Two-dimensional distribution of $G_{i2\alpha}$ in the plasma membrane: a critical evaluation by immunocytochemistry

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Abstract Caveolae have been postulated as a center for signal transduction, because many signaling molecules are concentrated in caveolin-rich fractions. We took $G_{i2\alpha}$ as an example and examined whether it is constitutively concentrated in caveolae. First, the behavior of caveolin and $G_{i2\alpha}$ in density-equilibrium ultracentrifugation was reexamined. By collecting fractions efficiently, caveolin and $G_{i2\alpha}$ were found to distribute differently. Secondly, by novel immunocytochemical methods it was found that the labeling density of $G_{i2\alpha}$ was 2.29 times higher in caveolae than in the non-caveolar plasma membrane. The results indicate that the concentration of $G_{i2\alpha}$ in caveolae is lower than deduced from most biochemical studies.

Key words: Caveola; Plasma membrane; $G_{i2\alpha}$; Immunocytochemistry

1. Introduction

Caveolae have been hypothesized as a signal transduction center of the cell surface [1,2]. The hypothesis was based upon the findings that various receptors and signaling molecules were concentrated in caveola preparations obtained by biochemical methods. For example, scavenger receptors, GTP-binding proteins, Src family tyrosine kinases, etc., are enriched in Triton X-100-insoluble floating fraction (TIFF); because the TIFF is highly enriched with caveolin, it was assumed to be equivalent to purified caveolae [3,4]. However, more recent studies have revealed that some molecules concentrated in the TIFF are not densely distributed in caveolae in situ: GPI-anchored proteins, glycolipids, and sphingomyelin were shown to exist in a membrane domain distinct from caveolae [5–7]. The results suggest that detergent treatment may cause artificial accumulation of some molecules in the TIFF (for review, see [8]).

More sophisticated methods have been reported to obtain purified caveolae, and even with those methods, various signaling molecules were found enriched in the caveola preparation [9–13]. For example, the colloidal silica-coating technique produces morphologically well preserved caveola preparations [11]. The reliability of this method was shown by the exclusion of GPI-anchored proteins from the caveola preparations [6], which agreed with the result of immunocytochemistry [5]. The colloidal silica casting that is in direct contact with molecules in the outer leaflet probably preserves their in situ distribution. But the molecules in the inner leaflet may not be stabilized and retain their mobility; there is the possibility that Triton X-100 treatment, used to separate caveolae from the silica-coated membrane, caused their redistribution. Physical and/or chemical manipulations used in other methods might not be immune from redistribution either. Furthermore, a recent paper reported that an endothelial caveola preparation immunoisolated with anti-caveolin antibody was not enriched with signaling molecules [14].

Considering the possible influence of various treatments, methods with minimal membrane perturbation have to be applied to examine the distribution of signaling molecules in situ. Immunocytochemistry is appropriate for this purpose, but since signaling molecules are not exposed to the extracellular space, most studies used detergents to permeabilize the plasma membrane (examples for trimeric G proteins are in [15–19]). Even though cells were fixed before the detergent treatment in the above studies, differential solubility of membrane domains may have caused artificial distributional change [20,21]. In the present study, we took $G_{i2\alpha}$ as an example of the signaling molecules enriched in the caveola preparations and examined its localization in A431 cells without using detergents. For immunofluorescence and immunoelectron microscopy, plasma membrane preparations were subjected to immunolabeling with minimal chemical perturbation. Furthermore, we reexamined the behavior of the protein in density-equilibrium sucrose-gradient ultracentrifugation by collecting fractionated material with a higher centrifugal force and after more dilution than the published procedure [3]. The results indicate that the relative distribution density of $G_{i2\alpha}$ in and around caveolae is higher than in the non-caveolar membrane, but the difference is less than estimated from the past biochemical results.

2. Materials and methods

2.1. Cells and antibodies

A431 cells obtained from the Japanese Cancer Research Resources Bank were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Rabbit antibody was raised against $G_{i2\alpha}$ isolated from bovine lung, affinity-purified, and characterized as described previously [22,23]. For control experiments, antibodies that passed through the antigen-affinity column were used. Rabbit anti-caveolin (Transduction Lab., Lexington, KY) and mouse anti-caveolin antibodies (Zymed Lab., Inc., South San Francisco, CA), fluorescein- and rhodamine-conjugated donkey anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), colloidal gold (10 nm)-conjugated goat anti-rabbit IgG and anti-mouse IgG antibodies (British BioCell Int., Cardiff, UK), horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Pierce, Rockford, IL) were purchased from the respective suppliers.

