

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

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Abstract. By using an immunoisolation 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 caveolae-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 α 1-3 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

PLASMALEMAL 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 immunoadsorption on magnetic microspheres coated with anticaveolin 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 vesicles; PVDF, polyvinylidene 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 immunisolated caveolae with that of a fraction derived primarily from the plasmalemma proper. The results obtained with this immunisolation 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 , α subunits of heterotrimeric G proteins, α s, α i 1, 2, 3, and α q) 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 caveolar 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 immunisolated lung endothelial caveolae is PV-1 (formerly known as gp68) (37). Accordingly, 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 immunisolation on anti-caveolin antibodies) may be functionally significant, we decided to isolate it, obtain sequence information, and identify it. As 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. Ampholines pI 3–10 and DEAE-Sephacel were from Pharmacia. Agarose bound or biotinylated *Griffonia simplicifolia* I lectin (GS I) and melibiose were from

either Vector Laboratories or EY Laboratories. PVDF (polyvinylidene difluoride) membrane was purchased from Millipore and nitrocellulose membrane from MSI. Protogel (30% acrylamide solution) was obtained from National Diagnostics. The rat lung expression library and the rat multiple tissue Northern Blot™ were purchased from Clontech. Protran 82 nitrocellulose circles were purchased from Schleicher & Schuell. Bacto-Tryptone and Bacto-Agar were from Difco and ampicillin, agarose, 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 TACloning™ kit were from Invitrogen Corp. pQE-30 cloning vector and QIAprep™ plasmid DNA miniprep kits were from Qiagen. Luria-Bertani broth (LB-broth) and Luria-Bertani agar (LB-agar) were purchased from Bio101. MaxiScript™ and RPA II™ kits were purchased from Ambion. [³²P]dUTP and BSA were from ICN 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 5 mM MgCl₂ and protease inhibitors cocktail (10 μ g/ml each leupeptin, pepstatin, *o*-phenantroline, 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-glass homogenizer by 15 strokes at 1,800 rpm. The homogenate was filtered through a 53- μ m nylon mesh and separated into a nuclear pellet and a postnuclear supernatant by centrifugation (10 min, 1,800 g, 4°C) in a Beckman GPR centrifuge. The postnuclear supernatant was resolved into a total membrane pellet and a soluble supernatant by centrifugation (2 h, 100,000 g, 4°C) in a SW28 rotor. Next, the rat lung total membrane pellet was resuspended in 25 ml of ice-cold 0.1 M Na₂CO₃, 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 Ti70 rotor and the supernatant discarded. The pellet was further resuspended by gentle homogenization in 20 ml ice-cold 2% Triton X-100 in 5 mM Hepes, pH 7.2, supplemented with protease inhibitors followed by a 1-h incubation at 4°C with end-over-end rotation. The Triton X-100 extract was clarified by centrifugation (1 h, 45,000 rpm, 4°C) in a Ti70 rotor. The supernatant, containing the Triton X-100 soluble proteins, was further adjusted to 4% (wt/vol) urea, 2% (vol/vol) ampholines, pI 3–10, 1% (vol/vol) Triton X-114, and 5% (wt/vol) glycerol to a final volume of 35 ml and subjected to isoelectric focusing using a Rotofof 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 counting from the acidic end of the gradient –pH 3) were pooled and dialyzed (4 h, 4°C) against 4,000 volumes of HBS-T using a 50-kD cut-off dialysis membrane. The dialysate was diluted 10 \times with HBS-T and next adjusted to 1 mM each 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/GalNAc α 1,3 [12]). The beads were collected by centrifugation (10 min, 500 g, 4°C) and washed 3 \times 10 min with 45 ml 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) DEAE-Sephacel (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 \times reducing sample buffer, resolved by 8% SDS-PAGE, and transferred to either PVDF (for NH₂-terminal sequencing) or nitrocellulose (for internal sequencing) membrane.

