECRTP/DEP-1 is expressed at high levels in renal glomerular, peritubular capillary of mature and developing kidney. ECRTP/DEP-1 is expressed at high levels in renal glomerular, peritubular capillary, and arterial endothelial cells and shows a pattern of distribution in vivo and in vitro consistent with a role in cell-cell interactions, possibly to signal responses important in vascular cell targeting.

Materials and Methods

Cell Lines and Cell Culture

Primary human RMEC were isolated, cultured, and used at third or fourth passage after thawing, as described (17). Human dermal microvascular endothelial cells (HMEC-1 cells, CDC) were grown in MCDB131 media (Sigma Chemical Co., St. Louis, MO) containing 15% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), 10 ng/ml epidermal growth factor (Collaborative Biomedical Products; Becton Dickinson, Bedford, MA), and 1 μg/ml hydrocortisone (Sigma) (18). Human umbilical vein endothelial cells (HUVEC; Clonetech, San Diego, CA) were cultured in M199 (Sigma) supplemented with 15% FBS, 5% Nusereum (Collaborative Research, Bedford, MA), 10 ng/ml endothelial cell growth factor, and 20 U/ml heparin. For growth of endothelial-like line, EAhY926 cells (kindly provided by Dr. Edgel, University of North Carolina, Chapel Hill, NC), Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies BRL, Gaithersburg, MD) containing 15% fetal calf serum, 0.1% hypoxanthine-aminopterin-thymidine medium (Sigma) was used. Madin-Darby canine kidney (MDCK) cells (kindly provided by L. Limbird, Vanderbilt University) were grown in Dulbecco’s modified Eagle’s medium (Life Technologies BRL) containing 4.5% D-glucose and supplemented with 10% FBS. All growth medium was supplemented with 1 mM L-glutamine (Life Technologies BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies BRL).

Generation of Antibodies to Recombinant ECRTP/DEP-1 Proteins

Ectodomain (amino acids 175 to 536) and catalytic domain (amino acids 1048–1338) sequences of human ECRTP/DEP-1 (13) were subcloned into the pRSET vector (Invitrogen, Carlsbad, CA). Recombinant fusion proteins were expressed in bacteria, purified by Ni-agarose affinity (Invitrogen), and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as greater than 95% homogeneous proteins of 40 and 36 kD, respectively. Mouse hybridoma antibodies (ECRTA.Ab1, ECRTA.Ab2) were generated against the ECRTP/DEP-1 recombinant protein by intraperitoneal immunization, fusion with SP2-0 cells, enzyme-linked immunosorbent assay screening, selection, expansion, and purification by affinity chromatography on protein G-agarose. Rabbit antisera was raised by immunization with ECRTP/DEP-1 cryotosin and purified by affinity chromatography on ECRTP/DEP-1 cryo-coupled agarose.

Immunodetection of Exogeneously and Endogeneously Expressed ECRTP/DEP-1

MDCK cells grown in 100-mm plastic dishes (Falcon, Oxnard, CA) were transfected with an expression plasmid pSRα ECRTP/DEP-1/3xHA that drives high level expression of human ECRTP/DEP-1, modified by addition of three hemagglutinin (HA) peptide repeats to the carboxy terminus, using cationic lipid (LipofectamineTM, Life Technologies BRL) according to the manufacturer’s protocol. Forty-eight hours after transfection, cells were placed on ice, washed twice with ice-cold phosphate-buffered saline (PBS) and immediately lysed in 0.5 ml of lysis buffer (50 mM Heps, pH 7.5, 50 mM NaCl, 5 mM ethylenediaminetetra-acid [EDTA], 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonylfluoride [PMSF]). Lysates were clarified by centrifugation, and membrane receptors were recovered by batch adsorption to wheat germ agglutinin (WGA)-agarose (Sigma) for 4 h at 4°C. The resultant precipitates were resolved by 7% SDS-PAGE under reducing conditions, transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA), and blocked in 5% nonfat dry milk in Tris-buffered saline (50 mM Tris-HCl, pH 7.5, 157 mM NaCl) containing 0.2% Tween 20 (TBST) overnight at 4°C. Blots were incubated with murine monoclonal ECRTP.Ab1 or ECRTP.Ab2 (1 μg/ml, 5 μg/ml), rabbit polyclonal ECRTP/DEP-1 antibody (25.0 μg/ml), or anti-HA antibody (2.5 μg/ml, clone 12CA, Boehringer Mannheim) followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (Boehringer Mannheim). Membranes were washed with TBST, then developed using a chemiluminescent substrate (enhanced chemiluminescence; Amersham, Arlington Heights, IL) according to the manufacturer’s instructions.