2.2. Fractionation of Triton X-100-insoluble material

Triton X-100-insoluble material was separated by sucrose density-

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Abbreviations: BSA, bovine serum albumin; TIFF, Triton X-100-insoluble floating fraction

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gradient centrifugation. Briefly, A431 cells confluent in a 150 mm plastic dish were rinsed with ice-cold PBS three times and treated with 1.5 ml of TNE (25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, pH 7.5) containing 1% Triton X-100 for 20 min on ice. The sample was scraped with a silicon rubber piece, homogenized in a Dounce homogenizer with a Teflon pestle, and mixed with an equal volume of 80% sucrose in TNE (final volume, 3.8 ml). It was overlaid with 38% sucrose in TNE (5.5 ml) and 5% sucrose in TNE (3 ml) and centrifuged at 39 000 rpm for 15–20 h in a Beckman SW 40Ti rotor at 4°C. Fractions of 1.5 ml were taken by puncturing the bottom of the tube, and each fraction was divided into two. One was diluted threefold with TNE and centrifuged at 15 000 rpm (15 000 × *g*) for 10 min as described before [3]; the other was diluted sevenfold with TNE and centrifuged at 50 000 rpm (200 000 × *g*) for 60 min in a Beckman Ti 75 rotor at 4°C. Pellets and supernatants precipitated in cold acetone from both groups were dissolved in the same volume of the sample buffer for SDS-PAGE.

2.3. Immunoblotting

Samples were separated by SDS-PAGE in 10 or 15% acrylamide gels, electrotransferred to nitrocellulose paper and probed with rabbit antibodies to $G_{i2\alpha}$ and caveolin. After incubation with goat anti-rabbit IgG antibodies conjugated with horseradish peroxidase, the reaction was visualized using the Super Signal ULTRA Chemiluminescent substrate (Pierce) as described by the manufacturer. The intensity of the reaction was quantified by measuring the density developed on Hyperfilm-ECL (Amersham, Buckinghamshire, UK) using NIH Image (Ver. 1.55).

2.4. Immunofluorescence microscopy

The apical plasma membrane of A431 cells was adhered to poly-L-lysine-coated glass coverslips. In most experiments, the coverslips were further treated with 0.1% glutaraldehyde in distilled water for 5 min. The procedure was based on Rutter et al. [24] with minor modifications. Briefly, cells cultured in plastic dishes were washed extensively with PBS, rinsed twice with the cytosol buffer (20 mM PIPES-NaOH, 100 mM KCl, 5 mM $MgCl_2$, 3 mM EGTA, pH 6.8), incubated in the cytosol buffer diluted to 70% for 30 s, and overlaid with the adherent coverslips. After 5 min, the coverslips were gently separated from the dish. All solutions were precooled and the entire process was done on ice.

The plasma membrane preparation isolated on coverslips was labeled for immunofluorescence microscopy. For $G_{i2\alpha}$, the samples were treated with 1% bovine serum albumin (BSA), incubated with rabbit anti-$G_{i2\alpha}$ antibody (5 μg/ml) on ice for 30 min, and then with fluorescein-conjugated donkey anti-rabbit IgG antibody (10 μg/ml) for 30 min, and fixed immediately in 3% formaldehyde for more than 60 min in the dark. All the labeling steps were again done on ice. For caveolin, the membrane preparation was fixed immediately by 3% formaldehyde (freshly depolymerized from paraformaldehyde) in the cytosol buffer for 10 min on ice, treated with 3% BSA for 10 min, incubated with mouse anti-caveolin antibody (10 μg/ml) for 30 min, and then with fluorescein-conjugated donkey anti-mouse IgG antibody (10 μg/ml) for 30 min.

2.5. Immunoelectron microscopy

Cells cultured on thin gold foil were inverted upon thin copper foil and rapidly frozen by clamping with gold-plated copper blocks cooled by liquid nitrogen [25]. The cell sandwich was freeze-fractured in a Balzers BAF401 apparatus (Balzers High Vacuum Corp., Balzers, Liechtenstein), and the obtained platinum/carbon replicas were digested with SDS, treated with BSA for blocking, and then incubated with rabbit anti-$G_{i2\alpha}$ or mouse anti-caveolin antibodies (10 μg/ml) [26]. They were further incubated with colloidal gold-conjugated goat anti-rabbit IgG or anti-mouse IgG antibodies (diluted to 1/30) and observed under a JEOL 200CX electron microscope.

