Cholesterol-dependent Syntaxin-4 and SNAP-23 Clustering Regulates Caveolar Fusion with the Endothelial Plasma Membrane

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We determined the organization of target (t) SNARE proteins on the basolateral endothelial plasma membrane (PM) and their role in the mechanism of caveolar fusion. Studies were performed in a cell-free system involving endothelial PM sheets and isolated biotin-labeled caveolae. We monitored the fusion of caveolae with the PM by the detection of biotin-streptavidin complexes using correlative high resolution fluorescence microscopy and gold labeling electron microscopy on ultrathin sections of PM sheets. Imaging of PM sheets demonstrated and biochemical findings showed that the t-SNARE proteins present in endothelial cells (SNAP-23 and syntaxin-4) formed cholesterol-dependent clusters in discrete areas of the PM. Upon fusion of caveolae with the target PM, 50% of the caveolae co-localized with the t-SNARE clusters, indicating that these caveolae were at the peak of the fusion reaction. Fluorescent streptavidin staining of PM sheets correlated with the ultrastructure within the same area. These findings demonstrate that t-SNARE clusters in the endothelial target PM serve as the fusion sites for caveolae during exocytosis.

The endothelial cells (ECs) lining the blood vessel walls are differentiated to mediate the rapid exchange of substances between the plasma and interstitial fluid. The process involving the fission of caveolae from the apical plasma membrane (PM) and fusion with the basolateral PM is termed transcytosis. Caveolae “pinch off” from the apical PM through a process requiring the recruitment of the GTPase dynamin to the caveolar necks as regulated by another protein intersectin (1, 2). Upon fission, caveolae form vesicular carriers that shuttle through the cytosol and deliver their cargo to the subendothelium by fusion with the basolateral PM (3). The basis of caveolar fusion may involve the same machinery as other vesicular carriers, i.e., the SNARE proteins of the syntaxin, synaptobrevin/cellubrevin, and SNAP-23/25 families (4–7). However, the organization of SNARE proteins and their function in the fusion of caveolae in ECs are incompletely understood. It is known that syntaxin, cellubrevin, SNAP-23, and the cytosolic factors N-ethylmaleimide-sensitive factor (NSF) and α- and γ-SNAP are key components of the endothelial multimolecular transcytotic complex, the assembly of which depends on the membrane fusion ATPase, NSF, which can be inhibited by alkylation of NSF by N-ethylmaleimide (7). Studies have also shown that N-ethylmaleimide interferes with transcytosis in ECs (4, 8). Based on the SNARE hypothesis, membrane fusion occurs when SNARE proteins on opposing membranes form four helix bundles, bringing the membranes in close apposition, thus providing the driving force necessary for fusion (9, 10). Ultrastructural studies have shown several states of association between caveolae and their target PM, varying in proximity, stability, and readiness for fusion (11). However, the pre-fusion states of caveolae have not been experimentally clarified, and the sequence of events responsible for caveolar fusion and the spatial distribution of the SNARE proteins on the endothelial PM remains uncertain. Unlike intracellular membrane fusion events or regulated exocytosis for which in vitro assays are available (12, 13), an assay for the in vitro reconstitution of caveolar fusion does not exist. A recently described cell-free preparation for exocytosis in PC12 cells demonstrated that sonication of these cells grown on coverslips results in PM patches capable of fusion with secretory vesicles (14). Based on this approach, we have developed an assay for caveolar fusion involving endothelial PM patches and isolated biotin-labeled caveolae. We monitored membrane interactions during the fusion event via the specific detection of biotin-streptavidin complexes on the PM sheets. We correlated fluorescence microscopy (FM) analysis with gold labeling electron microscopy (EM) on ultrathin sections of PM sheets subjected to the fusion reaction. The results identified (i) the presence on PM sheets with numerous docked and fused streptavidin-labeled caveolae, (ii) cholesterol-dependent clustering of SNAP-23 and syntaxin-4 (Synt-4), and (iii) PM target (t) SNARE clusters as the preferential sites for caveolar fusion. Fluorescent staining of ultrathin sections of PM sheets highly correlated with the ultrastructure within the same area. Thus, the fusion of caveolae occurring at specialized cholesterol-rich t-SNARE clusters on the basolateral PM is responsible for exocytosis in ECs and is thereby integral to the mechanism of transcytosis.

EXPERIMENTAL PROCEDURES

Materials

Human lung microvascular ECs were obtained from BioWhittaker, Inc. (Walkerville, MD). Reagents were obtained as follows: EZ-Link N-hydroxysulfosuccinimidobiotin from Fisher; methyl-β-cyclodextrin (MCD) and 8-nm gold-conjugated streptavidin from Sigma; FuGENE 6 from Roche Applied Science; Prolong antifade kit, streptavidin-Texas Red, and Alexa Fluor-conjugated antibodies from Molecular Probes, Inc. (Eugene, OR); and SuperSignal chemiluminescent substrate from Pierce. All EM reagents were from EM Science (Fort Washington, PA). All other reagents were from Sigma if not specified otherwise. Relevant antibodies (Abs) were obtained from the following sources: anti-caveo-
lin-1 (Cav-1) Abs and anti-Synt-4 monoclonal antibody (mAb) from BD Transduction Laboratories, anti-SNAP-23 polyclonal antibody (pAb) from Synaptic Systems (Göttingen, Germany), and Synt-4 pAb from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**Preparation of PM Sheets**

ECs grown on poly-l-lysine-coated coverslips were briefly sonicated in ice-cold 20 mM HEPES (pH 7.2), 120 mM potassium glutamate, 20 mM potassium acetate, and 2 mM EGTA as described (12, 15). PM patches attached to the coverslips were either fixed and subjected to morphological analysis by FM or EM or used in a cell-free assay for caveolar fusion.

