Multiple PV1 dimers reside in the same stomatal or fenestral diaphragm

Radu V. Stan
Department of Cellular and Molecular Medicine, University of California-San Diego, La Jolla, California 92093-0651

Submitted 25 September 2003; accepted in final form 19 November 2003

One of the most critical functions of the vascular endothelium is to mediate microvascular permeability. The molecular mechanisms underlying this function are still largely unknown in both normal and disease states. Several endothelial subcellular structures have been involved in these processes: caveolae, transendothelial channels (TECs), fenestrae, vesiculovacuolar organelles (VVOs), and stomatal diaphragms. The intimate structure of these diaphragms has been shown to consist of a meshwork formed by radial fibrils. We have recently shown that PV1 is a key structural element of both types of diaphragms, with its expression being sufficient to form de novo stomatal and fenestral diaphragms in both endothelial and nonendothelial cell types in culture. We have further tested the role of PV1 in the structure of the diaphragms and demonstrate here that multiple PV1 homodimers reside in close proximity within the same diaphragm. Our data bring further support to the paradigm by which PV1 dimers would form the fibrils of the diaphragms with a function in the microvascular permeability.

Recently, we have shown by EM that endothelial SDs and FDs share at least one protein, namely, PV1 (15, 16), which is their only known biochemical marker. The PV1 gene product is encoded by the PLVAP gene in humans, and its presence has been documented in select endothelium of other several other mammalian species including the mouse, rat, and bovine (14). PV1 is an endothelium-specific, single-span, type II membrane glycoprotein that forms dimers in situ. The size of the monomer lacking posttranslational modifications is 50 and 60 kDa in the N-glycosylated, mature form (4, 16). PV1 has a short intracellular tail and a long extracellular domain. Although there is no conserved known consensus site in the extracellular domain across mammalian species, the extracellular domain contains four N-glycosylation sites near the membrane, a proline-rich region near the COOH-terminus, and two large coiled-coil domains, suggesting a rod-like protein (14).

Probing into the function of PV1 within the diaphragms, we have also shown that PV1 is a key structural component necessary and sufficient for the formation of both SDs and FDs (for a review, see Ref. 12). This is sustained by several lines of evidence, such as (1) reagents that induce the de novo formation of the diaphragms upregulate PV1, which is found in the newly formed FDs and SDs; (2) treatment of cells with PV1 siRNA prevents the formation of caveolar SDs and the TEC and fenestrae altogether; and (3) moreover, overexpression of PV1 in both endothelial and nonendothelial cell types that do not express either PV1 or diaphragms results in de novo formation of caveolar SDs.

In summary, the diaphragms seem to be present on four distinct endothelial microdomains where their functions in biogenesis and regulation are not clearly understood. By their localization at sites of transendothelial exchanges, one could assume that they function either as a sieving device (opposing the exchanges of select molecules) or a facilitating device (enabling selective transport of other molecules) or both. PV1 is expressed in select endothelia, where it is a key structural component of SDs and FDs. As for the presence of PV1 on such diaphragms, it could function as either a structural component, function modulator component, or both. The data so far obtained point to an essential structural role with probability of PV1 participating in the radial meshwork of the diaphragms. It should be noted, however, that this does not exclude the possibility of diaphragm function modulation.

Here, we continued to address the role of PV1 within the endothelial diaphragms and show that PV1 forms homodimers that occur in several copies in close proximity within the same diaphragm. These data support the working hypothesis that we have recently developed by which PV1 dimers would form part of the radial fibrils demonstrated to occur in these diaphragms.
MATERIALS AND METHODS

Materials. The cross-linkers diethio bis(succinimidyl)propionate (DSP), 3,3′-dithiobis(succinimidyl)propionate (DTSSP), sulfosuccinimidyl-6-[3-(2-pyridyldithio)propionamido]hexanoate (sulfo-LC-SPDP), and ECL reagents were from Pierce-Endogen (Rockford, IL). BSA-agarose and colloidal gold were from Sigma (St. Louis, MO). All the other general reagents were as reported in our previous work (15–17).

Cells and culture. Human umbilical vein endothelial cells (HUVECs) were from Clonetics (San Diego, CA). COS7 and HEK293 cells were from the American Type Culture Collection (Manassas, VA). HUVECs were grown on gelatin-coated plasticware in EG2 growth medium and used at passages 3–10. HEK293 and COS7 cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics.

