Glomerular Endothelial Fenestrae In Vivo Are Not Formed from Caveolae

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Abstract. Previous reports indicate that endothelial fenestrae in vitro can form by fusion of caveolae or caveolae-like vesicles. The principal aim of this study was to determine whether formation of glomerular endothelial cell fenestrae in vivo similarly involves caveolae and caveolin-1. Whereas caveolin-1 immunofluorescence was found around the circumference of human and mouse glomerular capillary loops, it co-localized only partially with the endothelium-specific lectin Ulex Europaeus I in human glomeruli, leaving portions of the endothelium devoid of caveolin-1. Immunogold electron microscopy, used to definitively localize caveolin-1 in glomeruli, showed that caveolin-1 was completely excluded from the fenestrated portion of the endothelium. Moreover, in caveolin-1–deficient mice, which cannot form caveolae, the ultrastructure of glomerular endothelial fenestrae appeared entirely normal. Interestingly, strong caveolin-1 immunogold labeling was observed in podocytes, where some caveolin-1 localized to filtration slits. Caveolin-1 co-immunoprecipitated with the podocyte slit diaphragm proteins nephrin and CD2AP, and dual immunofluorescence confirmed co-localization of caveolin-1 and nephrin. Nevertheless, in caveolin-1–deficient mice, podocyte ultrastructure appeared normal, and the podocyte proteins synaptopodin, nephrin, and podocin were expressed normally. In addition, blood urea nitrogen concentrations and urinary protein excretion in these mice were similar to those in wild-type mice. Thus, unlike caveola formation, glomerular endothelial cell fenestrae formation in vivo does not require caveolin-1, ruling out the previous hypothesis that endothelial fenestrae represent fused caveolae, at least for glomerular endothelial cells. Localization of caveolin-1 to podocytes and their filtration slits is consistent with the view that the filtration slit plasma membrane represents a type of lipid raft microdomain.

The endothelium lining glomerular capillaries is extremely flattened and densely perforated by fenestrae. Glomerular endothelial cell fenestrae are plasma membrane–lined transendothelial pores that measure 60 to 100 nm in diameter. They permit rapid flux of water and small solutes across the glomerular barrier (1,2). The molecular composition of glomerular endothelial cell fenestrae and the mechanisms regulating their formation are as yet poorly understood.

Several lines of evidence have suggested that endothelial fenestrae form through fusion of caveolae. Caveolae are diaphragmed, cholesterol-rich vesicular plasma membrane invaginations with a lipid composition similar to that of lipid raft microdomains (3). They contain high levels of the caveola-specific protein caveolin-1, which is required for their formation (4,5). Ultrastructural studies of cultured endothelial cells show that organelles resembling caveolae and containing caveolin-1 can undergo fusion to form transendothelial channels (6–8), a postulated stage in fenestrae formation. Like caveolae, most endothelial cell fenestrae in vivo contain bridging diaphragms (9,10), and their peristomal plasma membrane is cholesterol-rich, as in caveolae (11). Furthermore, the protein PV-1, initially isolated from the apical plasma membrane of pulmonary endothelium (12), is specifically associated with the bridging diaphragms of endothelial fenestrae and caveolae (9). In regard to this study, it is notable, however, that PV-1 was uniquely absent from glomerular endothelial cell fenestrae (9), possibly due to the lack of or a different molecular composition of fenestral diaphragms in these cells.

VEGF-mediated induction of endothelial fenestrae has also been linked to fusion of caveolin-1–containing vesicles (13). Typically, VEGF is highly expressed in epithelial cells neighboring fenestrated endothelia (14), and VEGF can rapidly induce fenestrae in capillaries in vivo (15). VEGF-mediated induction of fenestrae is accompanied by an increase in the number of fused caveolar vesicles (16), and such vesiculo-vacuolar organelles express caveolin-1 (13). Recently, Chen et al. (6) similarly found that caveolin-1–lined transendothelial channels can be induced by VEGF in cultured glomerular endothelial cells in vitro. However, Esser et al. (16), who observed caveolin-1 in fusing caveolae in vitro, noted that caveolin-1 is absent from fenestrae of cultured endothelial cells and fenestrae of choroid plexus endothelium in vivo.
Taken together, the data reported so far suggest that both caveolae and fenestrae are spanned by diaphragms and that the protein PV-1 is a component of caveolar diaphragms and diaphragms of some but not all fenestrae. Furthermore, fusion of caveolin-1-containing caveolae occurs in response to VEGF in vitro, and such fused caveolae have been reported to form transendothelial channels/fenestrae. However, fenestrae have not been shown to contain caveolin-1. If caveolae are indeed precursors of fenestrae, it would be expected that caveolin-1-deficient mice, which cannot form caveolae (4,5), would also exhibit defects in their fenestrae.