For Western blots of endothelial crude protein lysates, RMEC cells grown on 100-mm dishes were lysed in lysis buffer (50 mM Heps, pH 7.5, 50 mM NaCl, 5 mM EDTA, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF). Clarified lysates (10 μg) were subjected to 7% SDS-PAGE, transferred to membrane, and blocked with 10% normal mouse serum in TBST overnight at 4°C. Blots were incubated with biotinylated ECRTP.Ab1 or ECRTP.Ab2 (5 μg/ml, 50 μg/ml) for 60 min at room temperature, followed by incubation with horseradish peroxidase-conjugated streptavidin (1 μg/ml, Pierce, Rockford, IL). Membranes were washed with TBST, then developed using a chemiluminescent substrate (enhanced chemiluminescence; Amersham). To preabsorb the immunoreactivity of ECRTP antibodies, 50 μg of ECRTP/DEP-1 proteins (Ec) were preincubated with ECRTP antibodies for 4 h at 4°C and microcentrifuged at 15,000 rpm for 20 min, and the resultant supernatants were used.

Generation of Stably Transfected MDCK Cells and Cell Staining

MDCK cells were stably transfected with an expression plasmid pCDNA3 ECRTP/DEP-1/3xHA (Invitrogen) using cationic lipids (Li-fofectamineTM, Life Technologies BRL) according to the manufacturer’s protocol. Stable transfecants were selected by addition of G418 (Life Technologies BRL) to culture media at a final concentration of 800 μg/ml, and a single colony was obtained by limited
dilution cloning. The cells were grown on glass coverslips (Fisher Scientific, Springfield, NJ) and fixed with 100% methanol for 10 min at −20°C. Coverslips were washed with PBS, blocked with 5% goat serum for 30 min at room temperature, incubated with ECRTP.Ab2 (10 μg/ml) for 60 min, washed, then incubated with FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 60 min at room temperature. Coverslips were mounted and analyzed by confocal microscopy (Zeiss LSM 410). To preabsorb the immunoreactivity of ECRTP/DEP-1 antibodies, 50 μg of ECRTP/DEP-1 proteins (Ec or Cy) was preincubated with ECRTP.Ab2 for 4 h at 4°C and microcentrifuged at 15,000 rpm for 20 min, and the resultant supernatant was used to stain cells.

Immunoblots of Human Cultured Endothelial Cells

Human endothelial cells grown in 60-mm dishes were lysed at confluence in 0.5 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5% SDS, 0.5% deoxycholate, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM PMSF) on ice for 30 min. Cleared lysate protein (75 μg) was incubated with 10 μg/ml affinity-purified rabbit ECRTP/DEP-1 antibody or rabbit IgG (Sigma) at 4°C for 4 h, and immunoprecipitates were recovered using protein A-Sepharose (Sigma). SDS-PAGE and immunoblotting procedures were carried out as described above.

Tissue Immunolocalization

Human kidney tissue was snap-frozen in a dry ice-acetone bath. Cryostat sections (4 μm) were fixed in acetone at −20°C for 10 min, washed with PBS, and preadsorbed with avidin-biotin blocking reagents (Vector Laboratories) according to the manufacturer’s instructions. Sections were washed with PBS, blocked with 5% goat serum, incubated with monocular ECRTP/DEP-1 antibody (ECRTP.Ab1, 10 μg/ml, 60 min), washed, incubated with biotinylated goat anti-mouse IgG (Vector Laboratories, 7.5 μg/ml, 60 min), washed, incubated with FITC-conjugated streptavidin (Pierce, 4 μg/ml, 30 min), and finally washed with PBS. Coverslips were mounted (Vectorashield; Vector Laboratories) and analyzed by confocal microscopy (Zeiss LSM410).