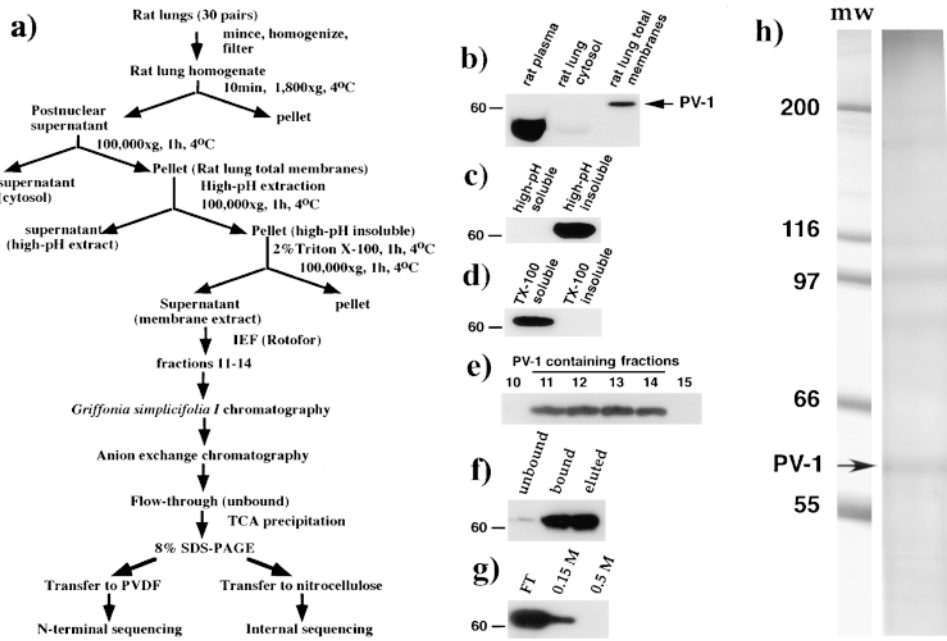


Figure 1. PV-1 isolation. (a) Schematic of the isolation procedure of PV-1 from rat lung. (b–g) Immunoblotting with anti-PV-1 21D5 mAb of equivalent volumes of fractions from different steps in the isolation procedure. (b) Rat lung homogenate separation into cytosol and total membranes. Rat plasma proteins (20 μ g) were resolved on the same gel to demonstrate the blood plasma origin of the lower (\sim 45–50 kD) band recognized by the 21D5 mAb in the cytosolic fraction; (c) high-pH soluble and insoluble material resulted from extraction of rat lung total membranes with 0.1 M Na_2CO_3 , pH 11; (d) Triton X-100 soluble and insoluble material resulted from the extraction of high-pH insoluble material in 2% Triton X-100; and (e) fractions resulted from the IEF

separation of the Triton X-100 extract in a Rotofor cell. (f) Equivalent amounts of fractions resulted from GS I chromatography of the PV-1-containing Rotofor fractions (fractions 11–14 from e). (g) DEAE chromatography of the glycoproteins eluted from GS I column (see Materials and Methods). To monitor PV-1 losses on the DEAE column, the column was washed with 10 ml HBS-T followed by 10 ml of HBS-T adjusted to 0.5 M NaCl final concentration. The proteins of the two washes were collected by TCA precipitation and solubilized in 0.5 ml $2\times$ reducing SDS-PAGE sample buffer. Equivalent volumes of the flow-through (FT), 0.15 M NaCl, and 0.5 M NaCl wash were resolved by 8% SDS-PAGE and immunoblotted with the anti-PV-1 21D5 mAb. (h) Silver staining of the 8% SDS-PAGE resolved proteins unbound to the DEAE-Sephacel column. The position of the reduced PV-1 is indicated by the arrow.

*NH*₂-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 *NH*₂-terminal sequencing was done on samples obtained from two separate experiments by the protein sequencing facility at the University of California, San Diego.

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 (Shrewsbury, MA).

Cloning of the Full Length PV-1 cDNA

We used the anti-PV-1 21D5 mAb 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 induced 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 by Western blotting with the anti-PV-1 21D5 mAb. The positive plaques were purified to homogeneity by three more screening rounds. The four longest inserts were either subcloned into pBluescript SK(–) vector or PCR amplified using *λgt11*-specific primers (sense: 5' TCCTGGAGCCCGTCAGTATCGGCG3' and antisense: 5' ATGGTAGCGACCGGCGCTCAGCTG3') and the PCR product inserted into pCR 2.1 vector. The resulting clones were sequenced in both directions which led to a partial sequence of PV-1 message. To obtain the full length cDNA we designed a 428-bp DNA probe (residues 841–1268 in the rat PV-1 full length cDNA) in the 5' region of the message obtained by screening with the antibody. This probe was ³²P-

labeled using PrimeIt™ kit (Stratagene) and used to screen another 500,000 phages. 24 positive phage clones were purified to homogeneity and the 5 longest inserts were sequenced after subcloning them into pBluescript II KS (+) vector. The sequencing of these later inserts yielded the full length PV-1 message. DNA sequencing was performed on an ABI Prism Sequencer (model 373XL) by either the Core Facility for AIDS Research at the University of California, San Diego or the Sequencing Facility at the Scripps Research Institute (La Jolla, CA). The resulting sequences were analyzed using the MacVector release 6.0 software from Oxford Molecular Group, Inc.

