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Endothelial Transcytotic Machinery Involves Supramolecular Protein–Lipid Complexes

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We have demonstrated that the plasmalemmal vesicles (caveolae) of the continuous microvascular endothelium function as transcytotic vesicular carriers for protein molecules >20 Å and that transcytosis is an *N*-ethylmaleimide–sensitive factor (NSF)-dependent, *N*-ethylmaleimide-sensitive process. We have further investigated NSF interactions with endothelial proteins to find out 1) whether a complete set of fusion and targeting proteins is present in the endothelium; 2) whether they are organized in multimolecular complexes as in neurons; and 3) whether the endothelial multimolecular complexes differ from their neuronal counterparts, because of their specialized role in transcytosis. To generate the complexes, we have used myc-NSF, cultured pulmonary endothelial cells, and rat lung cytosol and membrane preparations; to detect them we have applied coimmunoprecipitation with myc antibodies; and to characterize them we have used velocity sedimentation and cross-linking procedures. We have found that both cytosolic and membrane fractions contain complexes that comprise beside soluble NSF attachment proteins and SNAREs (soluble NSF attachment protein receptor), rab 5, dynamin, caveolin, and lipids. By immunogold labeling and negative staining we have detected in these complexes, myc-NSF, syntaxin, dynamin, caveolin, and endogenous NSF. Similar complexes are formed by endogenous NSF. The results indicate that complexes with a distinct protein–lipid composition exist and suggest that they participate in targeting, fusion, and fission of caveolae with the endothelial plasmalemma.

INTRODUCTION

We have recently shown that protein molecules >20 Å in diameter are transported across the continuous microvascular endothelium of the murine myocardium by plasmalemmal vesicles (PVs) or caveolae. Moreover, we have reported that this activity is inhibited by the alkylating reagent *N*-ethylmaleimide (NEM) by ~80% (Predescu, 1994). NEM is known to inactivate the trimeric ATPase NEM-sensitive factor (NSF), which is a critical component of the "membrane fusion machinery" (Rothman, 1994; Whiteheart, 1994). NSF interacts with soluble NSF attachment proteins (SNAPs) and their cognate membrane-targeting proteins (SNAREs, SNAP receptors) as parts of a common, conserved machinery for vesicular carrier targeting and fusion at different relays along the exocytic, endocytic, and transcytotic pathways (Waters, 1991; Sztul, 1993; Rothman, 1994) in other eukariotic cells (SNAREs appear to be "relay specific," whereas NSF and SNAPs are assumed to operate at every relay).

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Against this background, our findings suggest that membrane targeting and fusion equipment is also involved in caveolar transcytosis in the continuous microvascular endothelium. In fact, preliminary experiments have already shown that some of these proteins are present in microvascular endothelia (Predescu, 1994; Schnitzer, 1995). Because in other cell types examined, especially in neurons, these proteins are organized in macromolecular complexes with 7S and 20S sedimentation coefficients (Sollner, 1993b; Bajjalieh, 1995), we felt that searching for similar complexes in the continuous microvascular endothelium is warranted. To this intent, we used coimmunoprecipitation, velocity sedimentation in glycerol gradients, cross-linking procedures, and electron microscopy (EM) of negatively stained immunolabeled preparations and we found out that such complexes are indeed present in microvascular endothelial cells.

Because in transcytosis membrane fission is coupled tightly and efficiently with membrane fusion on the opposite domain of the plasmalemma, it can be expected that the complexes are different. Indeed, we found out that they contain dynamin, the fission GTPase (Takei 1995), and more significantly, caveolin, an integral membrane protein of the caveolar membrane, generally accepted as a caveolar marker

(Kurzchalia, 1992; Rothberg, 1992). We also found that the endothelial complexes comprise additional components, rab 5, and lipids such as cholesterol and ganglioside GM1, whose presence has not been reported so far in complexes isolated from other cell types. We propose to call them endothelial multimolecular transcytotic complexes (EMTCs) and we will use this term in the rest of this article.

MATERIALS AND METHODS

Lung human microvascular endothelial cells (HMECs) were obtained from Clonetics (San Diego, CA) and Sprague-Dawley rats were bought from the Harlan Sprague Laboratories (Indianapolis, IN). The high performance thin-layer chromatography plates (HPTLC) were bought from EM Science (Gibbstown, NY); other reagents were obtained from the following sources: polyvinylidene difluoride (PVDF) and nitrocellulose membranes (Micron Separations, Westboro, MA); [α-^{32}P]GTP (Amersham, Arlington Heights, IL); Kodak film X-OMAT-AR (Eastman Kodak, Rochester, NY); all EM grade reagents (Electron Microscopy Science, Forth Washington, PA); m-maleimidobenzoil-N-hydroxisuccinimide ester and sulfo DST (disulfosuccinimidyl tartrate) (Pierce, Rockford, IL); and lipid markers (Avanti Polar Lipids, Alabaster, AL). All other chemicals were from Sigma Chemical (St. Louis, MO).

Relevant antibodies were obtained from the following sources: anti-α-SNAP and anti-γ-SNAP polyclonal antibodies (pAbs) were a gift from Dr. J. Rothman (Memorial Sloan-Kettering Institute, New York, NY); anti p85 was a gift from Dr. L. Ghitescu (University of Montreal, Canada); anti-dynamin, anti-annexin II, and anti-caveolin-1 monoclonal antibodies (mAbs), and anti-Rab 5 pAb (Transduction Laboratories, Lexington, KY); anti-syntaxin mAb (Sigma Chemical); anti-GM1 pAb (Matreya, Pleasant Cap, CA); and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG (Cappel, Organon Teknika, Durham, NC). The following antibodies were prepared in our laboratory: anti-myc mAb as in Wilson (1992), anti-caveolin pAb as in Stan (1997), anti-PV-1 as in Stan (1999), and anti-cellubrevin and anti-NSF pAbs as described in MATERIALS AND METHODS. Colloidal gold suspensions (5–15 nm) were prepared according to Slot (1985), and stabilized with affinity-purified anti-myc antibody, anti-caveolin antibody, goat IgG anti-mouse IgG, or goat IgG anti-rabbit IgG as in Quagliarello (1991). The gold-labeled antibodies were stored as concentrates at 4°C.

Preparation of Membrane and Cytosol Fractions

From Rat Lung. Rat lungs were perfused with sPBS (supplemented PBS) as in Predescu (1993) and a homogenate was prepared using a Potter-Elvejem homogenizer (30–40 strokes) and a 1:4 ratio (9 g of wet weight tissue/36 ml of buffer containing 20 mM Tris/HCl, pH 8.0, 1 M KCl, 250 mM sucrose, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was filtered through four layers of cheesecloth and its final volume was adjusted to 20% (wt/vol) by using the same buffer. The postnuclear supernatant was used to prepare a membrane fraction according to a protocol published by Sollner (1993a), but we complemented it by the isolation and analysis of a cytosol fraction.

Briefly, the cytosol was obtained by centrifuging the postnuclear supernatant at 45,000 rpm (60 Ti rotor) for 1 h, at 4°C, and it was aliquoted and stored at −80°C. The resulting pellet was washed once in a buffer containing 10 mM HEPES, pH 7.8, 100 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, and then resuspended in the same buffer. Triton X-100 was added slowly to a final concentration of 4% and the suspension was incubated at room temperature (RT) with frequent mixing. After 45 min, the suspension was clarified by centrifugation at 45,000 rpm (60 Ti rotor), for 1 h, and the supernatant dialysed overnight against 100 vol of 25 mM Tris, pH 7.8, 50 mM KCl, 1 mM DTT, 1% Triton X-100. After dialysis, the material was clarified by centrifugation for 1 h, at 45,000 rpm (60 Ti rotor), aliquoted, and stored at −80°C.

From HMECs. Monolayers of HMECs, 2 d after confluence, were washed three times with phosphate-buffered saline (PBS), scraped in the homogenization buffer (Wilson, 1992), 20 mM HEPES/KOH, pH 7.4, 100 mM KCl, 2 mM DTT, 2 mM EDTA, 0.5 mM ATP, 1 mM PMSF (1 ml/100-mm^2 plate), collected by centrifugation (2000 rpm, for 10 min, at 4°C), and homogenized, first with a Dounce homogenizer (20–30 strokes) and then by 10–12 passages through a 27-gauge needle. The resulting postnuclear supernatant was used to prepare a cytosol fraction by centrifugation at 45,000 rpm (60 Ti rotor), for 1 h, at 4°C. The high-speed supernatant (the cytosol fraction) was aliquoted and stored at −80°C. The pellet, resuspended in homogenization buffer, was treated with Triton X-100 as described above, and the Triton X-100 membrane extract (membrane fraction) was aliquoted and stored at −80°C.

In some experiments, both cytosol and membrane fractions isolated from cultured HMECs or rat lung were treated with either Triton X-100 (to 4% final concentration) as in Sollner (1993a) or 60 mM 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS) for 1 h at RT. The cytosol was detergent treated only for symmetry reasons.

Permeabilization of Cultured HMECs with Streptolysin O

HMECs were grown and collected in homogenization buffer as described previously. The time needed for membrane permeabilization and the optimal SLO (streptolysin O)/endothelial cell ratio were determined as in Graham (1994). The degree of membrane permeabilization was assessed by measuring the increase in lactate dehydrogenase (LDH) activity in the supernatant versus the decrease of cell-associated LDH activity (Worthtington, 1993).

Coimmunoprecipitation Experiments

Coimmunoprecipitation experiments were performed by incubation of HMECs cytosolic and membrane fractions (100 μg of total protein) with 0.5 μg of purified myc-tagged NSF in assay buffer (20 mM HEPES/KOH, pH 7.4, 100 mM KCl, 2 mM DTT, 2 mM EDTA, 0.5 mM ATP, 1% poly(ethyleneglycol) 4000, 1 mM PMSF). Myc-tagged NSF was prepared and purified as in Sollner (1993a). The plasmid encoding His_6-NSF-myc was a gift from Dr. J. Rothman (Memorial Sloan-Kettering Institute). Myc-NSF and associated proteins were immunoprecipitated using either anti-myc mAb or anti-caveolin pAb, followed by protein A/G agarose beads. All the immunoprecipitation experiments performed in this study were preceded by a preclearing step by using a mouse IgG followed by protein A/G, and then the resulting supernatant was used for immunoprecipitation with anti-myc antibody. The immunoprecipitates were analyzed by 5–20% SDS-PAGE. The gels were either stained with Commassie blue R-250 or transferred to nitrocellulose membranes, which were immunoblotted as in De Maio (1994) with the antibodies against proteins known to be involved in the process of vesicular transport in other systems.

Velocity Sedimentation Studies

Rat lung or HMECs cytosol preparations (~250 μg of total protein/tube) were equilibrated in gradient buffer (20 mM HEPES/KOH, pH 7.4, 100 mM KCl, 2 mM DTT, 2 mM EDTA, 0.5 mM ATP) in a 1-ml final volume, for 30 min, at RT. Two micrograms of myc-NSF was added and the incubation was continued for one more hour, with occasional agitation. The reaction mixture was layered on top of 10–35% (wt/vol) glycerol gradients formed by layering 1-ml volumes of gradient buffer as in Wilson (1992). After centrifugation at 40,000 rpm (SW40 rotor), for 18–20 h, at 4°C, 1-ml fractions were

collected from the top of the gradient by using either a pipette or a density gradient fractionator (Isco, Lincoln, NE), and immunoprecipitated using anti-myc mAb or anti-caveolin pAb. The immunoprecipitates were analyzed by 5–20% SDS-PAGE. The electrophoretograms were transferred to nitrocellulose membranes and blotted with mAbs to myc tag, syntaxin, and dynamin and pAbs to α-SNAP, γ-SNAP, cellubrevin, and anti-caveolin.

