Isolation, Cloning, and Localization of Rat PV-1, a Novel Endothelial Caveolar Protein

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Abstract. By using an immunosolubilization procedure (Stan, R.-V., W.G. Roberts, K. Ihida, D. Predescu, L. Saucan, L. Ghitescu, and G.E. Palade. 1997. Mol. Biol. Cell. 8:595–605) developed in our laboratory, we have isolated a caveolar subfraction from rat lung endothelium and we have partially characterized the proteins of this subfraction which include an apparently caveola-specific glycoprotein we propose to call PV-1 (formerly known as gp68). The isolation and partial sequencing of PV-1, combined with the cloning of the full length PV-1 cDNA led to the following conclusions: (a) PV-1 is a novel single span type II integral membrane protein (438 amino acids long) which forms homodimers in situ; (b) the transmembrane domain of PV-1 is near the NH₂ terminus defining a short cytoplasmic endodomain and a large COOH-terminal ectodomain exposed to the blood plasma; (c) PV-1 is N-glycosylated and its glycan antennae bear terminal nonreducing galactosyl residues in α1-3 linkage. PV-1 is expressed mostly in the lung but both the messenger RNA and the protein can be detected at lower levels also in kidney, spleen, liver, heart, muscle, and brain. No signal could be detected in testis and two lower molecular weight forms were detected in brain. Immunocytochemical studies carried out by immunodiffusion on rat lung with an anti–PV-1 polyclonal antibody directed against a COOH-terminal epitope reveal a specific localization of PV-1 to the stomatal diaphragms of rat lung endothelial caveolae and confirm the extracellular orientation of the PV-1 COOH terminus.

Key words: plasmalemmal vesicles • Griffonia simplicifolia • lectin chromatography • rat lung • immunodiffusion

Plasmalemmal vesicles (PVs)¹ were first described in endothelial cells (23) as spherical vesicles of regular size (~70 nm) and shape associated with the plasma membrane (2). Similar structures were found in epithelial cells and called caveolae intracellulares (39). Subsequent studies have demonstrated the presence of caveolae at different surface densities in practically all types of mammalian cells with few exceptions (5, 11, 21).

Attempts to define the function of PVs have relied on several approaches: (a) perfusion experiments with a variety of tracers carried on microvascular beds provided with a continuous endothelium (9, 20, 27, 29, 30, 35) have established the role of PVs in transcytosis but have not provided information about their chemistry; (b) immunocytochemical tests carried on in situ have shown that certain antigens are either restricted in their distribution to PVs and absent or present at much lower density on the plasmalemma proper (6, 8) or only partially present in PVs (7, 13–15, 22); and (c) attempts to isolate caveolae by cell fractionation procedures from different cell types (3, 18, 19, 32, 36) including endothelial cells (33, 34). These cell fractionation procedures employ nonspecific physical parameters (Triton X-100 insolubility, size, buoyant density) of the caveolae and their membrane for their isolation and purification. The results obtained by this approach constitute already a sizable literature that ascribes to PVs a wide variety of components and, by implication, functions (for review see reference 1).

We have developed a caveolae purification procedure (37) that uses as starting preparation luminal plasmalemmal patches isolated from rat lung vasculature by the cationized silica procedure (16), detaches the vesicles by sonication, and isolates them by specific immunoabsorption on magnetic microspheres coated with anticleavin antibody.

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1. Abbreviations used in this paper: aa, amino acid; GS I, Griffonia simplicifolia I lectin; PBSA, 1% BSA in PBS; PEG, polyethylene glycol; PLP, paraformaldehyde-lysine-metaperiodate fixate; PV, plasmalemmal vesicle; PVDF, polyvinyl difluoride; RT, room temperature.
The procedure relies on the fact that caveolin is a generally accepted marker for caveolae (17, 25, 31). Our method also has the advantage of permitting the comparison of the protein and lipid content of immunoisolated caveolae with that of a fraction derived primarily from the plasmalemma proper. The results obtained with this immunoisolation procedure are different from those obtained with procedures that rely on physical parameters for caveolae purification. For instance, proteins involved in signal transduction (c-src, endothelial nitric oxide synthase, annexin II), protein kinase C, α subunits of heterotrimeric G proteins, α, α1, 2, 3, and αQ) and novel endothelial specific antigens, namely gp85/95 and gp95/115 (10), distribute preferentially to the plasmalemma proper in our procedure but are found enriched in caveolae fractions isolated by other methods. Our results agree with previous immunocytochemical findings on the distribution of several endothelial antigens such as thrombomodulin, functional thrombin receptor, podocalyxin (13, 14), or were later confirmed by immunolocalization studies in what concerns the α subunits of the heterotrimeric G proteins (15) and one novel endothelial antigen, namely gp85/95 (10).