Northern Blots

A premade rat multiple tissue Northern blot containing 2 μ g mRNA/lane from different rat tissues was probed with a ³²P-labeled 428-bp PV-1 cDNA fragment (residues 841–1268) for detection of the PV-1 message. The hybridizations were done using QuickHyb™ hybridization solution as per manufacturer's instructions.

RNase Protection Assay

A 283-bp fragment containing the nucleotides 1–283 of the full length PV-1 cDNA was PCR amplified, and the PCR product was gel purified and inserted into pCR 2.1 vector using the TACloning™ 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 μ g 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

NcoI and PstI sites of the pQE-30 vector and the construct used to transform competent *Escherichia coli* (M15 strain). Positive clones were cultured for 6 h at 37°C until the OD₆₀₀ ≈ 0.7 and the recombinant protein production was induced by the addition of IPTG to 1 mM final concentration. Aliquots of noninduced (control) and induced were collected by centrifugation at 10,000 *g* and solubilized in reducing SDS-PAGE sample buffer. The solubilized bacterial proteins were resolved by 10% SDS-PAGE, transferred to PVDF membrane, and immunoblotted using the 21D5 mAb.

Anti-PV-1C Polyclonal Antibody Production and IgY Purification

A 14 mer peptide (KGPPLVNPAVPPSG single letter amino acid [aa] code) corresponding to the 12 last COOH-terminal amino acids of rat PV-1 (to which a lysine and a glycine were added to facilitate the coupling and to improve solubility) was synthesized by SynPep Corp. The peptide was coupled to BSA via glutaraldehyde and this conjugate was used as antigen for polyclonal antibody production in chickens at Lampire Biologicals using the company's standard immunization protocol.

Total chicken IgY was purified from eggs laid by the immunized chickens using a modified procedure of Polson et al. (26). Briefly, egg yolks devoid of the amniotic sack were diluted 4× in PBS and brought to 3.5% polyethylene glycol (PEG). The precipitated lipoproteins were centrifuged for 25 min at 5,000 *g* in a JA-14 rotor and the supernatant was saved. The pellet was redissolved in 2× initial yolk volume of PBS and a second round of 3.5% PEG precipitation was performed as before. The two supernatants were pooled, filtered through four layers of cheesecloth, and centrifuged for an additional 30 min at 7,500 *g*. The supernatant was filtered through Whatman No. 4 filter paper, its volume measured (Z ml) and mixed with 0.085 × Z grams of PEG to precipitate IgY. The precipitate was collected by centrifugation at 5,000 *g* for 25 min. The pellet was solubilized in PBS (2.5× initial yolk volume) and IgY was again precipitated using 3.5% PEG. The precipitate was collected by 5,000 *g* centrifugation for 25 min and redissolved in PBS (0.25× initial yolk volume). This IgY solution was chilled on ice for 10 min and mixed with an equal volume of 50% ethanol which had been chilled at -20°C. The precipitated IgY was collected by 30 min of centrifugation at 10,000 *g*, redissolved in PBS (0.25× initial yolk volume), and dialyzed overnight against 20–40 vol of PBS to yield a total IgY fraction from egg yolk.

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 Hepes, pH 8.0, at a final concentration of 20 mg/ml and coupled to AffiGel-10™ beads (4 ml settled gel) (Bio-Rad) by incubation (16 h, 4°C) with gentle agitation. After quenching (1 h, room temperature [RT]) the remnant active sites with 0.2 M ethanolamine, pH 8.2, the matrix was packed into a column and washed with 100 bed vol of PBS. Total IgY fraction from egg yolk was incubated (12–14 h, 4°C) with the column with the help of a peristaltic pump. The column was washed with 100 bed vol of PBS and the bound antibodies were eluted with 10 bed vol of 0.1 M glycine, pH 2.5. Fractions of 1.4 ml were collected onto 100 µl of 1.5 M Tris, pH 8.8, and promptly mixed for antibody activity preservation. Each fraction was monitored for protein content by absorbance at 280 nm and for antibody specific activity by ELISA assays using PV-1C peptide-coated microwell plates. The fractions containing the antibody were pooled, concentrated, and dialyzed at 4°C against 2 changes (4 h each) of 100 vol of PBS using a 100 kD cut-off dialysis membrane.

