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Immunoisolation and Partial Characterization of Endothelial Plasmalemmal Vesicles (Caveolae)

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Plasmalemmal vesicles (PVs) or caveolae are plasma membrane invaginations and associated vesicles of regular size and shape found in most mammalian cell types. They are particularly numerous in the continuous endothelium of certain microvascular beds (e.g., heart, lung, and muscles) in which they have been identified as transcytotic vesicular carriers. Their chemistry and function have been extensively studied in the last years by various means, including several attempts to isolate them by cell fractionation from different cell types. The methods so far used rely on nonspecific physical parameters of the caveolae and their membrane (e.g., size-specific gravity and solubility in detergents) which do not rule out contamination from other membrane sources, especially the plasmalemma proper. We report here a different method for the isolation of PVs from plasmalemmal fragments obtained by a silica-coating procedure from the rat lung vasculature. The method includes sonication and flotation of a mixed vesicle fraction, as the first step, followed by specific immunoisolation of PVs on anticaveolin-coated magnetic microspheres, as the second step. The mixed vesicle fraction is thereby resolved into a bound subfraction (B), which consists primarily of PVs or caveolae, and a nonbound subfraction (NB) enriched in vesicles derived from the plasmalemma proper. The results so far obtained indicate that some specific endothelial membrane proteins (e.g., thrombomodulin, functional thrombin receptor) are distributed about evenly between the B and NB subfractions, whereas others are restricted to the NB subfraction (e.g., angiotensin converting enzyme, podocalyxin). Glycoproteins distribute unevenly between the two subfractions and antigens involved in signal transduction [e.g., annexin II, protein kinase Cα, the Gα subunits of heterotrimeric G proteins (αs, αq, αi2, αi3), small GTP-binding proteins, endothelial nitric oxide synthase, and nonreceptor protein kinase c-src] are concentrated in the NB (plasmalemma proper-enriched) subfraction rather than in the caveolae of the B subfraction. Additional work should show whether discrepancies between our findings and those already recorded in the literature represent inadequate fractionation techniques or are accounted for by chemical differentiation of caveolae from one cell type to another.

INTRODUCTION

Plasmalemmal vesicles (PVs)[1] were first described in endothelial cells as spherical vesicles of regular size (~70 nm) and shape (Palade, 1953; Bruns and Palade, 1968) associated with the plasma membrane. Similar structures were found in epithelial cells and called caveolae intracellulars (Yamada, 1955). High-resolu-

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[1] Abbreviations used: PVs, plasmalemmal vesicles; IIB (immunoisolation buffer); P1, luminal endothelial plasma membrane patches coated with silica; Ps, material that remains attached to silica after P1 sonication and sucrose density gradient centrifugation; HST, supernatant of high salt-treated, sonicated endothelial membrane patches; C, fraction of vesicles used as starting material for immunoisolation; B, bound subfraction; NB, nonbound subfraction; mAb, monoclonal antibody; pAb, polyclonal antibody; BSA, bovine serum albumin; HBS, HEPES-buffered sucrose; eNOS, endothelial nitric oxide synthase.

tion scanning electron microscopy (Peters *et al.*, 1985) and rapid freeze deep-etch techniques (Rothberg *et al.*, 1992) have revealed on the cytoplasmic face of the caveolae the presence of characteristic ridges disposed as meridians (Peters *et al.*, 1985) or spiral patterns (Rothberg *et al.*, 1992). Similar ridge patterns are found on flat areas of the plasmalemma proper as well as on partially invaginated PVs (Rothberg *et al.*, 1992). Subsequent studies have demonstrated the presence of caveolae at different surface densities in practically all types of mammalian cells with few exceptions (Fra *et al.*, 1994; Gorodinsky and Harris, 1995).

Attempts to define the function of PVs have relied on perfusion experiments with a variety of tracers carried on microvascular beds provided with a continuous endothelium (Milici *et al.*, 1987; Ghitescu *et al.*, 1988; Predescu *et al.*, 1994, 1997). These studies showed that all tracers above 2 nm diameter were restricted to PVs and that their transport was inhibited by agents known to interfere with the mechanism of membrane fusion with target membranes (Predescu *et al.*, 1994). These findings established the role of PVs in transcytosis but did not provide information about their chemistry. Another approach aimed at finding the function of PVs relied on immunocytochemistry tests carried on in situ; it has been shown that certain antigens are restricted in their distribution to PVs and absent or present at much lower density on the plasmalemma proper (Fujimoto *et al.*, 1992; Fujimoto, 1993).

Still another approach has relied on attempts to isolate caveolae from different cell types (Chang *et al.*, 1994; Lisanti *et al.*, 1994; Scherer *et al.*, 1994; Lisanti *et al.*, 1995; Smart *et al.*, 1995) including endothelial cells (Schnitzer *et al.*, 1995a–c; Shaul *et al.*, 1996). These procedures relied either 1) on the extraction of crude membrane fractions with Triton X-100 (Chang *et al.*, 1994; Lisanti *et al.*, 1994) to yield, after centrifugation in density gradients, a light membrane preparation assumed to consist primarily of caveolae; or 2) on mechanical disruption (e.g., sonication) to detach the caveolae and separate them by flotation in density gradients (Smart *et al.*, 1995; Shaul *et al.*, 1996); or 3) on mechanical disruption (e.g., shearing) in the presence of Triton X-100 followed by flotation in sucrose density gradients (Schnitzer *et al.*, 1995a–c). The results obtained with cell fractionation procedures already constitute a sizable literature that ascribes to PVs a wide variety of components and, by implication, functions (Chang *et al.*, 1994; Lisanti *et al.*, 1994, 1995; Scherer *et al.*, 1994; Shenoy-Scaria *et al.*, 1994; Schnitzer *et al.*, 1995a,b; Smart et al., 1995; Stahl and Mueller, 1995; Garcia-Cardena *et al.*, 1996; Shaul *et al.*, 1996). It is possible that these results reflect differential detergent extraction of membranes, and it is also likely that differences in physical parameters between different classes of vesicles might not be large enough to permit separation by centrifugation in density gradients.

To obviate these problems we have developed a procedure that uses luminal plasmalemma patches isolated from rat lung vasculature by the cationized silica procedure (Jacobson *et al.*, 1992) as starting preparation, detaches the vesicles by sonication, and isolates them by immunoabsorption on magnetic microspheres coated with anticaveolin antibody. The procedure takes advantage of the fact that caveolin is a generally accepted marker for caveolae (Glenney and Soppet, 1992; Kurzchalia *et al.*, 1992; Rothberg, *et al.*, 1992; Dupree *et al.*, 1993; Parton, 1994).