To determine whether glomerular endothelial fenestrae in vivo represent fused caveolae, we explored caveolin-1 localization in mouse and human renal glomeruli in situ and determined the ultrastructure of glomerular endothelial fenestrae in caveolin-1-deficient mice. We report here that caveolin-1 is expressed by glomerular endothelial cells but is excluded from the fenestrated region of these cells. Furthermore, although caveolae do not form in caveolin-1-deficient mice, glomerular endothelial cell fenestration is normal, implying that glomerular endothelial cell fenestrae in vivo do not form through fusion of caveolae.

Although the emphasis of the study was on the role of caveolin-1 in fenestrae formation, we also noted that caveolin-1 is highly expressed by podocytes. Caveolin-1 localizes, at least partially, to podocyte filtration slits and interacts with the podocyte slit diaphragm proteins nephrin and CD2AP, findings consistent with the lipid raft nature of the podocyte filtration slit plasma membrane.

Materials and Methods

Tissue Preparation

For immunofluorescence and immunoelectron microscopy studies, mouse kidneys (strain 129, male, 36- to 50-d-old; Charles River Laboratories, Wilmington, MA) were perfusion-fixed with 2% paraformaldehyde in PBS for 3 min followed by perfusion with 18% sucrose for 5 min. Sections of the perfusion-fixed tissues were frozen in OCT compound over dry ice. For transmission electron microscopy studies, mouse kidneys (strain 129, male, 36- to 50-d-old; Charles River Laboratories, Wilmington, MA) were perfusion-fixed with 2% paraformaldehyde and 0.5% tannic acid and counterstained with 2% osmium tetroxide. This was followed by staining with 1% uranyl acetate in dd H2O. The sections were air-dried in RT and then observed with a JEOL 1200EX transmission electron microscope.

Immunogold Electron Microscopy

Ultrathin (100 to 120 nm) frozen sections were prepared from perfusion-fixed mouse kidneys on a Leica Cryo Ultra Cut cryostat. Blocking was done with 2% FCS, 2% BSA, and 0.2% fish gelatin in PBS for 30 min at RT. Polyclonal rabbit anti-caveolin-1 IgG at a concentration of 1 μg/ml for 30 min at room temperature. After washing, some of the sections were incubated with rhodamine-labeled UEA at a concentration of 10 μg/ml for 30 min at RT. For experiments in caveolin-1-deficient mice, synaptopodin monoclonal antibody was applied for 1 hr (1:100) at RT, the sections were washed and polyclonal anti-nephrin antibody (1:100) was applied for 1 hr. After washing, Cy3 anti-mouse IgG (1:150) and Alexa 488 anti-rabbit IgG (1:500) were applied for 45 min at RT. For experiments in caveolin-1-deficient mice, synaptopodin monoclonal antibody was applied for 1 hr (1:100), nephrin (1:100), and caveolin-1 (1:200) antibodies were applied to frozen kidney sections of both wild-type and caveolin-1-deficient mice for 1 h. As secondary antibody, Alexa 488 anti-rabbit IgG (1:500) was applied for 45 min at RT. The blocking and washing steps were done as described above. The sections were mounted using ProLong Antifade kit (Molecular Probes). They were then evaluated by confocal microscopy using a Bio-Rad MR600 confocal fluorescence microscope.

Morphometry

The density of caveolin-1 immunogold labeling was determined for tissue sections from three separate mice processed independently. Digital photomicrographs (magnification, ×15,000 to ×30,000) were overlaid with a grid (5 × 5 mm). The area representing podocyte cell body, podocyte foot processes, endothelial cell body, fenestrated portion of endothelium, and background for each photomicrograph was determined by point-counting, using the intersects of the grid as evenly spaced points. Counts from each photomicrograph were converted to μm2 by correcting for the magnification. Gold particles associated with each structure were then quantified and expressed per μm2. Data represent the mean gold particle density/μm2 for each structural component from 4 to 10 photomicrographs per animal. Comparisons were made by the t test.
Transmission Electron Microscopy

Immersion-fixed mouse kidneys were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, postfixed with 1% osmium tetroxide followed by 1% uranyl acetate, dehydrated through a graded series of ethanol, and embedded in LX112 resin (LADD Research Industries, Burlington, VT). Ultrathin sections were cut on a Reichert Ultracut E, stained with uranyl acetate followed by lead citrate, and viewed on a JEOL 1200EX transmission electron microscope at 80 kV.