Anti-PV-1C Polyclonal Antibody Validation

ELISA. Affinity 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 anti-PV-1C antibody was detected using an anti-chicken IgY HRP-conjugated reporter antibody (Bioscience Resource Project) and TMB (3, 3', 5, 5' tetramethylbenzidine) substrate (KPL) for colorimetric reaction. Either preimmune IgY or an irrelevant peptide was used as negative controls.

Immunoblotting and Peptide Competition. Whole rat lung lysate proteins (200 µg) were resolved by preparative 10% SDS-PAGE and transferred to a PVDF membrane which was subsequently blocked (30 min, RT) in 5% nonfat dry milk in PBS and 0.1% Tween 20. Strips, containing ~20 µg protein, were cut and incubated (1 h, RT) with either serial dilu-

tions of the anti-PV-1C pAb or 21D5 mAb or preimmune IgY (as positive and negative controls, respectively). The bound antibody was detected by incubation (30 min, RT) with a rabbit anti-chicken IgY HRP-conjugated antibody (Bioscience Resource Project) 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 immunoprecipitation buffer (20 mM Tris-HCl, pH 7.5, containing 1% NP-40, 0.4% deoxycholate, 0.1% SDS, 300 mM NaCl, 1 mM EDTA, 1 mM PMSF, and protease inhibitor cocktail). The extract was clarified by centrifugation at 100,000 *g* for 1 h in a TLA45 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 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 × 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 again 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. For visualization of the immunoprecipitated protein, the solubilized antigen-antibody complexes were resolved in nonreducing conditions on 12% SDS-PAGE and silver stained. Control experiments were carried out by replacing the anti-PV-1C pAb with preimmune total IgY or by omitting it.

Western Blotting of Different Rat Tissues. Total membranes from different rat organs or tissues were prepared as in the case of the lung and high-pH extracted. The high-pH insoluble material (containing PV-1) was extracted in 0.5% SDS in 50 mM Tris, pH 6.8, and protease inhibitor cocktail, clarified by centrifugation for 30 min at 12,000 *g*, and the protein content determined. Equal amounts of protein (300 µg) from different rat tissues and 10 µg in the case of the lung were separated by 8% SDS-PAGE, transferred to PVDF, and immunoblotted using the anti-PV-1 21D5 mAb.

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 Hank's balanced salt solution, then fixed in situ by a 30-min perfusion of paraformaldehyde-lysine-sodium metaperiodate (PLP) fixative. The tissue was excised, cut into small blocks (~3 × 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 cryostat sections (~45 µm) cut from the fixed blocks were rinsed (5 × 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 × 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 acetate veronal buffer, pH 6.8 (1 h on ice), stained in the dark (1 h at RT) with Kellenberger uranyl acetate, dehydrated through graded ethanols, and finally embedded in Epon 812 resin. Tissue blocks were cured for 48 h at 90°C, and ~50-nm sections, 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 CM10 electron microscope.

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

Postembedding Immunogold. Small blocks (1 × 1 mm) from PLP fixed lungs, cryopreserved and stored as above, were sectioned on a Reichert ultramicrotome equipped with a F4 cryoattachment. Ultrathin cryosections 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-1C pAb 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, pI 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. 1 a and detailed in Materials and Methods. Aliquots 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. 1 b, 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. 1 b, 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. 1 c) 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. 1 d 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 pI gradient (fractions 11–14 in Fig. 1 e) corresponding to a pI 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. 1 f). 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-Sephacel chromatography and the flow-through was collected, as PV-1 binds very weakly (Fig. 1 g) 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. 1 h). 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. 2 c). To obtain the sequence of the full length message, the same library was re-screened with a ³²P-labeled DNA probe (residues 841–

Table I. Peptide Sequences Obtained by PV-1 Microsequencing

Sequence	Position	Full length PV-1 residues
MGLSMDRSPYSRTGDR	NH ₂ -terminal	1–16
FQADVNLNVR	Internal 1	231–240
SLDNIGYHYSMLMPEFSS	Internal 2	247–264