MATERIALS AND METHODS

Materials

Sprague Dawley male rats (250–300 g) were used in all experiments. Positively charged colloidal silica was kindly provided by Dr. B. Jacobson (University of Massachusetts, Amherst, MA). The other reagents were purchased from the following sources: xylazine, ketamine, and acepromazine from Victor Medical Laboratories (Irvine, CA); Nycodenz[5-(*N*-2,3-dihydroxypropylacetamido)-2,4,6-triiodo-*N*,*N*-bis(2,3-dihydroxy) isophtalamide] from Accurate Chemical (Westbury, NY); polyacrylic acid from Polysciences (Warrington, PA); 2-(*N*-morpholino)ethansulfonic acid, (MES) *N*-2-hydroxyethylpiperazine-*N*′-2-ethansulfonic acid (HEPES), phenylmethylsulfonyl fluoride, leupeptin, pepstatin A, *O*-phenanthroline, E-64 [transepoxysuccinyl-L-leucynamido(4-guanidino)butane], EDTA, heparin, sodium nitroprusside, ATP, and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, MO); DMEM from Life Technologies (Grand Island, NY); bovine serum albumin (BSA) was from Boehringer Mannheim (Indianapolis, IN); the other general reagents were obtained from either Fischer Scientific Co. (Pittsburgh, PA) or Sigma Chemical Co.; and the magnetic microspheres were purchased from Dynal (Lake Success, NY). A BCA protein assay kit and SuperSignal enhanced chemiluminescence (ECL) substrates were purchased from Pierce (Rockford, IL). [α-^{32}P]dGTP was purchased from Amersham Co. (Arlington Heights, IL).

Antibodies

The anti-caveolin polyclonal antibody (pAb) and monoclonal antibody (mAb), anti-annexin II heavy chain mAb, anti-endothelial nitric oxide synthase mAb, and anti-protein kinase Cα were purchased from Transduction Laboratories (Lexington, KY). Anti-c-src mAb was obtained from Oncogene Sciences (Cambridge, MA). The mAb against podocalyxin and anti-Gα pAbs was a kind gift from Dr. M. Farquhar (University of California, San Diego, La Jolla, CA); the endothelium specific mAbs (21D5, 28D5, and 30B3) were from Dr. Lucian Ghitescu (University of Montreal, Montreal, Ontario, Canada); the anti-rat thrombomodulin pAb was from Dr. D. Stern (Columbia University, NY); the anti-thrombin functional receptor pAb was from Dr. Shaun R. Coughlin (University of California, San Francisco); the anti-angiotensin-converting enzyme pAb was from Dr. R. Skidgel (University of Illinois, Chicago, IL); and the pAbs against αi2, i3, and q subunits of heterotrimeric G proteins were from Dr. T. Fischer, Dr. R. Zumbihl, and Dr. B. Rouot (INSERM U431, Montpellier, France). The unlabeled, horseradish peroxidase coupled, and biotinylated goat anti-rabbit IgG, Fc specific, and horseradish peroxidase-coupled goat anti-mouse IgG were purchased from Biodesign (Kennebunk, ME). Two and 10 nm gold-conjugated donkey anti-mouse antibodies were purchased from The Jackson Laboratory (Bar Harbor, ME). The streptavidin-biotinylated

horseradish peroxidase (ABC) complex was from Vector Laboratories (Burlingame, CA).

Antibody Production

Polyclonal sera were raised in New Zealand female rabbits against synthetic peptides covalently coupled to keyhole limpet hemocyanin using the N-terminal residues 1–14 of chicken caveolin (MSGGKYUSDSEGHLYC, single-letter code). Sera were collected after the fourth antigen boost. For affinity purification, the N-terminal peptide was linked directly to cyanogen bromide-activated Sepharose 4B according to the instructions of the manufacturer (Pharmacia, Uppsala, Sweden). The serum was incubated with the Sepharose beads overnight at 4°C, and the bound antibody (anticaveolin-N) was eluted with 0.2 M glycine (pH 2.8); the collected fractions were neutralized with unbuffered Tris.

Buffers and Solutions

Buffers and solutions used are as follows: buffer A: 0.25 M sucrose, 2 mM EDTA, 10 mM HEPES (pH 7.2); solution B: 2.3 M sucrose, 0.5 M KCl; solution C: 1.09 M sucrose, 0.5 M KCl; and solution D: 0.25 M sucrose, 0.5 M KCl. KCl (0.5 M) was added to solutions B–D to minimize the nonspecific binding of the proteins to the membranes. The immunoisolation buffer (IIB) contained 0.1% BSA and 2 mM EDTA in phosphate-buffered saline (PBS). HEPES-buffered saline (HBS) had 150 mM NaCl, 2 mM EDTA, and 10 mM HEPES (pH 7.4).

Purification of PVs

Figure 2 shows our general scheme for the isolation of PVs.

Step I (Surgery, Perfusion, Homogenization, and Filtration). The surgical procedures, rat lung perfusion with cationized silica, lung homogenization, and homogenate filtration were carried out as described previously (Jacobson *et al.*, 1992). The only modification was that the anesthetic mixture comprised ketamine:xylazine: acepromazine (6:2:1).

Step II (Nycodenz Density Gradient Centrifugation). The entire procedure was done as described previously (Jacobson *et al.*, 1992) except that the bottom cushion was 80% Nycodenz supplemented with 10 mM HEPES (pH 7.4) and 125 mM sucrose, and the centrifugation time was increased to 60 min.

Step III (Sonication). All P1 pellets were pooled in buffer A and silica-coated membranes were collected by 10 min centrifugation at 4°C in an Eppendorf 5403 centrifuge. The supernatant was discarded and the pellet was resuspended in 0.7 ml of ice-cold buffer A and sonicated three times for 30 s using a Microson device (Heat Systems, Farmingdale, NY) while keeping the sample on an ice-water slurry. After taking out a small sample of the resulting preparation, the remainder was adjusted with solution B to 1.72 M sucrose and 0.5 M KCl final concentration. This suspension was loaded at the bottom of a prechilled SW40 centrifuge tube, overlaid with 1.5 ml of solution C, and topped with 8 ml of solution D. The ensuing gradient was centrifuged in a SW40 rotor (Beckman Instruments, Palo Alto, CA) at 82,000 × *g* for 14 to 20 h at 4°C. This step yielded three fractions: a pellet (Ps), a supernatant fraction in the load region (1.72 M sucrose; supernatant of high-salt treated (HST), and a light-scattering band at the interface between 1.09 and 0.25 M sucrose (C). The C fraction was used as starting material for immunoisolation of the vesicles on anticaveolin-coated magnetic microspheres.

