Separation of Caveolae from Associated Microdomains of GPI-anchored Proteins

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In situ coating of the surface of endothelial cells in rat lung with cationic colloidal silica particles was used to separate caveolae from detergent-insoluble membranes rich in glycosyl phosphatidylinositol (GPI)-anchored proteins but devoid of caveolin. Immunogold electron microscopy showed that ganglioside G_{M_{1}}-enriched caveolae associated with an annular plasmalemmal domain enriched in GPI-anchored proteins. The purified caveolae contained molecular components required for regulated transport, including various lipid-anchored signaling molecules. Such specialized distinct microdomains may exist separately or together in the plasma membrane to organize signaling molecules and to process surface-bound ligands differentially.

Cholesterol and glycolipids self-associate in lipid bilayers to form organized compositionally microdomains (1). GPI-anchored and other lipid-linked proteins may preferentially partition into glycolipid microdomains that are resistant to nonionic detergent solubilization (2–5). GPI-anchored proteins appear to be sorted into glycolipid, detergent-resistant “rafts” in the trans-Golgi network for polarized delivery to the cell surface by caveolin-rich smooth exocytic carrier vesicles (3, 5–8). On the cell surface, they are thought to reside in smooth membrane invaginations known as caveolae (9, 10), which are apparently also rich in glycolipids, cholesterol, and caveolin (7, 11–14). Antibody cross-linking of cell surface glycolipids (1) and GPI-linked proteins (15) can increase sequestration into clusters and induce cell activation (1, 16), apparently through lipid-anchored nonreceptor tyrosine kinases (NRTKs) (17). Caveolae have been implicated not only in signaling but also in transport via endocytosis, transcytosis, and potassium (14, 18–21). The physiological function of and interaction between caveolae, detergent-resistant microdomains, and various lipid-anchored molecules remain undefined.

To explore the relation between GPI-anchored proteins and caveolae under conditions that avoid potential influences of antibody effectors, cell culture (22), and contamination from intracellular compartments, we purified the plasma membranes of rat lung endothelial cells and then subfractionated them into specific microdomains. The rat lung vasculature was perfused in situ at 10° to 13°C with a suspension of cationic colloidal silica particles, which coated the luminal endothelial cell plasma membranes normally exposed to the circulating blood. This coating created a stable pellicle that specifically marked this membrane and enhanced its density, which allowed its purification from tissue homogenates by centrifugation (13, 23). The silica-coated membrane pellets (P) were enriched in endothelial cell surface markers, with little contamination from other tissue components (13, 23).

Caveolae attached to the cytoplasmic side of the plasma membranes, opposite to the silica coating, were stripped from these membranes by shearing during homogenization at 4°C in the presence of Triton X-100. They were then isolated by sucrose density gradient centrifugation to yield a homogeneous population of biochemically and morphologically distinct caveolar vesicles (Figs. 1 and 2). As with caveolae present on the endothelial cell surface in vivo (7, 12, 24), these purified caveolae (V) were enriched in caveolin, plasmalemmal Ca^{2+}- dependent adenosine triphosphatase, and the inositol 1,4,5-trisphosphate receptor (13). In contrast, other markers present amply in P, including angiostatin-converting enzyme, band 4.1, and β-actin, were almost totally excluded from V.

The purified caveolae were not rich in GPI-anchored proteins. First, detergent extraction studies performed on P revealed differences in the ability of various detergents to solubilize caveolin and 5'-nucleotidase (5'-NT). Caveolin was partially solubilized by β-octyl glucoside, CHAPS, deoxycholate, NP-40, and SDS (but not Triton X-100), whereas 5'-NT was rendered soluble only by SDS and deoxycholate (Fig. 1A). Second, like caveolin, 5'-NT and uridineplasmigen activator receptor (uPAR) were enriched in P relative to the starting rat lung homogenate (H) (Fig. 1C). However, unlike caveolin, these proteins were not enriched in V; they remained almost totally associated with the resedimented silica-coated membranes stripped of the caveolae (P–V) [which contain few, if any, remaining caveolin (13)]. More than 95% of the signal for caveolin was detected in V, with <4% remaining in P–V. Conversely, >95% of 5'-NT and uPAR remained in P–V, with <3% present in V. Thus, these GPI-anchored proteins were neither cou-

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plied to caveolin nor concentrated in the isolated caveolin-enriched caveolae.

Other methodologies not based on the silica-coating technique have been developed for isolating low density, detergent-resistant membranes from a variety of cultured cells and tissues (3–5, 25, 26). By performing similar isolations with rat lung tissue as in (26), we showed, consistent with previous studies (5, 25, 26), that caveolin and GPI-anchored proteins (in this instance, 5’-NT) were both present in the isolated Triton X-100–insoluble membranes (TI) (Fig. 1B). In addition, differential detergent extraction studies were performed on TI. As expected (3–5), GPI-anchored proteins were solubilized effectively by β-octyl glucoside, CHAPS, deoxycholate, and SDS (27), different from the pattern of solubility for 5’-NT in P but similar to that for caeinol in P.