Protein Extraction and Immunoprecipitation

Glomeruli from adult C57 black mice were isolated as described in (17). They were pelleted by centrifugation for 5 min at 1000 x g at 4°C and resuspended in homogenization buffer (20 mM Tris, 0.1% NP-40, 150 mM NaCl, pH 7.5) supplemented with protease inhibitors as described (18). Glomeruli were then homogenized at 4°C with 15 strokes of a Dounce homogenizer. Insoluble material was pelleted at 14,000 x g for 10 min at 4°C and reprocessed once as described. The soluble material from both extractions was pooled and passed 20 times through a 27-gauge needle before use. For each immunoprecipitation, samples containing 0.5 mg protein were precleared with protein A-sepharose 4B (Sigma, St. Louis, MO) in 0.5 ml TNE (250 mM NaCl, 5 mM EDTA, 10 mM Tris, protease inhibitors, pH 7.4) for 1 h at 4°C. The extracts were then incubated with 1 µg of anti-nephrin IgG (19), anti-CD2AP IgG (20), or anti-snRNP IgG (Progen, Heidelberg, Germany) for 2 h at 16°C in the presence of 0.4% SDS. Alternatively, the extracts were incubated with 2 µg of caveolin-1 IgG (N-20, Santa Cruz) or 1 µg of caveolin-1 IgG (2234, BD Transduction Laboratories) overnight at 4°C in the presence of 0.4% SDS. The SDS was included to disrupt lipid-protein interactions in this raft microdomain fraction. The nonspecific aggregates were pelleted at 14,000 x g for 5 min at 4°C. Protein A/G-sepharose 4B beads were then added to the supernatants and incubated for 90 to 180 min at 4°C. The beads were washed five times in TNE buffer and eluted by boiling at 95°C for 5 min in Laemmli sample buffer (21) containing 5% β-Mercaptoethanol.

SDS-PAGE and Western Blotting

Samples containing equal protein concentrations were separated on 4 to 15% Tris-HCl gradient gels (Bio-Rad, Hercules, CA) in a standard manner. The resolved proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). The blots were blocked in 5% nonfat dry milk in TBS-Tween for 1 h at room temperature followed by overnight incubation with rabbit polyclonal anti-caveolin-1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) or monoclonal anti-eNOS (1:800; BD Transduction Laboratories, Lexington, KY) at 4°C. Alternatively, the blots were incubated with rabbit polyclonal antinephrin (1:2000) or rabbit polyclonal anti-CD2AP (1:2000). After washing in TBS-Tween (3 times for 10 min), horseradish peroxidase-conjugated donkey anti-rabbit IgG or donkey anti-mouse IgG at a concentration of 1:4000 was applied (in TBS-Tween) for 1 h. Bands were visualized using enhanced chemiluminescence (Renaissance; NEN Life Science Products, Boston, MA).

Blood Urea Nitrogen Assay

Blood was collected from the clipped tail of three female caveolin-1−/− deficient and three female littermate wild-type mice, all 3 mo of age. Serum was prepared by centrifugation (6000 x g; 6 min at RT). Three separate assays (BUN Infinity Reagent; Sigma, St Louis, MO) were run according to the manufacturer’s instructions. Standard concentrations of urea were used for reference.

Urinary Protein Excretion

Urine was collected from the same three female caveolin-1−/− deficient and three littermate wild-type mice. The urinary protein excretion was evaluated by electrophoresis of the urine samples on a 4 to 15% gradient SDS-PAGE gel followed by staining with Coomassie blue in a standard manner. Albumin (60 kD; 0.1 µg/µl) was included as a molecular size control.