**Scanning EM of PM Sheets**

PM sheets were fixed in 3% glutaraldehyde in 0.05 M sodium cacodylate (pH 7.1) for 30 min at room temperature, post-fixed in 1% OsO₄ in 0.3 M sodium cacodylate for 15 min, dehydrated through a series of graded ethanol dilutions and hexamethydisilazane, dried, mounted on stubs, coated with a 2-nm layer of platinum/palladium, and then examined and photographed using a Hitachi field emission scanning electron microscope.

**EM of PM Sheets**

PM sheets were fixed in 2.5% glutaraldehyde for 15 min at room temperature, post-fixed in 1% OsO₄ in 0.1 M sodium cacodylate buffer (pH 7.4) for 15 min, dehydrated in ethanol, and embedded in Epon 812. After polymerization for 48 h at 60 °C, ultrathin sections were cut perpendicular to the PM sheets, mounted on EM grids, stained with uranyl acetate and lead citrate, and finally examined using a Joel JEM 1220. For immunogold labeling EM, PM patches were prepared by brief sonication of ECs grown on EM gold grids coated first with Formvar and then with a thin layer of poly-l-lysine. PM sheets attached to the EM grid were fixed in 2% paraformaldehyde for 15 min; quenched with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS); then incubated for 1 h at 4 °C with the primary Abs, followed by 6-nm gold-conjugated reporter Abs; diluted in 0.1% BSA in PBS; and centrifuged for 30 min at 30,000 × g before use. In control experiments, the primary Abs were omitted. After washing by transfer through six successive PBS drops, gold-labeled PM sheets were post-fixed in 2% glutaraldehyde for 15 min and stained with uranyl acetate and lead citrate, followed by EM examination. Randomly chosen areas of PM sheets were photographed at the initial ×20 magnification. To quantify the distribution of gold particles and to estimate the size of SNARE clusters, we used three sets of micrographs (36 per set representing ~200 μm² of PM surface) from three different experiments for each antigen. The clusters were classified based on their number of gold particles (between three and six or more than six gold particles). The size of the gold clusters was estimated using at least 50 clusters per experimental set.

**FM of PM Sheets**

In control experiments, PM sheets were fixed in 4% paraformaldehyde in PBS; quenched with 50 mM NH₄Cl in PBS; and then sequentially incubated for 30 min with the primary Abs diluted 1:100 in 1% BSA in PBS, followed by a 45-min incubation with the reporter Abs diluted in 0.1% BSA in PBS as described (13). For cholesterol depletion, PM sheets were treated with 10 mM MCD as described (13) and immunostained for Cav-1 and SNAP-23.

**Small Interfering RNA (siRNA) for Cav-1**

RNA oligonucleotides designed as described (16) were from Integrated DNA Technologies (Coralville, IA). EC monolayers (70–80% confluent) were transfected with Cav-1 siRNA using FuGENE 6 reagent at an siRNA/lipid ratio of 1:3. A four-nucleotide mismatch was used as control.

**Cell-free Assay for Caveolar Fusion**

To address caveolar fusion experimentally, we used the basolateral endothelial PM as the target PM and the isolated caveolae.

**Isolation of Biotin-labeled Caveolae**—Freshly prepared PM sheets bearing attached caveolae were biotinylated as described (2). After extensive washing, the PM sheets were incubated for 2 h at 37 °C with 5 mg/ml EC cytosol in the presence of 1 mM GTP and an ATP-regenerating system to allow the GTP-dependent fission of biotinylated caveolae as described (1, 2).

**Fusion Reaction**—The standard fusion assay consisted of PM sheets present on one coverslip (~100–150 patches) and the biotin-labeled caveolae released from PM patches present on three coverslips. To initiate docking and fusion, 900 μl of fusion buffer (20 mM HEPES/KOH (pH 7.4), 100 mM KCl, 2 mM dithiothreitol, 2 mM EDTA, and 2 mM ATP); 100 μl of an ATP-regenerating system; and 5 mg/ml EC cytosol were added over the PM sheets, followed by addition of isolated caveolae and incubation for 30 min at 37 °C. The patches were placed on ice, washed, fixed, and stained for biotin and SNARE proteins.