The only protein found so far to strictly colocalize with caveolin on the immunoisolated lung endothelial caveolae is PV-1 (formerly known as gp68) (37). A corollary, this protein qualifies for a novel caveolar marker in rat lung endothelium. PV-1 was discovered recently (10) as the antigen recognized by a novel endothelial-specific monoclonal antibody (21D5 mAb). Partial characterization of the PV-1 molecule, carried on in the same study, revealed that: (a) PV-1 is an integral membrane protein (resistance to extraction by high pH and high salt); (b) PV-1 forms dimers (the 21D5 mAb recognizes a band at ~60–70 kD apparent molecular mass in reducing conditions and a band ~110–120 kD in nonreducing conditions); (c) PV-1 has an extracellular domain demonstrated by the possibility of biotinylation by perfusion in situ; (d) PV-1 is heavily N-glycosylated (15% mass reduction after PNGase F treatment); (e) PV-1 was detected in rat lung capillaries and rat kidney peritubular capillaries by immunofluorescence; and (f) attempts to localize this protein to caveolae isolated from rat lung endothelium by the Triton X-100 based purification procedure of Schnitzer et al. (33) have failed as the protein is Triton X-100 soluble (10).

Since PV-1 strict colocalization with caveolin in lung endothelial caveolae (obtained by immunoisolation on anti-caveolin antibodies) may be functionally significant, we decided to isolate it, obtain sequence information, and identify it. At the peptide sequences we initially obtained pointed to a completely novel protein, we cloned the full length cDNA of PV-1 and localized its mRNA in different rat tissues. We raised antibodies against a COOH-terminal peptide of the translated sequence and detected it in different rat tissues by Western blotting and localized it by immunocytochemistry at EM level in the rat lung.

Materials and Methods

Frozen rat lungs were purchased from Pel-Freez Biologicals. A mopholines pH 3–10 and DEAE-Sepharose were from Pharmacia. A garose bound or biotinylated Griffonia simplicifolia I lectin (GS I) and melibiose were from either Vector Laboratories or EY Laboratories. PVDF (polyvinylidifluoride) membrane was purchased from Millipore and nitrocellulose membrane from M.S. Protogel (30% acrylamide solution) was obtained from National Diagnostics. The rat lung expression library and the rat multiple tissue Northern Blot™ was purchased from Clontech. Protran 82 nitrocellulose circles were purchased from Schleicher & Schuell. Bacto-Tryptone and Bacto-Agar were from Difco and ampicillin, argar, and Trizol™ from Life Technologies. Restriction enzymes were from New England Biolabs and cloning vectors pBluescript SK (+) and pBluescript II KS (+), pfu DNA polymerase, and QuickHyb™ hybridization solution were from Stratagene. pCR 2.1 vector and TA Cloning™ kit were from Invitrogen Corp. pE6-30 cloning vector and QIAprep™ plasmid DNA mini prep kits were from Qiagen. Luria-Bertani broth (LB-broth) and Luria-Bertani agar (LB-agar) were purchased from Bio101. MaxScript™ and RPA II™ kits were purchased from Amaxa Biomedicals. Hybond-N™ nylon membrane and [32P]dCTP were from Amersham. All other reagents were either from Sigma Chemical Co. or Fischer.

Buffers

Buffers were as follows: Hepes-buffered sucrose: 250 mM sucrose, 20 mM Hepes, pH 7.2, supplemented with 1 mM CaCl2 and protease inhibitors cocktail (10 μg/ml each leupeptin, pepstatin, o-phenanthroline, E-64, and 1 mM PMSF); Hepes-buffered saline (HBS): 150 mM NaCl in 10 mM Hepes, pH 7.2; Hepes-buffered saline/Triton X-100 (HBS-T): 0.2% Triton X-100, 150 mM NaCl in 10 mM Hepes, pH 7.2; lectin binding buffer: 0.2% Triton X-100, 150 mM NaCl in 10 mM Hepes, pH 7.2, supplemented with 1 mM each of CaCl2, MgCl2, and MnCl2.