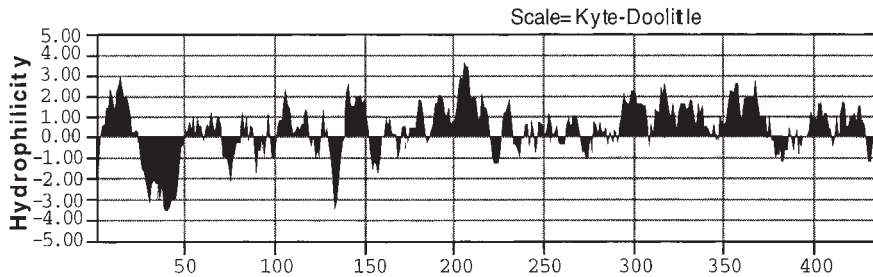
a)

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1 G TGGGC CCCTCC GGACA CTGGG CAAMT GGGGC TTCCTC ATGGA CCAGCA GCCTGG TACTCC CCAGC GGGGG GAAGG GAGTC GCAGC TGCTGG GTACT ACCTG CCG
100 T ATTTT TTCTCT CTCTG TGCTG CTCTC CCAGT TCCTC ATCAT CCCTG CCAGC GTCTT CTACA TATCG TATCG TGACG GCAGC CAAGG AGTCC AGT
26 Y F F L F V S L I Q F L I L G Q V L F M I Y G N V H A T T E S S
199 C TGCGG GCAGC GGACA TCCTG GCAGC CAAMC TATTC AGCCA GGTGG TGGGA CTGTC GGCGG CAAGC GCTGA CCTGA CCAGA CAAGT GAACA TCAGC ACC
59 L R A T E I R A D N L Y S Q V V G L S A A Q A N L S K Q L N I S T
298 C TCCTC AAGGA CAAGG TCATG CAGCA GCTCC TGACC ACAGG CCAGG AGGAG GAGG CATCA ACCTC AGCTT CCAGC AGTCC CAGG CGACC TGATC ACC
92 L V K D T V M Q Q L L T T R R E V E R I N A S F R Q C Q G D L I T
397 T ACATA AACCA TATTC GATTC ATGCG CCCTA TATTC CTGAG CAGCA AGCAG TGCCA GGACC AGCTG AAGGA GGCCA ACAGC ACCTG CGAGC CCCTG CTC
125 Y I N Y N R F I A A I L S E K Q C Q E Q L K E G N K T C E A L L
496 T TCANG CTGGG AGAGA AGGTT AAGC ACCTG AAGTG GAGGT GGTCA AGGAG GAGGC GGTAT CCTCC AAGGA CAGG ACAGC CTCTT GCAGG GAAGG CCG
158 F K L G E K V K T L E M E V V K E E A V C S K D K D S L L A G K R
595 C AGGGG GAAMT GCAGC AGGGG GCCTG TGCCA AAGCG CGAGA GCAGC AANG CAGCA CCAGC AGGTC ACAGC GGACC AACTG CCAGA GGTCC AGTCC CTG
191 Q A E M Q Q E A C G K A R E Q Q K Q D Q Q V T E E Q L R K V Q S L
694 T GCCTC CCAGT GCAGC AGGGG AAGTT CCAGG CCAGC GTCTT GAGCG TGCTG CGGGA TTCCG TGATC TACCG TTCCG TGATC AATAT AGGTT ACCTC TAC
224 C L P L D Q E K [F Q A D V L N V W R] D S L V Y R [S L D N I G Y H Y]
793 T CCCTG ATGCG TCTGG TCCTT CCAGC GAACA TGCCA GAGCC TGCCG GGAT CATCA CCAGC AAGGT AAGAG AGCTG CCAGG CCAGT TGCCG CCG
257 [S L M P E F S S] L R R T C E S L P G I M T T K V E E L A R G L R A
892 G CCATP GAAGG CPTCA CCCTT GAANA TGAGG AGCTG CGAGC CCAGA AGCTG GAGCT GGAGC CCAGC ATACA GGCG AGCGG GAGCC GCAGC CCAGC GCC
290 G I E R V T R E N G E L L R Q K L E L E R A I Q G E R E A R T R A
991 G GGACC GAAGC CCAGC CCAGC GAAGC CCAGC TACCG ACAGC GTGGG CCAGC CAAGC ACAGC TGCTG CTGGA GGAGA AGGCA CCAGC GCAGC CAAGC CCG
323 G T E A Q A R E T Q L R T E C A R Q T Q L A L E E K A A L R T Q R
1090 G ACAGC CTGGA CCAGC AGCTG GAAGC CCAGC AGCGG GAGCC AACTG CCAGC CCAGG TGATC GTGGC CATTA CCAGC CTGGA CCAGC CCAGC AAG
356 D L E R Q L E A R K R E L E Q L R T E V D V R I S A L D T C V K
1189 G CCANG TGCTT CCAGC CCAGC CAGCC GAGC TCACA GGTCC ACCTC CCAGC CCCTC ACCTA TTGAT CCAGC TACCG TGAGG GAAMT CAAGA AAGGG ATC
389 A C S L P A I P R L P G P P P N P P P I D P A S L E E F K R I
1288 C TAGAG TCACA CCAGC CCCTT CTAGT CAAMC CTCTT GTCTC ACCTA GTGGC TGAGG ATGGA GTCTC TGCCG GAGCC TGCCG ACAGC AATCG CCAGC CCG
422 L E S Q R P P L V N P A V P P S G
1387 T GGATG GGGCC AGCCA GATCC CCACA GTGCT GGACA CCAGC TGTCG CCAGC CAGG ACCTC CAACC CCACA CCTCT TCTGG CAACC CTACA CTGTC CCG
1486 T GCACC CCAGT GTCTT CCAGC ACAGT GGATC TAGAG CAGC GCAGC TGTCG GGTCG CPTCA CATCG CCCTC TTCCG ACATG AAGTG CCAGC AGCTC AGC
1585 T GCCTA GGAGC CTCTT CCAGT TTCTT ATCAC TCTCC TCCTG ACAGG GGAGG TGAGA ACAGG AAGCC CGAGC TTCCG CTCCG TTGGG AGTAT CCAGC CTC
1684 T CTCTT AAGG TTCTC AGGTT CTCTT TGAGA GTTTC CAGC CTCTC CTGGA GGATP CTCTC CCCTA TTCCG CCAGA ATATT CATTG GATTC TAGAA CCG
1783 T TCTA GAGC TGCCG GTCTC CCCTC TCCTC ACAGG GAGG ACCTC AACTG TATGT GAGTC CCCTG TGATC CTCCG GCCTC GGAGG GGTCC CTCCG GCC
1882 T CAGTC TGCTT GAGC CCTTA AAGC ATCAC TGCCA CCTGA TATAT ATCAT GAAGG TCCTT TGGA AAAAA AAAAA AAAAA AAAAA A