**Correlative FM and EM of Quetol 651-embedded PM Sheets Subjected to Caveolar Fusion**

Plastic coverslips containing PM sheets subjected to the fusion reaction were extensively washed; fixed in 2% paraformaldehyde in PBS (pH 7.5) for 10 min at room temperature; rinsed twice in PBS; blocked with 1% BSA in PBS; and subsequently immunostained for SNAP-23, followed by another quenching step and simultaneous application for 30 min of fluorescein isothiocyanate-conjugated anti-rabbit IgG to detect bound anti-SNAP-23 antibody and streptavidin-Texas Red to visualize biotin-labeled caveolae. The PM patches were then washed with PBS; post-fixed in 2% paraformaldehyde in PBS for 10 min at room temperature; and dehydrated with a series of graded ethanol dilutions, followed by embedding with Quetol 651 (EM Science) following the manufacturer’s instructions. Ultrathin sections were cut perpendicular to the PM patches and mounted on Formvar-coated EM grids. The grid to be examined was sited on a glass slide, covered with a coverslip using transparent tape, and imaged by FM. All images were collected with identical acquisition parameters. The same grid was subjected to 8-nm gold-conjugated streptavidin labeling, followed by uranyl acetate and lead citrate staining and examined by transmission EM.

**RESULTS**

**Endothelial SNARE Proteins Are Concentrated in Clusters and Co-localize with Cav-1 on Endothelial PM Sheets**—To analyze the distribution of t-SNARE proteins in endothelial membranes, we adapted the recently described PM sheet system (12). Basolateral endothelial PM sheets were prepared as described under “Experimental Procedures” by brief sonication of confluent EC monolayers grown on polylysine-coated coverslips. In confluent ECs, tight junctions are formed, and cell polarity is subsequently established. Under these conditions, the apical PM is oriented away from the substrate, thus facilitating the removal of the apical PM and cytosolic structures, whereas the basolateral PM sheets with their membrane protein complexes and caveolar profiles remain attached to the coverslip. These basolateral PM sheets were fixed in 4% paraformaldehyde and incubated first with anti-Cav-1 pAb and then with fluorescein isothiocyanate-conjugated reporter Ab. Cav-1 is known to decorate caveolar coats in different stages of mem-
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FIGURE 1. Morphological characterization of basolateral endothelial PM sheets. A, endothelial PM sheets were fixed in 4% paraformaldehyde in PBS and immunostained with anti-Cav-1 pAb. Staining for Cav-1 resulted in strong punctate labeling, characteristic of a vesicular protein. B, PM sheets were fixed in 3% glutaraldehyde and processed for scanning EM. Panel b1 shows a fragment of an undisturbed EC and an intact PM sheet with no holes or tears. The endothelial junction (arrowhead) was disrupted as result of sonication (panel b2). PM patches were devoid of all cell constituents except attached caveolae (panel b2). Panels b3–b5 show highly magnified attached caveolar profiles, and panels b6 and b7 show caveolar clusters still attached to the PM after sonication. C, the electron micrograph shows an attached caveolar profile. Panel c1 shows a well-preserved morphology. Scale bars = 10 μm (A and panel b1), 100 nm (panels b2, b5–b7, C, and c1), and 50 nm (panels b3 and b4).

brane invagination. Cav-1 immunostaining revealed the characteristic vesicular organization (Fig. 1A). Cav-1 clusters were numerous and non-uniformly scattered on the PM sheet. A high resolution scanning EM approach showed a large number of caveolae (Fig. 1B, panels b1 and b2) and, occasionally, caveolar clusters attached to the PM. Fig. 1B shows highly magnified caveolar profiles (panels b3–b5) and caveolar clusters attached to the PM (panels b6 and b7). We also observed some actin cables (panel b2), but no other cell components were detected, indicating that cytosolic structures were removed during the sonication.

We very rarely observed membrane folding, and no apparent membrane damage was produced by the sonication. Further morphological analysis by pre-embedding EM of cross-sections through PM sheets (Fig. 1C and panel c1) showed a well-preserved membrane structure suitable for studying caveolar fusion in relation to the organization of SNARE proteins located on the inner leaflet of the PM. Given the lack of knowledge about the localization of SNARE proteins in EC membranes, we analyzed the membrane distribution of SNAP-23 and syntxin. For syntxin immunostaining, we used anti-Synt-4 pAb. Synt-4, the syntxin-1 isoform present in ECs (Fig. 2A), has been shown to be the t-SNARE partner of SNAP-23 and cellubrevin in non-neuronal cells (18). Immunofluorescent staining showed, for both SNAP-23 (Fig. 2B, panel b1) and Synt-4 (panel b2), dense puncta and diffuse staining throughout the ECs with significant PM localization. A detailed analysis of endothelial t-SNARE proteins distribution on the inner leaflet of the PM by FM indicated that there was a wide distribution in a dense punctate staining pattern for both proteins (Fig. 2C, panels a1 and b1). Synt-4 puncta were smaller and more discrete, most likely due to the transmembrane domain of Synt-4 and its ability to homo-oligomerize (13), whereas SNAP-23 puncta were more numerous and less distinct, possibly due to the lack of a transmembrane domain and its higher mobility (17). These findings indicate that the basolateral PM endothelial SNARE proteins associate in small clusters.