PV-1 Isolation Procedure

Fig 1. A gives the schematic of the PV-1 isolation procedure. Frozen rat lungs were weighed, minced on ice in Hepes-buffered sucrose (1:4 wt/vol), and homogenized in a motor driven Thomas type “C” Teflon pestle-plate homogenizer by 15 strokes at 1,800 rpm. The homogenate was filtered through a 53-μm nylon mesh and separated into a nuclear pellet and a postnuclear supernatant by centrifugation (10 min, 15,000 g, 4°C) in a Beckman GPR centrifuge. The postnuclear supernatant was resuspended into a total membrane pellet and a soluble supernatant by centrifugation (2 h, 100,000 g, 4°C) in a SW 28 rotor. Next, the rat lung total membrane pellet was resuspended in 25 ml of ice-cold 0.1 M NaCl, pH 11, by low speed homogenization using a Thomas type “B” homogenizer followed by incubation (15 min, 4°C) with gentle agitation. The insoluble material was collected by centrifugation (1 h, 45,000 rpm, 4°C) in a T70 rotor. The superantigen containing the Triton X-100 soluble proteins, was further adjusted to 4% (wt/vol) urea, 2% (vol/vol) ampholines, pH 3–10, 1% (vol/vol) Triton X-100, and 5% (wt/vol) glycerol to a final volume of 35 ml and subjected to isoelectric focusing using a Rotofor chamber (Bio-Rad) as per manufacturer’s instructions. 20 fractions of ~1.7 ml were collected, subjected to 8% SDS-PAGE, and analyzed for the presence of PV-1 by Western blotting using the anti-PV-1-21D5 mAb and by silver staining for visualization of the protein content. The fractions containing the antigen (usually fractions 11–14) were dialyzed (1 h, 4°C) against 4,000 volumes of HBS-T using a 50-kD cut off dialysis membrane. The dialysate was diluted 10× with HBS-T and next adjusted to 1 mM each of CaCl2, MgCl2, and MnCl2. The resulting mixture was incubated (10–12 h, 4°C) with 2 ml (settled gel) of GS I-agarose with gentle rotation (GS I lectin specificity: Gal/GalNAcα1,3 [12]). The beads were collected by centrifugation (10 min, 500 g, 4°C) and washed 3× with 10 ml of 45 mM lectin binding buffer. GS I bound proteins were eluted twice by incubation with 2.5 ml elution buffer (HBS-T supplemented with 0.2 M melibiose and 5 mM EDTA) for 1 h and the two eluates pooled. The eluted glycoproteins were further incubated with 2 ml (settled gel) of DEAE-Sepharose (previously equilibrated with HBS-T) for 4 h at 4°C. The flow-through was collected and subjected to 15% TCA precipitation for 1 h on ice. The precipitated proteins, representing the purified material, were solubilized in 2× reducing sample buffer, resolved by 8% SDS-PAGE, and transferred to either PVDF (for NH2-terminal sequencing) or nitrocellulose (for internal sequencing) membrane.
NH₂-terminal Sequencing
The PVDF membrane containing SDS-PAGE resolved proteins was stained with 0.1% Coomassie brilliant blue G 250 in 40% methanol and 10% acetic acid and the PV-1 band was excised and used for Edman degradation. The NH₂-terminal sequencing was done on samples obtained from two separate experiments by the protein sequencing facility at the Worcester Foundation for Biomedical Research (Shrewsbury, MA).

Internal Sequencing
The SDS-PAGE resolved proteins transferred to nitrocellulose membrane were stained with 0.1% Ponceau S in 1% acetic acid and the PV-1 band was excised and digested with trypsin. Two of the resulting peptides were purified, sequenced, and the sequence confirmed by mass spectrometry by the protein sequencing laboratory at the Worcester Foundation for Biomedical Research at the University of California, San Diego.

Cloning of the Full Length PV-1 cDNA
We used the anti-PV-1 21D 5 mA b to screen a rat lung expression library (Clontech catalog No. RL5002b) cloned into the bacteriophage λgt11 as per manufacturer's instructions. Briefly, 500,000 phages were plated and incubated with 10 mM IPTG (isopropyl β-D-thiogalactopyranoside) to express the proteins encoded by their inserts. The proteins were transferred to nitrocellulose membranes which were probed with Western blotting with the anti-PV-1 21D 5 mA b. The positive plaques were purified to homogeneity by three more screening rounds. The four longest inserts were either subcloned into pBluescript II KS (+) vector or PCR amplified using anti-PV-1 21D 5 mA b and the PCR product was gel purified and inserted into pCR 2.1 vector using the TA Cloning™ kit. The cloned insert was checked by DNA sequencing and a 32P-labeled complementary RNA probe was synthesized with T7 RNA polymerase using the MaxiScript™ kit. Total RNA from rat lung, spleen, kidney, and liver were purified using the Trizol™ reagent as per manufacturer's instructions. 20 µg of total RNA from the above tissues was used in an RNase protection assay carried out using RPA II™ kit and following the manufacturer’s standard protocol. The reactions were resolved by 5% denaturing polyacrylamide gel electrophoresis and the gel exposed to autoradiography film for 6-24 h.