Antibodies. Anti-rat PV1 21D5 mAb and anti-ratPV1C pAb (α-rPV1C) were as previously described (16). α-rPV1C was made against a 12-amino acid COOH-terminal peptide (rat PV1C peptide) coupled to BSA, affinity purified on the rat PV1C peptide coupled to Affi-Gel10, followed by extensive cross-absorption on BSA-agarose. Anti-FLAG mAb (clone M2) and anti-FLAG-agarose were from Sigma (St. Louis, MO).

In situ cross-linking. The procedure is shown in Fig. 3. All the procedures involving animals were carried out under a protocol approved by the local Institutional Animal Care and Use Committee. Sprague-Dawley male rats (250–300 g) were anesthetized, followed by a thoracotomy, bolus administration of 200 units heparin in their right ventricle, and catheterization of the pulmonary artery. The blood was flushed by perfusion [5 min, room temperature (RT)] with PBS supplemented with 2 mM each of CaCl2 and MgCl2 (PBS/Ca/Mg) at a perfusion rate of 1.5 ml/min using a peristaltic pump. Next, the lungs were perfused (10 min, RT) with the cross-linker of choice dissolved in PBS/Ca/Mg. The perfusion time was determined in preliminary experiments as the minimal amount of time at which a high cross-linking efficiency was achieved. Excess cross-linker was flushed (10 min, RT) with 150 mM NaCl in 20 mM Tris·Cl, pH 7.4; this step also ensured the quenching of the remnant active sites of the cross-linkers via interactions with the amino groups of Tris. The lungs were homogenized (15 strokes, 1,800 rpm, 4°C) in a motor-driven Thomas type C Teflon pestle-glass homogenizer in 20 mM HEPES (pH 7.4), 25 mM sucrose, 5 mM MgCl2, 10 mM iodoacetamide, and protease inhibitor cocktail (containing AEBSP, pepstatin A, E-64, bestatin, leupeptin, and apropin) at a ratio of 1:4 (wt/vol) and homogenized. The homogenate was passed through a 30-μm Nytex nylon mesh, and the filtrate was centrifuged (10 min, 500 g, 4°C) to eliminate cell debris and nuclei. The postnuclear supernatant was further separated into a total membrane pellet and cytosolic fraction by centrifugation (1 h, 100,000 g, 4°C) with a SW60 rotor. The cytosolic fraction was discarded, and the membrane pellet was solubilized in either reducing (containing β-mercaptoethanol) or nonreducing SDS-PAGE sample buffer followed by protein separation by 6% SDS-PAGE, transfer to a polyvinylidene difluoride (PVDF) membrane, and immunoblotting with anti-rPV1 mAb clone 21D5 (4, 15).

Purification of cross-linked complexes. The in situ cross-linking procedure was carried out as described above except that the total membrane pellet was resuspended in 4 ml of 100 mM Na2CO3 (pH 11) by homogenization (10 strokes, 4°C) in a Thomas BB homogenizer at low speed, followed by collection of the insoluble material by centrifugation (1 h, 100,000 g, 4°C) with a SW60 rotor. The resulting pellet was resuspended (10 strokes, low speed, 4°C) in a Thomas AA homogenizer in 1 ml of solubilization buffer containing 0.5% SDS in 125 mM NaCl, 50 mM Tris·Cl (pH 7.4), 10 mM iodoacetamide, 2 mM EDTA, and protease inhibitors, followed by incubation (15 min, RT) under gentle rotation. The mixture was laid over a 10–40% (wt/vol) continuous sucrose gradient made in 50 mM Tris·Cl (pH 7.4) containing 0.2% SDS followed by centrifugation (16–20 h, 100,000 g, 18°C) with a SW60 rotor. Eight fractions (0.5 ml each) were collected from the bottom of the centrifugation tube. The fraction containing the heaviest cross-linked complexes (fraction 1) was diluted with 4 volumes of water and brought to 0.4% Triton X-100, 2 mM each CaCl2, MgCl2, and ZnCl2 final concentration. The PV1-containing complexes were isolated by incubation (O/N, 4°C) with 0.1 ml (settled gel) Griffonia simplicifolia I (GSI)-agarose, followed by three washes (10 min, 4°C) and heating (10 min, 40°C) in 200 μl of nonreducing SDS sample buffer containing 1% SDS, 1% Triton X-100. The cross-linked complexes were eluted on a 5% SDS-PAGE containing 8 M urea and stained with Coomassie G-250. The band at the bottom of the well and the one at the top of the resolving gel were excised, subjected to in-gel cleavage (2 h, 37°C) of the cross-linker in 50 μl of 50 mM diithiothreitol (DTT), followed by the separation of its protein components by 10% SDS-PAGE, and the proteins were visualized by silver staining.