Sedimentation coefficients of endothelial complexes were estimated by comparison of the protein profile of their gradients with a gradient profile obtained under similar conditions by using standard proteins, bovine serum albumin (BSA) (4.6S), catalase (11.4S), and α-2 macroglobulin (20S).

Cross-linking Experiments of EMTC Proteins by Using Sulfo DST, a Periodate-cleavable Bifunctional Reagent

The cross-linking reaction was started by adding directly disulfosuccinimidyl tartrate, a water-soluble, homobifunctional *N*-hydroxysuccinimidyl ester cross-linker, periodate cleavable, to 2 ml of cytosolic fraction obtained by SLO permeabilization of the cell membrane and containing 250 μg of total protein in assay buffer to give a final concentration of 1.5 mg of sulfo DST/ml as in Smith (1978). The specific cross-linked products present in the cytosolic fraction were analyzed after immunoprecipitation with anti-myc antibody by two-dimensional gel electrophoresis as in Smith (1978).

TLC

The lipids present in the immunoprecipitates obtained from glycerol gradient fractions were extracted as in Murata (1995). To detect the phospholipids and cholesterol, possibly present in the chloroform phase, the corresponding dried residues were dissolved in 30 μl of chloroform/methanol (2:1), by vortexing, and then applied (1 μl/spot) on a HPTLC plate. A mixture of chloroform/methanol/water (65:25:4) was used as the mobile phase as in Murata (1995). For detection of gangliosides and sulfatides, possibly present in the methanol/water phase, the corresponding dried residues were dissolved in 30 μl of chloroform:methanol:28% ammonia (65:25:5), and applied to the HPTLC plate (1 μl/spot) by using the same solvents mixture as the mobile phase as in Hamilton (1992). The lipids present in our preparations were detected by charring, after spraying the plates with methanol/sulfuric acid (1:1).

Immunodotblotting for the Ganglioside GM1

GM1 was extracted from gradient fractions in the methanol/water phase as described above. Then 1 μl/spot from this mixture was applied on PVDF membranes by using a dot blot apparatus and treated as in Chabraoui (1993).

GTP Overlay Assay

Immediately after the electrotransfer to PVDF membranes of 5–20% electrophoretograms containing the gradient fractions immunoprecipitated with anti-myc antibody, the transfers were rinsed in binding buffer (50 mM phosphate buffer, pH 7.5, 10 mM $MgCl_2$, 0.3% Tween 20, 2 mM DTT, 4 mM ATP). Then, the PVDF membranes were incubated for 2 h in 30 ml of binding buffer containing 2 μCi[α-^{32}P]GTP/ml. The radioactive GTP-containing buffer was removed; the membranes were extensively washed with fresh binding buffer and then exposed to x-ray film.

Electron Microscopy: Negative Staining of Endothelial Multimolecular Transcytotic Complexes Isolated from Detergent-free Preparations of HMEC Cytosol

Samples of endothelial multimolecular complexes, resolved by velocity sedimentation, were incubated with 1) 5 nm gold-conjugated anti-myc antibody and/or 15 nm gold-conjugated anti-caveolin antibody for 2 h at 4°C; 2) anti-NSF pAb and anti-syntaxin mAb for 1 h, at 4°C each, followed by 15 nm gold-conjugated anti-rabbit IgG and 5 nm gold-conjugated anti-mouse IgG applied together, for 2 h, at 4°C; and 3) anti-NSF pAb and anti-dynamin mAb for 1 h, at 4°C each, or 4) anti-caveolin pAb and anti-dynamin mAb, for 1 h, at 4°C each, followed by the two reporter antibodies, as described above. The EMTCs were adsorbed onto formvar-coated nickel grids, recently exposed to glow discharge, fixed with 1.5% glutaraldehyde, negatively stained with 2% uranyl acetate, and examined and micrographed in a Philips CM 10 electron microscope.

Polyclonal Anti-Cellubrevin and Anti-NSF Antibody Production

A peptide corresponding to the first 13 amino acids of the N terminus of rat cellubrevin, MSTGVPSGSSAATC (McMahon 1993), was synthesized at a core facility on our campus, purified by high performance liquid chromatography, and kept at 4°C when not in use.

New Zealand female rabbits were immunized with the synthetic peptide coupled to BSA by m-maleimidobenzoil-*N*-hydroxisuccinimide ester as in Muller (1988). The coupled peptide was mixed (1:1) with complete Freund adjuvant and injected (2 ml/animal) as in Muller (1986). The antibody was obtained by $(NH_4)_2SO_4$ precipitation at different bleedings followed by absorption on a protein A/G affinity column as in Andrew (1992). The specificity was established by 1) enzyme-linked immunosorbent assay against the synthetic peptide; 2) immunoblotting of endothelial microvascular cell homogenate; and 3) immunoprecipitation from whole cell homogenates, followed by enhanced chemiluminescence (ECL) detection. The antibody recognizes a single band of ~18 kDa in HMECs and rat lung preparations.

Polyclonal antibody directed against full-length NSF-His was raised in New Zealand White female rabbits as in Muller (1986). The recombinant protein was expressed and purified as in Morgan (1995). The antibody recognizes a single protein band of ~76 kDa in homogenates of HMECs.

RESULTS

Endothelial Cells Contain the Factors Needed for Transendothelial Transport

The work reported was done on lung HMECs maintained in culture and on rat lung preparations. HMECs have a large population of PVs opened on either side of the cells or present, apparently free, within the cytoplasm (Figure 1). They have typical Weibel-Palade bodies and the usual set of subcellular components, i.e., a relatively large endoplasmic reticulum, a Golgi complex, endosomes, and mitochondria. They have the important advantage of providing a homogenous cell population, but the disadvantage of low yield. To obviate this limitation we used rat lungs, after flushing them free of blood by perfusion with sPBS (supplemented PBS) as in Predescu (1993), and prepared from them a crude membrane fraction and a supernatant. The membrane fraction is a crude preparation that contains in addition to endothelial membranes, membranes from all other cellular elements of the lung. Because the endothelium represents ~50% of the lung cell population, the preparation can be used as a starting material for biochemical studies. It has the disadvantage of heterogeneity but it is a better approximation of the situation in situ and in vivo.

To investigate the presence in cultured endothelial cells of the factors so far identified as required for fusion-fission of

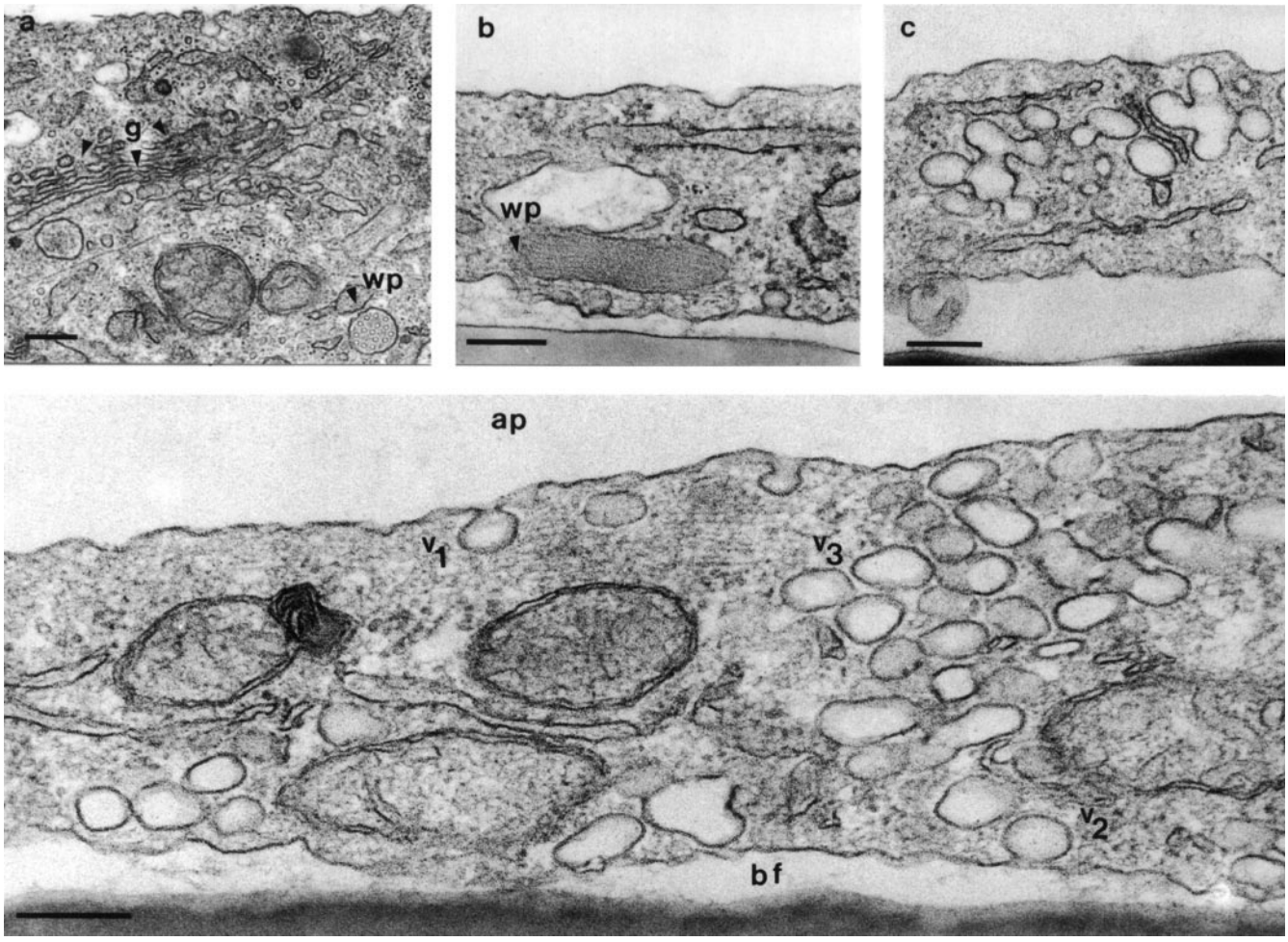

Figure 1. Electron micrographs of cultured HMECs. It shows a partial view of a HMEC in culture (passages 3–4). It maintains a large population of plasmalemmal vesicles open to the two sides of the cell (v_1, v_2) or apparently free in the cytosol (v_3). The exposed (apical surface) is marked ap. The one facing the cultured dish is marked bf. The insets a and b show a Golgi region (g) and a Weibel-Palade (w-p) body, and the inset c illustrates the tendency of vesicles to fuse with one another. Bar, 200 nm.