Step IV (Immunoisolation of PVs). The preparation of the magnetic microspheres was done according to Saucan and Palade (1994) with the modification that the immunoisolation buffer (IIB) contained 0.1% BSA in PBS (pH 7.4) supplemented with 2 mM EDTA. Briefly, the beads were activated using the tosyl chloride method as specified by the manufacturer. After washing, the magnetic microspheres were incubated for 12 to 24 h at room temperature with a Fc-specific goat anti-rabbit IgG antibody; the next step was to block unspecific binding sites using IIB for 1 h at room temperature. This was followed by overnight incubation at 4°C with anticaveolin pAb.

One-half volume of the C fraction, as collected from the sucrose gradient, was diluted threefold in IIB, added to anticaveolin-coated magnetic microspheres, and incubated overnight at 4°C with gentle agitation. The magnetic separation of the microspheres from their supernatant yielded two subfractions: vesicles bound to the beads by anticaveolin antibody [the bound subfraction (B)] and remnant vesicles and membranes which did not bind to the anticaveolin-coated beads [the nonbound subfraction (NB)]. The magnetic beads were washed three times for 10 min at 4°C in 300 mM NaCl and 50 mM sodium phosphate (pH 7.4) supplemented with 2 mM EDTA. NB and the washes were pooled and their membranes collected by centrifugation at 105,000 × *g* for 1 h at 4°C in a TLA45 rotor.

The other half of the C fraction was diluted threefold with HBS and its vesicles and membranes were collected by centrifugation at 105,000 × *g* for 1 h in the same conditions as above and labeled C-starting material.

All samples were lysed for 15 min at room temperature in solubilization buffer [0.5% SDS in TBS, pH 6.8, supplemented with pepstatin A, leupeptin and E-64 (10 mg/ml each), 1 mM *O*-phenanthroline, 2 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA].

Biochemical Procedures

The protein content of each fraction was determined by the bicinchoninic acid method (Pierce) using BSA standards with 0.5% SDS in 50 mM Tris (pH 6.8) supplemented with 1 mM EDTA. The protein amount in the B fraction was estimated by subtracting the protein amount in NB from the protein content of C (B = C − NB); reliable, direct measurements were not possible because of the presence in B of IgG and albumin used in the immunoisolation procedure.

SDS-PAGE, Silver Staining, and Immunoblotting. Proteins were separated by SDS-PAGE and either silver stained or transferred to Immobilon P or nitrocellulose membranes which were immunoblotted with various antibodies. ECL was used as a detection system. For quantitation assays, radioiodinated protein G was used, the blots were exposed to a PhosphorImager screen for 1 to 3 days, and

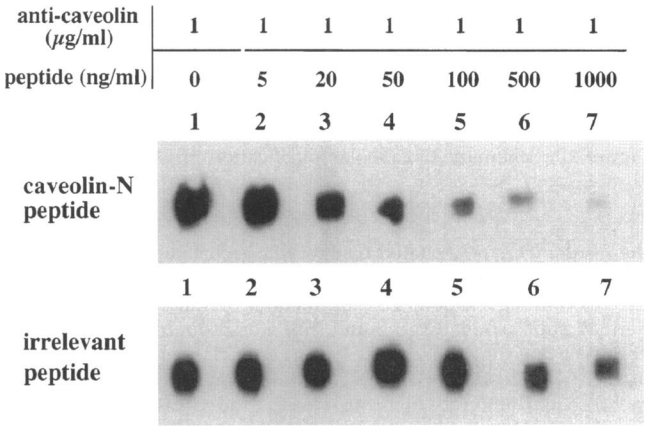

Figure 1. Anticaveolin antibody validation. P1 proteins were resolved using a preparative 12% SDS-polyacrylamide gel and then transferred to a PVDF membrane that was treated for 1 h at room temperature with blocking buffer (5% nonfat dry milk, 0.1% Tween 20 in PBS). Strips were cut and incubated for 1 h at room temperature with 1 μg/ml anticaveolin-N pAb alone (lane 1) or antibody incubated beforehand for 30 min at room temperature with increasing concentrations of relevant (top) or irrelevant (bottom) peptide (lanes 2–7).

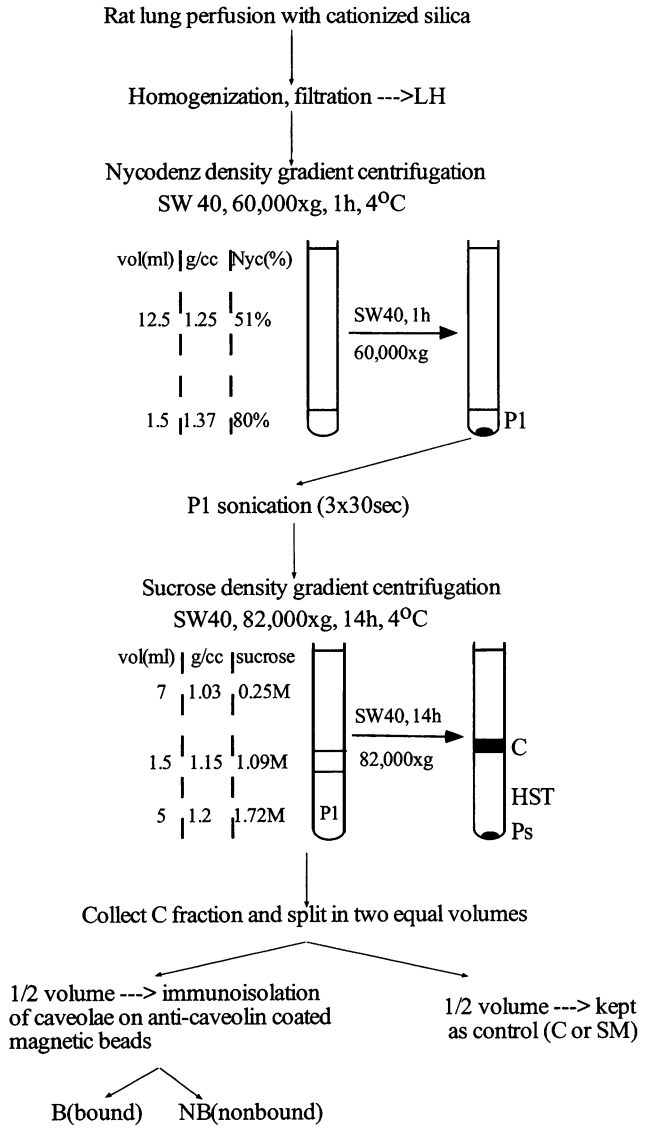

Figure 2. Schematic of caveolae purification procedure.

the results were quantitated using a PhosphorImager 445 (Molecular Dynamics, Sunnyvale, CA). To determine distribution of various antigens among fractions and subfractions of interest, the volume of derived subfractions (e.g., B and NB) was brought up to the level of the starting material (e.g., C-starting material) and equal volumes of each were loaded onto gels. To assess concentration (or enrichment), equal amounts of protein were loaded onto gels for each fraction or subfraction.