For colocalization experiments, acetone-fixed frozen sections were blocked with 5% donkey serum and incubated with a mixture of ECRTP.Ab1 (1 μg/ml) and goat anti-human vascular endothelial (VE)-cadherin antibody (5 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA) or rat anti-mouse VE-cadherin antibody (2.5 μg/ml, Pharmingen, San Diego, CA) at room temperature for 60 min. Specific antibodies were detected using a mixture of FITC-conjugated donkey anti-mouse and rhodamine-conjugated donkey anti-goat or anti-rat antibodies (Jackson ImmunoResearch Laboratories) at room temperature for 30 min. In colocalization experiments of murine kidney, tissue sections were blocked with 5% donkey serum containing unconjugated donkey anti-mouse antibody (50 μg/ml) to reduce backgrounds. Specific immunostaining for each antigen was identified in overlapping images generated by analysis of the same section at 488 nm and 568 nm wavelengths, respectively, on a Zeiss LSM410 confocal microscope.

Immunolabeled murine kidney sections showed high background and required an alternative technique. The anti-ECRTP/DEP-1 monoclonal antibody ECRTP.Ab1 was directly coupled to FITC. Briefly, ECRTP.Ab1 (0.55 ml of 0.94 mg IgG/ml in 0.1M sodium carbonate buffer, pH 9.0) was conjugated to 0.03 ml of FITC solution (Sigma, 1.0 mg/ml in DMSO) overnight at 4°C. The reaction was stopped by adding ammonium chloride to 50 mM final concentration. After incubation for 2 h at 4°C, the mixture was dialyzed exhaustively against PBS to remove unbound FITC. A mouse monoclonal IgG against rat glomerular basement membrane coupled to FITC using the identical protocol was used as a control (19). Acetone-fixed sections were blocked with 0.5 M ammonium chloride, incubated for 30 min with ECRTP.Ab1-FITC conjugates, washed, and examined by epifluorescence microscopy. In some additional control experiments, the anti-ECRTP-FITC conjugate was mixed with a molar excess of the immunization peptide before incubation with the sections.

Immunocytochemistry of Human Endothelial Cell Lines

Endothelial cells were grown on uncoated glass coverslips (Fisher), then fixed with 50% methanol for 10 min at 4°C. Coverslips were washed with PBS, blocked with 5% goat serum for 30 min at room temperature, incubated with ECRTP.Ab2 antibody (10 μg/ml) or VE-cadherin monoclonal antibody (2 μg/ml, Transduction Laboratories, Lexington, KY) for 60 min, washed, then incubated with biotinylated goat anti-mouse IgG (1 μg/ml, Vector Laboratories) for 60 min, washed, and finally incubated with FITC-conjugated streptavidin (4 μg/ml, Pierce) for 30 min. Coverslips were mounted and analyzed by confocal microscopy (Zeiss LSM410). For HA staining of transfected endothelial cells, 90% confluent HMEC-1 cells cultured on 35-mm dishes were transfected with 2.2 μg of pSRα ECRTP/DEP-1/3xHA expression plasmids using adenovirus-assisted Lipofectamine™ transfection as described (20). The transfected cells were replated on uncoated glass coverslips at 48 h after transfection. The cells were fixed with 2% methanol-free formaldehyde (Polysciences, Warrington, PA) for 10 min at 4°C and permeabilized with 0.02% saponin (Sigma), stained with mouse HA monoclonal antibody (1 μg/ml, Babco, Richmond, CA) as described above.