Expression of PV-1 as a His-tagged Protein
The PV-1 cDNA (residues 36-1797) was directionally subcloned in the
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Affinity Purification of Anti-PV-1C Polyclonal Antibodies

The PV-1 COOH-terminal peptide used for immunizations (PV-1C peptide) was solubilized in 0.1 M Heps, pH 8.0, at a final concentration of 20 mg/ml and coupled to AffiGel-10™ beads (4 ml settled gel) (Bio-Rad) by reaction in a buffer containing 0.1 M Hepes, pH 8.0, 1% NP-40, 0.4% deoxycholate, 0.1% SDS, 300 mM NaCl, 1 mM EDTA, 1 mM PM SF, and protease inhibitor cocktail. The precipitate was centrifuged at 20,000 g for 30 min. The supernatant containing the solubilized antigen-antibody complexes was collected by centrifugation and dialyzed overnight against 20-40 vol of PBS to yield a total IgY fraction from egg yolk.

Anti-PV-1C Polyclonal Antibody Validation

ELISA. Afinity purified anti-PV-1C pAb was checked for specificity and activity in an ELISA assay using serial dilutions of the antibody on 10 ng PV-1C peptide/plate well. The bound antibody was detected by incubation (30 min, RT) with a rabbit anti-chicken IgY HRP-conjugated antibody (Biodesign) and enhanced chemiluminescence (Super Signal™; Pierce). In the case of the peptide competition, 1-µg aliquots of anti-PV-1C pAb were incubated (1 h, RT) with different amounts of the peptide prior to the incubation with the strips.

Immunoprecipitations. Rat lung membranes were extracted for 1 h on ice in 100 µl of immunoprecipitation buffer (20 mM Tris-HCl, pH 7.5, containing 1% NP-40, 0.4% deoxyxylol, 0.1% SDS, 300 mM NaCl, 1 mM EDTA, 1 mM PM SF, and protease inhibitor cocktail). The extract was clarified by centrifugation at 100,000 g for 1 h in a TLA 45 rotor. The extracted proteins (200 µg) were incubated (14 h, 4°C) with 5 µg of anti-PV-1C pAb with gentle agitation. This mixture was further incubated (4 h, 4°C) with rabbit anti-chicken IgY antibodies insolubilized onto agarose beads to precipitate the antigen-antibody complexes. The beads were collected by centrifugation (500 g, 5 min) and washed (3 x 5 min) with immunoprecipitation buffer followed by one final wash in 50 mM Tris, pH 6.8. The beads were boiled in nonreducing SDS-PAGE sample buffer (2.3% SDS, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8) and collected by centrifugation. The supernatant containing the solubilized antigen-antibody complexes was saved, adjusted to 5% 2-mercaptoethanol, and boiled for 2 min. The solubilized proteins were resolved by 8% SDS-PAGE, transferred to PVDF membrane, and immunoblotted using the anti-PV-1 21D5 mAb.

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Electron Microscopy

Preembedding Immunocytochemistry. Preembedding immunocytochemistry was performed as previously described in Predescu et al. (28). Briefly, the tissue was flushed free of blood by a 10-min perfusion with Hanks’ balanced salt solution, then fixed in situ in 3% paraformaldehyde–lysole-sodium metaperiodate (PLP) fixative. The tissue was excised, cut into small blocks (3 x 3 mm), further fixed in fresh PLP for 1 h at RT, and then fixed overnight at 4°C with a fresh PLP change. Fixed specimens were cryoprotected by infiltration (12-16 h at 4°C followed by 1 h at RT) with a solution containing 1.5 M sucrose, 50% polyvinylpyrrolidone in PBS, and stored frozen in liquid nitrogen. Thick cryosections (45 µm) cut from the fixed blocks were rinsed (5 x 5 min) and incubated overnight at 4°C in 10% goat serum in PBS, quenched in 1% BSA in PBS (PBSA) for 30 min at RT and incubated overnight at 4°C with the anti-PV-1C pAb diluted (1:50-1:250) in PBSA. The sections were washed 3 x 30 min in PBSA at RT, incubated with the rabbit anti-chicken IgY 5 nm gold-conjugated antibody (1:100 dilution in PBSA) for 12-16 h at 4°C. After final washes as above the antibody-antigen complexes were stabilized by fixation (1 h at RT) in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, postfixed in 1% OsO4 in acetone veronal buffer, pH 6.8 (1 h on ice), stained in the dark (1 h at RT) with Kellenberger uranyl acetate, dehydrated through graded ethanol, and finally embedded in Epon 812 resin. Tissue blocks were cut on a Reichert microtome, mounted on formvar-coated nickel grids, and stained with 2% uranyl acetate and saturated lead citrate. The stained sections were examined and photographed in a Philips CM 10 electron microscope.

This approach for immunolabeling relying on the diffusion of the antibodies along vascular and perivascular spaces provides adequate sampling of immunologically well preserved vascular endothelia.