There has been a tendency to equate low density, Triton-insoluble membranes with caveolae (5, 25, 26). Electron microscopy of the V and TI membrane preparations revealed that V comprised a relatively homogeneous population of vesicles (≤100 nm) with the typical morphology of caveolae, whereas TI contained caveolae, linear membrane sheets, and many larger vesicles (≥150 nm) (Fig. 2). In many favorable cross sections, a characteristic flask-shaped caveola attached to a larger, spherical vesicle was apparent, suggesting that these two detergent-resistant membrane domains were associated with each other as a unit before fractionation in the membrane (Fig. 2, D and E).

Because the larger noncaveolar vesicles in TI contained the GPI-linked proteins (Fig. 3), it appeared that silica coating of the outer membrane surface altered the way in which the GPI-anchored proteins interacted with various detergents and thus prevented the separation of noncaveolar, detergent-resistant microdomains from the cell membranes. Cationic silica particles interact with the anionic cell surface to stabilize it against vesiculation or lateral rearrangement by immobilizing membrane molecules (28). Because the silica particles uniformly coated the cell surface but were rarely associated with or present inside the caveolae because of their size, it is likely (13, 23) that the plasma membrane was stabilized by being firmly attached on one side to most, if not all, nonvesiculated regions. This adherent pellicle would allow the caveolae on the opposite side of the membrane to be sheared away by homogenization, with little contamination from other membranes, including other detergent-resistant domains. Conversely, without silica coating, both caveolar and noncaveolar detergent-resistant membranes would be coisolated. In addition, without isolating the plasma membrane first, such preparations of cells and tissues would also contain intracellular detergent-insoluble caveolin-rich domains, such as those present in the trans-Golgi network (3, 7).

As a test, we increased the salt concentration during the isolation procedure, which reduced electrostatic interactions sufficiently to detach the plasma membrane from the silica pellicle in P and to allow the coisolation of caveolae with GPI-linked protein microdomains (29). More importantly, because the silica coating did indeed prevent the release of the detergent-insoluble membranes rich in GPI-anchored proteins, it was possible to isolate these domains separately from the caveolae. We incubated the silica-coated membranes already stripped of caveolae (P−V) with 2 M K2HPO4, followed by homogenization in Triton X-100 at 4°C. This procedure allowed the isolation by sucrose density centrifugation of a membrane fraction (G) that contained vesicles of >150 nm in diameter with no apparent caveolae (27). G lacked caveolin but was enriched in several GPI-anchored proteins: 5’-NT, uPAR, and carbonic anhydrase (CA) (Fig. 1D). Little of these proteins remained behind in the resedimented membrane pellet (RP). The G/RP ratio ranged from ~3 for 5’-NT and uPAR to 12 for CA. Thus, distinct deter-

![Fig. 1. Protein analysis of various membrane subfractions. (A) Differential detergent extraction performed on silica-coated endothelial cell membranes (P). Equal portions of resuspended P were incubated with rotation for 1 hour at 4°C with various detergents (β-OG, β-octylglucoside; Deoxy, sodium deoxycholate; TX-100, Triton X-100) before centrifugation at 13,000g for 2 hours. The soluble proteins (S) and the sedimented, insoluble proteins (I) were fractionated by SDS–polyacrylamide gel electrophoresis (10 μg per lane), transferred to nitrocellulose or Immobilon (Millipore) filters, and subjected to immunoblot analysis with equivalent amounts of specific antibodies for the indicated proteins and the appropriate 125I-labeled secondary antibodies as described (13, 20). Other proteins tested included angiotensin-converting enzyme, which was solubilized by all of these detergents, and carbonic anhydrase, which was solubilized similarly to 5’-NT (27). (B) Coisolation of caveolin and 5’-NT in detergent-resistant membranes derived without silica coating. Proteins from rat lung homogenate (H), the Triton X-100–insoluble membranes isolated by sucrose density gradient centrifugation (TI), and the sedimented pellet (R) were subjected to immunoblot analysis as in (A), with the exception that the secondary antibodies were conjugated to horseradish peroxidase (HRP) and binding was detected with ECL chemiluminescent substrate (Amersham). (C) Lack of GPI-anchored proteins in the purified caveolae enriched in caveolin and ganglioside GM1~. Whole-lung homogenate (H), silica-coated luminal endothelial membranes (P), purified caveolae (V), and the resedimented silica-coated membranes after stripping of the caveolae (P−V) were subjected to immunoblot analysis as in (B). GM1~, was detected not only by immunoblotting but also by direct blotting with HRP-conjugated cholera toxin. Ratios of the signals detected in V versus P−V are shown. (D) Separate isolation of the GPI-anchored protein microdomain from the silica-coated membranes. Immunoblot analysis was performed as in (B) with P−V, G [the detergent-resistant membranes derived from P−V after detecting the membrane from the silica (46)], and RP (the resedimented pellet of silica-containing material). The caveolin in P−V is equivalent to that seen in (C), representing the small residual signal after stripping of the caveolae (compare V and P−V), except that the exposure here is much longer. GM1~, could not be detected in P−V [see (C)] nor, as expected, in G or RP (27). G is rich in GPI-linked proteins (5’-NT, uPAR, and CA) but lacks caveolin and GM1~. Control experiments performed identically but without high salt did not yield any detectable membranes in the sucrose gradient (27). (E) Immunoblot analysis of caveolae isolated without Triton X-100. Caveolae were purified without any exposure to detergent (34). These caveolae (V’) and the membrane stripped of them (P’−V’) were subjected to immunoblot analysis as in (B).
Detergent-resistant plasma membranes rich in GPI-anchored proteins but lacking caveolin could be isolated separately from the caveola. Similar detergent-resistant membranes, consisting of large vesicles rich in GPI-anchored proteins but devoid of caveolin, have also been isolated from lymphocytes and neuroblastoma cells, both of which lack caveola and do not express caveolin (30).