caveolae to their plasmalemmal targets, we have explored the interactions of NSF, the fusion ATPase, with other endothelial proteins. To this intent, we have incubated the cytosol and membrane fractions (obtained by mechanical disruption of endothelial cells) with myc-NSF, for 1 h, at RT as specified in MATERIALS AND METHODS. Triton X-100 was added to a final concentration of 0.5% for 1 h, at RT. The samples precleared with a mouse IgG as mentioned in MATERIALS AND METHODS were immunoprecipitated with anti-myc antibody. The immunoprecipitates were analyzed by 5–20% SDS-PAGE, followed by Commassie blue staining or Western blotting with antibodies against some of the proteins known to be involved in targeting, fusion, and fission of vesicular carriers in other systems (Sollner, 1993a; Takei, 1995; Sollner, 1996). In our previous work (Predescu, 1993, 1994, 1997, 1998) we have demonstrated that PVs or caveolae are involved in transcytosis of proteins across the continuous microvascular endothelium. Because caveolin is a generally accepted caveolar marker (Kurzchalia, 1992) we also used anti-caveolin antibody for immunoblotting. Figure 2A shows that anti-myc antibody recognizes myc-NSF (lanes a and a′) and in addition brings down some other endothelial proteins, from both cytosolic and membrane fractions. Among them we identified α-SNAP, lanes b and b′; γ-SNAP, lanes c and c′ (proteins known to be components of the general fusion machinery); syntaxin, lanes d and d′ (protein identified as a t-SNARE in neurons); cellubrevin, lanes e and e′ (protein identified as a v-SNARE in other cells than neurons); Rab 5, lanes g and g′; annexin II, lanes i and i′ (proteins involved in different steps of vesicular transport in other systems); and dynamin, lanes h and h′ (assumed to be the fission GTPase). Of particular interest is the presence of caveolin (lanes f and f′) as a myc-NSF– interacting protein in both cytosol and membrane fractions. When anti-caveolin antibody was used for immunoprecipitation, the same endothelial proteins were identified in the immunoprecipitate (Figure 2B). In control experiments, the cytosol and membrane fractions were incubated with anti-myc antibody, either 1) in the absence of ATP, 2) in the absence of myc-NSF from the reaction mixture, or 3) in the presence of myc-NSF

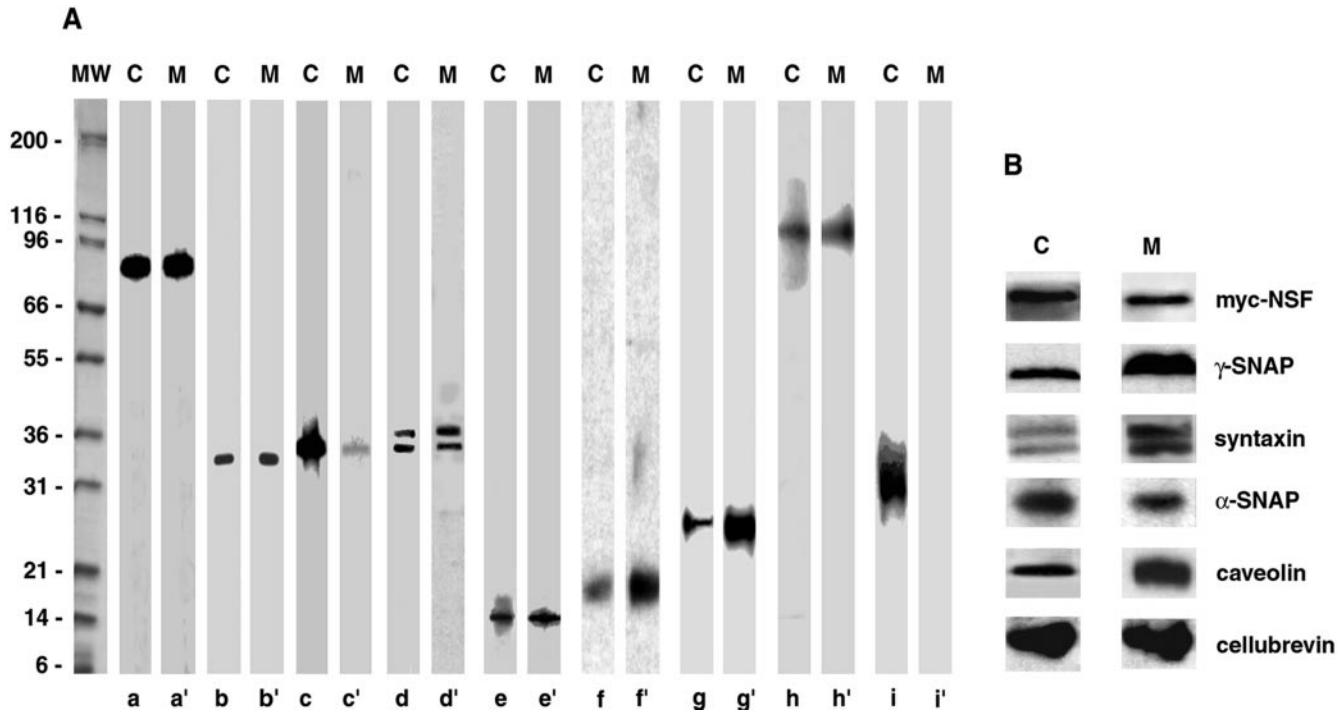

Figure 2. Protein complexes formed by adding myc-NSF to endothelial cytosolic and membrane fractions prepared from cultured HMECs. Samples of cytosol and Triton X-100 extract of the membrane preparation (100 μg of total protein) were incubated with 0.5 μg of purified myc-NSF, in the presence of 0.5 mM ATP and 2 mM EDTA, as described in MATERIALS AND METHODS. Proteins associated with myc-NSF were immunoprecipitated with 2 μg of anti-myc mAb (a) or anti-caveolin pAb (b). The immunoprecipitates were resolved on 5–20% SDS-PAGE. The electrophoretograms were transferred to nitrocellulose membranes that were immunoblotted with antibodies against proteins known to be involved in fusion-fission processes of vesicular carriers. An ECL detection system was used for visualizing the bound antibodies. All findings were confirmed in at least eight different experiments. (A) Anti-myc antibody recognizes myc-NSF (lanes a and a′), and in addition brings down other endothelial proteins from both cytosol (C) and Triton X-100 extracts of the membrane fractions (M). By immunoblotting these proteins were identified as α-SNAP (lanes b and b′), γ-SNAP (lanes c and c′), syntaxin (lanes d and d′), cellubrevin (lanes e and e′), caveolin (lanes f and f′), and rab 5 (lanes g and g′), and dynamin (lanes h and h′). Annexin II was detected as a myc-NSF–associated protein only in the cytosol fraction (lanes i and i′). To investigate the presence of rab-5 as a myc-NSF–associated protein, anti-myc antibody has been immobilized on Fast Flow protein G Sepharose, as in Sollner (1993a). (B) Anti-caveolin pAb immunoprecipitates the same endothelial proteins, from both cytosol (C) and Triton X-100 extracts of the membrane preparations (M).

treated with 1 mM NEM (Figure 3a). No protein bands were detected in the absence of myc-NSF, in the absence of ATP or in the presence of NEM-treated myc-NSF. The bands seen at 25 and 55 kDa are the light and heavy chains of the anti-myc antibody. By contrast with the components revealed by immunoblotting, a large number of protein bands, many of them still unidentified, were coimmunoprecipitated from the complete mixture. This protein pattern was obtained from cytosol and membrane fractions after Triton X-100 treatment, but the same pattern was found after CHAPS treatment or in the absence of either detergent (PAGEs not shown). Figure 3b shows, for comparison, the protein pattern of EMTCs and the even more complex protein pattern of a whole cytosolic fraction. Moreover, anti-Rab 3, anti-Rab 4, and anti-Rab 8 antibodies were used for immunoprecipitation as mentioned in MATERIALS AND METHODS and they did not bring down the endothelial particles.

To check the results obtained in cultured endothelial cells and to explore the possible occurrence of phenotypic drift in cell culture, we extended our investigation to rat lung. In this case, in which we have a heterogeneous cell population, but with a high endothelial cell content, all findings documented above were confirmed. These results demonstrate that 1) endothelial proteins interact with myc-NSF in both cytosol and membrane preparations, as shown by similar protein pattern on Commassie-stained gels and Western blot analysis; 2) the interaction between myc-NSF and other endothelial proteins is NEM-sensitive and requires ATP; 3) when myc-NSF was omitted, and anti-myc antibody was used for detection of endothelial complexes containing myc-NSF, the protein interactions described above were not detected; and 4) caveolin, the caveolar marker, a protein with a central 33 hydrophobic amino acid sequence, is a myc-NSF interacting protein, in both cytosol and membrane preparations.

Cytosolic Pools of Syntaxin, Caveolin, and Cellubrevin Are Present in Cultured Endothelial Cells

The presence of syntaxin, caveolin, and cellubrevin, usually considered as integral membrane proteins, in soluble com-

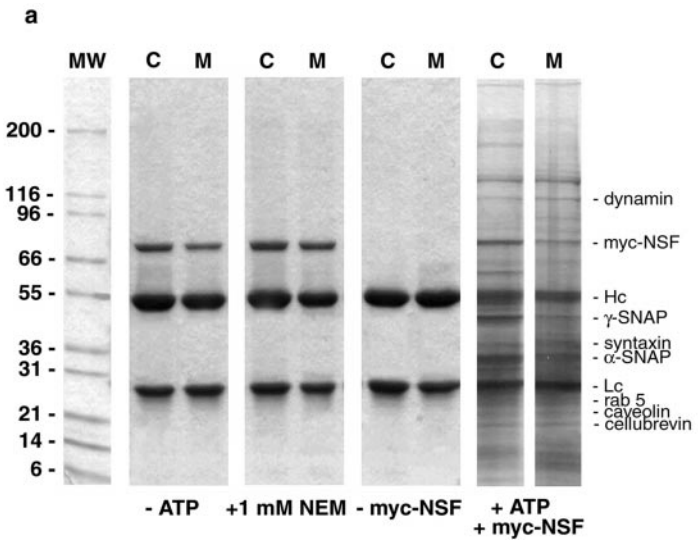

Figure 3. ATP and NEM effect on EMTCs formation. (a) Commassie blue R-250 staining of a 5–20% SDS-PAGE showing that in the absence of ATP or myc-NSF, or in the presence of 1 mM NEM-modified NSF, EMTCs do not form. The only protein bands detected are the light (Lc) and the heavy (Hc) chains of the anti-myc antibody and myc-NSF, when present. When 0.5 mM ATP and myc-NSF are present EMTCs are assembled in both cytosol (C) and Triton X-100 extract of membrane fraction (M). (b) Comparison between the protein composition of EMTCs (C) and a protein pattern obtained from a whole HMECs cytosolic preparation (C_1) on a one-dimensional 5–20% SDS-PAGE.

plexes isolated in the absence of detergent from a cytosolic fraction of cultured endothelial cells, is an unexpected and unusual finding. It may be an artifact generated by membrane fragmentation during the mechanical disruption of the cells. Yet, the validity of this finding is supported by 1) the EM survey of the cytosolic fraction that failed to reveal any membrane fragments (our unpublished results); 2) the control experiments in which the rat lung cytosol was analyzed by immunoblotting for the presence of PV-1, an integral membrane protein, a new marker for endothelial plasmalemmal vesicles from rat lung (Stan, 1999). PV-1 was not detected in the cytosol or EMTCs. The presence of an 85-kDa integral membrane protein, a novel endothelial antigen present on the plasma membrane in lung alveolar capillaries (Ghitescu, 1999), also has been investigated. This protein also was not found in the cytosol or EMTCs; 3) the assembly of the endothelial particles from endothelial cytosolic or membrane preparations is NEM-sensitive and it requires the presence of magnesium-ATPγS (our unpublished results) or the presence of ATP in the absence of magnesium (i.e., in the presence of EDTA), as already shown for the neuronal fusion complexes by Wilson (1992). To get more insight into this issue and to rule out the possibility of caveolar membrane fragmentation as a possible explanation for the presence of integral membrane proteins in a cytosolic fraction obtained by mechanical disruption of cultured endothelial cells, we decided to use an alternative procedure for obtaining a cytosolic preparation, namely, permeabilization of cell membranes with SLO. SLO binds to cholesterol-containing target membranes, and assembles into supramolecular curved rod structures forming rings that penetrate into the apolar domain of the lipid bilayer, thereby generating large transmembrane pores of up to 30 nm in diameter (Bhakdi 1985, 1993); seldom, if ever, it passes the plasmalemma to attack the intracellular membranes. Permeabilization was achieved by incubating the cells ($\sim 2 \times 10^6$ cells/ml) with 70 HU (hemolytic units) SLO/ml, for 20 min, at 37°C. After cell permeabilization was performed as mentioned above, the supernatant containing all LDH activity was centrifuged for 1 h, at 45,000 rpm (60 Ti rotor). No pellet was detected. We also have examined at the EM level the SLO-treated cells and the results indicate that they retain their intracellular membrane systems swollen and deformed but still in recognizable form; the pores generated are limited to the plasmalemma and are small enough to retain the intracellular membranes while allowing the efficient release of LDH.