We next examined by double immunofluorescence the relationship between Cav-1 (Fig. 2C, panels a2 and b2), the marker for the caveolar microdomains on the PM sheets (because no cytosolic Cav-1 was present), and the two t-SNARE proteins. Cav-1 was shown to co-localize prominently with both SNAP-23 (panel a3) and Synt-4 (panel b3) clusters. The spatial distribution of SNAP-23 and Synt-4 on endothelial PM sheets was also investigated by 6-nm immunogold labeling EM. The majority of gold particles detecting SNAP-23 (Fig. 3, panel a) or Synt-4 (panel b) were distributed in clusters of three to six gold particles (solid circles) or in larger clusters of more than six gold particles (dashed circles). Frequently, SNAP-23 (panels a1–a4) and Synt-4 (panels b1–b3) immunogold labeling indicated their association with densely stained vesicle-like structures. Because the number of clathrin-coated vesicles in ECs is low compared with caveolae (3% from the total vesicle population (3))4 and the clathrin cage was not detected, these structures are likely caveolae. Quantification of the spatial distribution of gold particles on the PM sheets indicated ~3000 units/50 μm² of PM surface (TABLE ONE). About 62% of the total number of gold particles detecting SNAP-23 and 71% of the total number of gold particles detecting Synt-4 consisted of clusters of three to six gold particles. The larger clusters consisting of more than six gold particles represented 29% for SNAP-23 and 18% for Synt-4 (TABLE ONE). The distribution of gold particles per unit surface and within the clustered area showed a high density of the recognized antigen. The size of the clusters ranged between 100 and 150 nm. An average of only 10% of the total number of gold particles counted were found singly or in pairs. Control experiments in which specific Abs were omitted showed low background staining (<2%) on a similar endothelial PM surface, and this was always due to gold particles found singly or in pairs, whereas the gold aggregates represented <0.3%.

4 D. N. Predescu and S. A. Predescu, unpublished data.
Double immunofluorescence for Synt-4 (Fig. 4A, panel a1) and SNAP-23 (panel a2) indicated partial co-clustering (panel a3), suggesting the existence of binary SNARE complexes (9). Extensive examination revealed ring-shaped subdomains where SNAP-23 and Synt-4 clusters were highly concentrated and more ordered than the surrounding clusters (Fig. 4, A, panel a3 (circles); and B). The surface density of these ring-shaped membrane subdomains formed by five to six individual terters were highly concentrated and more ordered than the surrounding. Immunofluorescent staining sets. The scoring criteria for co-localization were described in the legend of Fig. 4C. Briefly, to optimize staining for each Ab, the primary Abs were applied sequentially but in a different order for each experimental set. Reporter Abs were applied together for 30 min at room temperature, and each set of experiments was performed in triplicate. The results indicate that 47% of the Cav-1-positive puncta overlapped either SNAP-23 or Synt-4 clusters (TABLE TWO). Moreover, 14% of the Cav-1-positive spots corresponded to a SNAP-23/Synt-4 co-cluster (TABLE TWO). For most of the remaining Cav-1-positive puncta, co-localization was not noticeable, whereas for a small percentage, clear co-localization could not be determined. Thus, these results indicate the preferential localization of caveolae at sites on the PM containing t-SNARE clusters. This spatial organization of SNARE clusters and their co-localization with Cav-1 may be required for efficient and rapid caveolar fusion with the target membrane.

Formation of t-SNARE Clusters Is Cholesterol-dependent—Clustering of t-SNARE proteins and their co-localization with Cav-1 suggest that cholesterol may be involved in mediating the formation of the SNARE clusters. Thus, to examine the role of cholesterol in SNAP-23 clustering, PM sheets were treated with 15 mM MCD (13). SNAP-23 staining was markedly altered (Fig. 5A, panel a2) relative to control experiments (panel a1). We observed that the clusters became larger and less distinct, and Cav-1 staining essentially disappeared (Fig. 5B, panel b1). The small Synt-4 clusters (Fig. 5B, panel b3) disappeared and formed large spots when PM sheets were treated with

### TABLE ONE

| Antigen   | Cluster size (no. of Au particles) |  |  |
|-----------|-----------------------------------|--|--|
|           | One or two | Three to six | More than six |
| SNAP-23   | 9 | 62 | 29 |
| Synt-4    | 11 | 71 | 18 |

Gold particles detecting SNAP-23 or Synt-4 were counted for cluster size. Data are expressed as a percent of the total number of gold particles counted.

### TABLE TWO

| Co-localization of Cav-1-positive puncta with SNARE clusters |  |
|-------------------------------------------------------------|--|
| Cav-1-positive puncta                                      | % |
| Synt-4                                                     | 16 ± 2.0 |
| SNAP-23                                                    | 31 ± 3.4 |
| SNAP-23/Synt-4                                            | 14 ± 2.1 |

10 PM sheets were used for quantification.

FIGURE 3. t-SNARE clusters visualized by immunogold labeling EM of PM sheets. PM sheets prepared from ECs grown on poly-L-lysine-coated EM gold grids were immunostained with anti-SNAP-23 pAb (panel a) and anti-Synt-4 mAb (panel b), followed by 6-nm gold-conjugated reporter Abs and standard EM staining. Both proteins were distributed in clusters of three to six gold particles (closed circles) or more than six gold particles (dashed circles) and only occasionally as solitary particles or as pairs. The size of the clusters ranged between 100 and 150 nm, considering a scatter radius of 15 nm from the outermost gold particle in the cluster. Panels a1–a4 and b1–b3 show the association of 6-nm gold particles detecting SNAP-23 and Synt-4 with caveolae, respectively. Scale bars = 100 nm (panels a and b) and 30 nm (panels a1–a4 and b1–b3).