Although several studies that have examined the immunolocalization of GPI-anchored proteins in cultured cells have concluded that these proteins reside in caveola (9), reexamination of the published electron micrographs reveals little gold labeling directly inside the caveola. Almost all of this labeling is actually adjacent to the caveola on the flat plasma membrane directly attached to, but not a part of, the neck of the caveola. The small amount of labeling apparent inside the caveola and the extent of clustering observed may be induced artifactually by antibody cross-linking (15, 31). In contrast, another lipid-anchored molecule, the cholera toxin–binding ganglioside $G_{M1}$, has been localized with gold labeling inside the caveola (11, 14). The fractions isolated directly from the silica-coated membranes mimic these observations, with $V$ containing >90% of $G_{M1}$ (Fig. 1C). The remaining membrane devoid of caveola lacked detectable $G_{M1}$, although it was rich in GPI-anchored proteins. Hence, $G_{M1}$ was used as a caveolar marker.

The amounts of GPI-anchored proteins that partition into microdomains remain unclear, partially because antibody cross-linking can increase the clustering of GPI-linked proteins from ≤10 to >90%; again, gold immunolabeling is not in the caveola but on the adjacent annular membrane (15, 31). Other studies that have examined membrane diffusion by fluorescence recovery after photobleaching have detected a larger fraction of GPI-anchored proteins (20 to 60%) present in an immobile fraction (8, 32). By examination of detergent solubility, we have detected similar percentages for the GPI-linked proteins in the detergent-resistant microdomains, suggesting

![Fig. 2.](image-url) Electron microscopy of the vesicles (V) purified from the silica-coated rat lung endothelial membranes (A) and the detergent-resistant membranes (T) isolated without silica coating (B to E). Electron microscopy was performed on membrane isolates as described (13). Typical low- and high-power fields are shown. (A) Membranes of the V isolate, showing a homogeneous population of small vesicles with typical caveolar morphology (13). Despite the isolation procedure, many caveola retained their characteristic flask shape. (B to E) Membranes in fraction T1, consisting of many larger vesicles (>150 and <700 nm in diameter) interspersed with smaller caveolar vesicles (<100 nm) and some nonvesiculated, linear membrane sheets. A typical caveola was often apparent attached to the inside of a larger vesicle (arrowheads). Bars, 500 nm (A to D) and 300 nm (E).

![Fig. 3.](image-url) Colloidal gold localization of CA to large vesicles and $G_{M1}$ to caveola. Detergent-resistant membrane isolates (T) were embedded in agarose for gold labeling of CA (A to E) or $G_{M1}$ (F to I) (45, 47). (A and E) Low-magnification electron micrographs showing immunolabeling of CA. All gold is attached to membranes with little, if any, background labeling. The gold particles are located primarily on the surface of the larger vesicles and some linear membrane sheets, but are not associated with the smaller caveola. (C to E) Higher magnification images revealing unlabeled caveolar (arrowheads) apparently attached to a large vesicle labeled with gold (C) or associated with labeled membrane strands attached to the neck of the caveola (D and E). Control experiments with nonimmune serum showed little labeling of membranes; only an occasional gold particle was detected per field examined and appeared equivalent to background labeling of agarose alone. (F to I) Higher magnification micrographs revealing immunogold labeling of $G_{M1}$ inside caveola (arrowheads), with little labeling of the caveolar-associated larger vesicles (F) or remnant membranes (G). (I) Direct labeling of caveola (arrowhead) with cholera toxin–gold conjugates (47). Control experiments performed with conjugates plus a 10-fold molar excess of monomeric cholera toxin showed almost complete absence of gold. Overall, a size criterion was obvious in distinguishing the caveolar vesicles from the larger noncaveolar vesicles. Therefore, we divided the vesicles clearly observed in the electron micrographs into two groups; those with diameters of <80 nm and those with diameters of >150 nm. This size criterion cannot be considered absolute in separating caveola from noncaveolar vesicles, because, for instance, a few caveola could remain attached to each other and form a larger vesicle. Nevertheless, 86% of the vesicles of <80 nm were labeled for $G_{M1}$, with a range of one to nine gold particles per caveola, whereas only 2% were labeled for CA (all labeled with only one gold particle). For the larger vesicles, 80% were labeled for CA and only 13% for $G_{M1}$ (≥50 vesicles were counted in each category). These results support the use of $G_{M1}$ as a caveolar marker, substantiate the size criterion, and are consistent with previous studies on $G_{M1}$ localization (11, 14). Bars, 100 nm.
equivalence of this fraction with the immo-
le fraction detected in the diffusion stud-
ies (33). Thus, it appears that a substantial