Calcium Chelation to Disrupt Inter-Endothelial Cadherin Complexes

Confluent HMEC-1 cells grown on glass coverslips in Dulbecco’s modified Eagle’s media supplemented with 15% FBS were exposed to addition of ethyleneglycol-bis-aminoethylther-Ν,Ν,Ν,Ν′-tetra-acetic acid (EGTA; Sigma) to reach a final concentration of 5 mM. Cells were incubated for an additional 20 min, then fixed with 50% methanol at 4°C for 10 min, washed with PBS, and stained with ECRTP.P.Ab2 antibody (10 μg/ml) or VE-cadherin monoclonal antibody (2 μg/ml, Transduction Laboratories), as described above.

Results

Monoclonal Antibodies Recognize Recombinant and Expressed ECRTP/DEP-1

Recombinant fusion proteins representing either ectodomain (Ec) or cytoplasmic domain (Cy) ECRTP/DEP-1 sequences were expressed in bacteria and used to immunize rabbits and/or mice. As shown in Figure 1A, the monoclonal antibodies ECRTP.Ab1 and ECRTP.Ab2 specifically identify the ectodomain but not the cytoplasmic domain recombinant proteins, and ECRTP polyclonal antibody specifically reacts to ECRTPcy protein. To ascertain whether these antibodies recognize the full-length protein expressed in mammalian cells, MDCK cells were transiently transfected with either an empty expression plasmid (SRα) or one driving expression of a full-length ECRTP/DEP-1, tagged on the carboxy terminus with a hemagglutinin epitope (SRα ECRTP/HA). Membrane proteins recovered by WGA lectin were probed with the antibodies indicated, including ECRTP.Ab1 and ECRTP.Ab2, polyclonal rabbit anti-ECRTP/DEP-1 (PolyAb), and monoclonal anti-HA.
Figure 1. Antibodies ECRTP.Ab1 and ECRTP.Ab2 recognize recombinant, overexpressed, and endogenous ECRTP/DEP-1. (A) Recombinant proteins representing extracellular (Ec) or cytoplasmic (Cy) domains of ECRTP/DEP-1 were expressed in bacteria and purified. Proteins (100 ng) were separated on a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF) membrane, and probed with monoclonal antibodies ECRTP.Ab1, ECRTP.Ab2, and rabbit ECRTP polyclonal antibody (PolyAb) as indicated. ECRTP.Ab1 and ECRTP.Ab2 specifically reacted to 40-kDa ECRTP Ec protein and PolyAb to 36-kDa ECRTP Cy protein. (B) Madin-Darby canine kidney (MDCK) cells were transfected with empty pSRα vector (SRα) or pSRα-ECRTP/DEP-1/HA (SRα-ECRTP/HA) expression constructs, and membrane receptor proteins recovered from 75 μg of lysate protein by lectin wheat germ agglutinin (WGA)-conjugated agarose were subjected
Each antibody recognized the expressed 220-kD ECRTP/DEP-1/HA. In addition, ECRTP.Ab1 and ECRTP.Ab2 each recognized endogenous ECRTP/DEP-1 in immunoblots of crude lysate proteins from RMEC. On immunoblots of endogenous protein recovered from endothelial cells, ECRTP.P.Ab1 was more effective, suggesting that ECRTP.Ab2 binds a discontinuous epitope preserved in recombinant antigen, but is lost in the glycosylated endogenous protein displayed on blotting membranes. Binding of both antibodies to the 220-kD ECRTP/DEP-1 band was eliminated by preincubation with the immunizing antigen ECRTP/DEP-1Ec (Figure 1C).