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b)



c)

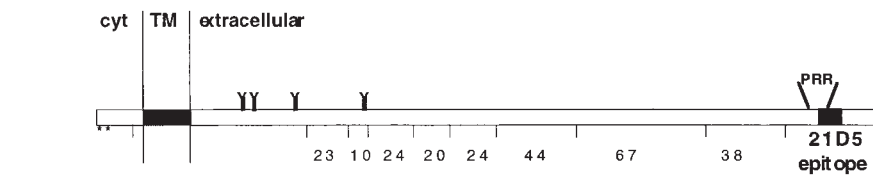


Figure 2. (a) Sequence of the full length rat PV-1 cDNA and protein. The positions of the peptides obtained by protein microsequencing (square boxes) and the putative transmembrane domain (oval box) are indicated. The portion of the PV-1 message that was obtained by rescreening of the rat lung library with a DNA probe (see Materials and Methods) is underlined. These sequence data are available from GenBank/EMBL/DDBJ under accession number AF154831. (b) Hydrophilicity plot and (c) schematic of the protein sequence. The positions of the consensus phosphorylation sites (*), N-glycosylation sites (Y), cysteines (I), the 21D5 mAb epitope (filled box), and the proline-rich region (PRR) are marked. The number of amino acids in between cysteines is indicated.

1268 of PV-1 full length cDNA). Upon sequencing of seven longest inserts isolated through the second screening we obtained an 1968-nucleotide message (Fig. 2 a). 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 Δ 116–252 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 1,341 bases long and contains all three peptides obtained by protein sequencing of the purified PV-1 (Fig. 2 a). 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. 2 b) 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

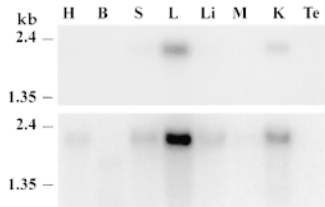


Figure 3. Northern blotting of rat tissues. Rat lung total RNA (20 μ g) was probed with a 428-bp 32 P-labeled PV-1 DNA fragment as described in Materials and Methods. A single band corresponding to the PV-1 mRNA was obtained at