Postembedding Immunogold. Small blocks (1 x 1 mm) from PLP fixed lungs, cryopreserved and stored as above, were sectioned on a Reichert ultramicrotome equipped with a F4 cryoattachment. Urithrin crossections transferred to nickel grids were stained and then washed by floating the grids, specimen down, on drops of filtered solutions (41). After quenching (30 min at RT) with 1% BSA and 0.01 M glycine in PBS, the
sections were incubated (1 h, RT) with anti-PV-1 mAb diluted 1:100 in PBSA, washed (3 × 15 min) in PBSA, and further incubated with a rabbit anti-chicken IgY antibody conjugated to 5 nm colloidal gold (diluted 1:100 in PBSA) for 2 h at RT, then washed as above. The immune complexes were stabilized (30 min at RT) in 2% glutaraldehyde in PBS, post-fixed in 1% OsO4 in acetate-veronal buffer, pH 7.6, absorption-stained (5 min, RT) with 0.002% lead citrate in 2.2% polyvinylalcohol, and finally examined and photographed in a Philips CM10 electron microscope. This procedure has the disadvantage of a limited yield of useful sections and suboptimal structural preservation, but the advantage of providing information on the intracellular distribution of the antigen.

**General Procedures**

**Protein Determination.** The protein content was determined by the BCA method (Pierce) against either BSA or IgY standards made in appropriate buffers.

**Immunoblotting.** The membranes were blocked (30 min, RT) in 5% nonfat dry milk in PBS and 0.1% Tween 20 (blocking buffer), incubated (1 h, RT) with the first antibody diluted in blocking buffer, washed (3 × 5 min) in PBS containing 0.1% Tween 20, incubated (30 min, RT) with a HRP-conjugated reporter antibody, washed again as above, and the signal detected by using ECL reagents.

**Results**

**Isolation of PV-1 from Rat Lungs**

As the monoclonal antibody 21D5 is not a useful reagent for affinity purification of PV-1, we explored alternative approaches taking advantage of the already known properties of this protein (molecular weight, occurrence as a dimer, and resistance to high-pH and high-salt extraction). Pilot experiments as Triton X-100 differential solubility, pl determination, lectin binding and elution, and anion exchange chromatography were carried out using rat lung total membranes to assess the behavior of PV-1 in any of the isolation steps.

Based on the pilot experiments, the isolation of PV-1 from rat lungs was carried out according to the protocol depicted in Fig. 1a and detailed in Materials and Methods. A liquids of different fractions throughout the purification procedure were monitored for presence of PV-1 by immunoblotting using the 21D5 mAb.

The frozen rat lungs were minced, homogenized, and resolved by centrifugation in a tissue debris and nuclei pellet and a postnuclear supernatant. The latter was used for obtaining a total membrane fraction containing PV-1. As documented in Fig. 1b, PV-1 distributes exclusively in the total membrane fraction. However, the anti-PV-1 21D5 mAb recognizes another band of ~45–50,000 molecular weight which we determined to be a soluble rat blood plasma protein (see Fig. 1b, rat plasma and rat lung cytosol lanes) which is efficiently eliminated by the centrifugation step used to separate membranes from cytosol. To eliminate the membrane associated proteins, the rat lung total membranes were high-pH extracted, the insoluble material which contained PV-1 (Fig. 1c) collected by centrifugation, and the supernatant discarded. This high-pH insoluble material was further extracted in 2% Triton X-100 and the extract clarified by centrifugation. Fig. 1d shows the efficiency of PV-1 extraction which was essentially complete. The clarified Triton X-100 extract was further subjected to isoelectric focusing in a Rotofor chamber. PV-1 was recovered in 3–4 fractions of the pl gradient (fractions 11–14 in Fig. 1e) corresponding to a pl interval 6.85–7.9. These fractions were pooled, dialyzed, and incubated with GS I lectin bound to agarose. After washing nonspecifically interacting proteins the bound glycoproteins were eluted by competition with melibiose and EDTA (Fig. 1f). GS I lectin was chosen for the isolation procedure for two reasons. First, the binding efficiency and especially elution efficiency were the highest compared to other lectins tested (our unpublished data); and second, the binding sites for this lectin have been documented at the electronmicroscopic level in rat lung by previous work done in our laboratory (2). This study has shown some of the binding sites of GS I to be located in the caveolae of the rat lung endothelium and endothelia of other microvascular beds. The eluate from GS I column was further subjected to DEAE-Sephadex chromatography and the flow-through was collected, as PV-1 binds very weakly (Fig. 1g) to this anion exchange column. We introduced this step because DEAE binds proteins in the 50–70 kD range, thereby improving the separation of PV-1 from other bands and making possible its excision free of contaminants. The proteins from the flow-through were precipitated by TCA, solubilized in 2× reducing sample buffer, and resolved by 8% SDS-PAGE to obtain maximum resolution in the 50–60,000 molecular weight range (Fig. 1h). The resolved proteins were transferred to either a PVDF or a nitrocellulose membrane, the PV-1 band identified, excised, and sent for NH2-terminal or internal sequencing. The sequences of three peptides, one representing the NH2 terminus of PV-1 (NH2-terminal) and the other two representing internal sequences (Internal 1 and Internal 2) were obtained (Table I). Mass spectrometry analysis was carried out on the internal peptides for confirmation of the sequence. Searches of protein databases and mass spectrometry databases showed that these sequences are novel, as very little homology with other known proteins was found.