As shown in Figure 1D, ECRTP.Ab2 was quite effective in recognizing ECRTP/DEP-1 expressed in a stable MDCK transfectant cell line. Staining localized ECRTP/DEP-1 to lateral cell membranes in confocal Z plane sections of MDCK cells grown to confluence on permeable membrane supports (Figure 1D, Panel e), in a pattern identical to that of staining with antibodies against the HA epitope (not shown). Competition with the immunizing antigen ECRTP/DEP-1Ec, but not ECRTP/DEP-1Cy, blocked immunostaining (Figure 1D, Panels b and c). Immunoblots of ECRTP/DEP-1 recovered by immunoprecipitation from endothelial cells derived from different vascular sites showed abundant expression of ECRTP/DEP-1; included are RMEC from which it was cloned, a dermal microvascular endothelial cell line, HMEC-1 (18), human umbilical vein endothelial cells, and a HUVEC-derived cell line, EAhy926 (Figure 1E) (21). A range of other cell lines, including rat vascular smooth muscle cells, rat mesangial cells, P19 embryonic carcinoma cells, 4T1 murine breast cancer cells, mouse NIH 3T3

Figure 2. ECRTP/DEP-1 is abundant in endothelial cells of adult human kidney. Acetone-fixed frozen sections (4 μm thickness) of human kidney were incubated with ECRTP.Ab1 (Panels A, B, D, and E), anti-vascular endothelial (VE)-cadherin (Panel C), or a class-matched control monoclonal antibody (Panel F) and bound antibody were detected by epifluorescence microscopy, as described in Materials and Methods. ECRTP.Ab1 prominently labeled glomerular (G), peritubular (arrow, Panel B), and arterial (A) endothelial cells. Panels B and C show that ECRTP/DEP-1 and VE-cadherin staining patterns overlap in the same section, suggesting endothelial localization of ECRTP/DEP-1 in human kidney. Note that glomerular capillary and arterial ECRTP/DEP-1 labeling revealed punctate labeling patterns (arrowheads, Panel E). Magnification: ×100 in A and F; ×400 in B and C; ×600 in D and E.
fibroblast cells, and Chinese hamster ovary lines failed to show detectable expression using this method.

**ECRTP/DEP-1 Immunoreactivity Localizes to Endothelial Cells of Glomerular Capillaries, Peritubular Capillaries, and Renal Arteries**

To localize distribution of ECRTP/DEP-1 in mature mammalian kidney, we used indirect and direct immunofluorescence staining experiments on frozen sections from human and mouse tissues. As shown in Figure 2, ECRTP.Ab1 stains ECRTP/DEP-1 in arterial, glomerular, and peritubular capillary endothelium of human kidney. Higher magnification frames show that ECRTP/DEP-1 labeling is predominantly along the luminal membranes of endothelial cells, at least in the arterial sites where endothelial membrane definition is most reliable (Figure 3).

The punctate characteristic of the staining in the glomerular microcirculation led us to evaluate whether ECRTP/DEP-1 was engaged in inter-endothelial junctional complexes. In double-labeling studies of a human intraparenchymal renal artery using ECRTP.Ab1 and VE-cadherin antibodies, overlap was evident (Figure 3). In addition to the luminal endothelial membrane staining, a regional accumulation of ECRTP/DEP-1 was evident at points of inter-endothelial contact, overlapping, but not limited to, the endothelial junctional complexes that include VE-cadherin (22). This pattern was evident in both arterial and peritubular capillaries. In extrarenal sites, capillary and large vessel endothelial staining is evident in brain, heart, and spleen, as well as in murine endocardium (T. Takahashi, K. Takahashi, and T. O. Daniel, unpublished results).