Results

Caveolin-1 Expression in Mouse and Human Kidney Cortex

We first reasoned that caveolin-1 might be expressed in the fenestrated region of glomerular endothelial cells if fenestrae are indeed derived from fused caveolae. Indeed, by immunofluorescence, caveolin-1 immunoreactivity was observed around the circumference of glomerular capillary loops in mouse (Figure 1, I-A, thin arrows) and human (Figure 1, I-B) glomeruli, consistent with previously published work (22). Staining was also observed in collecting duct (Figure 1, I-A, thick arrow) and in glomerular arterioles (Figure 1, I-B, arrow) in both species. No staining was detected in control sections treated with secondary fluorescence antibody or blocking solution in the absence of primary antibody (data not shown).

To determine whether the circumferential labeling of glomerular capillary loops by caveolin-1 antibodies observed in Figure 1I represents endothelial cell caveolin-1 expression, dual labeling with an endothelial cell-specific marker was explored. Human glomeruli were therefore co-labeled with caveolin-1 antibodies and the endothelial-specific lectin UEA. Representative glomerular loops from human cortex show circumferential staining of the endothelial marker UEA (Figure 1, II-A and II-D). Caveolin-1 staining is observed in the same glomerular loops with a characteristic punctuate pattern (Figure 1, II-B and II-E). However, dual labeling with caveolin-1 and UEA reveals only partial co-localization of the two markers (Figure 1, II-C and II-F, arrows). Whereas UEA staining always followed the glomerular capillary loop contour, caveolin-1 immunoreactivity was also observed outside the loops (Figure 1, II-C and II-F, arrowheads). These findings suggest that caveolin-1 is excluded from a portion of the endothelium and that podocytes, which line the extraluminal surface of glomerular capillaries, may also express this protein.

Glomerular Caveolin-1 Localization by Immunogold Electron Microscopy

To definitively localize caveolin-1 expression in glomerular capillary cells, immunogold labeling followed by transmission electron microscopy was performed. Caveolin-1 immunogold labeling was observed in glomerular endothelial cell bodies (EB) (Figure 2, I-B) but never in the fenestrated portion of the glomerular endothelium, (Figure 2, I-A through I-C). Caveolin-1 immunogold labeling was also observed in podocytes, both in the podocyte cell body and in foot processes (Figure 2, I-A through I-C). In foot processes, caveolin-1 was often, but not invariably, found at the filtration slit (Figure 2, I-C). This
Immunofluorescence studies. I: Frozen sections from mouse (I-A) and human (I-B) kidney were labeled with rabbit polyclonal anti-caveolin-1 IgG. The peripheral loop pattern (small arrows, I-A) of caveolin-1 expression in glomeruli is similar in the two species. Glomerular arterioles (I-B, large arrow) and collecting duct (I-A, large arrow) were also stained. II: Human kidney cortex sections were labeled with Ulex Europaeus Agglutinin I (UEA) (II-A, II-D) and caveolin-1 antibody (II-B, II-E). Partial co-localization of UEA and caveolin-1 was observed (II-C and II-F, arrows). However, portions of endothelium labeled with UEA were devoid of caveolin-1 immunoreactivity (*); conversely, caveolin-1 was found without UEA in other areas (arrowheads). Thus, caveolin-1 is not exclusively localized to the endothelium of the glomerular capillary loops. Omission of the primary antibody yielded no staining (data not shown).
is the first demonstration that caveolin-1 is highly expressed by renal glomerular podocytes.

Morphometric analysis of immunogold density in three separate mice showed that caveolin-1 immunogold labeling of the fenestrated portion of the glomerular endothelium was similar to background, whereas immunogold labeling was much greater than background ($P < 0.01$) in the endothelial cell bodies (Figure 2, II-A). The total area counted for fenestrated and nonfenestrated portions of endothelium was 6.27 and 11.33 $\mu m^2$, respectively, whereas the total background area counted was 168.63 $\mu m^2$. Hence, the low level of labeling in the fenestrated portion of endothelium is not accounted for by evaluation of a much smaller area than that for the endothelial cell body.

The caveolin-1 immunogold density for podocytes is shown in Figure 2, II-B. The total area of podocyte foot processes and cell bodies counted was 16.25 and 31.74 $\mu m^2$, respectively. Some but not all of the caveolin-1–linked gold particles were found at the podocyte filtration slit.