Protein identification by mass spectroscopy. Proteins were resolved by SDS-PAGE, and the gels were stained with silver by a mass spectroscopy-compatible method (16). Gel slices containing the interesting bands were excised, destained, and in-gel digested with trypsin (Roche). The resulting peptides were extracted in acetonitrile, and the mass map of the mixture was obtained by Matrix Assisted Laser Desorption Ionization-Time of Flight. Sequence information was obtained by postsource decay for two or three representative peptides.

Constructs. Full-length PV1 cDNA was PCR amplified using pfu DNA polymerase (Stratagene; San Diego, CA) and forward 5'-TGAATTCATATGAATGCATGGAAGAC3' and reverse 5'-AAAGCTTCAGCCACTGTTGGCTTCATGAC3'-primers. The resulting DNA fragment was inserted using the EcoRI and SalI restriction sites (in bold) in the pIREs-hrGFP-1a or -2a bicistronic vectors (Stratagene) upstream and in frame with sequences encoding either 3xFLAG or 3xHA epitopes, respectively (the Start codon is underlined). These vectors also encoded for the humanized Revilis green fluorescent protein (hrGFP) gene.

Transfections. Cells were transfected by the calcium phosphate precipitation method (COS7, HEK293) or by using reagents such as Targeffect F1 (HUVEC, HEK293) and Targeffect F2 (CO7) (Targeting Systems; San Diego, CA) as per the manufacturer’s instructions. Transfection efficiency was judged by the percentage of cells expressing hrGFP.

Immunoprecipitations. At 48 h posttransfection, cells (plated in 60-mm dishes) were extracted for 1 h on ice in 1 ml of immunoprecipitation buffer (20 mM Tris·HCl (pH 7.5) containing 1% Triton X-100, 300 mM NaCl, 1 mM EDTA, and protease inhibitors cocktail (10 μg/ml each of leupeptin, pepstatin, o-phentanilin, E-64, and 1 mM PMSF)). The extract was clarified by centrifugation at 100,000 g for 1 h with a TL-A45 rotor at 4°C. The supernatants containing the extracted proteins were incubated (14 h, 4°C) end over end with 10 μl of either anti-HA-agarose or anti-Flag-agarose, as noted. The beads were collected by centrifugation (500 g, 5 min) and washed (3 × 5 min) with immunoprecipitation buffer, followed by one final wash in 50 mM Tris (pH 6.8). The beads were incubated (1 min, 95°C) in nonreducing SDS-PAGE sample buffer [2.3% SDS, 10% glycerol, and 62.5 mM Tris·HCl (pH 6.8)] and collected by centrifugation. The supernatant containing the solubilized antigen was saved, adjusted with 5% 2-mercaptoethanol, and boiled again for 2 min. The solubilized proteins were resolved by 8% SDS-PAGE, transferred to a PVDF membrane, and immunoblotted using either the anti-HA or anti-Flag mAb. Control experiments were carried out from cells that were either nontransfected or mock transfected with empty vector.

Conjugation of anti-ratPV1C pAb to 10-nm gold. Chicken anti-rat PV1C pAb was absorbed onto 10-nm monodisperse colloidal gold (obtained by tannic acid oxidation and reduction) by a method described elsewhere (10). The gold particles were absorbed onto affinity-purified anti-rat PV1C pAb (50 μg/ml) and diluted to AS20 = 0.8–1. Further stabilization of the gold suspensions was obtained by
incubation with 200 μg/ml poly-L-glutamic acid. Immediately before use, the concentrated IgG-Au stock solutions were diluted in PBS to give A520 = 0.2–0.3 and centrifuged for 1 h at 40,000 g to remove gold aggregates.