**PV-1 Cloning from a Rat Lung Library**

The anti-PV-1 21D5 mAb was used for screening an oligo-dT and random primed rat lung expression library cloned in the bacteriophage λgt11 and the inserts of the positive phages were sequenced as described in Materials and Methods. Only 1,274 nucleotides of the PV-1 full length message were obtained by this approach. Additionally, we were able to narrow down the region where the epitope of the 21D5 mAb is located as the overlap of the cloned phage inserts amounted to a sequence of 39 nucleotides (residues 1240–1278 of the PV-1 full length cDNA) which encodes for the amino acid residues 405–417 of the PV-1 translated protein sequence (Fig. 2c). To obtain the sequence of the full length message, the same library was rescreened with a 32P-labeled DNA probe (residues 841–

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<th>Table I. Peptide Sequences Obtained by PV-1 Microsequencing</th>
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1268 of PV-1 full length cDNA). Upon sequencing of seven longest inserts isolated through the second screening we obtained a 1968-nucleotide message (Fig. 2a). The size of the message was confirmed by Northern blotting of mRNA from different rat tissues using the same DNA probe that had been used for screening of the library (Fig. 3).

Along with the full length PV-1 cDNA we found one alternatively spliced form lacking the region in between residues 116–252. An RNase protection assay (see Materials and Methods) with a probe appropriate for D116–252 form showed that 99% of the naturally occurring message consisted of the full length RNA in rat lung, kidney, spleen, and liver (our unpublished results).

The longest region of the full length PV-1 cDNA which encodes for protein is 1341 bases long and contains all three peptides obtained by protein sequencing of the purified PV-1 (Table I). Even though there is no stop codon before the initiator ATG and the consensus Kozak sequence is incomplete (G is present in the position +4 but no A or G is found in position –3) we set the beginning of the coding region at residue 25 of the PV-1 full length cDNA based on the sequence obtained by NH2-terminal sequencing of the purified PV-1 (Table I).

The full length PV-1 cDNA encodes for a protein with a calculated 50 kD mass and an estimated pI 9.0. Hydrophilicity plot (Fig. 2b) shows only one hydrophobic region (aa residues 25–50) which could qualify for a transmembrane domain. As no signal peptide could be found, the hydrophobic region (aa residues 25–50) could act as a signal and anchoring peptide thus defining PV-1 as a type II membrane protein with its NH2 terminus intracellular and COOH terminus extracellular. Searches of protein databases with the translated protein sequence show little homology with known proteins. No known long protein patterns and few protein motifs were detected. Noteworthy features of PV-1 are the four consensus N-glycosylation sites (arginines 82, 88, 112, and 150), an odd number (nine) of cysteines in the extracellular domain which would imply the possibility of intermolecular disulfide bonds, a short proline-rich region (residues 397–411) at the COOH terminus, and two casein kinase consensus phosphorylation sites (Table I).
sites (the serine 4 [SMD] and threonine 13 [TGD]) in the cytoplasmatic domain. No consensus O-glycosylation sites were found. A striking feature of PV-1 is the regular pattern formed by the spacing of the cysteines in the extracellular domain starting with the cysteine in position 117 (C-23X-C-10X-C-24X-C-20X-C-24X-C-44X-C-67X-C-38X), whose significance, if any, is not known. A schematic of PV-1 monomer and the representation of different features is given in Fig. 2 c.

To prove that this cDNA encodes indeed for PV-1, we expressed recombinant PV-1 as a His-tagged protein in bacteria (see Materials and Methods). The 21D5 mAb detected by immunoblotting a single band at $\sim 50$ kD (the size of the unglycosylated PV-1 monomer) only in the induced transformant bacterial clones but not in the noninduced (our unpublished results).