\sim 2000 nt in heart (H), spleen (S), lung (L), liver (Li), muscle (M), and kidney (K). No message was obtained in the testis (Te). Exposure time: upper panel, 4 h; lower panel, 12 h.

sites (the serine 4 [SMD] and threonine 13 [TGD]) in the cytoplasmic 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 (KGPPLVN-PAVPPSG 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 immuno-

blotting 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

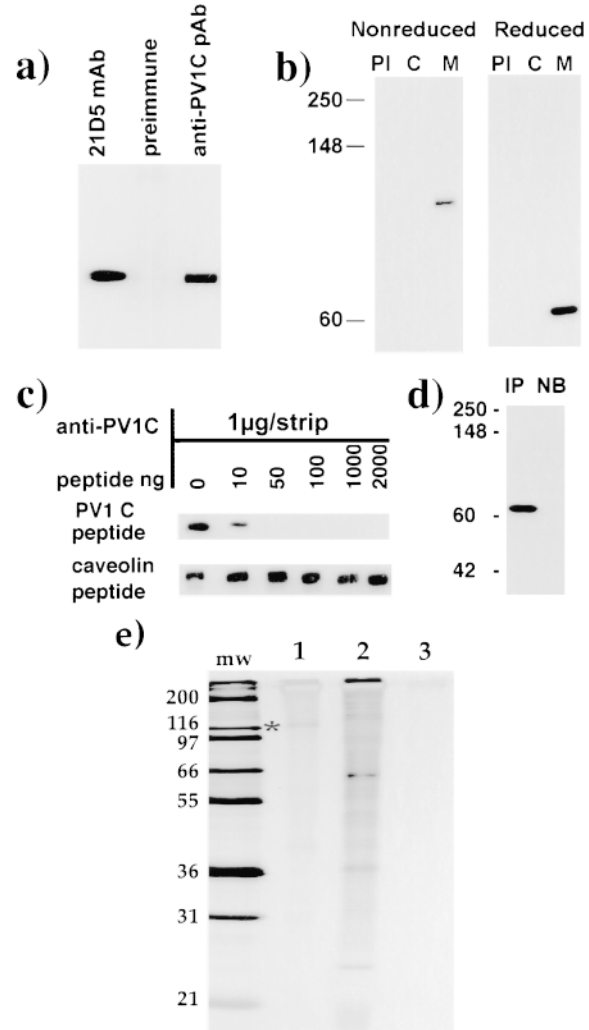


Figure 4. Anti-PV-1C chicken polyclonal antibody specificity. (a) Immunoblotting of rat lung total membrane proteins containing strips with 1 μ g of each 21D5 mAb, preimmune IgY, and anti-PV-1C polyclonal antibody as indicated in the figure. (b) Immunoblotting of rat blood plasma (PI), rat lung cytosol (C), and rat lung membranes (M) (10 μ g each) in nonreducing (left) and reducing (right) conditions. (c) Competition of the anti-PV-1C pAb by the PV-1C peptide compared with an irrelevant (caveolin) peptide. (d) Immunoprecipitation of rat PV-1 using the anti-PV-1 C pAb followed by immunoblotting of equivalent amounts of the immunoprecipitated (IP) and the unbound (NB) material using the 21D5 mAb. (e) Immunoprecipitation of rat lung PV-1 with anti-PV-1C pAb as described in Materials and Methods. The immunoprecipitated proteins by anti-PV-1C pAb (lane 1), preimmune total IgY control (lane 2), and anti-chicken IgY-agarose control (lane 3) were resolved by 12% SDS-PAGE in nonreducing conditions and silver stained. The asterisk marks the position of the PV-1 dimer.

pAb is highly specific for PV-1; by immunoblotting it detects a single band of either $\sim 110,000$ or $\sim 60,000$ 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 $\sim 120,000$ (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 immunoisolation 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 plas-

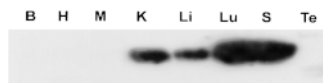


Figure 5. Western blotting of different rat tissues using the anti-PV-1C pAb. Proteins from total membranes from

different rat tissues were prepared as in Materials and Methods and immunoblotted with the anti-PV-1C pAb (1:500 dilution) followed by an HRP-conjugated anti-chicken IgY reporter antibody and the signal was detected by enhanced chemiluminescence. B, brain; H, heart; M, muscle; K, kidney; Li, liver; Lu, lung; S, spleen; Te, testis.

malemma 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 immunoisolation on anti-caveolin 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 immunisolated 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 pI of the dimer and the presence of terminal, nonreduced galactosyl residues in $\alpha 1-3$ 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. As the NH_2 -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 $\sim 50,000$ apparent molecular weight band (which would represent the nonglycosylated form of PV-1 monomer) by the 21D5 mAb only in the transformed bacterial clones.