**Glomerular Ultrastructure and Function in Caveolin-1–Deficient Mice**

To obtain more conclusive evidence of whether caveolae are required glomerular endothelial cell fenestrae precursors, we examined the morphology of the glomerular endothelium in caveolin-1–deficient mice. These mice have previously been extensively characterized (4); they neither express caveolin-1, nor can they form caveolae. Transmission electron microscopy revealed that glomerular endothelial cell fenestrae are indistinguishable in density and size in the caveolin-1–deficient mice compared with wild-type mice (Figure 3, I-A and I-B, $n = 3$/group).

In the caveolin-1–deficient mice, the glomerular basement membrane and podocyte ultrastructure also appeared normal (Figure 3, I-A and I-B). Caveolae were observed in podocytes and endothelial cells of wild-type (Figure 3, I-C), but not caveolin-1–deficient mice. Immunofluorescence staining for the podocyte proteins synaptopodin (Figure 3, II-A and II-B), podocin (Figure 3, II-C and II-D), and nephrin (Figure 3, II-E and II-F) did not differ between wild-type and caveolin-1–deficient mice. As expected, caveolin-1 was completely absent in the caveolin-1–deficient mice (Figure 3, II-H) compared with wild-type (Figure 3, II-G). To determine whether there is any defect in glomerular function in the caveolin-1–deficient mice, urinary protein excretion was determined using Coomassie blue staining of polyacrylamide gels after urine protein electrophoresis. Albumin (0.1 $\mu g/\mu l$) was included as a size control. The urinary albumin concentration was similar in wild-type and caveolin-1–deficient mice (Figure 3, III). In addition, the blood urea nitrogen concentration did not differ between the caveolin-1–deficient and the wild-type mice (Figure 3, IV). Hence, even though there is significant caveolin-1 expression in podocytes in normal mice, the absence of caveolin-1 has no evident effect on podocyte ultrastructure or on glomerular function.

**Interaction of Caveolin-1 with Nephrin and CD2AP**

We observed some caveolin-1 localization to podocyte filtration slits; therefore, we evaluated nephrin and CD2AP immunoprecipitates from whole mouse glomerular lysates for the presence of caveolin-1. The immunoprecipitation was done in the presence of SDS to preclude nonspecific associations of these proteins due to their location in lipid raft microdomains (18,23). Western blotting with caveolin-1 antibody of nephrin and CD2AP immunoprecipitates shows that caveolin-1 is co-immunoprecipitated from the lysates with both nephrin and CD2AP antibodies (Figure 4, I-A, lanes 2 and 4) but not with antibodies to the nuclear protein snRNP (Figure 4, I-A, lane 6). Also, the supernatants of the nephrin and CD2AP immunoprecipitates contained almost no detectable caveolin-1 (Figure 4, I-A, lanes 3 and 5), whereas caveolin-1 remained in the supernatant after immunoprecipitation with snRNP (Figure 4, I-A, lane 7). To control for possible nonspecific interaction of non-podocyte caveolin-1 with nephrin and CD2AP in the lysates, we determined whether nephrin and
CD2AP immunoprecipitates contain eNOS, a protein known to interact with caveolin-1 in endothelial cells. As shown in Figure 4 (I-B, lanes 2 and 4), eNOS was not immunoprecipitated with anti-nephrin or anti-CD2AP antibodies but remained in the lysates. In addition, immunoprecipitates prepared with anti-caveolin-1 polyclonal or monoclonal antibodies were probed for the presence of nephrin and CD2AP. This experiment was conducted in a similar manner to the previous co-immunoprecipitations. Immunoprecipitates prepared with anti-caveolin-1 polyclonal antibodies and blotted for nephrin (Figure 4, II-A) or CD2AP (Figure 4, II-B) show co-immunoprecipitation of both proteins with caveolin-1 (Figure 4, II, lane 2 in A and B). Not shown, immunoprecipitates prepared with monoclonal anti-caveolin-1 antibodies gave identical results. It therefore seems unlikely that the results in Figure 4, I-A, can be explained by nonspecific association of endothelial cell caveolin-1 with nephrin or CD2AP.

Immunofluorescent labeling of glomeruli with anti-nephrin and anti-caveolin-1 antibodies revealed a peripheral loop pattern (Figure 4, III-A and III-B, respectively). In many loops, nephrin and caveolin-1 immunoreactivity co-localized (Figure 4, III-C), confirming that nephrin and caveolin-1 interact in podocytes.