**Detection of the PV-1 mRNA by Northern Blotting**

The presence of the PV-1 mRNA in several rat tissues was checked by Northern blotting (see Materials and Methods). As PV-1 was implied to be an endothelial antigen (10) we screened tissues containing several endothelial types (e.g., continuous, fenestrated, and sinusoidal). We found the PV-1 message to be present mostly in the lung and at much lower levels in kidney, spleen, liver, heart, and muscle (in decreasing order of the signal intensity) (Fig. 3). A very weak signal, if any, of the PV-1 full length mRNA along with very low levels of two shorter mRNA species was found in the brain only after long exposure of the blot to the film (Fig. 3, lane B). No message was detected in testis.

**Anti–PV-1 Polyclonal Antibody Production**

Since we found that the 21D5 mAb was not a useful reagent for immunoprecipitation or immunolocalization of PV-1 at the EM level (our unpublished results) we raised polyclonal antibodies in chickens against a 12 mer COOH-terminal peptide representing residues 427–438 of rat PV-1 to which a lysine and a glycine were added for coupling purposes (PV-1C peptide). The peptide (KGPPLVNPAPSGL single letter amino acid code) was coupled to BSA via glutaraldehyde, and the resulting conjugate was used as antigen for antibody production in chickens. A total yolk IgY fraction was purified and used for anti–PV-1C pAb affinity purification as described in Materials and Methods.

The chicken anti–PV-1C pAb was checked in immunoblotting on PVDF strips containing 10% SDS-PAGE resolved rat lung total membranes proteins. As seen in Fig. 4 a the anti–PV-1C antibody recognizes with high specificity a band of the same molecular weight as the one seen by the 21D5 mAb when reacted with the same material. The specificity of the binding is confirmed by competition with the peptide antigen (Fig. 4 c). Moreover, the anti–PV-1C
pAb is highly specific for PV-1; by immunoblotting it detects a single band of either ~110,000 or ~60,000 apparent molecular weight in nonreducing or reducing conditions, respectively, and does not detect any protein in either rat plasma or rat lung cytosol (Fig. 4 b). By immunoprecipitation, the chicken anti–PV-1C pAb binds a protein which is recognized by the 21D5 mAb by immunoblotting on the immunoprecipitated material (Fig. 4 d). Silver staining of the immunoprecipitated material detects a single band of ~120,000 (Fig. 4 e, lane 1) apparent molecular weight in nonreducing conditions.

Taken together, these data show that the new chicken anti–PV-1C pAb recognizes PV-1 with high specificity in immunoblotting and immunoprecipitation thus being an appropriate reagent for attempts to localize PV-1 in the rat lung by immunocytochemistry techniques.

**Localization of PV-1 by Western Blotting**

The anti–PV-1C pAb was used in an immunoblotting assay to check for the presence of PV-1 in different rat tissues (see Materials and Methods and Fig. 5 legend). When equal amounts of protein from different rat tissue total membranes were loaded, the antibody detected the protein only in the lung in the linear part of the film, and in the lung and faintly in the spleen and kidney when the exposure time was highly increased. When the protein amount ratio of lung to other tissues was 1:30 (Fig. 5) the signal was easily detected in spleen, kidney, and liver, thereby matching the data obtained by Northern blotting. No signal was detected in brain, testis, heart, and muscle.

**Immunolocalization of PV-1 in the Rat Lung**

Rat lung specimens processed and labeled as described in Materials and Methods were examined by transmission electron microscopy.

In the case of preembedding immunocytochemistry, which documents the distribution of PV-1 on the cellular surface, the gold particles were found primarily on the endothelial plasmalemmal vesicles (caveolae) in agreement with the results previously obtained by the immunosolation procedure of endothelial caveolae (37). Interestingly, the label was found mostly associated with the neck of the caveolae or their stomatal diaphragms at both fronts of the endothelial cells, although the frequency of the label was considerably higher on the luminal side presumably reflecting higher accessibility from the lumen (Fig. 6, a–e). In oblique or en face views, the diaphragms occasionally appeared labeled by a cluster of gold particles (Fig. 6 d). Very little label, if any, was found on the endothelial plasmalemma proper, coated pits, or other cellular types to which the label had access in our lung preparations (e.g., epithelial cells lining alveoli and other airways structures). This finding is taken to indicate the specificity of PV-1 localization to endothelial caveolae. Control experiments in which the anti–PV-1 antibody was either omitted or replaced with an irrelevant antibody or preimmune IgY confirmed the specificity of the localization. Moreover, the immunocytochemical findings reinforced the conclusion that PV-1 is a type II membrane protein with the COOH terminus accessible from the microvascular lumina.

By immunogold labeling of ultrathin (~60 nm) rat lung cryosections the label was found at comparable frequencies on the caveolae at both fronts of the endothelial cell as well as vesicles within the cytoplasm (our unpublished results), pointing to the fact that PV-1 is a caveolar resident protein.