To test whether cytosolic pools of syntaxin, caveolin, and cellubrevin are present in this cytosol preparation, 100 μg of total protein from cytosol and Triton X-100 membrane extracts was resolved by SDS-PAGE and immunoblotted for syntaxin, caveolin, and cellubrevin. The results shown in Figure 4a indicate the existence of cytosolic pools of syntaxin, caveolin, and cellubrevin in the cytosol fraction obtained by SLO permeabilization of cultured endothelial cells. To test whether these proteins are part of the EMTCs, we generated endothelial complexes by using myc-NSF, as previously described. The samples were immunoprecipitated by using anti-myc, anti-dynamin, or anti-Rab 5 antibodies. The results are shown in Figure 4b. All three antibodies bring down myc-NSF and the other endothelial proteins shown in our previous experiments performed on mechanically disrupted HMECs to be part of the fusion-fission endothelial complexes: α-SNAP, γ-SNAP, cellubrevin, syntaxin, caveolin, rab 5, and dynamin. Because SLO-treated membranes are poorly solubilized by Triton X-100 and for the purpose of this inquiry we studied only the cytosolic fraction. The presence of cytosolic endothelial transcytotic complexes containing integral membrane proteins is an element of novelty.

Endothelial Cells Contain Multimolecular Transcytotic Complexes

To get more information regarding the chemical makeup of these complexes and to study their distribution patterns, we used velocity sedimentation procedures. Rat lung or HMECs cytosol (detergent-free or detergent-treated preparations) and Triton X-100 and CHAPS membrane extracts were incubated with myc-NSF for 30 min, at 4°C, with occasional agitation. The reaction mixtures were loaded onto

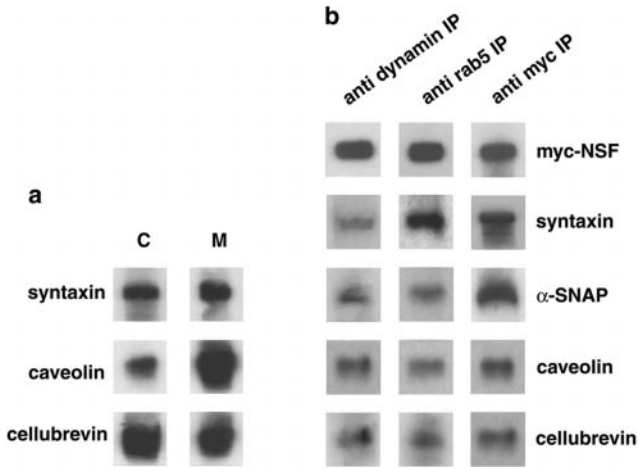

Figure 4. Alternative procedure to prepare the cytosol and to isolate EMTCs. (a) Cytosolic pools of syntaxin, caveolin, and cellubrevin. Cultured HMECs (2×10^6 cells/ml) were permeabilized with SLO (70 HU (hemolytic units)/ml) to prepare cytosol and membrane fractions as described in MATERIALS AND METHODS. Total protein (100 μg) from the cytosol (C) and Triton X-100 membrane extract (M) were resolved by SDS-PAGE and immunobloted for syntaxin, caveolin, and cellubrevin. An ECL detection kit was used for visualizing the bound antibodies. The membranes were exposed for 30 s. (b) Immunoprecipitation of cytosolic EMTCs. Three equal aliquots (~100 μg of total protein) from the cytosolic fraction obtained by SLO permeabilization of HMECs were used to generate endothelial transcytotic complexes with exogenously added myc-NSF (1 μg of myc-NSF/100 μg of total protein). Endothelial transcytotic complexes were immunoprecipitated with 2 μg each of anti-dynamin mAb, anti-rab 5 pAb, and anti-myc mAb. Each immunoprecipitate was resolved by SDS-PAGE, and transferred to nitrocellulose membranes on which the bound antibodies were visualized with an ECL system (exposure time 20 s.) Representative data from two separate experiments are shown.

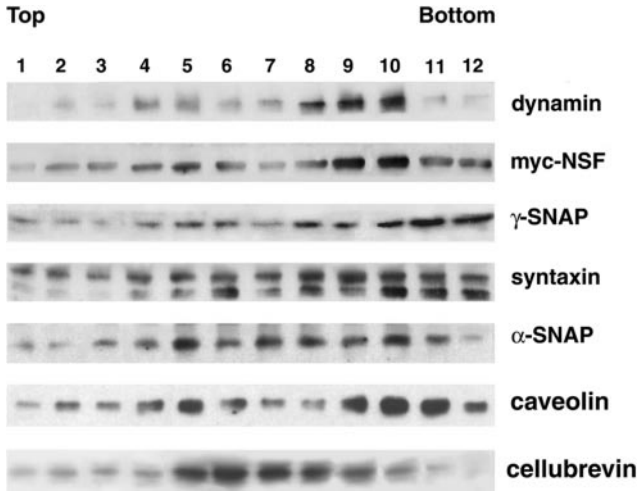

Figure 5. Assembly of myc-NSF and associated proteins in EMTCs. Detergent-free preparation of HMECs cytosol (250 μg of total protein) was incubated for 30 min, on ice, with 2 μg of myc-NSF in a buffer containing 0.5 mM ATP and 2 mM EDTA (1-ml total volume). The mixture was layered on top of a 10–35% glycerol gradient and centrifuged for 18–20 h, at 4°C and 40,000 rpm in a SW40 rotor (Beckman, Fullerton, CA). Fractions (1 ml) were collected from the top of the gradient and immunoprecipitated using anti-myc mAb (1 μg of Ab/fraction). After 5–20% SDS-PAGE and electrotransfer on nitrocellulose membrane, the transfers were probed for the presence of the proteins indicated above. Bound antibodies were visualized using an ECL detection system. The majority of the proteins present in the EMTCs display a wide distribution along gradient fractions. They appear to be more concentrated in fractions 5, 6 and 9, 10, 11. However, the different components are not perfectly aligned, which would suggest heterogeneity of the complexes or technical problems. Sedimentation coefficients estimated as given in MATERIALS AND METHODS are 4.6S for fraction 4, 11.4S for fraction 7, and 20S for fraction 10.

10–35% glycerol gradients, which were subjected to centrifugation for 18 h, at 40,000 rpm (SW40 rotor) at 4°C. When detergent-treated preparations were used, the gradient buffer contained 0.5% Triton X-100 or 10 mM CHAPS. One-milliliter fractions were collected from those gradients and myc-NSF–associated proteins were immunoprecipitated using either anti-myc mAb or anti-caveolin pAb. Each gradient fraction was analyzed by 5–20% SDS-PAGE followed by electrotransfer to nitrocellulose membranes and Western blotting with antibodies against myc (for myc-NSF), α-SNAP, γ-SNAP, syntaxin, cellubrevin, caveolin, and dynamin. The results of these experiments, shown in Figure 5, indicate that the majority of the interactions identified by coimmunoprecipitation among myc-NSF and its associated proteins are preserved. The proteins that coimmunoprecipitate with myc-NSF are widely distributed along the gradient, but most of them (yet not all of them) appear to be most concentrated in fractions 5, 6, and 9, 10, and 11. Notwithstanding this wide distribution, the results suggest the existence of two groups of endothelial complexes: small complexes, present primarily in fractions 5 and 6, and large ones, present mostly in fractions 9–11 of glycerol gradients. The wide distribution could reflect the presence of unstable complexes or of complexes continuously in the making. Their assembly on exogenous myc-NSF, in fact, indicates the dynamic character of these complexes.

The presence of integral membrane proteins, e.g., caveolin, syntaxin, and cellubrevin in these complexes, raises the possibility that they might be artificially produced during the preparation procedures, especially when detergents are used. To rule out this possibility, we compared by the velocity gradient procedure cytosolic fractions prepared without any detergent or with Triton X-100 or CHAPS at different concentrations. To obtain a strong signal, we pooled the gradient fractions 4–6 and 9–11 and used the anti-caveolin antibody for immunoprecipitation. The results obtained indicate the presence of the same interactions among myc-NSF and endothelial proteins in both detergent-free and detergent-treated preparations of HMECs cytosol (our unpublished results). Moreover, the same complexes were found in both cytosol (Figure 5) and membrane preparations obtained from cultured endothelial cells and rat lung (our unpublished results). These findings rule out the presence of detergent artifacts and suggest, in fact, that the protein interactions within the complexes are strong enough to resist dissociation by the detergents so far tested.

The presence of small GTP binding proteins was investigated by the GTP overlay procedure. The results of these

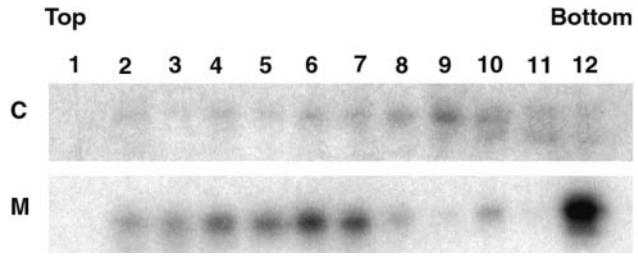

Figure 6. Rab 5 is a protein component of EMTCs. GTP overlay reveals the presence of GTP binding proteins along gradient fractions derived from both cytosol (C) and Triton X-100 extract of the membrane preparation (M). Rab 5 (25 kDa) has been identified by coimmunoprecipitation and immunoblotting experiments as a protein component of EMTCs. Rab 5 seems to be more concentrated in membrane than in cytosol gradient fractions. The highest concentration was found at the bottom of the membrane gradient.