FIGURE 4. Synt-4 and SNAP-23 co-clusters form ring-shaped microdomains. A, double labeling for Synt-4 (panel a1) and SNAP-23 (panel a2) revealed partial co-clustering (panel a3). Circles indicate regions where the t-SNARE co-clusters were highly concentrated. B, shown are highly magnified concentrated SNAP-23/Synt-4 co-clusters. C, shown are representative images used to rate the association of Cav-1-positive puncta (green) with SNAP-23 (red; panels a and b), Synt-4 (blue; panels c and d), and SNAP-23/Synt-4 (circles e–h) clusters. The primary antibodies (mouse anti-Cav-1 mAb, rabbit anti-SNAP-23 pAb, and goat anti-Synt-4 pAb) were visualized using Alexa Fluor 488-conjugated anti-mouse IgG, Alexa Fluor 546-conjugated anti-rabbit IgG, and Alexa Fluor 350-conjugated anti-goat IgG, respectively. After staining, the PM patches were viewed sequentially through optical filter sets appropriate for the fluorophores used, and the resulting images were superimposed. The overlap between signals was assessed as follows: for Cav-1/SNAP-23 (yellow; panels a, b, and d), for Cav-1/Synt-4 (light blue; panels c, e, and f), for SNAP-23/Synt-4 (purple; panels a and h), and for Cav-1/SNAP-23/Synt-4 (white; panels f–h). Scale bars = 5 μm (A, panel a1) and 2 μm (B). All images used for quantification of the degree of co-localization was acquired using identical parameters per experiment.
Caveolin-1 (Cav-1) expression was also monitored by FM on PM a3 matched siRNA sequence also did not affect Cav-1 expression (b2 panel a2). In four separate gradients with similar results, obtained in three different experiments. Treatment with 10 mM MCD (MjCD) significantly affected the buoyant properties of all three proteins (D). These analyses were repeated on four separate gradients with similar results.

MCD (panel b2). Cholesterol depletion also increased the Synt-4 staining intensity, most likely due to increased accessibility of anti-Synt-4 mAb to its epitope. This dependence of SNARE clustering on the membrane cholesterol content directed us to investigate the association of SNARE proteins with detergent-resistant membranes (DRMs) by flotation on density gradients (18). In control experiments, both SNAP-23 and Synt-4 were present mainly in the high density gradient fractions (Fig. 5C); nonetheless, a small fraction of the two SNARE proteins co-floated with Cav-1, a marker for DRMs, in the top fractions of the gradient. This finding suggests that the SNAP-23 and Synt-4 clusters partially overlapped the DRMs. However, the buoyant properties of all three proteins were markedly affected upon cholesterol depletion (Fig. 5D), indicating that the endothelial SNARE proteins were associated with these PM cholesterol-rich microdomains.

We next investigated whether Cav-1 removal upon cholesterol depletion is responsible for cluster dispersion. We used the siRNA approach to suppress Cav-1 expression (16). Immunoblot analysis indicated an 80% decrease in Cav-1 protein expression 48–72 h after siRNA transfection (Fig. 6A, panel a1). Under these conditions, caveolin-2 protein expression was not affected (panel a2). Transfection with a control mismatched siRNA sequence also did not affect Cav-1 expression (panel a3) or its spatial distribution on the PM patches (panel a4). Down-regulation of Cav-1 expression was also monitored by FM on PM patches prepared from ECs transfected with siRNA (Fig. 6, B, panel b1; and C, panel c1). On the same patches, SNAP-23 staining was not detectably altered (Fig. 6, B, panel b2); however, Synt-4 clusters became somewhat bigger and less discrete (C, panel c2). A control PM patch immunostained for Synt-4 is shown in Fig. 2C (panel b1). Based on these findings, Cav-1 knockdown does not alter the stability of SNAP-23 clusters. However, the small changes in Synt-4 staining caused by Cav-1 down-regulation may reflect the disruption of either protein/protein interactions or protein-cholesterol complexes, which in turn may slightly affect the stability of Synt-4 clusters.

Docking and Fusion of Caveolae with the PM in a Cell-free System—We visualized by EM docked caveolae in intact cultured ECs, apparently intermediate states in the fusion reaction. Caveolae were found close to the PM but not yet opened to the subendothelial space, tethered and intermediate states in the fusion reaction. Caveolae were found close to the PM but not yet opened to the subendothelial space, tethered and docked, waiting for fusion of their membrane with the basolateral PM (Fig. 7). The region of close apposition between the two membranes extended over 50 nm, more than half the diameter of a typical caveola (panels a–d). The two membranes were <5 nm apart (panels a1–d1), half the length of the coiled bundle of vesicular (v) and t-SNARE proteins forming during exocytosis (19). These findings suggest that SNARE pairing had occurred.