[α-^{32}]PdGTP Overlay. Proteins from different samples were resolved by Tricine-SDS-PAGE (Schagger and von Jagow, 1987) and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was soaked for 30 min in a buffer containing 50 mM Na_2PO_4, 1 mM $MgCl_2$, 0.3% Tween 20, 2 mM dithiothreitol, and 10 μM ATP, followed by incubation for 2 h in the same buffer containing 1.2 μCi/ml [α-^{32}P]dGTP. The radioactive buffer was aspirated and the membrane was washed for 5 min six times in buffer without GTP. After drying, the membrane was exposed to film for 6 to 48 h.

Electron Microscopy

Samples of all fractions were monitored by electron microscopy. HST, C, and NB were fixed in suspension in 1% OsO_4, and their membranes and vesicles were pelleted for 30 min at 13,000 rpm in a microcentrifuge. The resulting pellets were stained en block in Kellenberger's uranyl acetate for 1 h or overnight, in the dark, and further processed for electron microscopy. P1 and Ps pellets and the B fraction were fixed in 1.5% glutaraldehyde and 3% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M cacodylate buffer (pH 7.4), postfixed in 1% OsO_4, and further processed for electron microscopy using standard procedures.

Immunogold Labeling. A sample of P1, resuspended in PBS, was applied onto a positively charged filter paper (Whatman Int. Ltd., Madstone, United Kingdom), labeled with anticaveolin mAb followed by a donkey anti-mouse IgG antibody conjugated to 2 nm gold, and then processed for electron microscopy. Vesicles immunoisolated onto magnetic beads were labeled on the beads with mAb anticaveolin antibody followed by 10 nm gold-conjugated donkey anti-mouse IgG antibody.

Tannic Acid Treatment. Treatment was performed by including in the standard procedure a 1-h incubation step with 1% tannic acid in 0.1 M cacodylate buffer (pH 7.4) after OsO_4 postfixation. The samples were washed three times for 10 min in 0.1 M cacodylate buffer (pH 7.4) before and after the tannic acid.

RESULTS

Antibody Characterization

The specificity of our caveolin-N antibody was tested using recombinant caveolin and the P1 fraction. The same band at 22 kDa was recognized in both cases (our unpublished results). Addition of antigen (N-terminal peptide) in incremental amounts reduced (Figure 1) and finally abrogated the signal. Our antibody was used exclusively for immunoisolation of caveolae on magnetic beads and commercial antibodies were used for monitoring (by Western blotting) different experimental samples.

Figure 3 (facing page). Electron micrographs of samples collected at different steps of the procedure: (A) P1, an example of the sheets of endothelial luminal membranes still bearing PVs (arrowheads) on their cytoplasmic side, found in this pellet and (B) immunogold labeling with an anticaveolin mAb followed by a gold-tagged reporter antibody of a membrane sheet from the P1 fraction (see MATERIALS AND METHODS). The gold particles of the reporter antibody (arrowheads) were found only on membrane invaginations representing PVs; (C) C fraction obtained by flotation in sucrose gradients of P1 sonicates; it consists of a mixed population of vesicles, many of them in the range of 50–100 nm; (D) B subfraction consists of regular, spherical membrane-bound vesicles immunoadsorbed on anticaveolin-coated magnetic beads. Free-edge membrane fragments are only occasionally encountered (arrow); (E) NB subfraction (vesicles and membranes which did not bind to anticaveolin) contains vesicles of various sizes, some of them with a characteristic fibrillar content (arrow), free-edge membrane fragments (arrowheads), and occasionally contaminating mitochondria (m); (F and G) labeling with anticaveolin mAb followed by a gold-tagged reporter antibody of the B subfraction while the vesicles were still attached to magnetic beads (see MATERIALS AND METHODS). The vesicular profiles appear at a distance from the bead surface in the oblique section shown in F whereas they are clearly attached to the bead in the normal section shown in G. Bars, 100 nm.