**ECRTP/DEP-1 Expression in Developing Mouse Kidney**

Based on the prominent ECRTP/DEP-1 expression in vascular endothelium of mature kidney, we were motivated to evaluate temporal and spatial expression of this receptor during renal vascular development in mouse embryos. As shown in Figure 4, ECRTP.Ab1 binds an antigen that we presume is murine ECRTP/DEP-1, based on its similar pattern of staining in mature murine and human kidneys, and based on the effect of the recombinant human immunogen ECRTP/DEP-1\textsubscript{ec} to block staining of the mouse tissue (not shown). In developing mouse kidneys at embryonic day 14, day 16, and postnatal day 6 (Figure 4, A through C), conjugates of ECRTP.Ab1-FITC displayed a pattern of immunoreactivity that was strikingly similar to the pattern observed previously using antibodies against the VEGF receptor flk-1, and the EphB1/ephrin-B1 receptor ligand (4,9). Notably, ECRTP.Ab1-FITC bound to endothelial cells of developing glomeruli and microvessels in the fetal kidney cortex. Small but intense foci of bound antibody were observed on isolated cortical mesenchymal cells that we have previously speculated to be angioblasts (Figure 4, A and B, arrows). Within vascular clefts of comma- and S-stage developing glomeruli, a subpopulation of cells consistent with glomerular endothelial precursors was labeled. Immunolabeling for ECRTP/DEP-1 on sections of neonatal kidney produced a distinct vascular labeling pattern (Figure 4C). Arteriolar, glomerular, and peritubular capillary endothelia all labeled intensely. Glomerular endothelial cells were also brightly labeled in adult mouse kidney (Figure 4D), as they were in sections of human kidney (Figure 2). Other cells within the immature and mature kidneys did not bind ECRTP.Ab1-FITC, and sections labeled with control monoclonal IgG-FITC conjugates, or mixtures of ECRTP.Ab1-FITC and the immunization peptide (Ec), showed no staining (not shown).

Double-labeling studies of neonate kidney using ECRTP.P.Ab1 and VE-cadherin antibodies (Figure 5) showed substantial overlap. Many, if not most, VE-cadherin-positive cells newly incorporating into early S stage developing glomeruli were also expressing ECRTP/DEP-1 (evident by yellow staining in Panel C), as were isolated mesenchymal cells in the yet undeveloped neocortex. As noted above, a subpopulation of ECRTP/DEP-1-positive cells appears not to stain prominently for VE-cadherin.

**ECRTP/DEP-1 Recruitment to Inter-Endothelial Cell Contacts**

Immunofluorescence staining experiments using ECRTP.P.Ab1 or ECRTP.Ab2 showed similar patterns of ECRTP/DEP-1 localization in human renal microvascular endothelial cells, RMEC (Figure 6A), and human dermal microvascular endothelial cells, HMEC-1 (Figure 6B). Confluent RMEC cultures displayed prominent staining with ECRTP.Ab1 and ECRTP.Ab2 at points of inter-endothelial contact (Figure 6A,

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**Figure 3. Confocal localization of ECRTP/DEP-1 and VE-cadherin in human kidney vasculature.** Acetone-fixed human kidney sections were labeled with ECRTP.Ab1 (green) and anti-VE-cadherin (red) as described in Materials and Methods. ECRTP.Ab1 (green) staining was distributed over the entire endothelial membrane in large artery (A) and glomerular capillaries (D), whereas VE-cadherin labeling (red) was restricted to endothelial junctions (B and E). Overlapping confocal images (C and F) demonstrated colocalization of ECRTP/DEP-1 with VE-cadherin at inter-endothelial junctions (arrows). Magnification: \(\times600\).
Panels a and b). Serial confocal images of RMEC staining and X-Z axis analysis of transfected HMEC-1 cells showed that ECRTP/DEP-1 was distributed to inter-endothelial contacts. In addition, there were punctate accumulations of apical membrane staining in confocal planes capturing the apical surface (Figure 6A, Panel f), but not on the basal membrane surface. Endothelial cells plated at sufficiently low density to be isolated from contact with one another did not show the prominent pattern of cell border staining seen in contacting cells (not shown). It should be noted that ECRTP.Ab1 demonstrated the receptors on the entire surface membranes, but ECRTP.Ab2 preferentially stained the inter-endothelial junctions. We presume that this reflects differences in accessibility of the ECRTP epitopes recognized by each antibody, based on conformation and interaction with other proteins. Similar differences in epitope recognition among antibodies against platelet endothelial cell adhesion molecule 1 (PECAM-1) have recently been reported (23).