Immunogold labeling was performed by a preembedding method, as in our past work (15, 16). Rat lungs or kidneys were flushed (5 min, RT) free of blood followed by fixation in perfusion (10 min, RT) in 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Next, tissues were excised, trimmed into 3 × 3-mm blocks, further fixed (16–20 h at 4°C) in fresh fixative, cryoprotected in 2.3 M sucrose, snap frozen, and stored in liquid nitrogen. Thick sections (50 μm) were cut, rinsed in three changes (5 min, RT) in 0.07 M glycine in PBS and incubation (1–2 h, RT) in 10% fetal calf serum in PBS. This was followed by incubation (overnight, 4°C) with 10-nm gold-conjugated anti-rat PV1C pAb diluted in 1% BSA in PBS (PBSA). Sections were washed (3 × 30 min, RT) in PBSA, and the antibody-antigen complexes were stabilized by fixing (30 min, RT) in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). After osmication (1 h, on ice, in the dark) and incubation (30 min, RT, in the dark) in Kellenberger’s uranyl acetate, the sections were collected as a pellet, dehydrated in graded ethanol, and processed for LX-112 resin embedding. Sections (~50 nm thickness) were cut using a Reichert microtome, mounted on formvar-coated nickel or copper grids, poststained with uranyl acetate and lead citrate, and examined with a Philips CM 10 EM. Images were acquired using a Gatan charge-coupled device camera and processed using either DigitalMicrograph 3.0 (Gatan; Pleasanton, CA) or Adobe Photoshop software (Adobe Systems; San Jose, CA). The preembedding immunocytochemistry approach allowed a larger sampling with better preserved morphology than the classical labeling of ultrathin cryosections.

RESULTS

PV1 forms homodimers in situ. PV1 has been suggested to form dimers in situ by results obtained by immunoblotting in reducing and nonreducing conditions (4, 15). Silver staining of PV1 immunoprecipitated from rat lungs with anti-PV1 pAb showed a band at ~120 kDa in nonreducing conditions and a band at ~60 kDa in reducing conditions, which strongly suggests homodimer formation (15). However, the possibility of heterodimer formation with another protein of roughly the same size still remains valid and has not been formally addressed.

To clarify this, PV1 was tagged with either HA or Flag epitopes, and the resulting constructs were cotransfected into cells. If PV1 would form homodimers, both forms of tagged PV1 (i.e., HA and Flag) could be found in the same dimer. For this, we performed immunoprecipitation with either anti-HA or anti-Flag antibody followed by immunoblotting with either anti-Flag or anti-HA, respectively. As shown in Fig. 1A, a signal was detected only in the transfected cells compared with mock-transfected cells or cells transfected with only one of the constructs (Fig. 1A). This demonstrates that the monomers of PV1 tagged with different epitopes (i.e., HA and Flag) formed dimers, implying that PV1 indeed forms homodimers. To show that this is not a result of nonspecific binding, the samples were reduced and alkylated before immunoprecipitation. This disassociated the dimers found in the lysates, and no signal was detected by the immunoblotting (Fig. 1A) using the same strategy (e.g., precipitation with anti-HA and immunoblotting with anti-Flag). The data shown in Fig. 1, A and B, were obtained in HUVECs. The same results were obtained by transfections in COS7 or HEK293 cells (Fig. 1C).