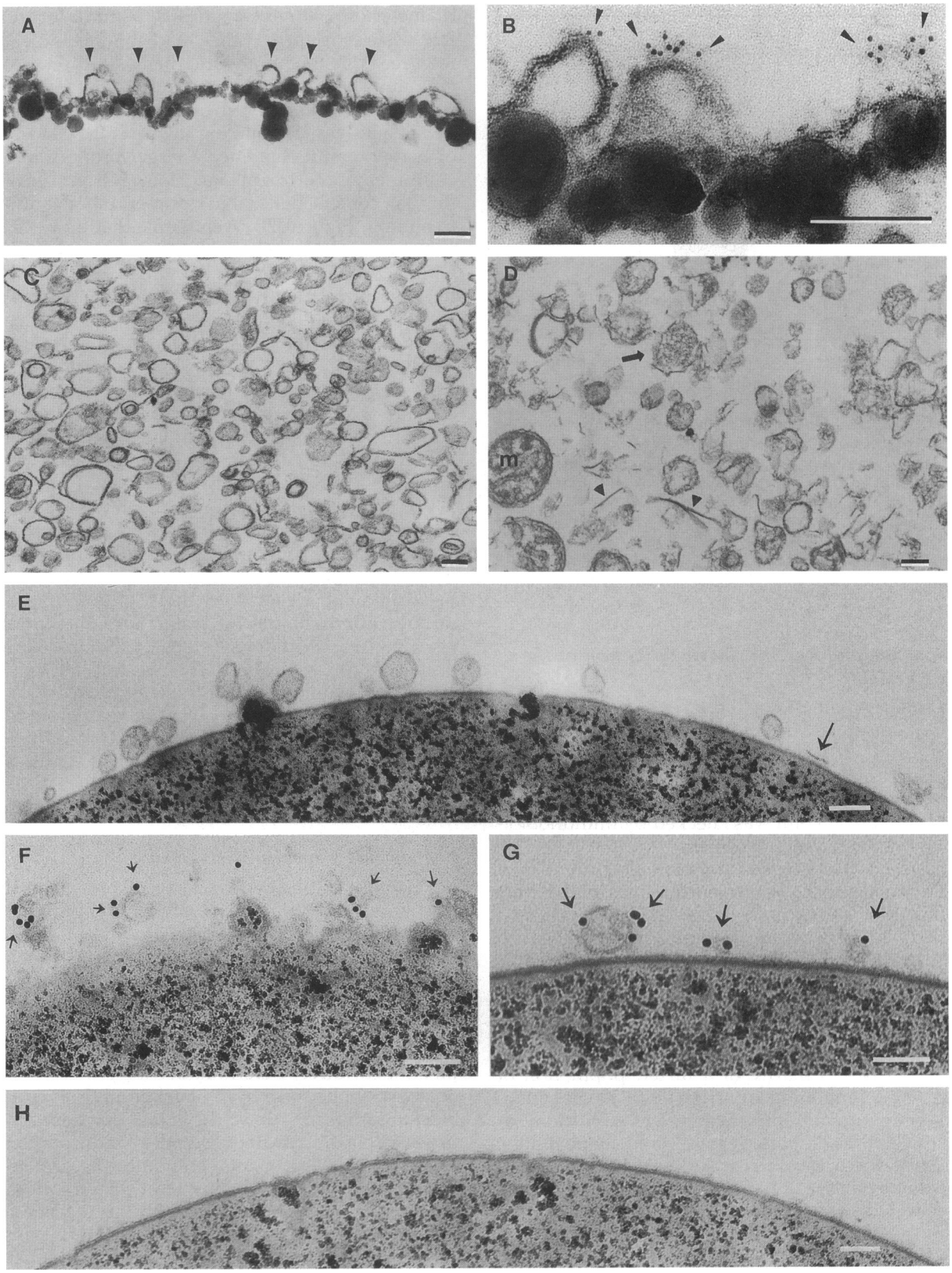

Figure 3.

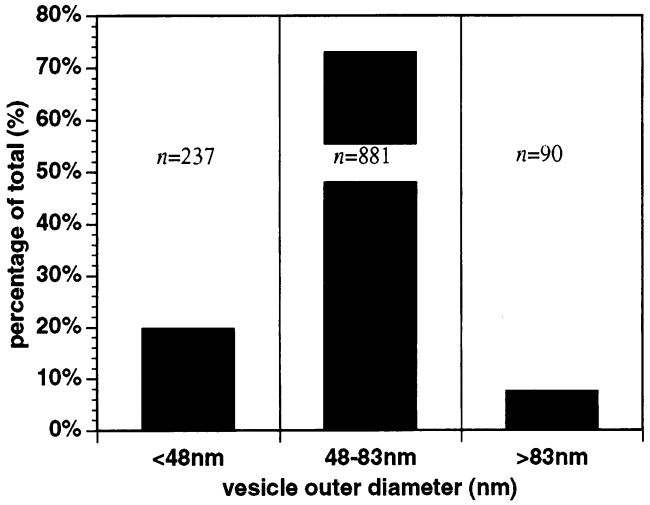

Figure 4. Morphometric analysis of the vesicles' diameters in the bound (B) subfraction. Aliquots from the best three experiments (>85% caveolin recovery in the B) were processed for electron microscopy. Blocks (two per experiment) were cut and random photographs at standard magnification (15,500×) were taken from sections (five per each block). Negatives were printed at standard magnification (2.75×) and the dimensions of the vesicles were measured directly on the micrographs (n, number of vesicles counted). Fragmentation and microtomy are assumed to account for vesicles of diameters <48 nm.

PVs Isolation and Partial Characterization of the Purified Vesicles

Figure 2 is a schematic of the PV purification procedure from rat lung vasculature as described in MATERIALS AND METHODS.

All of the fractions were monitored by electron microscopy, their protein content was determined, and the presence of caveolin was checked by immunoblotting with anticaveolin antibodies.

Figure 3 illustrates by transmission electron microscopy the subcellular components found in the fractions obtained at successive steps in the procedure. Figure 3A is an example of a plasmalemmal patch with PVs still attached to its cytoplasmic side. The PVs are less uniform in size and shape than in situ, presumably because of the homogenization trauma, but they still retain their caveolin as shown by the immunogold test in Figure 3B. Figure 3C shows that the C fraction consists primarily of a mixed population of vesicles (96%) ranging in size from 40 to 300 nm and a few free edge membrane fragments (<4%). Many of these vesicles are comparable in size and shape to PVs; the larger vesicles are of irregular shape. Occasionally, a few morphologically recognizable contaminants were detected. The vesicles in the C fraction were compared with those in ensuing NB (Figure 3D) and B subfractions (Figure 3, E–G). The bound subfraction (Figure 3, E–G) was homogenous and consisted of closed spherical vesicles (94%) with an average outer diameter of ~60 nm (range, 40–150 nm). It contained few vesicles of forms other than spherical (6%) and a few free edge membrane fragments. It did not contain any morphologically recognizable contaminants. In contradistinction, the NB subfraction was heterogeneous with vesicles ranging in size from 40 to 300 nm or higher, and it contained a larger proportion of free edge membrane fragments. Many NB vesicles had a fibrillar content detectable upon en block tannic acid treatment (Figure 3D). We assume that they are right-side out sealed vesicles derived from the plasmalemma proper, which is known to be backed by a fibrillar infrastructure in between PVs (Peters *et al.*, 1985). A fibrillar content was not revealed in B vesicles by en block tannic acid treatment (our unpublished results). NB appears, therefore, to be enriched in vesicles derived from the plasmalemma proper. It also contains a few recognizable mitochondria (Figure 3D) which come from the subcellular fragments of endothelial cells known to contaminate P1 (Jacobson *et al.*, 1992).

The results of a morphometric analysis of the vesicles in the B subfraction are given in Figure 4. We assume that the difference between the size of the PVs in situ (Bruns and Palade, 1968; 55–80 nm, ~70 nm mean outer diameter) and the PVs immunopurified through our method is the result of fragmentation and resealing during different steps of the procedure, especially upon sonication. This assumption is clearly supported by immunogold tests (Figure 3, F–G) which show that the immunoadsorbed vesicles contain caveolin in their membranes irrespective of their sizes (Figure 3G). Further details are given in the figure legend.