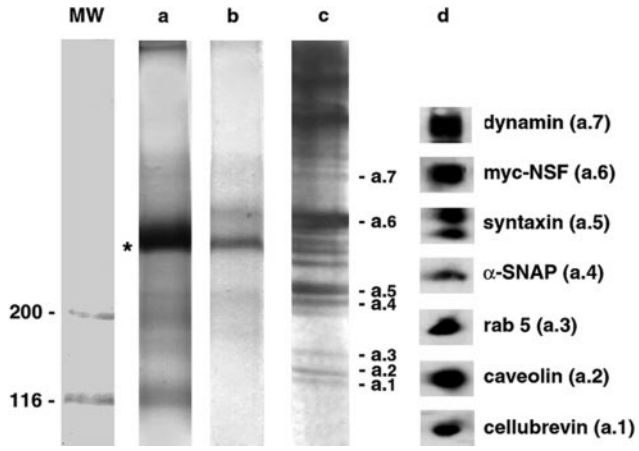

Figure 7. Cross-linking of EMTCs proteins. First dimensional analysis by 6% SDS-PAGE in 8 M urea, of the cross-linked products generated by sulfo DST. (a) EMTCs were assembled from the cytosolic fraction obtained by SLO permeabilization of endothelial cells (250 μg of total protein) by using 2 μg of myc-NSF. The protein components of endothelial complexes were cross-linked with sulfo DST (2.5 mg) and then immunoprecipitated with anti-myc mAb. The most prominent band seen by Commassie R-250 staining indicates the presence of an aggregate >200 kDa (marked by an asterisk). (b) A similar experiment was performed to cross-link the protein components of endothelial particles containing endogenous NSF. The aggregates of interest generated by sulfo DST were immunoprecipitated with anti-caveolin pAb. A similar protein cross-linking pattern was obtained. Molecular weight scale applies only for lanes a and b. Second dimensional analysis of the major cross-linked product generated by sulfo DST. (c) Silver stained 5–20% SDS-PAGE shows the protein bands generated by cleaving with sodium periodate the major endothelial cross-linked product containing myc-NSF. (d) Protein bands marked a.1 to a.7 were identified by immunoblotting as cellubrevin, caveolin, rab 5, α-SNAP, syntaxin, myc-NSF, and dynamin, respectively. Three other significant bands (40–70 kDa) remain unidentified. The higher M_r protein bands are most probably, uncleaved or partially cleaved aggregates.

experiments, shown in Figure 6, confirm Rab 5 as a myc-NSF–associated protein and reveal 1) its wide distribution along gradient fractions; 2) differences in distribution in the cytosolic complexes versus the membrane complexes; and 3) its heavy concentration in the heavy cytosolic gradient fractions, and in gradient fractions 4–7 from membranes. These results suggest that Rab 5 is a protein component of these endothelial complexes, supposedly involved in transcytosis. As far as we know, this is the first report regarding the presence of a small GTP binding protein in complexes potentially involved in transendothelial transport. In addition, using the GTP overlay, we detected in the cytosol another small GTP binding protein (~23 kDa) present predominantly in the heavy fractions obtained from the glycerol gradient (Figure 6). We have similar results on the presence of endothelial complexes in membrane preparations obtained from both HMECs and rat lung.

Admittedly, these findings need further documentation, but they already strongly suggest that the cells of the continuous microvascular endothelium contain the molecular machinery required by the fusion-fission processes involved in transcytosis by plasmalemmal vesicles.

Cross-linking of EMTCs Proteins by Using Disulfosuccinimidyl Tartrate

Sulfo DST, a water-soluble, periodate cleavable cross-linker, was used in this study to get independent evidence regarding the presence and molecular composition of EMTCs. The experiments focused on the cytosolic detergent-free fraction obtained from SLO-permeabilized HMECs. From this fraction multimolecular complexes were generated by two different procedures. The first was our usual procedure that relies on the addition of exogenous myc-NSF, followed by immunoprecipitation with anti-myc antibody. It was applied as described in MATERIALS AND METHODS, except that after myc-NSF, 2.5 mg of sulfo DST was added for 30 min to the reaction mixture to cross-link the proteins of the complexes and thereby achieve symmetry of preparation with the complexes produced by the second procedure. For this second procedure, the cytosolic fraction (250 μg) was incubated with the cross-linker in the absence of myc-NSF to cross-link the constituents of the preformed endogenous NSF-containing particles. The cross-linking reactions were quenched by adding 1 M Tris, pH 7.5, to a final concentration of 40 mM, and the cross-linked products from the two cross-linking reactions were immunoprecipitated by using anti-myc antibody (when myc-NSF was used) or anti-caveolin antibody (when myc-NSF was omitted). The immunoprecipitates containing the cross-linked products generated by sulfo DST were solubilized in sample buffer with 8 M urea, heated for 3 min, at 100°C, and analyzed by 6% SDS-PAGE in the presence of 8 M urea. The results are shown in Figure 7, a and b. The cross-linking pattern of the two experiments is similar. A major band indicating the existence of an aggregate >200 kDa is generated by sulfo DST in both cases. The first dimension containing the most prominent cross-linked product (Figure 7a) was subjected to a second dimensional analysis. Briefly, the first dimension strip was soaked overnight in 0.02 M sodium phosphate buffer containing 0.1% SDS to remove urea and then for 2 h, with several changes, in the same solution containing 0.015 mM sodium periodate, to cleave the cross-linked products. Then, the strip was washed once in distilled water and cemented to a 5–20% SDS-PAGE by a thin layer of acryl-

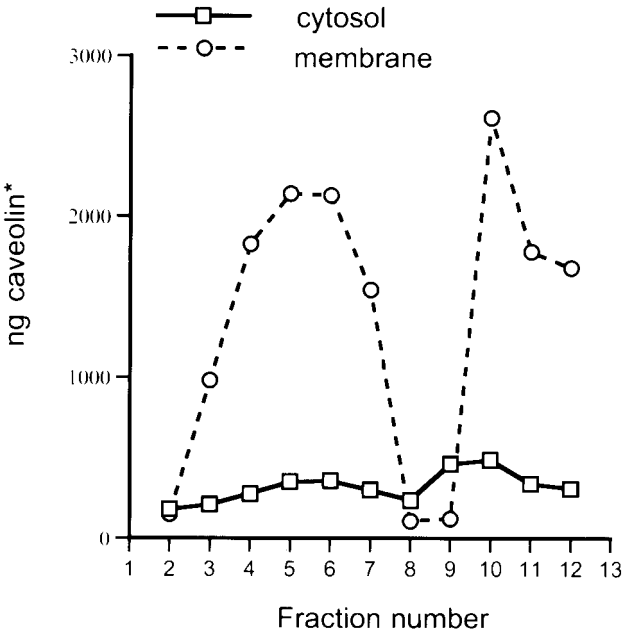

Figure 8. Caveolin quantitation. Caveolin is also present in gradient fractions obtained from cytosol and Triton X-100 extracts of the membrane preparations used in this study. It was quantitated by enzyme-linked immunosorbent assay and found particularly concentrated in two peaks (fractions 5 and 10). *Nanograms of caveolin immunoprecipitated with anti-myc antibody/1-ml fraction.

amide. The second dimension protein profile, shown in Figure 7c, was already suggestive of the presence of EMTCs and subsequent immunoblotting showed that, indeed, dynamin, myc-NSF, syntaxin, α-SNAP, rab 5, caveolin, and cellubrevin are present (Figure 7d). Some significant protein bands (40–70 kDa) remain still unidentified (Figure 7c).

These results confirmed 1) the molecular composition of EMTCs, 2) the efficient assembly of endothelial complexes in the presence of myc-NSF, and 3) the existence of endogenous NSF-containing endothelial complexes.

Caveolin Quantitation

Quantitative data on caveolin distribution in the cytosol, EMTCs, and membrane fractions were obtained using an established capture enzyme-linked immunosorbent assay as in Crowther (1995). An anti-caveolin pAb was the capture antibody, whereas an anti-caveolin mAb was used as detecting antibody. A standard curve was generated using known concentrations of the detecting antibody and a total endothelial cell homogenate. With this approach we found that the rat lung cytosol preparation used in this study contains 25% of the entire amount of endothelial caveolin, whereas the remaining 75% is present in the Triton X-100 extract of the membrane fraction. We have also calculated that 75% of the cytosolic caveolin could be immunoprecipitated with anti-myc antibody. Apparently, this caveolin is part of the cytosolic EMTCs. Figure 8 shows quantitative data on caveolin distribution among glycerol gradient fractions. The caveolin profile displays two peaks located in fractions 5 and 10, assumed to contain the small and large endothelial complexes, potentially involved in transcytosis.

Endothelial Transcytotic Complexes Contain Lipids

Caveolin is a protein with a 33 hydrophobic amino acids sequence, supposed to form a hairpin structure within the caveolar membrane (Kurzchalia, 1992). A possible explanation for the presence of caveolin in soluble cytosolic complexes, and especially in complexes isolated from cytosol without any detergent, could be its association with polar lipids. Data already published have shown that caveolin is a cholesterol binding protein (Murata, 1995), and that caveolae concentrate the ganglioside GM1 in their membranes (Parton, 1994; Fra, 1995). To check for the presence of lipids in EMTCs, we analyzed them by TLC. Cytosol fractions isolated from cultured HMECs or rat lung were incubated with myc-NSF to allow the detection of the endothelial complexes. After velocity sedimentation, the gradient fractions expected to contain EMTCs were immunoprecipitated using anti-myc antibody and the immunoprecipitates were solubilized in electrophoresis sample buffer without bromphenol blue. The lipids present in these preparations were extracted according to Murata (1995) and analyzed by TLC. The results are shown in Figure 9. They indicate that cholesterol is present in the chloroform phase (Figure 9a), and ganglioside GM1 in the methanol/water phase (Figure 9b). The sampling used was against the possibility of showing the variation in the intensity of the signal obtained. The presence of GM1 in the EMTCs also was investigated by immunodotblotting, (Figure 9c). As described in MATERIALS AND METHODS, the fractions from glycerol gradients, expected to contain EMTCs, were immunoprecipitated using anti-myc antibody and the lipids were extracted as described above. To detect GM1 we used 1-μl extract/spot, anti-GM1 antibody, and an ECL detection kit. Figure 9c shows a strong specific staining indicating GM1 presence in these complexes. No staining was observed in control experiments in which 1 μl of 1 mg/ml BSA or myc-NSF was applied per spot.

The results suggest that the EMTCs contain beside proteins (myc-NSF, α-SNAP, γ-SNAP, syntaxin, cellubrevin, Rab 5, caveolin, dynamin), cholesterol, the ganglioside GM1, and other lipids that remain to be identified.

Electron Microscopy: Negative Staining of EMTCs

The morphology of the small and large EMTCs present in detergent-free preparations of HMECs cytosol (fractions 5 and 10 of glycerol gradients) was examined by EM, by using a negative-staining procedure with uranyl acetate as described in MATERIALS AND METHODS. Aliquots of EMTCs present in fractions 5 and 10 of glycerol gradients were incubated with 5 nm gold-conjugated anti-myc antibody and/or 15 nm gold-conjugated anti-caveolin antibody. Figure 10a shows a low magnification of negatively stained EMTCs present in fraction 10 of glycerol gradients. It is a polidisperse preparation containing relatively regular structures, some of them labeled with 5 nm gold-conjugated anti-myc antibody. Structures with similar morphology remained unlabeled by anti-myc antibody. Their presence could be explained by 1) limited efficiency of the labeling procedure, 2) the presence of similar complexes containing