**Discussion**

The present study takes advantage of: (a) the specific procedure we (37) devised for the purification of caveolae from rat lung endothelium by immunosolation on antica-oveolin antibody-coated magnetic beads; and also (b) the availability of a novel monoclonal antibody (21D5 mAb) (10) directed against a rat lung endothelial antigen (PV-1). We found that this antigen colocalizes strictly with caveolin on immunosolated caveolae, thus qualifying for a novel caveolar marker in rat lung endothelium. In light of this fact we decided to isolate this protein in order to obtain sequence information for its identification and further characterization.

The PV-1 isolation method presented in this study employs several of its already documented properties: PV-1 is an integral membrane protein resistant to extraction by high salt and high pH, that forms dimers, is N-glycosylated, and is Triton X-100 soluble. Novel findings are the slightly alkaline pl of the dimer and the presence of terminal, nonreduced galactosyl residues in α-1-3 linkage (proven by the binding to GS I lectin) on PV-1 glycan antennae. Even though the isolation method yields several protein bands at its final step, as judged by the silver staining of SDS-PAGE resolved proteins from the purified material, it permits a sufficient separation of PV-1 band from other peptides to be useful for band excision and protein sequencing. A single NH2-terminal and internal sequences obtained from the PV-1 protein band pointed to a novel protein, we went further for cloning of the PV-1 full length cDNA from a rat lung expression library.

The deduced protein sequence encoded by PV-1 full length cDNA contains all three peptides obtained by protein sequencing. Further confirmation that this cDNA encodes for PV-1 came from the expression of PV-1 as a His-tagged protein in E. coli and the detection of an ~50,000 apparent molecular weight band (which would represent the nonglycosylated form of PV-1 monomer) by the 21D5 mAb only in the transformed bacterial clones.

A analysis of the primary structure of the PV-1 confirms its already known properties such as dimer formation, membrane insertion, and glycosylation. A diatlonal information is represented by the possibility of phosphorylation of the cytoplasmic domain, which might be involved
in the regulation of the protein, and the presence of the putative protein-protein interaction motif (38) represented by the proline-rich region at the COOH terminus.

Sequence analysis of the PV-1 cDNA and the deduced protein sequence shows that PV-1 is a novel protein, as little homology with other known proteins was found by searches of DNA and protein databases. This is a notable finding when correlated with the results obtained by immunocytochemistry at EM level which demonstrate the strict localization of PV-1 to endothelial caveolae in rat lung: (a) it brings further proof for the special chemistry of the caveolar microdomains (in addition to lipids and caveolins); and (b) it provides another marker for caveolae in rat lung endothelium useful in further studies of these subcellular structures. Moreover, PV-1 strict localization to caveolae in the rat lung endothelium brings additional validation to the procedure of caveolae purification by specific immunosoliation as the results obtained by the two approaches are in agreement. By immunosoliation the label colocalizes strictly with caveolin on immunosoliated caveolae (37). We assume that the protein is anchored in the membrane and is incorporated within the caveolae upon detachment by sonication. We recognize, however, that by immunocytochemistry the label is found mostly on the necks and the stomatal diaphragms as well as in the vesicles. PV-1 is a single span type II transmembrane protein and its extracellular domain is \(~390\) aa long, which would amount to \(~100\) nm long if fully extended. Considering the inner diameter of endothelial caveolae of \(~40-50\) nm (average), it might be that the protein is anchored at the level of the caveolar membrane and either: (a) participates in the formation of the diaphragms or (b) its COOH terminus protrudes through the stomatal diaphragm. The participation of PV-1 in the structure of the diaphragms is further sustained by the possibility of protein-protein interaction via the proline-rich region at the COOH terminus.

We have named PV-1 in light of its localization in caveolae in endothelium of rat lung. However, the data by Northern and Western blotting indicate that the messenger and the gene product is present in other organs which contain endothelia in part fenestrated and in part provided with caveolae. The association of PV-1 with the stomatal diaphragms of caveolae in the lung endothelium, and the findings that PV-1 is present in tissues where endothelial caveolae are known to have stomatal diaphragms (4, 24, and references therein) are consistent with a strict caveolar localization. If PV-1 is associated only with the endothelial caveolae in these tissues it might be that this protein has a function in the transport of macromolecules into or across the endothelium. If PV-1 is also associated with other structures (e.g., diaphragms of the transendothelial channels and fenestrae) in these endothelia, its distribution would suggest a sieving function for this protein. The latter hypothesis would be the most interesting as PV-1 would be the first protein of this type to perform such a function.
function. Continuation of this work will find out if PV-1 is restricted only to caveolae in these “mixed” microvascular beds. Precise information on the localization of PV-1 in different microvascular beds is a prerequisite for the formulation of any hypothesis as to its function.

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