Protein and Caveolin Distribution

We usually started with ~2 mg of protein in the P1 fraction representing ~0.2% of the total protein in the filtered lung homogenate. The amount of protein recovered in the C fraction was on average ~7.5% of the P1 value. After immunoisolation, ~80% of the protein from the C fraction was found in the NB subfraction (Figure 5A). The amount of protein in B was estimated at ~20% of the C content by subtraction. As already mentioned, direct determination of protein in B was not possible because of the background given by the blocking mixtures. Since the protein content of NB is probably overestimated (because of incomplete removal of blocking agents) and since the protein content of B is estimated rather than directly measured, we relied in subsequent work on the distribution of different components normalized to equivalent volumes (see MATERIALS AND METHODS) rather than on values normalized to protein. Enrichment could be, however, reliably determined in the first part (from

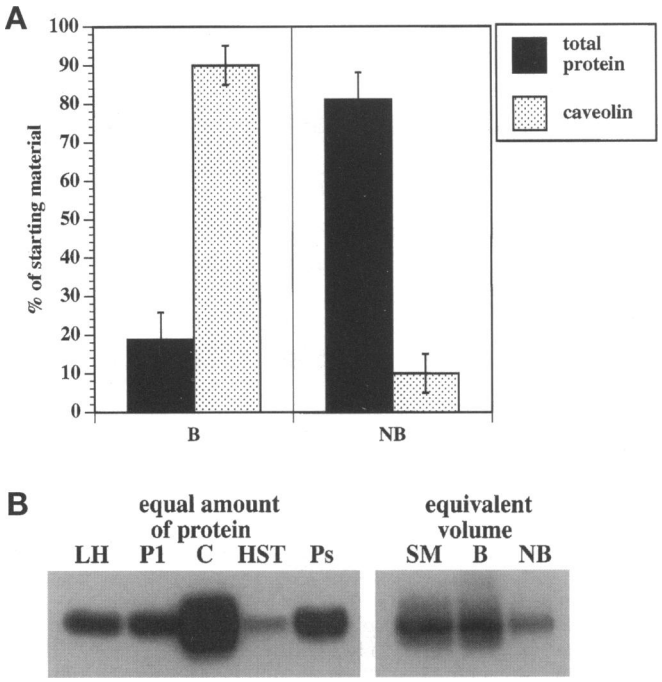

Figure 5. (A) Distribution of total protein and caveolin from C fraction among B and NB subfractions. Data for caveolin distribution were obtained by quantitative analysis of the blots of equivalent volumes from different fractions in which the anticaveolin pAb was followed by ^{125}I-labeled protein G. The membranes were exposed to a PhosphorImager screen (1–3 days) and the signal was quantitated using a PhosphorImager 445 (Molecular Dynamics); (B) immunoblotting with anticaveolin of the fractions obtained in the purification procedure. Either equal amounts of protein (2 μg/lane) or equivalent volumes (as indicated) were used from various samples. The proteins were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked for 1 h in blocking buffer (5% nonfat dry milk, 0.1% Tween 20 in PBS) at room temperature followed by a 1-h incubation with anticaveolin pAb, 1-h incubation with biotinylated goat anti-rabbit secondary antibody (Fc specific), and 15-min incubation with avidin-biotinylated horseradish peroxidase (ABC complex). ECL was used as the detection system.

lung homogenate to C) of the lung fractionation procedure (Figure 5B).

The distribution of caveolin in different samples was studied by immunoblotting using either horseradish peroxidase-coupled secondary antibodies and ECL or radioiodinated protein G as detection systems. Figure 5B, left panel, shows that the highest enrichment in caveolin is obtained in the C fraction. Some caveolin remains in HST and some is found in Ps (the final pellet). Given the high concentration of caveolin in the C fraction, we decided to use the latter for immunoisolation on anticaveolin-coated magnetic beads. Figure 5B, right panel, shows the distribution of caveolin between the B and NB subfractions, and Figure 5A gives the quantitation of this distribution; 90% of the caveolin is recovered in the B subfraction.

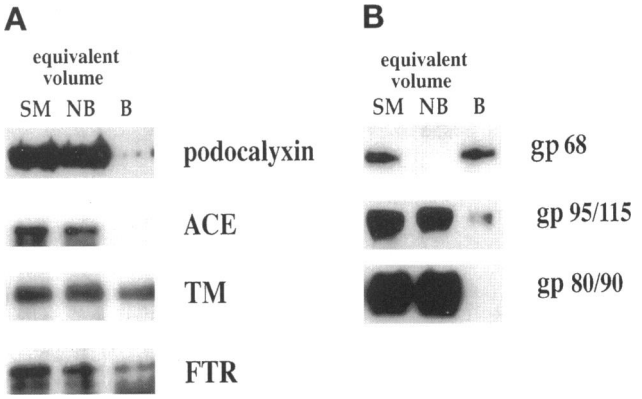

Figure 6. The distribution of different endothelial specific antigens between the bound (B) and nonbound (NB) fractions. Equivalent volumes from each sample were loaded, and proteins were separated by SDS-PAGE and transferred onto either nitrocellulose or PVDF membranes. (A) Immunoblotting using antipodocalyxin mAb (1:1000 dilution), anti-angiotensin-converting enzyme pAb (ACE; 1:250 dilution), anti-thrombomodulin pAb (TM; 1:500 dilution), antifunctional thrombin receptor pAb (FTR; 1:500 dilution). Note that the anti-FTR antibody recognizes both forms (cleaved and uncleaved) of the receptor. (B) Western blotting using anti-endothelium-specific mAbs21D5 (anti-gp68), 28D5 (anti-gp95/115), and 30B3 (anti-gp80/90) was performed. Horseradish peroxidase-coupled reporter antibodies and ECL were used as the detection system.

Presence of Endothelial-specific Markers

To establish a connection with previous electron microscopic immunocytochemical findings, we used several antibodies directed against integral membrane proteins known to be expressed at the level of endothelium in lung and/or other organs, e.g., thrombomodulin (Horvat and Palade, 1993), functional thrombin receptor (Horvat and Palade, 1995), podocalyxin (Horvat *et al.*, 1986), and the angiotensin-converting enzyme (ACE; Ryan *et al.*, 1975). Figure 6A shows the results obtained and compares the distribution of these antigens with that of caveolin. Two of them (i.e., ACE and podocalyxin) were found nearly exclusively in the NB subfraction which is enriched in vesicles derived from plasmalemma proper; the other two, thrombomodulin and the functional thrombin receptor, were present mainly in the NB but were also detected in the B subfraction. The results obtained with thrombomodulin and the thrombin receptor are in agreement with immunocytochemical data already published (Horvat and Palade, 1993, 1995).