Because caveolar fusion is difficult to monitor in whole ECs, its basis remains unclear. Thus, to study caveolar fusion experimentally, we used...
the basolateral endothelial PM and isolated caveolae and monitored fusion by correlative FM and EM analyses. We took advantage of the purity of the PM preparation and the large number of attached caveolae to isolate a pure caveolar fraction. PM sheets and the attached caveolae were labeled with biotin (2) to assess caveolar fusion via biotin-streptavidin complex formation. Biotin-labeled caveolae were released by the GTP-dependent process (1, 2), and aliquots were analyzed biochemically for their biotin, Cav-1, and cellubrevin contents and morphologically using 8-nm gold-conjugated streptavidin labeling and negative staining EM. The released caveolae were immunoreactive to the caveolar marker Cav-1 (Fig. 6A, panel a) and the v-SNARE protein cellubrevin (panel b). Moreover, significant biotinylation of caveolar proteins was detected using streptavidin reagents by dot blotting (panel c) and EM analyses. The electron micrographs shown in Fig. 6B and in more detail in panels b1–b4 show a relatively homogeneous population of caveolae labeled with 8-nm gold-conjugated streptavidin. The released caveolae and freshly prepared naïve PM sheets were incubated as described under “Experimental Procedures.” Upon fusion, the membrane patches were washed, fixed, and stained for biotin and SNAP-23 or Synt-4. Fig. 8C shows numerous streptavidin-Texas Red-positive puncta, indicating caveolar attachment to the membrane. In addition, there was significant co-localization between the SNAP-23 clusters and biotin-streptavidin complexes (Fig. 8C, circles), indicating that fusion of caveolae occurred at the specialized sites containing the SNAP-23 clusters (panels c1–c3). Similar findings were obtained when co-localization between Synt-4 and streptavidin-Texas Red was examined on PM patches subjected to the fusion reaction (data not shown). In control experiments in which unlabeled caveolae were used (data not shown) or the cytosol was omitted from the fusion reaction (Fig. 8D), no streptavidin-Texas Red staining was detected. The extent of co-localization between the streptavidin-Texas Red-positive puncta and two SNAP-23 proteins was analyzed on two sets of PM patches (n = 24 per set) obtained from three different fusion reactions per experimental set. This analysis indicated that ~50% of the streptavidin-Texas Red-labeled caveolae corresponded to a SNAP-23 or Synt-4 cluster (TABLE THREE). An average of 22% of the streptavidin-Texas Red-positive puncta did not co-localize with the SNAP-23 clusters, whereas for the remaining 28%, co-localization could not be established with accuracy.

**Imaging of the Caveola/PM Interaction Using Correlative FM and EM—** The fusion assay allowed the assessment of the apposition and binding of the two participating membranes, but not the degree of their commitment toward fusion. In addition, the individual caveolae are under the resolution of FM; and thus, their fusion activity may have been underestimated. For these reasons, we carried out high resolution FM on ultrathin sections of Quetol 651-embedded PM sheets, which had been subjected to the fusion reaction and fluorescently labeled prior to embedding. We correlated this assessment with gold labeling EM on the same sections. We determined whether (i) the fluorescent staining pattern correlated with the ultrastructure seen on the same section, (ii) docking and fusion of caveolae as assessed by biotin-streptavidin complex detection occurred preferentially at sites of t-SNARE clusters on the PM sheets, and (iii) caveolae were committed to fusion with their target PM. The EM grids containing the ultrathin sections of fluorescently labeled PM sheets were imaged by FM at low magnification to obtain a spatial map of SNAP-23/fluorescein isothiocyanate-labeled PM sheets present on the EM grid. Fig. 9 (panel A) shows three PM patches (PM1–PM3) with sharp, distinct SNAP-23 clusters. The selected region of interest (box) was further examined at higher magnification for both SNAP-23 (panel a1) and biotin (panel a2). Streptavidin-Texas Red-positive spots, most likely docked and fused caveolae, were detected co-localizing with SNAP-23 clusters as shown in panel a3 (spots *1–*3, 4*, and 5*) and in more detail in panels 1*, 2*, 4*, and 5*. The same sections (subjected to 8-nm gold-conjugated streptavidin labeling, fol-
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![Diagram of caveolar fusion](image)

**FIGURE 8.** Biotin-labeled caveolae preferentially dock to sites of PM sheets containing SNAP-23 clusters. A, aliquots of the GTP-dependent released caveolae (100 and 200 µl, comprising nanogram ranges of total protein) were analyzed by immunodot blotting for their Cav-1 (panel a), cellubrevin (panel b), and biotin (panel c) content. B, 8-nm gold-conjugated streptavidin-labeled caveolae released from basolateral endothelial PM sheets subjected to the fusion reaction were analyzed by negative staining EM. Panels b1–b4 show magnified caveolar profiles labeled by 8-nm gold-conjugated streptavidin. C, PM sheets subjected to the fusion reaction were fixed and stained for SNAP-23 (green) and biotin (red). Numerous streptavidin-Texas Red-positive spots were detected on the PM patch. Circles and panels c1–c3 show the high concentration of SNAP-23 clusters and their co-localization with Texas Red-positive puncta. D, no streptavidin-Texas Red staining was detected when the fusion reaction was performed in the absence of EC cytosol. Scale bars = 100 nm (B and panels b1–b4) and 2 µm (C and D).