Electron micrographs were printed to the same magnification, and the distribution density of gold particles in caveolae vs. non-caveolar plasma membrane was quantitated. As described in Section 3, caveolae took at least three different shapes in the freeze-fractured replica: deep dimple, shallow dimple, crater (definitions of the three categories are given in Fig. 3B). The caveolar area was defined by drawing a circle of 100 nm in diameter around the dimples. Craters were excluded from the quantitation since the major portion of the caveolar membrane was not retained on the replica. Gold particles in and around deep and shallow dimples were counted separately. The difference of the labeling density between caveolae and non-caveolar area was examined statistically with the Mann-Whitney *U*-test.

3. Results

3.1. Immunoblotting of the total lysate and detergent-insoluble fractions

By immunoblotting of the total A431 cell lysate, the antibody to $G_{i2\alpha}$ reacted with a single band at around 41 kDa (Fig. 1A). The mobility in SDS-PAGE matched with previous reports and confirms the specificity of the antibody. When fractions obtained by sucrose density-equilibrium centrifugation were pelleted with the protocol reported previously [3], positive reactions for both $G_{i2\alpha}$ and caveolin were restricted to a few low-density fractions, corresponding to the TIFF (left panel of Fig. 1B). When the same fractions were sedimented by ultracentrifugation, the reaction for $G_{i2\alpha}$ was still limited to a few light fractions; on the other hand, caveolin was detected in all the fractions, although the reaction was most intense in the low-density fractions (right panel of Fig. 1B). Supernatants obtained with the two protocols were also subjected to immunoblotting; those of the microspin showed an intense reaction for both $G_{i2\alpha}$ and caveolin, while those of the ultracentrifugation were negative for both proteins (data not shown). The difference between the two methods was whether fractions of the density-gradient centrifugation were collected by microcentrifugation (15 000 × *g*, 10 min) after 3-fold dilution or by ultracentrifugation (200 000 × *g*, 60 min) after 7-fold dilution. By densitometric quantitation, the total amount of caveolin recovered in the low-density fractions (fractions 6–8) was 90.9% by microcentrifugation, while it was only 47.7% by ultracentrifugation. On the other hand, comparable amounts of $G_{i2\alpha}$ were recovered in the low-density fractions with the two different protocols: 94.6% by microcentrifugation and 87.9% by ultracentrifugation (Fig. 1C). The result can be explained by assuming that membrane fragments equilibrated with the dense sucrose solution (fractions 1–7) were sedimented only by applying a high gravity force in a low-density solution. The experiment showed that the remarkable enrichment of $G_{i2\alpha}$ can be dissociated from the behavior of caveolin.

3.2. Immunofluorescence microscopy of plasma membrane preparation

A wide area of the apical plasma membrane of A431 cells was obtained on coverslips with the cytoplasmic surface exposed. For this preparation detergent treatment was not necessary before immunolabeling. Chemical fixation was also avoided to eliminate a possible risk of artifacts. To minimize the possibility that antigens cross-linked with antibodies may change distribution [5], incubation of unfixed specimens was done on ice, and then the specimens were fixed for more than 60 min with formaldehyde; the fixation protocol should be sufficient to stabilize lipid-anchored molecules even after cross-linking [5]. In the preparation, labeling with the anti-$G_{i2\alpha}$ antibody occurred as fine punctates which were distributed evenly without local concentration (Fig. 2A). As a control, when the antibody passed through the $G_{i2\alpha}$ affinity column was used, little fluorescence was observed (Fig. 2B). To observe the distribution of caveolae, anti-caveolin antibody was applied to the same membrane preparation fixed with formaldehyde. The labeling was seen as distinct punctates,