To expand the survey, we used three other mAbs raised against luminal endothelial plasmalemmal integral proteins (Ghitescu *et al.*, 1997; unpublished data). They recognize integral membrane glycoproteins [resistant to extraction with high salt and high pH (Ghitescu *et al.*, 1997)] whose function is still unknown (Figure 6B). One of these integral membrane proteins was restricted to the NB subfraction (gp80/90), another one appears to be in both NB and B

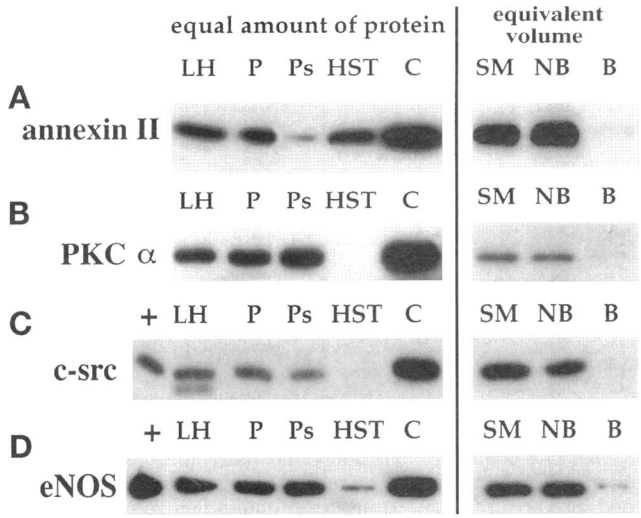

Figure 7. Immunoblotting of the fractions and subfractions of interest using antibodies against molecules involved in signal transduction. Either equal amounts of protein (2 μg/lane) or equivalent volumes (indicated in the figure) from different fractions and subfractions were separated by SDS-PAGE; the proteins were transferred to a PVDF membrane that was treated for 1 h at room temperature in blocking buffer (5% nonfat dry milk, 0.1% Tween 20 in PBS) and then blotted using mAbs against annexin II HC (A), PKCα (B), c-src protein tyrosine kinase (C), and eNOS (D). Horseradish peroxidase-coupled reporter antibodies followed by ECL were used as the detection system. A positive control (+) has been added for c-src and eNOS. The results show progressive enrichment of the antigens and preferential distribution in the NB subfraction.

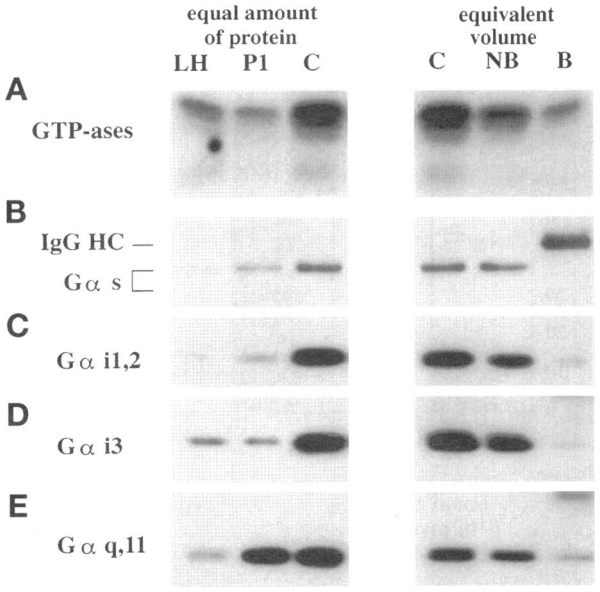

Figure 8. (A) [α-^{32}P]dGTP overlay of rat lung fractions and subfractions used as described in MATERIALS AND METHODS. (B–E) Immunoblotting of the fractions using antibodies against α subunits of the heterotrimeric G proteins (αs, αi2, αi3, and αq in B, C, D, and E, respectively). Left panel shows the concentration of all GTP-binding proteins tested in the C fraction. Right panel shows the distribution of the same proteins between B and NB subfractions and reveals that they are preferentially recovered in the NB subfraction. The heavy chain of IgG used in immunoisolation is detected by ECL in B, D, and E.

(gp95/115), and still another one distributed only in the B subfraction (gp68). Work in progress is trying to clarify the exact nature of these proteins.

The restricted distribution of podocalyxin, ACE, and the three integral membrane glycoproteins indicates that our procedure (which involves a sonication step) does not randomize the chemistry of the vesicles derived from the starting preparation.

Proteins Involved in Signal Transduction

Since previous work done in other laboratories claimed that the caveolae are centers of signal transduction in different cell types [e.g. Madin-Darby canine kidney cells (Lisanti *et al.*, 1995), smooth muscle cells (Chang *et al.*, 1994), endothelial cells (Schnitzer *et al.*, 1995a–c)] or tissues [e.g., mouse lung (Lisanti *et al.*, 1994)], we tested this hypothesis on our subfractions. We looked at the distribution of annexin II heavy chain, protein kinase Cα, the α subunits of the heterotrimeric G proteins (s, i2, i3, and q), small GTP-binding proteins, endothelial nitric oxide synthase (eNOS), and c-src protein tyrosine kinase which had been shown to be enriched in caveolae-enriched fractions obtained by different procedures involving either mechanical disruption (Smart *et al.*, 1995; Shaul *et al.*, 1996) or Triton X-100 extraction (Chang *et al.*, 1994; Lisanti *et al.*, 1994; Schnitzer *et al.*, 1995a–c; Garcia-Cardena *et al.*, 1996).

Our results show that annexin II, protein kinase Cα, protein tyrosine kinase c-src, eNOS (Figure 7,A–D, respectively), and the α subunits of the heterotrimeric G proteins Gα s, Gα i2, Gα i3, and Gα q (Figure 8,B–E, respectively) are highly concentrated in the C fraction; all of these proteins separate almost exclusively in the NB subfraction in tests in which we followed antigen distribution. In quantitation experiments using ^{125}I-labeled protein G showed that in the case of annexin II and protein kinase C (PKC) α, most of the signal [95 ± 4% (n = 3) and 96 ± 1% (n = 3), respectively] was found in the NB subfraction. When the fractions were tested for equal amounts of proteins, these antigens were also detected in smaller amounts in the B subfraction but, as already discussed, we attach less credence to enrichment figures because of uncertainties in protein determination in both B and NB. The pattern is similar for GTP-binding proteins (Figure 8A), the larger amount being detected in the NB and the smaller in the B subfraction.

Taken together, these data suggest that in endothelial cells we do not have an enrichment of these proteins in caveolae. Admittedly our survey is incom-

plete; it has to be extended and further consolidated. Possible reasons for the lack of agreement of our data with the data already recorded in the literature will be considered in DISCUSSION.