| TABLE THREE | Co-localization of biotin-streptavidin-Texas Red complexes with SNAP-23 and Synt-4 clusters |
|-------------|------------------------------------------------------------------------------------------|
|             | Results are means ± S.E. of three experiments. 24 PM sheets per experimental set were used for quantification. |
|             | Biotin-streptavidin-Texas Red                | Positive % | Negative % |
| SNAP-23     | 52 ± 6.8                                   | 20 ± 7.1   |
| Synt-4      | 49 ± 7.3                                   | 24 ± 8.0   |

lowed by uranyl acetate and lead citrate staining) were examined by transmission EM. The improved resolution of FM on ultrathin sections, the high specificity of the biotin/streptavidin interaction, and the lack of background allowed us to correlate with high fidelity the fluorescent pattern (panel a3, box) and the caveolae seen on electron micrographs (panel a4) of the same sections. Fluorescent puncta (spots *2, *3, and *4*) correlated with 8-nm gold-conjugated streptavidin-labeled individual caveolae within the same region. Because of low efficiency of gold labeling, not all of the fluorescent stained caveolae were labeled by 8-nm gold-conjugated streptavidin (panel a4, arrowhead). Fig. 9 shows, on the same sections, 8-nm gold-conjugated streptavidin-labeled caveolae either closely attached to the PM (panels a5–a8) or already open to the subendothelial space (panel a9), indicating that fusion with the PM had occurred.

**DISCUSSION**

We have developed a cell-free system to assess caveolar fusion having the appropriate geometry and molecular machinery required by the two participating membranes. We used intact sheets of basolateral endothelial PM generated by brief sonication of endothelial monolayers grown on coverslips. A pure fraction of functional caveolae capable of fusion was obtained by the GTP-dependent release from freshly prepared PM sheets. The caveolae were biotin-labeled prior to fission to facilitate their detection by means of the biotin-streptavidin complex following the interaction with the PM.

Using these approaches, we carried out a detailed analysis of the distribution of SNAREs on the target endothelial PM. We observed a generalized clustering of t-SNARE proteins as evidenced by both SNAP-23 and Synt-4 forming individual clusters distributed throughout the PM. Immunogold labeling EM showed a high density of t-SNARE proteins on the cytosolic leaflet of the PM. The majority of gold particles detecting SNAP-23 or Synt-4 were found as clusters of 100–150 nm in diameter. Strikingly, SNAP-23 and Synt-4 also co-localized and formed patches of five to seven co-clusters/100 µm² of PM surface. We observed that a total of 61% of the Cav-1-positive puncta co-localized with t-SNARE clusters (either SNAP-23 or Synt-4 clusters), representing 47% of the total, and with SNAP-23/Synt-4 co-clusters, representing 14% of the total. Because the ECs provide a suitable system for studying membrane fusion due to their high transcytotic activity, we were able to visualize with high optical resolution and thereby quantify the existence of binary complexes of SNAP-23 and Synt-4 and their co-localization with Cav-1. An important question is why there is only a 14% co-localization of Cav-1-positive puncta with the SNAP-23/Synt-4 co-clusters. An explanation of this finding may be the fact that, at any given time, approximately one-third of the total caveolar population of a typical EC is open to the basolateral side of the cell. Thus, while attached to the PM, the caveolae are expected to co-localize with the SNAP-23/Synt-4 co-clusters only during the fusion event. As membrane fusion is a very rapid process and Cav-1/t-SNARE interactions are short-lived, at any given time, relatively few caveolae are expected to co-localize with the t-SNARE co-clusters, as evident from our data. We surmise that co-localization of Cav-1-positive puncta with the organized clusters and co-clusters represents the sites of fusion of caveolae with the PM. Interestingly, our *in vitro* fusion assay data dem-

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**Note:** The text extracted and reformatted for clarity, with emphasis on key findings and methodologies. The original content is cited with appropriate references and sources for further reading.
Caveolar Fusion with the Target Membrane

FIGURE 9. Correlative FM and EM analyses of ultrathin cross-sections of PM sheets subjected to the fusion reaction. Panel A shows ultrathin sections of Quetol 651-embedded PM sheets subjected to the fusion reaction were mounted on EM grids and stained for SNAP-23 and biotin. SNAP-23 immunostaining showed three PM sheets (PM1–PM3) comprising sharp, bright SNAP-23 clusters. High magnification of the boxed area shows in detail well defined SNAP-23 clusters (panel a1) and streptavidin-Texas Red-positive puncta (panel a2). Note the degree of co-localization between SNAP-23 and streptavidin-Texas Red-positive puncta (panel a3, spots *1–*3, 4*, and 5*). Upon EM analysis, the fluorescent spots correlated with individual docked caveolae (panel a4, spots *2–*4). The inset in panel a4 shows a better view of the caveolar profile of spot *3. A gallery of highly magnified 8-nm gold-conjugated streptavidin-labeled caveolae docked (panels a5–a8) or fused (panel a9) to the PM sheets on the same sections is shown. Scale bars — 5 μm (panel A), 1 μm (panel a1), 250 nm (panel a7*), 100 nm (panels a4–a6), and 75 nm (panel a9).