**Discussion**

The principal aim of this study was to determine whether renal glomerular endothelial cell fenestrae represent fused caveolae as had previously been suggested by Palade and coworkers (8,9), and recently again by Chen et al. (6). Although we found that caveolin-1 antibodies labeled glomerular capillary loops circumferentially in mouse and human glomeruli, immunoelectron microscopy revealed that the circumferential pattern was due in large part to labeling of podocytes with caveolin-1 antibodies. By contrast, caveolin-1 immuno-
reactivity was not observed in the fenestrated portion of glomerular endothelial cells. Furthermore, in caveolin-1–deficient mice, which lack caveolae, glomerular endothelial fenestrae ultrastructure was normal, and renal function in these mice did not differ from their wild-type controls. These findings indicate that glomerular endothelial cell fenestrae in vivo do not represent fused caveolae.

The view that endothelial fenestrae could represent fused caveolae was not supported by the data. Immunoprecipitation studies showed that caveolin-1, nephrin, and CD2AP were co-immunoprecipitated under certain conditions, but not under others. Moreover, eNOS, which is not present in podocytes, did not co-immunoprecipitate with nephrin or CD2AP.

Figure 4. Association of caveolin-1 with the podocyte slit diaphragm-specific proteins nephrin and CD2AP. I: Immunoprecipitates from mouse glomerular lysates were prepared with anti-nephrin, anti-CD2AP, and anti-snRNP IgG (negative control) and probed with anti-caveolin-1 (I-A); lane 1, endothelial lysate (positive control for caveolin-1 molecular size); lane 2, anti-nephrin immunoprecipitate; lane 3, supernatant of the nephrin immunoprecipitate; lane 4, anti-CD2AP immunoprecipitate; lane 5, supernatant of the CD2AP immunoprecipitate; lane 6, anti-snRNP immunoprecipitate; lane 7, supernatant of the anti-snRNP immunoprecipitate. Hence, caveolin-1 was co-immunoprecipitated with nephrin and CD2AP but not with snRNP. To rule out nonspecific co-immunoprecipitation with endothelial caveolin-1, Western blots were also probed with eNOS antibodies (I-B). eNOS is not present in podocytes and therefore should not co-immunoprecipitate with nephrin or CD2AP; lane 1, endothelial lysate (positive control for eNOS molecular size); lane 2, anti-nephrin immunoprecipitate; lane 3, supernatant of the nephrin immunoprecipitate; lane 4, anti-CD2AP immunoprecipitate; lane 5, supernatant of the CD2AP immunoprecipitate. Hence, endothelial eNOS does not co-immunoprecipitate with nephrin or CD2AP. II: Immunoprecipitates from mouse glomerular lysates were prepared with anti-caveolin-1 and probed with anti-nephrin (II-A); lane 1, glomerular lysate; lane 2, anti-caveolin-1 immunoprecipitate; lane 3, supernatant of the caveolin-1 immunoprecipitate. The immunoprecipitates were also probed with CD2AP (II-B); lane 1, glomerular lysate; lane 2, anti-caveolin-1 immunoprecipitate; lane 3, supernatant of the caveolin-1 immunoprecipitate. Hence, nephrin and CD2AP was co-immunoprecipitated with caveolin-1. III: Human kidney cortex sections were labeled with rabbit polyclonal anti-nephrin antibody (II-A) and mouse monoclonal anti-caveolin-1 antibody (II-B). Both nephrin and caveolin-1 antibodies labeled peripheral capillary loops circumferentially. Frequent co-localization of caveolin-1 and nephrin immunoreactivity was observed (II-C, arrows). Omission of the primary antibody yielded no staining (data not shown).
caveolae came from the observations that VEGF, known to induce fenestrae in vivo (15,24), also stimulates fusion of caveolae in endothelial cells to form caveolin-1–containing vesiculo-vacuolar structures (13,16) and caveolin-1–lined transendothelial channels (6) in vitro. Furthermore, the protein PV-1 is found in both the bridging diaphragms of endothelial caveolae and in the fenestrae of most endothelia (9). However, Esser et al. (16) reported that caveolin-1 was not present in the fenestrated region of choroid plexus endothelial cells in vivo or in fenestrae of cultured endothelial cells. Our finding showing that caveolin-1 is excluded from glomerular fenestrae is consistent with the observation by Esser et al. (16) for choroid plexus. We also find that fenestrae ultrastructure is normal in caveolin-1–deficient mice. Caveolin-1 is essential for caveole formation (4,5); therefore, glomerular fenestrae must be distinct from endothelial caveolae, and glomerular fenestrae formation must proceed through mechanisms other than caveolar fusion.