Analysis of the primary structure of the PV-1 confirms its already known properties such as dimer formation, membrane insertion, and glycosylation. Additional information is represented by the possibility of phosphorylation of the cytoplasmic domain, which might be involved

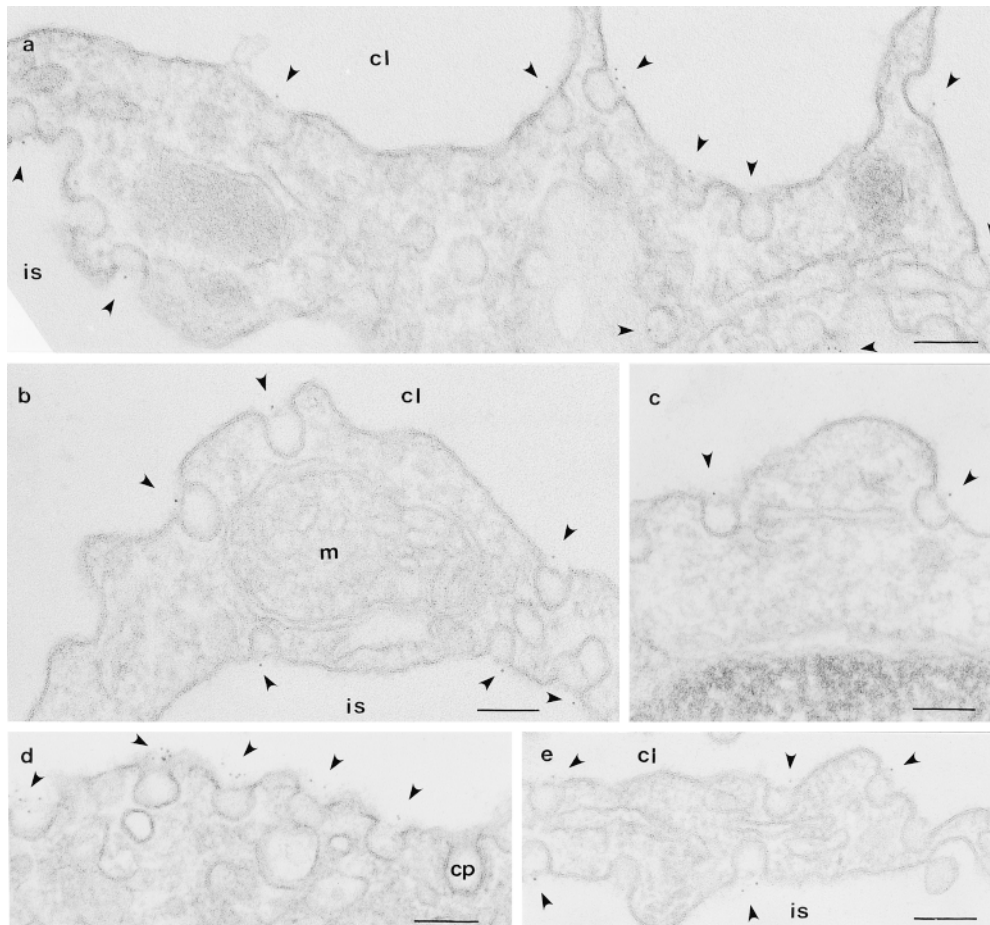


Figure 6. Immunolocalization of PV-1 in rat lung by preembedding immunocytochemistry. Rat lung specimens prepared as described in Materials and Methods were reacted with anti-PV-1C pAb followed by a 5 nm gold-conjugated reporter antibody. The label (arrowheads) was found associated specifically with the necks and stomatal diaphragms of endothelial caveolae (a-e) at both fronts of the endothelial cell (a, b, and e). Most caveolar stomatal diaphragms are marked by a single gold particle and occasionally by a cluster of particles (a, d, and e). No label was found on either plasmalemma proper (a-e) or coated pits (cp in d). Bars, 100 nm.

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 immunoisolation as the results obtained by the two approaches are in agreement. By immunoisolation the label colocalizes strictly with caveolin on immunisolated 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. Consid-

ering 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. 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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