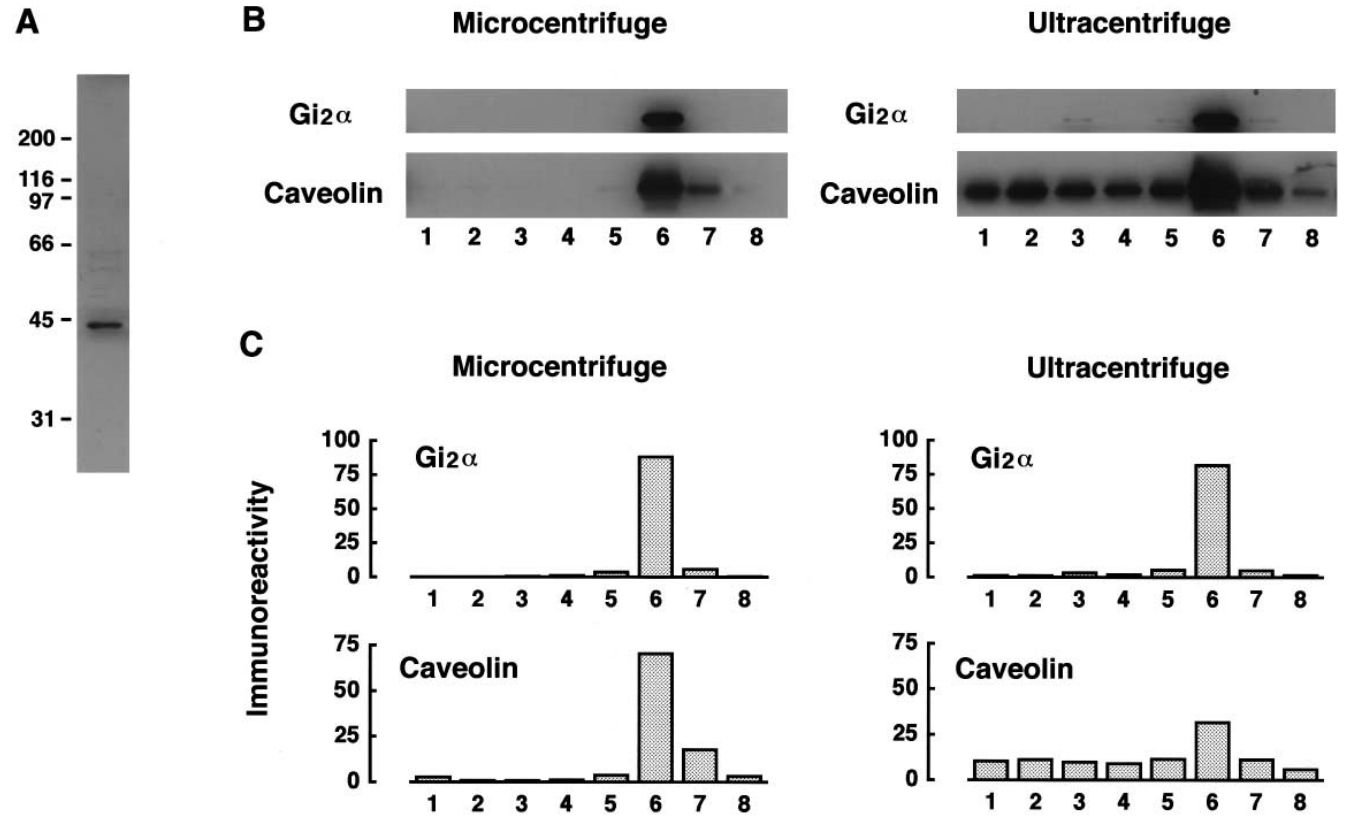

Fig. 1. A: Immunoblotting of the total lysate of A431 cells by the anti-$G_{i2\alpha}$ antibody. B: Fractions obtained by sucrose density-gradient centrifugation of Triton X-100-insoluble material from A431 cells were subjected to immunoblotting for $G_{i2\alpha}$ and caveolin. Fractions were: (left) diluted 3-fold and collected by microcentrifugation (15 000 × *g*, 15 min), or (right) diluted 7-fold and collected by ultracentrifugation (200 000 × *g*, 60 min). C: Densitometric quantitation of the immunoblotting. The intensity of reaction in each fraction is expressed as a percentage of the total sum of fractions 1–8.

which were coarser than those observed for $G_{i2\alpha}$ (Fig. 2C). Comparing the labeling by the two antibodies (Fig. 2A and 2C), there was no indication that $G_{i2\alpha}$ is concentrated in caveolae.