This accumulation of ECRTP/DEP-1 at sites of endothelial cell-cell contact is consistent with the punctate accumulations seen in mature human arteries and glomerular capillaries, suggesting that a subpopulation of receptors distribute to points of inter-endothelial contact, in vivo. This led us to compare the distribution of ECRTP/DEP-1 with that of VE-cadherin. Localization of ECRTP/DEP-1 and VE-cadherin immunoreactivity in double-labeling experiments showed modest overlap of ECRTP/DEP-1 staining with the VE-cadherin localized in inter-endothelial junctions (Figure 3). Finally, we conducted experiments to ascertain whether the intercellular accumulation of ECRTP/DEP-1 immunoreactivity required integrity of VE-

Figure 4. ECRTP/DEP-1 expression in developing murine glomeruli. Cryostat kidney sections of embryonic day 14 (A), day 16 (B), postnatal day 6 (C) and adult mice (D) were immunolabeled with ECRTP/DEP-1.Ab1 as described in Materials and Methods. In A and B, ECRTP.Ab1 binds to cells dispersed in the mesenchymal area (arrow) to endothelial precursor cells (arrowhead) migrating to the vascular cleft of comma-shaped glomeruli (Comma) or S-stage glomeruli (S) and to endothelium of capillary stage glomeruli (Cap.G). In C and D, ECRTP.Ab1 preferentially labels endothelial cells of the glomerulus (G), artery (A), and peritubular capillaries (arrow) in mature kidney. Magnification: ×400 in A; ×200 in B and C; ×350 in D.
cadherin interactions. As shown in Figure 6B, EGTA treatment of the HMEC-1 cells dissociates VE-cadherin from the inter-endothelial junctional complexes, but has no dramatic effect on ECRTP/DEP-1 localization over the 20-min time period of observation, indicating that inter-endothelial junctions may retain ECRTP/DEP-1 in the absence of VE-cadherin.

Discussion

Several of these observations extend information about ECRTP/DEP-1 tyrosine phosphatase in vascular development and in endothelial cell-cell interactions. ECRTP/DEP-1 is an abundant endothelial mRNA transcript (24) and is expressed in cultured endothelial cells of different vascular origins (Figure 1). Cultured RMEC express the protein on cell membranes, just as glomerular and peritubular capillaries do in intact kidney tissue. Indeed, capillary and arterial endothelium appear to be the dominant cellular sources of ECRTP/DEP-1 expression in mature human and mouse kidney. In contrast with the previous in situ hybridization experiments in rat kidneys (12), we find high level ECRTP/DEP-1 protein expression in glomerular endothelium of both mouse and human tissues.

Within arterial endothelium, ECRTP/DEP-1 distributes to apical membranes and accumulates in punctate aggregates at points of inter-endothelial contact that overlap VE-cadherin staining. Available resolution with frozen section material does not permit confirmation of our impression that the granular glomerular capillary endothelial pattern may represent a similar distribution. That possibility is tacitly supported by the lateral cell membrane distribution of ECRTP/DEP-1 in the transfected MDCK epithelial cell system (Figure 1D, Panel e) and in confluent RMEC cultures (Figure 6A). Since ECRTP/DEP-1 lateral membrane distribution is maintained in cultured endothelial cells in which VE-cadherin complexes have been dissociated by calcium chelation, we conclude that maintenance of ECRTP/DEP-1 in inter-endothelial contacts does require integrity of VE-cadherin complexes. It is possible that lateral ECRTP/DEP-1 membrane distribution may function to establish conditions permissive to assembly of inter-endothelial complexes as has been reported in PECAM-1-mediated inter-endothelial adhesions (25).

We speculate that the lateral membrane accumulation of ECRTP/DEP-1 may reflect interaction of its extracellular domain with a putative ligand expressed on contacting membranes that functions to redistribute receptors or stabilize them in ligand-receptor complexes created through juxtacrine engagement. Certainly there is evidence available that membrane-associated receptor tyrosine phosphatase activity is increased in cultured cells, including endothelial cells, that are in close contact (26,27). Recent findings suggest that ECRTP/DEP-1 may engage in homophilic interactions, because the fibronectin type III repeat domains of the neural adhesion molecule TAX-1 are reportedly necessary and sufficient for homophilic binding (28).