PV1 can be cross-linked in situ. Localization studies by immunocytochemistry have suggested that several PV1 dimers reside in the same diaphragm (15, 16). To confirm this by another approach and to investigate whether PV1 has other interacting partners, we performed in situ cross-linking by perfusing several cross-linkers in the vasculature of either rat lungs or rat kidneys. The lungs were chosen as an exclusive source for caveolar SDs, the endothelial cells here being virtually devoid of fenestrae or TECs. The kidneys were chosen as a source of predominantly FDSs, because the endothelial cells here show the highest density of fenestrae and the lowest density for caveolae of all fenestrated endothelia (7). The cross-linkers used had precise properties such as 1) small dimensions, between 0.6 nm (sulfo-LC-SPDP) and 1.5 nm (DSP and DTSSP), compared with the diameters of SDs <40 nm and FDs ~62–68 nm. The small size of the cross-linker ensured that only proteins that were in close contact could be bound together, provided that free NH2 groups would be available; 2) homobifunctional and amine reactive as to react with the available NH2 groups of lysines. There are 32 lysines in the whole human PV1 molecule, all in the extracellular domain and all with a good chance of being exposed on the surface of the molecule (14 lysines in the extreme COOH-terminal quarter of PV1); 3) cleavable (i.e., by thiols) as to allow the analysis of the cross-linked proteins; and 4) membrane impermeant, as to limit the targets to the extracellular (luminal) domain of PV1 (such as DTSSP and sulfo-LC-SPDP). We also used a membrane-permeant cross-linker (i.e., DSP) for comparison.
The respective vascular beds were perfused at low pressure to maintain the vessels intact. The cross-linkers were allowed to react for only 10 min, after which they were flushed out and the remnant active sites were quenched using the free NH₂ groups of Tris. The tissues were fractioned into cytosolic and total membrane fractions in the presence of iodoacetamide (to prevent SH group exchange) and protease inhibitors. As PV1 is an integral membrane protein, only the proteins of the total membrane pellet were immunoblotted with anti-rPV1 antibodies. (15). By separating the proteins in nonreducing conditions, which keep the cross-linking molecules intact, we succeeded in shifting the molecular mass of rPV1 with each of the cross-linkers mentioned up to molecular masses that do not enter the resolving gel or even the stacking gel (Fig. 2, right). The molecular mass of the band (asterisks in Fig. 2, right) above the one of the rPV1 dimer (120 kDa) is ~240 kDa, which suggests that it consists of two cross-linked rPV1 dimers. When the proteins are separated in reducing conditions, which cleave the cross-linkers, all the high-molecular-mass bands collapsed to a 60-kDa band corresponding to the size of rPV1 monomer (Fig. 2, left). These data showed that rPV1 could be cross-linked in situ. The size of the bands obtained (i.e., multiples of the mass of rPV1 dimer) also suggested that several rPV1 dimers could reside in close proximity as to be cross-linked to each other.

Several PV1 dimers reside in close proximity within one diaphragm as revealed by in situ cross-linking. However, cross-linking is a nonspecific approach. PV1 could have been cross-linked to other molecules than itself. Also, the method used for determination of the size of the cross-linked bands is not precise enough to allow definitive conclusions. Therefore, we decided to purify PV1-containing cross-linked complexes and to analyze their protein content. The schematic of the procedure and the results obtained are given in Fig. 3, A and B–G, respectively. Briefly, the cross-linking procedure was as follows:

**Fig. 3.** Purification of PV1-containing cross-linked complexes. A: schematic of the PV1-containing very high molecular mass complex purification procedure. PNS, postnuclear supernatant. Each step is numbered to facilitate correspondence with the results (B–G). Western blotting with anti-rat PV1 antibodies on membrane proteins isolated from rat lungs after cross-linking by perfusion of either 3,3'-dithiobis(succinimidyl)propionate (lanes 1), diiodobis(succinimidyl)propionate (lanes 2), or sulfosuccinimidyl-6-[3-(2-pyridyl)thio]propionamido]hexanoate (lanes 3) cross-linkers is shown. The proteins were separated by SDS-PAGE in either reducing (left) or nonreducing (right) conditions, which either cleave or keep intact the cross-linkers, respectively. As seen on the right, each of the cross-linkers was effective in shifting the molecular mass of PV1 up to very high molecular mass sizes.

The direction of the sucrose gradient is indicated on the bottom of the blot. Left, protein content of fractions 1–3 as seen by Coomassie staining. *Presence of bands that are not PV1 positive in fraction 1. E: Western blotting showing the efficiency of binding of PV1-containing very high molecular mass complexes by *Griffonia simplicifolia* I (GS I) lectin (bound (B) and nonbound (NB)). F: Coomassie staining of GS I-bound proteins separated by SDS/urea-PAGE in nonreducing conditions. The arrowhead marks PV1-containing cross-linked complexes. *Bands that are not PV1 positive. G: component(s) of very high molecular mass resolved by SDS-PAGE and detected by silver staining. As seen in the right lane, PV1 (large arrow) is the most abundant (98% by densitometry) protein found in these complexes along with a few other minor components (smaller arrows).
linking and tissue homogenization and fractionation in the cytosol and membranes were carried out as above using DTSSP as a cross-linker. DTSSP was chosen because it is membrane impermeant. To simplify the composition of the fraction analyzed and to minimize nonspecific interactions, the PV1-containing total membrane pellet (Fig. 3B) was once extracted in high pH, which solubilizes only the membrane-associated proteins. As previously shown (4, 15), PV1 is an integral membrane protein resistant to high pH extraction; therefore, all molecules cross-linked to PV1 should remain attached to the membranes that were collected by centrifugation (Fig. 3C).