3.3. Immunoelectron microscopy of freeze-fracture replicas

Localization of $G_{i2\alpha}$ and caveolin was examined by immunoelectron microscopy of SDS-treated freeze replicas [26]. Immunolabeling for caveolin showed that caveolae can take several different forms in the P face of the replica (Fig. 3A). Some were observed as deep dimples and their bottom did not appear coated with platinum/carbon (marked as **a** in Fig. 3A); because only the molecules physically stabilized by the metal shadowing are supposed to be retained after the SDS treatment, those at the bottom of deep dimples may have been lost. Other caveolae were seen as shallow dimples

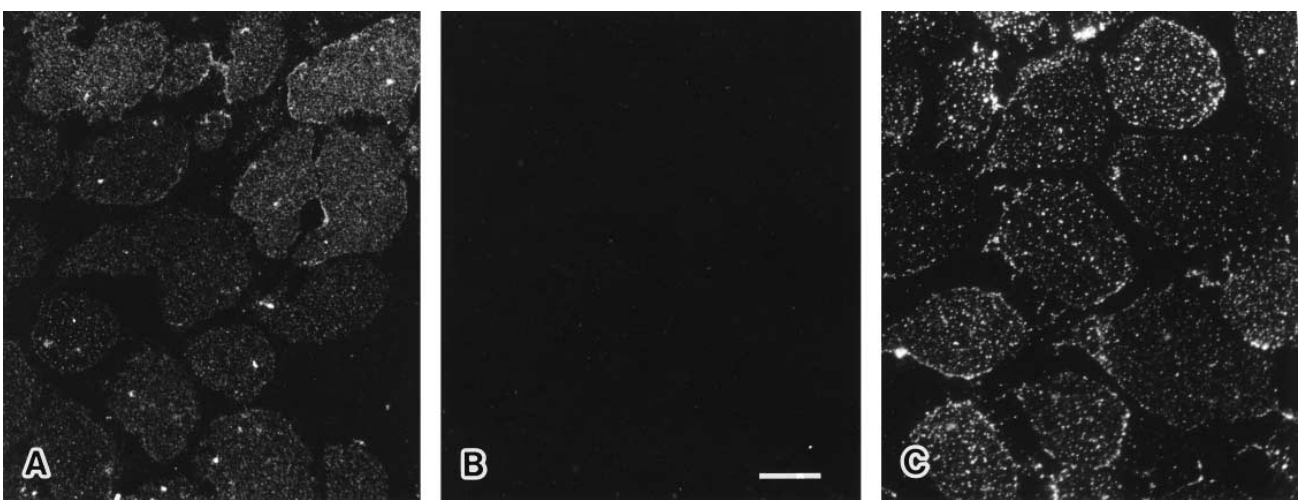

Fig. 2. Immunofluorescence microscopy of apical plasma membrane preparations. A: Unfixed preparations labeled with anti-$G_{i2\alpha}$ antibody; B: with anti-$G_{i2\alpha}$ antibody preabsorbed with the antigen. C: Fixed preparations labeled with anti-caveolin antibody. Bar, 10 μm.

and the entire contour was replicated (marked as **b** in Fig. 3A). The rest was fractured at the neck and appeared as craters (marked as **c** in Fig. 3A). The caveola area was defined as a circle of 100 nm in diameter around the dimples; the total area of caveolae was about 3% of the cell surface (Table 1).

The immunogold labeling for $G_{i2\alpha}$ was observed in an apparently random manner throughout the plasma membrane (Fig. 3B). Gold particles were seen singly or in clusters of more than two particles, and they were distributed without marked concentration. The same result was observed consistently in different experiments. The number of gold particles in the caveola area was 6.24% of the total gold particles. By measuring the relative distribution density of colloidal gold particles in caveolae vs. non-caveolar membrane, the density of gold particles for $G_{i2\alpha}$ was 2.29 times higher in caveolae compared to the non-caveolar area (Table 1). The density of gold particles for $G_{i2\alpha}$ was equivalent in deep and shallow caveolae. By the same method of counting, more than 99% of the labeling for caveolin was found in caveolae; the relative distribution density was 640 times higher in caveolae than in non-caveolar membrane (Table 1).