The apical membrane distribution of ECRTP/DEP-1 in arterial and apparently in capillary endothelium is intriguing, particularly in the context of data showing that platelets and all hematopoietic lineages express ECRTP/DEP-1 (29). If homophilic interactions between ECRTP/DEP-1 are possible between endothelial cells and circulating cells that may encounter it on luminal membranes of intact vessels, it seems likely that regulatory factors, or coreceptors, on each of the engaging cells may be important in modulating any downstream responses. Resolution of these issues awaits definition of the ligand and consequences of its engagement of receptor.

Finally, our data assessing the developmental pattern of ECRTP/DEP-1 expression on cells that contribute to assembly

Figure 5. Confocal localization of ECRTP/DEP-1 and VE-cadherin in developing murine kidney vasculature. Acetone-fixed neonate kidney sections were labeled with ECRTP.Ab1 (green) and anti-VE-cadherin (red) as described in Materials and Methods. ECRTP.Ab1 staining substantially overlapped to VE-cadherin distributions. Many VE-cadherin-positive cells (arrowheads) incorporating into early S-shaped developing glomeruli were also expressing ECRTP/DEP-1 (evident by yellow staining in C), as were isolated mesenchymal cells in the yet undeveloped neocortex. Magnification: ×400.
Figure 6. ECRTP/DEP-1 distributes to endothelial membranes at apical and inter-endothelial sites in cultured human endothelial cells, but does not dissociate from junctions with VE-cadherin. (A) Methanol-fixed RMEC cells were labeled with ECRTP.Ab1 or ECRTP.Ab2 as described in Materials and Methods. These antibodies brightly labeled endothelial membranes of RMEC cells, whereas class-matched antibody did not. Serial confocal images (Panels d, e, and f) labeled with ECRTP.Ab2 suggested that ECRTP/DEP-1 is distributed between points of inter-endothelial membrane contact and punctate regions of the apical membrane. HMEC-1 cells were transiently transfected with ECRTP/DEP-1/HA expression plasmids and stained with HA antibody detecting transfected (T) cells (Panel g). The accumulated signals were seen at the cell-cell contact points between transfected (T) and nontransfected cells (NT), and X-Z axis image of hatched line (top image) showed that overexpressed ECRTP/DEP-1/HA is distributed to cell-cell contact points. (B) HMEC-1 cells were grown to confluence, then incubated with media containing 5 mM ethyleneglycol-bis-aminoethylether-N,N′,N′,N′-tetra-acetic acid for 0 min (Panels a and c) or 20 min (Panels b and d) before fixation. The distributions of ECRTP.Ab2 and anti-VE-cadherin labeling were examined as described in Materials and Methods at each time point. While the distribution of ECRTP/DEP-1 immunoreactivity was not dramatically altered in the low Ca²⁺ medium, junctional VE-cadherin staining dissipated, consistent with dissociation of VE-cadherin junctions and redistribution across the cell membrane.
of the glomerular capillary network offer insight about possible roles for this receptor in this coordinated process. Receptor tyrosine phosphatases of the ECRTP/DEP-1 subclass, including DPTP10D, have been assigned important roles in the targeting of neurons to correct destinations during development (11). Previous reports have identified ECRTP/DEP-1 expression in hematopoietic progenitors, including erythroid, lymphoid, and myeloid series lineages (29). Furthermore, it appears that ECRTP/DEP-1 can function to promote differentiation of erythroid lineage cells that express it (30). With accumulating evidence that hemangioblasts serve as common precursors of both hematopoietic and vascular endothelial lineages, it now appears that ECRTP/DEP-1 expression is initiated early in the ontogeny of these precursors. At present, it is difficult to evaluate the biologic significance of growth inhibitory roles suggested in mammary carcinoma cells where DEP-1 is induced during differentiation and where overexpression inhibited cell growth (14). Additional studies will evaluate the importance of inter-endothelial engagement of ECRTP/DEP-1 in endothelial differentiation, glomerular capillary assembly, and cell contact-mediated growth arrest.

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