Next, the membrane pellet was solubilized in SDS and iodoacetamide to insure good solubilization and prevent nonspecific interactions, respectively, followed by separation of the cross-linked complexes by centrifugation in a SDS-containing, continuous sucrose gradient [5–40% (wt/vol)]. Proteins from the fractions collected from the gradient were separated by SDS-PAGE in nonreducing conditions and either stained with Coomassie (Fig. 3D, left) or immunoblotted with anti-rPV1 antibodies (Fig. 3D, right). Coomassie staining showed the characteristic smear due to cross-linking. The immunoblotting experiment showed that PV1 was present in the lower half of the gradient, the heaviest fraction included (fraction 1 on the gradient), cross-linked into complexes of molecular masses as high as not to enter the stacking gel (Fig. 3D, right). By its position in the gradient, the size of these complexes is estimated to be in the range of million(s) of Daltons.

We reasoned that these very high molecular mass complexes were the most informative with respect to the proteins PV1 might have been cross-linked to. Thus we devised a protocol for very high molecular mass isolation from fraction 1 of the gradient using lectin chromatography on GS-I-agarose. Please note that we have previously used GS-I to successfully purify rPV1 from rat lungs (15). We also preferred to use GS-I over anti-rPV1 antibodies as the lectin was much more efficient (∼50% efficiency, Fig. 3E) for binding of the very high molecular mass complexes than the anti-rPV1C pAb (unpublished data). This situation is possibly due to steric hindrance caused by cross-linking, which does not allow antibody binding.

The isolated complexes were analyzed by SDS-PAGE in nonreducing conditions. This obviated a fairly simple protein content in the lectin bound fraction (Fig. 3F). However, GS-I lectin brings down several bands that are not reactive with rPV1 antibodies (asterisks in Fig. 3F). To avoid possible contamination, the lectin-bound complexes were solubilized in very harsh conditions (2.5% SDS and 8 M urea) and separated in a 5% SDS-urea gel (18). The use of urea-containing gels was intended to minimize nonspecific interactions. Coomassie staining of the separated material revealed a band at the bottom of the well (Fig. 3F, arrow). As only this material was positive for PV1 (compare Fig. 3F, lane 1), the band was excised and subjected to in-gel cleavage of the cross-linker by DTT at 37°C (see MATERIALS AND METHODS), and the components of the cross-linked complex resolved by 10% SDS-PAGE. As seen in Fig. 3G, the major component (∼98% by densitometry) of these complexes was PV1 as revealed by silver staining of the separated proteins. However, three minor bands were also detected, of which we identified two by mass spectroscopy. The band above the 200-kDa marker is a myosin that is most likely a contaminant that could appear in the complex by SH group exchange, and the band at ∼50 kDa is a degradation product of PV1. The identity of the third band (∼20 kDa) could not be determined due to insufficient amounts in the gels.

In the case of the kidney, the results were the same throughout the procedure, although the yield of these complexes was much lower compared with the lung, which resulted in higher background on the silver-stained gels (Fig. 4). This lower yield might reflect either a lower content of endothelial structures in

Fig. 4. Components of PV1-containing very high molecular mass complexes in rat kidney as seen by silver staining. PV1 was found to be their major component as well (arrow), although the efficiency of cross-linking and the yield was by far lower than those in the lung.

Fig. 5. Preembedding immunocytochemistry by immunodiffusion with α-rPV1C pAb-10-nm Au. The electron micrographs of rat lungs (a–c, e and f, and i and j) show labeling of fenestral diaphragms of fenestrae (a–c) and stomatal diaphragms of transendothelial channels (e and f) and caveolae (d, g, and h). In favorable sections, several gold particles were detected in one diaphragm. The stomatal diaphragms of caveolae (d), transendothelial channels (i), and fenestrae (j) in control experiments in which nonimmune antibody coupled to 10-nm gold was used are devoid of gold particles. Bars = 100 nm.
the kidney or a lower cross-linking efficiency in the kidney or a combination of both.

Taken together, these data show that multiple PV1 dimers reside in close proximity in the same diaphragm. The results in the lung show that this is true for the stomatal diaphragms of caveolae, and the results in kidney show it could be also true for the diaphragms of fenestrae.