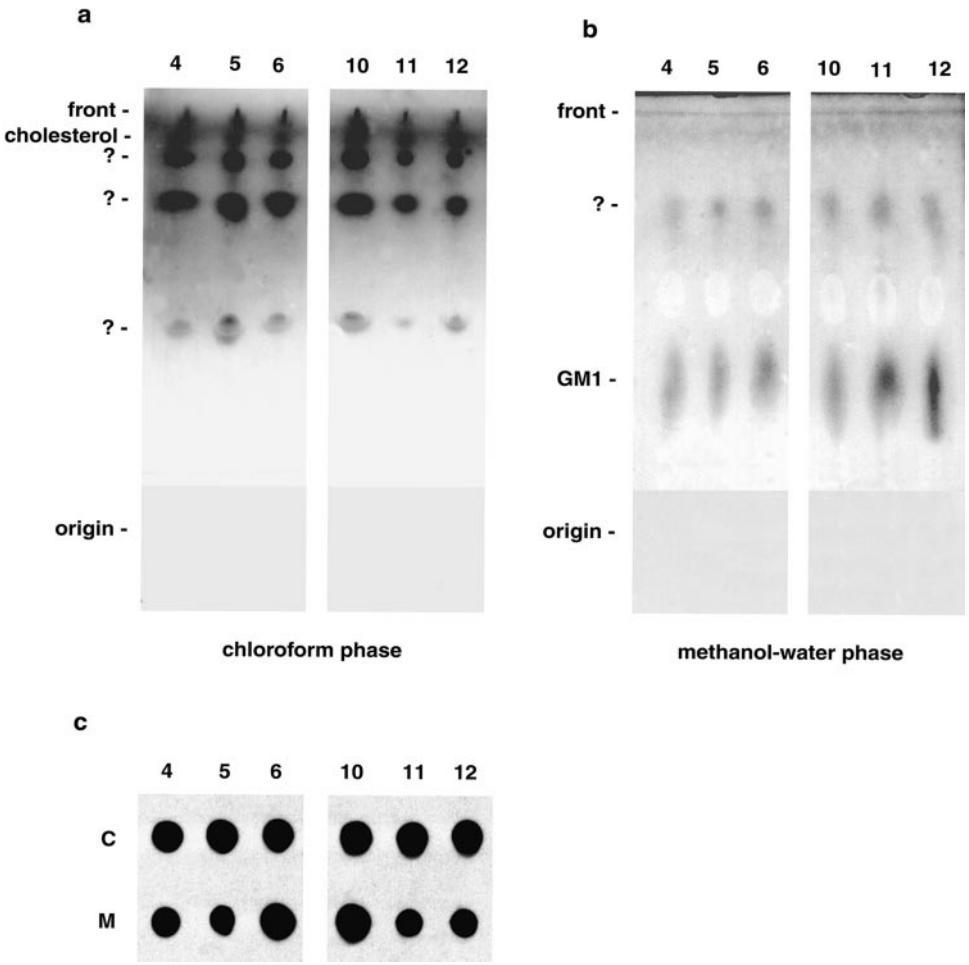

Figure 9. Lipid composition of EMTCs. (a) Chloroform phase obtained after lipids extraction from EMTCs containing fractions 4–6 and 9–12 of glycerol gradients was analyzed by TLC by using chloroform/methanol/water (65:25:4) as mobile phase. The lipids present were detected by charring after spraying the HPTLC plate with methanol/sulfuric acid (1:1). The R_f of the lipid migrating in the immediate proximity of the front line corresponds to R_f of the cholesterol standard. The identity of the other organic compounds is still under investigation. (b) Methanol/water phase obtained after lipid extraction from the same preparations as described above was analyzed by TLC by using methanol/chloroform/28% ammonia (60:25:5) as mobile phase. The lipids were detected by charring after spraying the plate with methanol/sulfuric acid (1:1). The R_f of the lowest band seen on the TLC plate corresponds to that of the GM1 standard. The other component detected was not yet identified. (c) Presence of GM1 in the EMTCs also was investigated by immunodotblotting by using anti-GM1 pAb, anti-rabbit IgG HRP-conjugated as reporter antibody and ECL for detection. One microliter from lipid extracts (methanol/water phase) was applied on the PVDF membrane by using a dot blot apparatus. The strong signal obtained indicates that GM1 is a component of EMTCs.

endogenous NSF that is not recognized by anti-myc antibody, or 3) the presence of other protein complexes. To investigate the existence of EMTCs containing endogenous NSF, we used aliquots of EMTCs present in fraction 10 of glycerol gradients containing detergent-free preparations of HMECs that were not incubated with myc-NSF. Using the same immunogold-labeling and negative-staining procedures we were able to show the existence in the endothelial cells of multimolecular protein complexes containing endogenous NSF (see MATERIALS AND METHODS; Figure 10b).

Figure 11 shows a gallery of negatively stained cytosolic EMTCs present in fraction 5 (Figure 11, a, c, d, and e) of glycerol gradients, labeled with 5 nm gold-conjugated anti-myc antibody and/or 15 nm gold-conjugated anti-caveolin antibody or 15 nm gold-conjugated anti-NSF antibody (Figure 11b). Figure 11, f–o, shows negatively stained EMTCs present in fraction 10 of glycerol gradients. We have detected in these complexes, singly or in pairs, NSF, syntaxin, dynamin, caveolin, and myc-NSF. See Figure 11 legend for details. The gold-labeled structures are consistent in appearance and display a relatively regular compact structure, suggesting a high concentration of proteins and lipids. They have a more regular shape when labeled only with one antibody and become less regular when reacted with a second antibody.

Data regarding the morphometric analysis of large EMTCs present in detergent-free preparations of HMECs cytosol are summarized in Table 1. Based on these findings we conclude that the large EMTCs have the average dimensions of 40 × 26 nm. A morphometric survey performed on small EMTCs labeled with either 5 nm gold-conjugated anti-myc antibody and/or 15 nm gold-conjugated anti-caveolin antibody (n = 17) or anti-NSF antibody (n = 16) indicates an average size of 26 × 20 nm for protein complexes present in fraction 5 of glycerol gradients. Comparable data were obtained when small- and large membrane-derived EMTCs were subjected to the same morphometric analysis (our unpublished results). We should mention that the endothelial particles subjected to the morphological analysis were not prepared by the critical point drying procedure.

DISCUSSION

Key evidence supporting the role of plasmalemmal vesicles or caveolae as vesicular carriers for protein molecules across the continuous microvascular endothelium came from tracer experiments performed with DNP (dinitrophenyl)-derivatized tracers, in the presence (or absence) of NEM (Predescu, 1994, 1997, 1998). The tracers, detected directly and individ-

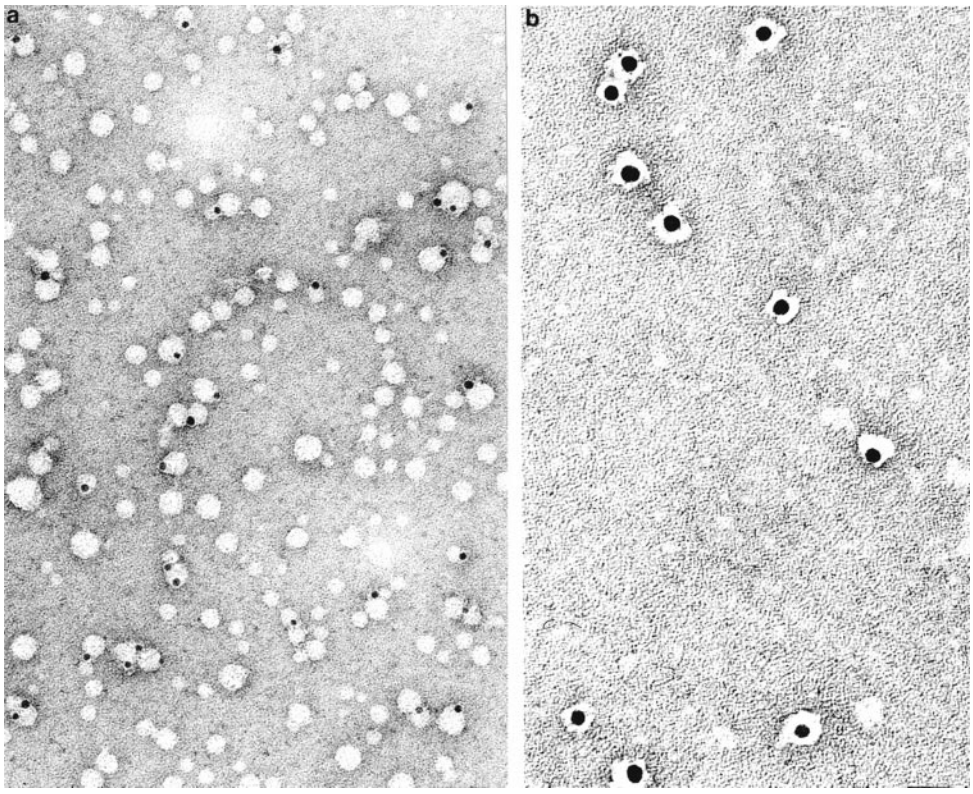

Figure 10. Negative staining of EMTCs present in detergent-free preparations of HMECs cytosol. Low magnification of EMTCs present in fraction 10 of glycerol gradient immunolabeled with 5 nm gold-conjugated anti-myc mAb (a) and 15 nm gold-conjugated anti-NSF pAb (b). Bar, 50 nm.

ually, were limited to PVs while in transit across the endothelium and their transport was inhibited >80% by NEM. These findings strongly suggest that the derivatized proteins reach the pericapillary spaces by transcytosis, an NSF-dependent process of vesicular transport involving membrane fusion-fission events. In addition to NSF, there is evidence that other proteins involved in membrane fusion and targeting (γ-SNAP and cellubrevin) are present in an endothelial membrane fraction isolated from rat lung tissue (Schnitzer, 1995). However, no data regarding the presence of other important components of the fusion machinery and no biochemical and morphological evidence concerning the organization of these proteins in multimolecular complexes, especially in the cytosol, have been presented so far. These findings raised two questions: 1) Are these endothelial fusion, fission, and targeting components organized in multimolecular complexes or "fusion particles" as in synaptosomes? and 2) Are these endothelial complexes different in some respects from their neuronal counterparts, considering the tight coupling of endocytosis to exocytosis that characterizes transcytosis, the apparent different chemistry of the vesicular carriers involved (synaptic vesicles versus caveolae), and considering also the extensive morphological evidence for homotypic fusion-fission of PVs?

In this study, we have used cytosol and membrane preparations from rat lung and cultured human lung microvascular endothelial cells, myc-tagged NSF (to detect the interactions of NSF with endothelial proteins with an anti-myc antibody), and a technology that worked out for other investigators in other systems, especially in synaptosomes (Sollner, 1993a,b). We believe that the results obtained can provide tentative answers to these questions.

Multimolecular complexes containing general components of the fusion machinery (NSF, α-SNAP, γ-SNAP) and targeting components (cellubrevin, syntaxin, or equivalents thereof) are present in the cells of the continuous microvascular endothelium. They contain SNAPs and SNAREs but, in addition, they also contain Rab 5, dynamin, and caveolin. The existence of an endothelial cytosolic SNARE complex is a novelty of this study.

The presence of caveolin, an integral membrane protein of the caveolar membrane, in EMTCs derived from both cytosol and membrane fractions is an unexpected finding. Quantitative data on caveolin distribution indicate that ~75% of the total endothelial caveolin is present in the membrane fraction, whereas ~25% is present in the cytosol. How could be explained the presence of caveolin, an integral membrane protein, in the cytosol? This "cytosolic" caveolin could reflect the presence of caveolar membrane fragments. As documented, this does not appear to be the case. We showed that the complexes are experimentally formed in the presence of myc-NSF, 0.5 mM ATP, and 0.2 mM EDTA by using both Triton X-100 or CHAPS, cytosol and membrane preparations, as well as detergent-free preparation of HMECs cytosol. Moreover, we have not detected by electron microscopy membrane fragments in the fractions collected from glycerol gradients. In addition, in preliminary experiments, we have found that the complexes dissociate specifically when ATP hydrolysis occurs (our unpublished data) and they do not assemble in the presence of 1 mM NEM.