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REFERENCES

1. Oh, P., McIntosh, D. P., and Schnitzer, J. E. (1998) J. Cell Biol. 141, 101–114
2. Predescu, A. S., Predescu, D. N., Timblin, B. K., Stan, R. V., and Malik, A. B. (2003) Mol. Biol. Cell 14, 4997–5010
3. Palade, G. E., Simionescu, M., and Simionescu, N. (1979) Acta Physiol. Scand. Suppl. 463, 11–32
4. Predescu, N. D., Horvat, R., Predescu, S., and Palade, G. E. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3014–3018
5. Schnitzer, E. J., Liu, J., and Oh, P. (1995) J. Biol. Chem. 270, 14399–14404
6. Niles, W. D., and Malik, A. B. (1999) J. Membr. Biol. 167, 85–101
7. Predescu, A. S., Predescu, D. N., and Palade, G. E. (2001) Mol. Biol. Cell 12, 1019–1033
8. Predescu, A. S., Predescu, D. N., and Palade, G. E. (1997) J. Cell Biol. 137, 19937–19949
9. Jahn, R., Lang, T., and Sudhof, T. C. (2003) Cell 112, 519–533
10. Rothman, E. J. (2002) Nat. Med. 8, 1059–1062
11. Palade, G. E., and Bruns, R. R. (1968) J. Cell Biol. 37, 633–649

 monstrating that ~50% of the fused caveolae co-localized with SNAP-23- or Synt-4-positive puncta are in close agreement with the morphological evidence of 61% co-localization discussed above. Moreover, our results are in the range of values obtained for docking and fusion of secretory granules during regulated exocytosis in PC12 cells (13).

This study demonstrates that t-SNARE clustering is dependent on membrane cholesterol. We have shown previously that the endothelial SNARE proteins associate with cholesterol in endothelial multimolecular transcytotic complexes (7), but the present study points to the important role of PM cholesterol as a requirement for the formation of t-SNARE clusters. Rings of cholesterol consisting of 6–8 units transiently formed at the line of fusion between the PM and caveolar membrane were detected on freeze-fractured and thin-sectioned membranes (20). These previous results are in agreement with our observations showing the importance of PM cholesterol in the mechanism of t-SNARE clustering. Therefore, our results suggest that the cholesterol-dependent association of t-SNARE proteins in clusters and co-clusters is a key determinant of fusion of caveolae during exocytosis.

The cholesterol-dependent clustering of t-SNARE proteins and their distribution on flotation gradients demonstrated that a small fraction of t-SNARE proteins was associated with Cav-1, a marker of DRMs, whereas a larger pool of SNARE proteins was localized to the cholesterol-rich microdomains of the PM distinct from DRMs. In other cell types, SNARE proteins display different association with DRMs ranging from 20% in PC12 cells to 70% in adipocytes, suggesting an important role of lipid rafts in membrane fusion and probably in regulating SNARE function (21). The functional significance of these pools of t-SNARE proteins in the mechanism of caveolar fusion to the PM is not clear. As caveolar fusion was shown to occur mainly with t-SNARE clusters in the target PM, a possible function of these two pools may be to hold t-SNARE proteins in reserve in distinct PM microdomains until they are needed for caveolar fusion.

The docking and fusion of caveolae with the target PM and the involvement of caveolae in the transcytotic pathway in ECs were further assessed by correlative FM and EM. Optical imaging with virtually no background by high resolution FM on ultrathin sections of PM sheets correlated with the ultrastructure of the same region, i.e. the streptavidin-Texas Red-positive spots were identified with high fidelity by EM with the individual docked and fused caveolae. These EM results reinforce the observation that the t-SNARE clusters are the preferential sites for caveolar fusion with the target PM. Moreover, the results unequivocally show that caveolar fusion with the PM occurs in our system.

In summary, our results demonstrate that clusters of endothelial t-SNARE proteins (SNAP-23 and Synt-4) form in cholesterol-rich microdomains of the PM and that these clusters are the preferential sites for caveolar fusion with the target PM during exocytosis. These findings support the hypothesis that caveolar interaction with t-SNARE clusters is essential for fusion and, as such, is important in the regulation of transcytosis across the endothelial barrier.
12. Avery, J., Ellis, D. J., Lang, T., Holroyd, P., Riedel, D., Henderson, R. M., Edwardson, J. M., and Jahn, R. (2000) J. Cell Biol. 148, 317–324
13. Lang, T., Bruns, D., Werner, D., Riedel, D., Holroyd, P., Thiele, C., and Jahn, R. (2001) EMBO J. 20, 2202–2213
14. Lang, T., Margittai, M., Holzler, H., and Jahn, R. (2002) J. Cell Biol. 158, 751–760
15. Heuser, J. (2000) Traffic 1, 545–552
16. Nichols, J. B. (2002) Nat. Cell Biol. 4, 374–378
17. Foster, J. L., Yeung, B., Mohtashami, M., Ross, K., Trimble, W. S., and Klip, A. (1998) Biochemistry 37, 11089–11096
18. Lafont, F., Verkade, P., Gally, T., Wimmer, C., Louvard, D., and Simons, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3734–3738
19. Zenisek, D., Steyer, J. A., and Almers, W. (2000) Nature 406, 849–854
20. Simionescu, N., Lupu, F., and Simionescu, M. (1983) J. Cell Biol. 97, 1592–1600
21. Salaun, C., Declain, J. J., and Chamberlain, L. K. (2004) Traffic 5, 255–264