DISCUSSION

The main purpose of the work we report in this article was to devise a specific procedure for the isolation of PVs or caveolae since these structural elements have been of considerable interest during the last 4 years and since there is still uncertainty as to their function, notwithstanding the already large body of published claims and findings.

We concentrated the work on the continuous microvascular endothelium which has a large population of PVs since, in this case, we have already shown that the PVs function in transcytosis (in the myocardium vasculature; Milici *et al.*, 1987; Ghitescu *et al.*, 1988; Predescu *et al.*, 1994), and since a procedure to obtain patches of luminal plasmalemma with attached PVs from the lung microvasculature was already devised in our laboratory (Jacobson *et al.*, 1992). That preparation, designated P1, is enriched in caveolae and plasmalemma proper and drastically de-enriched in other cytoplasmic membranes. Moreover, in previous work we have localized a number of endothelial-specific membrane proteins that can be used to check, by a different approach, the effectiveness of our cell fractionation procedure.

P1, the starting preparation, was sonicated to detach the PVs which were subsequently floated by centrifugation in sucrose density gradients and collected in a mixed vesicle fraction which comprised, in addition to PVs, vesicles derived from the plasmalemma proper as well as from cytoplasmic contaminants in P1. This mixed vesicle fraction was the starting material for the immunoisolation of a population of spherical vesicles (the bound subfraction) of regular shape (ranging in diameter from 40 to 150 nm) on anti-caveolin antibody-coated magnetic microspheres, on which the presence of caveolin could be demonstrated by immunogold labeling (Figure 3, F and G). The NB subfraction was heterogeneous. We assume that it contains primarily vesicles produced by sonication at the expense of plasmalemma proper in addition to cytoplasmic membranes present as contaminants in the starting material (P1). We believe, however, that the predominant components of NB are vesicles derived from the plasmalemma proper.

The distribution of caveolin (the generally accepted biochemical marker of caveolae) shows considerable enrichment in the mixed vesicles fraction (C) with incomplete recovery (some of the antigen is lost to the final pellet Ps). From the C fraction, as starting material, ~90% of caveolin (by quantitative determination) is recovered in the B subfraction which, therefore, appears to represent the caveolae on account of the morphology, immunocytochemical tests, and biochemical assays of its vesicular components.

As far as we know, this report is the first detailed description of the immunoisolation of endothelial PVs, their separation from plasmalemma proper derived vesicles, and their partial biochemical characterization. However, a recent article (Schnitzer *et al.*, 1995a) mentioned, without describing or referencing the procedure, the immunoisolation of endothelial PVs on anticaveolin-coated magnetic beads. The approach was used to colocalize caveolin with VAMP-2, a protein involved in vesicular carrier targeting.

We first investigated the distribution of endothelial-specific integral membrane proteins in these subfractions and found that some of them (e.g., podocalyxin and the angiotensin-converting enzyme) are exclusively present in the NB subfraction (Figure 6A) while others (e.g., thrombomodulin and the functional thrombin receptor) are found in both B and NB (Figure 6A). Previous immunocytochemical studies (Horvat *et al.*, 1986; Horvat and Palade, 1993, 1995) have shown that these receptors are present in the plasmalemma proper as well as in PVs apparently involved in the transport of thrombin from the cell surface to an endosomal compartment or in transcytosis, in addition to endocytosis. Immunoisolation data are, therefore, in agreement with immunocytochemical tests (Horvat *et al.*, 1986; Horvat and Palade, 1993, 1995). Moreover, these findings indicate that the caveolae are implicated in intracellular transport in addition to their extensive involvement in transcytosis (Milici *et al.*, 1987; Ghitescu *et al.*, 1988; Ghitescu and Bendayan, 1992; Predescu *et al.*, 1994, 1997). Caveolae were involved in transport in other cell types (e.g., Madin-Darby canine kidney cells; Dupree, *et al.*, 1993; Lafont *et al.*, 1995). We have also studied the distribution of a number of endothelial integral membrane glycoproteins of still unknown function. One of them appears to be pulmonary endothelial specific (gp 95/115; Ghitescu *et al.*, 1997) and to be present predominantly in the NB (but not only), another one (gp 80/90) is present only in the NB, while the other one (gp 68) distributes only in the B subfraction (Figure 6B).

We have found a number of proteins involved in signal transduction in the C fraction but upon immunoisolation some of them (such as annexin II, eNOS, c-src protein tyrosine kinase, PKCα, α subunits of the heterotrimeric G proteins, and small GTP-binding proteins) distributed predominantly in the noncaveolar (NB) subfraction (Figures 7 and 8, B–D). These findings would argue against the hypothesis that caveolae function as centers of signal transduction in the vascular endothelium. Since some of the proteins involved in signal transduction are peripheral rather than integral membrane proteins, it could be argued that they might be removed by the high salt treatment

involved at certain steps of our procedure, but our data show that at least PKCα, eNOS, c-src, and Gα subunits remain associated with the membrane (Figures 7, B–D, and Figure 8, B–E, respectively). A certain amount of annexin II is found in a cytosol equivalent (HST fraction) but most of it is recovered in the C fraction (Figure 7A).

Obviously there is a lack of agreement between our data and those already published in the literature. This discrepancy may be explained by the fact that we are comparing a caveolar fraction obtained by immunoisolation with caveolae-rich fractions obtained by nonspecific purification procedures (Chang *et al.*, 1994; Lisanti *et al.*, 1994, 1995; Schnitzer *et al.*, 1995a–c; Smart *et al.*, 1995). In other words, the reasons may be of a technical nature: they may reflect the heterogeneity of the caveolae-rich fractions obtained by nonspecific procedures. Our data are suggestive of this point of view in the sense that when we used nonspecific steps in the caveolae purification procedure (such as sonication and flotation in density gradients) we obtained a mixed vesicular fraction (C) highly enriched in caveolin and, moreover, enriched in all of the antigens that had been previously reported as enriched in caveolae. When a specific step (such as immunoisolation on anticaveolin antibodies) is included, there is an obvious difference between PVs (caveolae) and vesicles derived primarily from the plasmalemma proper.

However, this lack of agreement may also reflect a special situation: the caveolae may not be subcellular components chemically invariant from one cell type to another. One can think of them as specialized structural elements which, by analogy with clathrin-adaptin-coated pits and vesicles, are able to accommodate different proteins in a common structural framework in different cell types. Considering this possibility, we would like to limit our conclusions to the system we have used, namely, the PVs of the continuous microvascular endothelium. However, our procedure can be applied to other sources of caveolae to further investigate this alternative.

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