but variable fraction of GMP-anchored pro-
tains exists on the cell surface dynamically

partitioned into detergent-resistant glyco-

lipid microdomains that are not likely to be

simply a consequence of detergent extrac-

tion and that the size of this fraction may

depend on cell type, culture, and ligand or

antibody exposure.

Immediately after more so after cross-

linking, GPI-anchored proteins can parti-

tion into diffusion-restrictive microdo-
mains, some of which may associate with

caveola as an annular region at the open-
ing. Our experiments with detergent-resis-
tant membranes support this model in sev-

eral ways. Because both domains are resis-
tant to detergent solubilization, the normally

and flat membrane region surrounding the

opening of the cavea is essentially excised

from the plasma membrane to form an intact

large vesicle with a caveola still attached

and located usually inside but sometimes

outside of the vesicle. These structures were

often detected by electron microscopy (Fig.

2, C to E). The silica coating prevents the

colloidal gold. This will be included in the

discussion section. Thus, when preparing for

this project, it is important to ensure that

everyone understands the correct

methods and procedures. This includes,

but not limited to:

- Proper lab attire
- Safety glasses
- Hand protection
- Use of appropriate cleaning solutions

Finally, it is crucial to maintain a clean

work environment to prevent contaminations

and to ensure the accuracy of the results.

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24. Isolation of Caveolae was performed under high-salt

conditions to minimize the electrostatic interaction

between the cationic silica particles and the polyan-

cationic cell surface. Under these conditions, intact

membranes were separated from the silica pellicle,

and, with the addition of Triton X-100, low density,

detergent-resistant membranes were isolated as

usual by sucrose density gradient centrifugation (13).

GPI-linked proteins were not normally associated

with caveolae, such as α-1 NT, these were not present

in this isolate (27). Furthermore, EM microscopy

revealed that the specimens, which appeared bio-

chemically impure by this criterion, contained cavo-

leae mixed with larger vesicular structures, including

single caveolae attached to the inside of the larger

vesicles, similar to those present in TI (Fig. 2).
Eukaryotic messenger RNA synthesis is a complex biochemical process controlled in part by the concerted action of a set of general transcription factors that regulate the activity of RNA polymerase II (Pol II) at both the initiation and elongation stages of transcription. At least six general initiation factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIH) have been identified in eukaryotic cells and found to promote selective binding of RNA polymerase II to promoters and to support a basal level of transcription (1).

In addition to the general initiation factors, three general elongation factors [SII, TFIIH, and Elongin (SII)] from eukaryotes have been defined biochemically and shown to increase the overall rate at which Pol II transcribes duplex DNA (2–4). SII is an ~38-kD elongation factor (5) that promotes passage of Pol II through transcriptional impediments such as nucleoprotein complexes and DNA sequences that act as introns (6). Elongin is a heterodimer composed of ~70-kD (RAP74)

**Elongin (SII): A Multisubunit Regulator of Elongation by RNA Polymerase II**

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The Elongin (SII) complex activates elongation by mammalian RNA polymerase II by suppressing transient pausing of the polymerase at many sites within transcription units. Elongin is a heterotrimer composed of A, B, and C subunits of 110, 18, and 15 kDa, respectively. Here, the mammalian Elongin A gene was isolated and expressed, and the Elongin (SII) complex reconstituted with recombinant subunits. Elongin A is shown to function as the transcriptionally active component of Elongin (SII) and Elongin B and C as regulatory subunits. Whereas Elongin C assembles with Elongin A to form an A3 complex with increased specific activity, Elongin B, a member of the ubiquitin-homology gene family, appears to serve a chaperone-like function, facilitating assembly and enhancing stability of the Elongin (SII) complex.