4. Discussion

By separating the Triton X-100-insoluble material by sucrose density-gradient centrifugation, caveolin and $G_{i2\alpha}$ have been reported to be highly enriched in the TIFF [3]. But the contention that the TIFF is equivalent to isolated caveolae is not supported by more recent results [6,27,28]. There are two possibilities for the origin of the TIFF which appears as small vesicles by electron microscopy [3,29]. One possibility is that some of the vesicles are caveolae while others are derived from detergent-insoluble glycolipid-rich domains outside caveolae [30]. The other possibility is that the vesicles do not reflect membrane domains in situ, but are formed by artificial accumulation of detergent-insoluble materials. Whichever possibility is true, if caveolin and $G_{i2\alpha}$ are always contained in the same vesicles, they are likely to be distributed in the same membrane domains in situ. However, in contrast to previous results, we found that more than half of the caveolin was recovered from the high-density portion of the gradient, whereas most of the $G_{i2\alpha}$ was in the low-density fractions. The result indicates that at least for materials which were pelleted only by ultracentrifugation, the ratio of caveolin versus $G_{i2\alpha}$ is very high. The origin of the high-density caveolin-rich membrane is not clear; it may be derived from intracellular organelles, or may reflect some compositional difference among caveolae, e.g. between deep and shallow caveolae. The study does not exclude the possibility that $G_{i2\alpha}$ is concentrated in caveolae, but at the same time, it does not support that most caveolin and $G_{i2\alpha}$ is localized in the same membrane domain in situ.

As mentioned in Section 1, biochemical methods have shown that G proteins are concentrated in caveolin-enriched fractions [10,12,13,31]. But considering the two-dimensional mobility of lipid-anchored G proteins, it is difficult to prove that treatments used in the above methods, i.e. detergents [3,11], high pH [13], or ultrasonication [9,12], did not affect the distribution. Methods based on different principles, especially immunocytochemistry, may be helpful to resolve the issue. But to the best of our knowledge, the past immunolocalization studies of G proteins were done by first fixing cells

Table 1
Quantification of immunogold distribution for $G_{i2\alpha}$ and caveolin

	Area measured (μm^2)			Total number of caveolae	Number of gold particles			Labeling density in caveolae (gold particles/μm^2 area)			Labeling density in non-caveolar area (gold particles/μm^2 area)
	Total	Caveolae	Non-caveolar area		Total	Caveolae	Non-caveolar area	Total caveolae	Deep caveolae[a]	Shallow caveolae[b]	
$G_{i2\alpha}$	170.66	4.82	165.84	614	3653	228	3425	47.30 ± 19.96*	43.30 ± 29.18	51.20 ± 14.18	20.65 ± 3.00*
Caveolin	171.18	5.35	165.83	681	1508	1438	70	268.99 ± 53.50	341.54 ± 64.17	211.53 ± 74.68	0.42 ± 0.27

[a]Caveolae which appear as deep dimples with a white bottom.
[b]Caveolae which appear as shallow dimples with the entire contour replicated.
*The difference was significant by Mann-Whitney *U*-test ($P = 0.0253$).

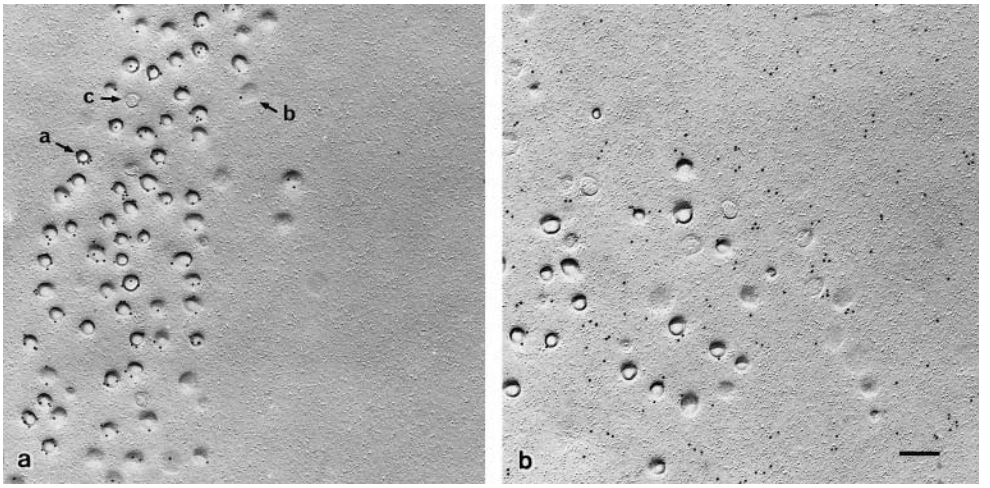

Fig. 3. Immunoelectron microscopy on freeze-replicas of quick-frozen A431 cells by SDS-FRL technique. A: Caveolin. B: $G_{i2\alpha}$. Caveolae are seen in three different shapes: deep dimples (marked as **a** in A), shallow dimples (**b**), and craters (**c**). The labeling density around shallow and deep dimples was measured separately. Bar, 200 nm.