Our study does not rule out the possibility that fenestrae in glomerular endothelial cells differ from those in other fenestrated endothelia. In fact, studies with cationic markers have long suggested that the composition of endothelial fenestral diaphragms differ in different organ beds (25). Also, the observation that the caveolar diaphragm protein PV-1 localizes to peritubular fenestral diaphragms but not glomerular fenestrae is consistent with the view that the molecular composition of bridging diaphragms differs between renal peritubular and glomerular fenestrae or that glomerular endothelial fenestrae do not contain bridging diaphragms (9). Therefore, it is still possible that caveolar fusion could form fenestrae in nonglomerular endothelial cells.

Caveolin-1 immunoreactivity in glomeruli has previously been described and was thought to represent mainly endothelial cell expression (22). We found a similar pattern of caveolin-1 immunofluorescence as that reported by Breton et al. (22), (Figure 1, I-A and I-B) but noted that the endothelial-specific marker UEA co-localized only partially with caveolin-1 (Figure 1, II-C and II-F). We postulate that those regions labeled with UEA but not with caveolin-1 likely represent the fenestrated portion of the glomerular endothelial cells. The regions labeled by caveolin-1 antibodies but not by UEA could represent podocyte or mesangial cell labeling. Indeed, Tamai et al. (26) recently reported that caveolin-1 is present in mesangial cells. Still, the pattern of glomerular caveolin-1 immunofluorescence (Figure 1) does not suggest a predominant mesangial location. Using caveolin-1 immunogold electron microscopy, we find that caveolin-1 is expressed at high levels in glomerular podocytes. Caveolin-1 expression in podocytes has not previously been described.

Caveolin-1 immunogold localization in podocytes was often, but not exclusively, found at the podocyte filtration slits (Figure 2, I-C). The filtration slit proteins, including nephrin (19), CD2AP (27), and podocin (18), are associated with lipid rafts (23). These filtration slit proteins all are required to maintain the glomerular barrier to protein filtration, as deletion of nephrin, CD2AP, and podocin all result in congenital proteinuria and progressive focal and segmental glomerular sclerosis (20,28,29). Caveolae, like membrane regions at the filtration slit, also represent a type of lipid raft microdomain; therefore, we explored whether caveolin-1 can associate with some of the known slit diaphragm proteins. Immunoprecipitation of nephrin and CD2AP from glomerular lysates resulted in co-immunoprecipitation of caveolin-1, (Figure 4, I-A). By contrast, eNOS, known to be associated with caveolin-1 in endothelial cell caveolae (30), did not co-immunoprecipitate with nephrin or CD2AP (Figure 4, I-B). Hence, nonspecific association of endothelial caveolin-1 with nephrin or CD2AP in the glomerular lysates was ruled out. Immunoprecipitation of caveolin-1 from glomerular lysates resulted in co-immunoprecipitation of both nephrin and CD2AP as well, further validating the experiments described above (Figure 4, II-A and II-B). In addition, dual labeling with anti-nephrin and anti-caveolin-1 antibodies results in partial co-localization of the antibodies around the capillary loops of the glomeruli, further increasing the evidence for nephrin and caveolin-1 association (Figure 4, III-C). Nevertheless, podocyte foot process ultrastructure was normal in mice deficient in caveolin-1, no increase in urinary protein concentration was observed, and their BUN was normal (Figure 3). In addition, expression of the podocyte proteins, synaptopodin, podocin, and nephrin, did not differ between caveolin-1–deficient mice and their wild-type controls. It is therefore evident that caveolin-1 is not critical for the formation or function of podocyte slit diaphragms. The exact role of caveolin-1 in podocyte slit diaphragm function remains to be defined.

In conclusion, caveolin-1 is not found in glomerular endothelial cell fenestrae, nor is it necessary for the formation of normal fenestrae. Hence, the possibility that endothelial fenestrae represent fused caveolae has been ruled out, at least for glomerular endothelial cells. The interaction of caveolin-1 with nephrin and CD2AP in podocytes is consistent with the previous findings that the filtration slit plasma membrane represents a type of lipid raft microdomain.

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