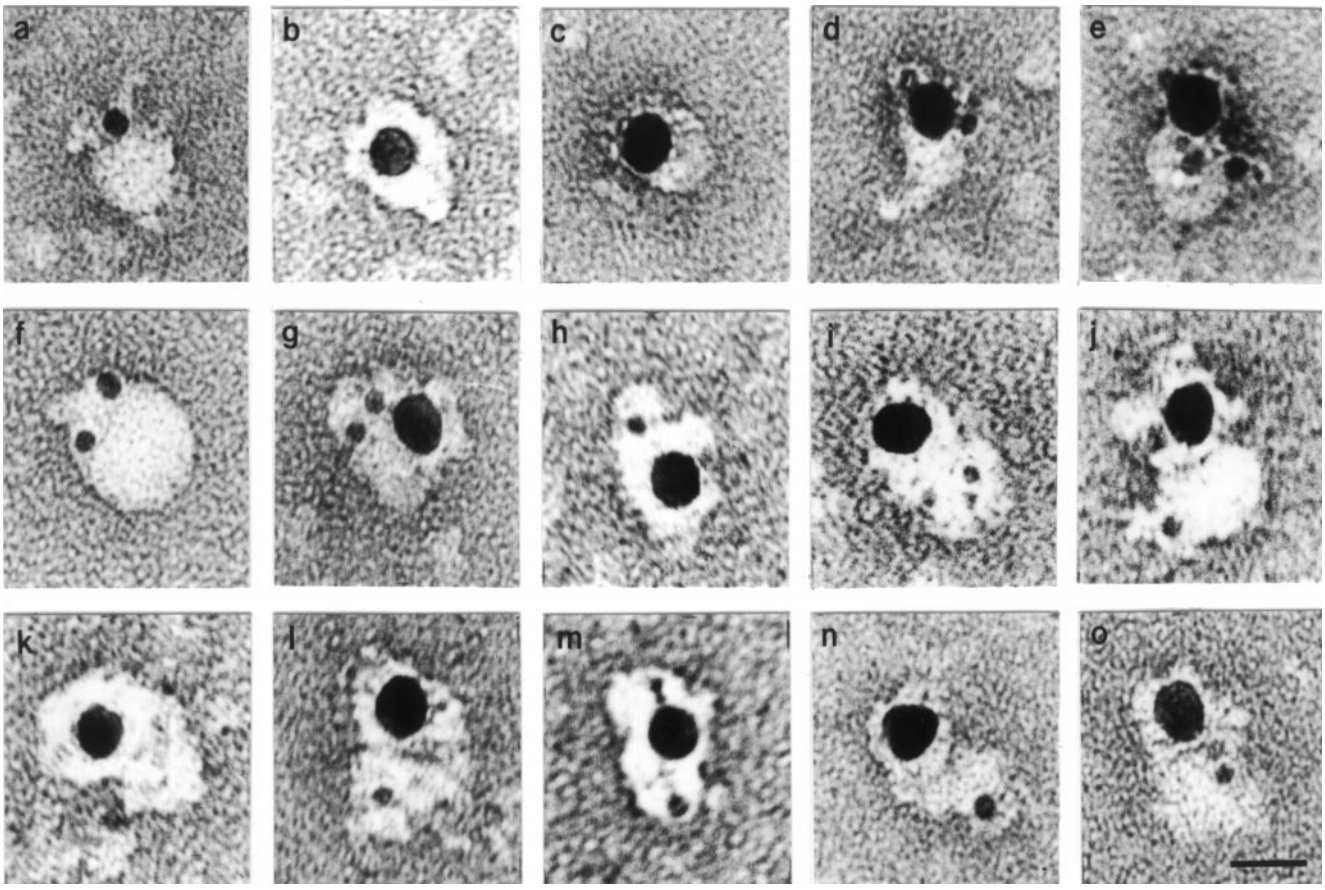

Figure 11. EMTCs morphology and immunocytochemistry at the electron microscope level. EMTCs present in fraction 5 (a, c, d, and e) of glycerol gradient have been immunolabeled as described in MATERIALS AND METHODS with 5 nm gold-conjugated anti-myc mAb and/or 15 nm gold-conjugated anti-caveolin pAb and with 15 nm gold-conjugated anti-NSF pAb (b). Cytosolic EMTCs present in fraction 10 from glycerol gradient have been immunolabeled with 5 nm gold-conjugated anti-myc mAb (f), 5 nm gold-conjugated anti-myc mAb and 15 nm gold-conjugated anti-caveolin pAb (g and h), 5 nm gold-conjugated anti-dynamin mAb and 15 nm gold-conjugated anti-caveolin pAb (i and j), 15 nm gold-conjugated anti-NSF pAb (k), 5 nm gold-conjugated anti-dynamin mAb and 15 nm gold-conjugated anti-NSF pAb (l and m), and with 5 nm gold-conjugated anti-syntaxin mAb and 15 nm gold-conjugated anti-NSF pAb (n and o). Comment: in general, NSF and caveolin localized to the "cores" of the particles; dynamin and syntaxin localized on the "wings." Bar, 20 nm for the entire gallery of highly magnified particles.

We also believe that the complexes are not artifacts produced by partial solubilization of membranes because they can be obtained at different concentrations of Triton X-100 or CHAPS or in the absence of any detergent treatment of the cytosolic fraction.

Table 1. Morphometric analysis of large EMTCs

Gold label	n_1	n_2	Size (nm)
Anti-myc and/or Anti-caveolin	34	15	36 × 24 (SD ± 3.5)
Anti-NSF and/or Anti-syntaxin	31	17	42 × 30.5 (SD ± 4)
Anti-NSF and/or Anti-dynamin	31	16	40 × 27 (SD ± 4)
Anti-caveolin and/or Anti-dynamin	7	6	41 × 24 (SD ± 4)

The endothelial particles subjected to the morphometrical analysis were not prepared by the critical drying point procedure.

n_1, number of EMTCs labeled with either one of the two antibodies; n_2, number of EMTCs labeled with both antibodies.

Moreover SLO permeabilization of the endothelial cell membrane used to obtain the cytosolic fraction, to isolate, and characterize the transcytotic endothelial complexes, and cross-linking experiments used to get independent evidence regarding the near-neighbor relationship among EMTC proteins, confirmed that cultured endothelial cells contain cytosolic endothelial transcytotic complexes, which comprise general components of the fusion machinery (NSF, α-SNAP), targeting components (syntaxin, cellubrevin), and proteins expected to fulfill the requirements of a highly specialized process such as endothelial transcytosis (dynamin).

Finally, we have used for convenience, in most biochemical investigations, myc-NSF but similar morphological and immunocytochemical EM evidence also has been obtained in the presence of endogenous NSF (Figures 10b and 11b, k, l–o).

Figure 3a shows that EMTCs contain in addition to proteins already identified by immunoblotting other still un-

identified proteins, especially high M_r components. Figure 3b shows, however, that the proteins present in the entire cytosolic fraction are much more numerous and diverse. Low- and medium-M_r proteins predominate. An equivalent electrophoretogram of the membrane fraction shows an even greater complexity (our unpublished results). This is another indication that the complexes are not membrane fragments or random aggregates of cytosolic proteins. EMTCs contain just a subfraction of the starting material.

Taking into account all these findings, our tentative conclusion is that EMTCs are not fragmentation or partial detergent solubilization artifacts. They appear to be macromolecular complexes normally present in the endothelial cells from the continuous microvascular endothelium. We don't know whether these complexes are functional but we have evidence that they are NSF- and ATP-dependent, and NEM-sensitive properties found in our previous work as being characteristic to transcytosis. Transcytosis apparently uses as the other major transport pathways do the same related fusion-fission molecular machinery.

Besides the presence of common components with the neuronal counterparts, there are some particular features of these EMTCs that indicate that the system is different. Endothelial complexes are heterogeneous particles, different from neuronal complexes. The heterogeneity of EMTCs is suggested not only by the distribution of different protein components along gradient fractions but also by their stoichiometry. The presence of caveolin is one of the important elements of the difference. The presence of caveolin, an integral membrane protein, in the soluble fraction in the absence of detergent is an unusual finding. To explain it, we hypothesized the existence of protein–lipid interactions in the EMTCs. TLC analysis indicated that, indeed, EMTCs contain in addition to proteins, cholesterol, GM1, and other still unidentified lipids. Results from other investigations indicate that 1) caveolin is a cholesterol binding protein (Murata, 1995), and 2) a component of a cytosolic heat shock protein chaperone complex (Uittenbogaard, 1998). Caveolin also has been implicated in the intracellular cholesterol trafficking (Fielding, 1995; Smart, 1996) and has been claimed to be present in lipoprotein particles secreted by exocrine cells (Ping Sheng, 1999). These findings raise the possibility that EMTCs or subclasses of EMTCs are involved in the transport of caveolin from its site of synthesis to the plasmalemma, a possibility that deserves to be explored by future work. In addition, GM1 is concentrated in caveolae (Parton, 1994) and it seems to be located in proximity of caveolin in the caveolar membrane (Fra, 1995). The presence of interactions among lipids and proteins in the fusion complexes also was suggested by previous work (Steel, 1997). It has been reported that α-SNAP, general component of the fusion machinery behaves in a way characteristic of hydrophilic, lipid-associated protein. The lipid binding activity of α-SNAP enhances the ATPase activity of NSF.

Two SNARE proteins, syntaxin and cellubrevin, or equivalents thereof were found present in EMTCs isolated from detergent-free cytosolic preparation of cultured endothelial cells mechanically disrupted or streptolysin O permeabilized. So far, little is known about how the different SNAREs are targeted to the various organelles and retained there. Every syntaxin isoform identified so far exists predominantly as an integral membrane protein that is anchored to membranes by a carboxyl terminal hydrophobic transmembrane domain. Current evidence indicates a multiplicity of syntaxins and VAMPs (vesicle associated membrane proteins), which includes variants without a membrane-anchoring domain (Bennett, 1993; Ibaraki, 1995; Mandic, 1997; Insenman, 1998; Simonsen, 1998; Tang 1998a,b; Berglund, 1999; Quinones, 1999). It has been suggested that they could function in different steps of the intracellular traffic in an unusual manner (Tang, 1998a). The antibodies we have used recognize generally common rather than specific domains of SNAREs, thereby leaving open the possibility that there are VAMPs (vesicle associated membrane proteins) and syntaxins specific to the endothelium and that the EMTCs contain soluble variants of SNAREs. This possibility remains to be explored by further work.

Another special feature of the endothelial transcytotic complexes is the presence of dynamin, believed to be primarily involved in clathrin-mediated endocytosis (Baba, 1995; Hinshaw, 1995). Dynamin is a 100-kDa GTPase that displays interesting properties, significant for its function. It oligomerizes around microtubules, it forms oligomeric rings in low-salt solutions (Hinshaw, 1995), and it interacts with phospholipids by its C terminus and pleckstrin domain (Warnock, 1996).

In the present work we have found dynamin in endothelial supramolecular protein–lipid complexes, together with proteins of the fusion machinery. Its presence in these transcytotic complexes containing proteins involved in fusion-fission processes in other systems of vesicular carriers could be required by a process that couples rapidly and efficiently endocytosis to exocytosis.

Data from previous investigations have indicated that caveolae pinch off from the plasma membrane in a GTP-dependent manner (Schnitzer, 1996) and that dynamin is a critical component of this fission process (Oh, 1998). Although the mechanism by which dynamin mediates the fission of caveolae from plasma membrane remains to be understood by further investigation, all these findings taken together, strongly suggest that dynamin is involved also in the caveolae-mediated endocytosis.

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REFERENCES

Andrew, S.M., and Titus, J.A. (1992). Affinity chromatography using Protein A Sepharose. In: Current Protocols in Immunology, vol. 1, eds. J.E. Coligan, A.M. Kruisbek, D.H. Margulies, and E.M. Shevak, Toronto: Wiley & Sons, 2.7.4–2.7.6.

Baba, T., Damke, H., Hinshaw, J.E., Ikeda, K., Schmid, S.L., and Warnock, D.E. (1995). Role of dynamin in clathrin-coated vesicle formation. Cold Spring Harb. Symp. Quant. Biol. *60*, 235–242.

Bajjalieh, M.S., and Scheller, R.H. (1995). The biochemistry of neurotransmitter secretion. J. Cell Biol. *270*, 1971–1974.

Bennett, M., Garcia-Arraras, J.E., Elferink, L.A., Peterson, K., Fleming, A.M., Kazuka, C.D., and Scheller, R.H. (1993). The syntaxin family of vesicular transport receptors. Cell *74*, 863–873.

Berglund, L., Hoffmann, H.J., Dahl, R., and Petersen, T.E. (1999). VAMP-1 has a highly variable C-terminus generated by alternative splicing. Biochem. Biophys. Res. Commun. *264*, 777–780.