Several PV1 molecules reside in the same diaphragm as shown by EM. Previous data on PV1 localization at the EM level showed several gold particles on the same diaphragm (15, 16). However, these results were obtained with a reporter system consisting of a primary anti-PV1 antibody followed by a gold-conjugated secondary antibody. The multiple labels found in the same diaphragm could have been the result of multiple reporter antibodies binding to one primary antibody.

To circumvent this, we performed the same experiments as in the past (15, 16), this time using a primary antibody directly conjugated to 10-nm gold particles. We labeled rat lung and kidney specimens by a preembedding method we call immunodiffusion. As seen in Fig. 5, in favorable sections several PV1 particles specifically labelled the same diaphragm. This is true in the case of both SDs (Fig. 5, d–h) as well as diaphragms of fenestrae (Fig. 5, a–c). Little label, if any, was found on the plasmalemma proper, coated pits, or intercellular junctions as well as on any other nonendothelial cell type. No label was found on control specimens, where a 10-nm gold coupled nonimmune antibody was used (Fig. 5, i–k). Together with the cross-linking data, these results suggest that in the endothelium in situ, several PV1 dimers reside in the same diaphragm.

DISCUSSION

The function of the SDs and FDs is not known. By their localization at the sites of transendothelial exchanges, one could assume that they function either as a sieving device (opposing the exchanges of select molecules) or a facilitating device (enabling selective transport of molecules) or both. As for the presence of PV1 on such diaphragms, it could function as either a structural component, a function modulator component, or both.

The work presented in here is a result of our interest on the elucidation of the molecular components of SDs and FDs. Using our purification method of endothelial caveolae (17) and taking advantage of a novel anti-endothelium antibody (4), we identified and cloned PV1, an endothelium-specific protein (16), which was further shown to be specifically associated with the SDs of caveolae and TECs as well as with FDs. This was the first demonstration that these structures, besides being morphologically similar, were also biochemically related sharing at least one protein, namely, PV1. In a subsequent study we have shown that, moreover, PV1 is a key structural component of both FDs and SDs, being necessary and sufficient for their formation, because 1) reagents that induce de novo formation of the SDs and FDs upregulate PV1, which is found in these new diaphragms; 2) downregulation of PV1 mRNA by a siRNA approach prevents the formation of SDs and FDs; and 3) overexpression of PV1 in endothelial or nonendothelial cell types leads to de novo formation of SDs (R. V. Stan, unpublished observations).

Here, we present several experiments that continue to address the role of PV1 in the formation of SDs and FDs. We first show formal evidence that PV1 indeed forms homodimers. Next, we show that PV1 homodimers could be cross-linked in situ into high-molecular-mass complexes using several crosslinkers in perfusion of either the lung or kidney. Moreover, upon isolation of these complexes we show that their most abundant (~98% by densitometry) protein is PV1. The data obtained with this approach strongly suggest that several PV1 dimers reside in the same diaphragm, this being in agreement with the data by immunocytochemistry with anti-PV1 antibodies directly conjugated to gold where several gold particles were found per diaphragm. They also suggest that several PV1 dimers reside in close proximity as to be bound by the cross-linker into such very high molecular mass complexes. The size of these complexes was in the range of millions of Daltons, which suggests that they can accommodate more than 10 PV1 dimers. This is in keeping with the number of fibrils that were described in the diaphragms (1), further suggesting that PV1 might form the meshwork of the diaphragm.

These data are supportive of a paradigm we have recently formulated (12, 14), by which PV1 would form (alone or together with other proteins) the fibrils of the diaphragms. The absence of other proteins from the cross-linked complexes is no proof that only PV1 forms the diaphragms. The cross-linking depends on the existence of functional groups capable of reaction with the cross-linker at the right distance one from another. As already mentioned in the results, PV1 has many available amino groups, especially in its COOH-terminal third, which would form the central mesh/density according to our model. Thus the absence of other proteins from the complexes might mean that PV1 just cross-links better. Further work should clarify whether PV1 dimers form the fibrils of the diaphragms alone or in combination with other proteins. However, it seems clear that PV1 participates in the formation of the meshwork of the diaphragms. This propels it as a protein that functions in microvascular permeability.

ACKNOWLEDGMENTS

The author thanks Dr. G. Palade for insightful discussions and critical reading of the manuscript.

GRANTS

This study was supported by National Heart, Lung, and Blood Institute Grant HL-65418 and American Heart Association Grant 0060080Y.

REFERENCES