Bhakdi, S., Tranum-Jensen, J., and Sziegoleit, A. (1985). Mechanism of membrane damage by streptolysin O. Infect. Immunol. *47*, 52–60.

Bhakdi, S., Weller, U., Walev, I., Martin, E., Jonas, D., and Palmer, M. (1993). A guide to use pore-forming toxins for controlled permeabilization of cell membranes. Med. Microbiol. Immunol. *182*, 167–175.

Chabraoui, F., Derrington, E.A., Mallie-Didier, F., Confavreux, C., Quincy, C., and Caudie, C. (1993). Dot-blot immunodetection of antibodies against GM1 and other gangliosides on PVDF-P membranes. J. Immunol. Methods *165*, 225–230.

Crowther, J.R. (1995). ELISA - theory and practice. In: Methods in Molecular Biology, vol 42, ed. J.M. Walker, Totowa, New Jersey: Humana Press, 161–177.

De Maio, A. (1994). Protein blotting and immunoblotting using nitrocellulose membranes. In: Protein Blotting, ed. B.S. Dunbar, Oxford: Oxford University Press, 141–150.

Fielding, P.E., and Fielding, C.J. (1995). Plasma membrane caveolae mediate the eflux of cellular cholesterol. Biochemistry *34*, 14288–14292.

Fra, A.M., Masserini, M., Palestini, P., Sonnino, S., and Simons, K. (1995). A photo-reactive derivative of ganglioside GM1 specifically cross-links VIP21 caveolin on the cell surface. FEBS Lett. *375*, 11–14.

Ghitescu, L., Jacobson, B.S., and Crine, P. (1999). A novel, 85 kDa endothelial antigen differentiates plasma membrane macrodomains in lung alveolar capillaries. Endothelium *6*, 241–250.

Graham, J.M., and Higgins, J.A. (1994). Biomembrane protocols II. Architecture and function. In: Methods in Molecular Biology, vol 27, ed. J.M. Walker, Totowa, New Jersey: Humana Press, 295–323.

Hamilton, R.J., and Hamilton, S. Lipid analysis. (1992). A Practical Approach. Oxford: IRL Press.

Hinshaw, E.J., and Schmid, S.L. (1995). Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding. Nature *374*, 190–192.

Ibaraki, K., Horikawa, H.P.M., Morita, T., Mori, H., Sakimura, K., Saisu, H., and Abe, T. (1995). Identification of four different forms of syntaxin 3. Biochem. Biophys. Res. Commun. *211*, 997–1005.

Insenman, S., Khew-Goodall, Y., Gamble, J., Vadas, M., and Wattenberg, B.W. (1998). A splice-isoform of vesicle-associated membrane protein-1 (VAMP-1) contains a mitochondrial targeting signal. Mol. Biol. Cell *9*, 1649–1660.

Kurzchalia, T.V., Dupree, P., Parton, R.G., Kellner, R., Virta, H., Lehnert, M., and Simons, K. (1992). VIP-21, a 21 kD membrane protein is an integral component of trans-Golgi-network-derived transport vesicles. J. Cell Biol. *118*, 1003–1014.

Mandic, R., Timble, W.S., and Lowe, A.W. (1997). Tissue-specific alternative RNA splicing of rat vesicle-associated membrane protein-1 (VAMP-1). Gene *199*, 173–179.

McMahon, H.T., Ushkariov, Y.A., Edelmann, L., Link, E., Binz, T., Niemann, H., Jahn, R., and Sudhof, T. (1993). Cellubrevin is a ubiquitous tetanus-toxin substrate homologous to a putative synaptic vesicle fusion protein. Nature *364*, 346–349.

Morgan, A., and Burgoyne, R.D. (1995). A role for soluble NSF attachment proteins (SNAPs) in regulated exocytosis in adrenal chromaffin cells. EMBO J. *14*, 232–239.

Muller, S., Plane, S., Couppez, M., and Van Regenmortel, M.H.V. (1986). Comparison of different methods for localization of antigenic regions in histone H2A. Mol. Immunol. *23*, 593–597.

Muller, S. (1988). Peptide carrier conjugation. Immunization with peptides. In: Synthetic Peptides as Antigens, eds. M.H.V. Van Regenmortel, J.P. Briand, S. Muller, and S. Plaue, Amsterdam: Elsevier, 95–144.

Murata, M., Peranen, J., Schreiner, R., Wieland, F., Kurzchalia, T.V., and Simons, K. (1995). Vip 21/caveolin is a cholesterol binding protein. Proc. Natl. Acad. Sci. USA *92*, 10339–10343.

Oh, P., McIntosh, D.P., and Schnitzer, J.E. (1998). Dynamin at the neck of caveolae Mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. J. Cell Biol. *141*, 101–114.

Parton, R. (1994). Ultrastructural localization of gangliosides; GM1 is concentrated in caveolae. J. Histochem. Cytochem. *42*, 155–166.

Ping Sheng, L., Machleidt, T., and Anderson, R.G.W. (1999). Identification of caveolin-1 in lipoprotein particles secreted by exocrine cells. Nat. Cell Biol. *1*, 369–375.

Predescu, N.D., and Palade, G.E. (1993). Plasmalemmal vesicles represent the large pore system of the continuous microvascular endothelium. Am. J. Physiol. *265*, H725–H733.

Predescu, N.D., Horvat, R., Predescu, S., and Palade, G.E. (1994). Transcytosis in the continuous endothelium of the myocardial microvasculature is inhibited by N-ethylmaleimide. Proc. Natl. Acad. Sci. USA *91*, 3014–3018.

Predescu, N.D., Predescu, S.A., McQuistan, T., and Palade, G.E. (1998). Transcytosis of α_1-acidic glycoprotein in the continuous microvascular endothelium. Proc. Natl. Acad. Sci. USA *95*, 6175–6180.

Predescu, S.A., Predescu, D.N., and Palade, G.E. (1997). Plasmalemmal vesicles function as transcytotic carriers for small proteins in the continuous microvascular endothelium. Am. J. Physiol. *272*, H937–H949.

Quagliarello, V.J., Ma, A., Stukenbrok, H., and Palade, G.E. (1991). Ultrastructural localization of albumin transport across the cerebral microvasculature during experimental meningitis. J. Exp. Med. *174*, 657–672.

Quinones, B., Riento, K., Olkkonen, V.N., Hardy, S., and Bennett, M.K. (1999). Syntaxin 2 splice variants exhibit differential expression patterns, biochemical properties and subcellular localizations. J. Cell Sci. *112*, 4291–4304.

Rothberg, K.G., J.E. Heuser, W.C. DonzelL, Y.S. Ying, J.R. Glenney, and R.G.W. Anderson. (1992). Caveolin, a protein component of caveolae membrane coats. Cell *68*, 673–682.

Rothman, J.E. (1994). Mechanisms of intracellular protein transport. Nature, Lond *372*, 55–62.

Schnitzer, J.E., Liu, J., and Oh, P. (1995). Endothelial caveolae have the molecular transport machinery for vesicle budding, docking and fusion, including VAMP, NSF, SNAP, annexins and GTPases. J. Biol. Chem. *270*, 14399–14404.

Schnitzer, J.E., Oh, P., and McIntosh, D.P. (1996). Role of GTP hydrolysis in fission of caveolae directly from plasma membrane. Science *274*, 239–242.

Simonsen, A., Bremnes, B., Ronning, E., Aasland, R., and Stenmark, H. (1998). Syntaxin 16, a putative Golgi t-SNARE. Eur. J. Cell Biol. *75*, 223–231.

Slot, J.W., and Geuze, H.J. (1985). A new method of preparing gold probes for multiple labeling cytochemistry. Eur. J. Cell Biol. *38*, 87–93.

Smart, E.J., Ying, Y., Donzell, W.C., and Anderson, R.G.W. (1996). A role for caveolin in transport of cholesterol from endoplasmic reticulum to plasma membrane. J. Biol. Chem. *271*, 29427–29435.

Smith, R.J., Capaldi, R.A., Muchmore, D., and Dahlquist, F. (1978). Cross-linking of ubiquinone cytocrome c reductase (complex III) with periodate-cleavable bifunctional reagents. Biochemistry *17*, 3719–3723.

Sollner, T.H., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J.E. (1993a). SNAP receptors implicated in vesicle targeting and fusion. Nature *362*, 318–324.

Sollner, T.H., Bennett, M.K., Whiteheart, S.W., Scheller, R.H., and Rothman, J.E. (1993b). A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicles docking, activation, and fusion. Cell *75,* 409–418.

Sollner, T.H., and Rothman, E.J. (1996). Molecular machinery mediating vesicle budding, docking and fusion. Experientia *52*, 1021–1025.

Stan, R.V., Roberts, W.G., Predescu, N.D., Ihida, K., Saucan, L., Ghitescu, L., and Palade, G.E. (1997). Immunoisolation and partial characterization of endothelial plasmalemmal vesicles (caveolae). Mol. Biol. Cell *8*, 595–605.

Stan, R.V., Ghitescu, L., Jacobson, B.S., and Palade, G.E. (1999). Isolation, cloning, and localization of rat PV-1, a novel endothelial caveolar protein. J. Cell Biol. *145*, 1189–1198.

Steel, G.J., Buchheim, G., Edwardson, J.M., and Woodman, P.G. (1997). Evidence for interaction of the fusion protein α-SNAP with membrane lipid. Biochem. J. *325*, 511–518.

Sztul, E., Colombo, M., Stahl, P., and Samanta, R. (1993). Control of protein traffic between distinct plasma membranes domains. Requirement for a novel 108,000 protein in the fusion of transcytotic vesicles with the apical plasma membrane. J. Biol. Chem. *268*, 1876–1885.

Takei, K., McPherson, P.S., Schmid, S.L., and De Camilli, P. (1995). Tubular membrane invaginations coated by dynamin rings are induced by GTPγS in nerve terminals. Nature *374*, 186–190.

Tang, B.L., Low, D.Y.H., and Hong, W. (1998a). Syntaxin 11: a member of the syntaxin family without a carboxyl terminal transmembrane domain. Biochem. Biophys. Res. Commun. *245*, 627–632.

Tang, B.L., Low, D.Y.H., Lee, S.S., Tan, A.E.H., and Hong, W. (1998b). Molecular cloning and localization of human syntaxin 16, a member of the syntaxin family of SNARE proteins. Biochem. Biophys. Res. Commun. *242*, 673–679.

Uittenbogaard, A., Ying, Y., and Smart, E.J. (1998). Characterization of a cytosolic heat-shock protein-caveolin chaparone complex. J. Biol. Chem. *273*, 6525–6532.

Warnock, D.E., and Schmid, S.L. (1996). Dynamin GTPase, a force-generating molecular switch. BioEssays *18*, 885–893.

Waters, M.G., Griff, I.C., and Rothman, J.E. (1991). Proteins involved in vesicular transport and membrane fusion. Curr. Opin. Cell Biol. *3*, 615–620.

Whiteheart, S.W., Rossnagel, K., Buhrow, S.A., Brunner, M., Jaenicke, R., and Rothman, J.E. (1994). N-ethylmaleimide-sensitive fusion protein: a trimeric ATP-ase whose hydrolysis of ATP is required for membrane fusion. J. Cell Biol. *126*, 945–954.

Wilson, D.W., Whiteheart, S.W., Wiedmann, M., Brunner, M., and Rothman, J.E. (1992). A multisubunit particle implicated in membrane fusion. J. Cell Biol. *117*, 531–538.

Worthtington, V. (1993). Worthington Enzyme Manual, Freehold, New Jersey: Worthington Biochemical, 236–237.