with aldehyde fixatives and then permeabilizing them with detergents [15–19]. However, as shown for GPI-linked proteins [5], some membrane molecules are not immobilized by a short fixation with formaldehyde alone; although formaldehyde reacts with lipids as well as proteins [31], the fluidity of the lipid bilayer is assumed to remain. When detergents are applied to such specimens, trimeric G proteins, which are anchored to the membrane through prenyl groups and acyl chains (for review, see [32]), may have more chance of redistribution than transmembrane proteins. Therefore, immunocytochemical methods which do not use detergent had better be applied. In the present study, immunoelectron microscopy of freeze-fracture replicas was done. In this method, cells were rapidly frozen with liquid nitrogen and the fracture plane was shadowed with platinum and carbon. Because integral membrane proteins and membrane lipids were physically stabilized by the casting [26,33], subsequent SDS digestion and antibody treatment should not cause their redistribution. Furthermore, since two-dimensional distribution of antigens can be observed in a large plane, quantitative evaluation can be done more efficiently than using ultrathin sections.

In interpreting the result of the replica labeling, two points must be considered. One is, as deduced from the principle of the method, that only those molecules stabilized by the platinum/carbon casting were retained on the replica. Since phosphatidylcholine has been shown to remain on SDS-treated replicas quantitatively [33], $G_{i2\alpha}$ is likely to be kept as far as they are anchored by fatty acid chains. But if there exists a population of $G_{i2\alpha}$ that binds to the membrane in a different manner, for example by electrostatic force, it must be lost from the replica by the SDS treatment. The other point is that, as described in Section 3, there are several ways that caveolae are fractured and/or replicated. If $G_{i2\alpha}$ were more concentrated in the bottom portion of deep dimples (**a** in Fig. 3A) than at their orifice or in shallow dimples (**b** in Fig. 3A), it must have been lost from the replicas and thus we underestimated its caveolar concentration. Although we think it unlikely based on the immunofluorescence result, the possibility remains.

The relative concentration of trimeric G protein subunits deduced from different caveola preparation methods is variable: ~3- ($G_{i\alpha1,2}$) and 6- ($G_{i\alpha3}$) fold (compared to the plasma membrane; chicken smooth muscle cell in vivo) [9]; 25-fold ($G_{i\alpha}$) (compared to the plasma membrane; human fibroblast in culture) [12]; ~20–40-fold ($G_{s\alpha}$) (compared to non-caveolar plasma membrane; MDCK cells) [34]; 2.8–8.6-fold ($G_{i\alpha1-3}$) (compared to non-caveolar plasma membrane; rat lung endothelium in vivo) [10]. For an immunoisolated caveola preparation, the relative enrichment or depletion was not calculated since the total protein amount could not be measured accurately [14]. The above ratios were calculated as relative enrichment of G proteins per unit weight of the membrane preparation. On the other hand, our result is expressed as distribution density per unit area of the plasma membrane. The two densities can be converted as follows: assuming that caveolae and non-caveolar membranes are equilibrated with 10% sucrose (approximate density: 1.04 g/ml) and 30% sucrose (approximate density: 1.13 g/ml), respectively, the 2.29 times difference in distribution density per unit area corresponds to 2.49 ($=2.29\times1.13/1.04$) in relative content per weight. Thus the relative caveolar enrichment of $G_{i2\alpha}$ deduced from the present study is smaller than the lowest number obtained by biochemical experiments [10].

Several lines of evidence have shown that receptors, G proteins, and effector molecules in the plasma membrane are not freely mobile, but are organized two-dimensionally for efficient interaction (for review, see [35]). In addition, G protein-linked receptors have been reported to concentrate in caveolae [36] or translocate to caveolae when bound with agonists [37,38]; bacterial toxins which modify G proteins are known to be internalized through caveolae [39–41]. More recently, caveolins were shown to interact with the inactive form of G_{α} and were thought to function as a GDP dissociation inhibitory protein or a GTPase-activating protein [34,42,43]. In conjunction with enrichment of G proteins in various caveola fractions, the results appear to suggest that G proteins are constitutively and highly concentrated in caveolae. However, the above observations can also be explained by assuming that G proteins are preferentially localized in some non-caveolar membrane domain as well as caveolae: that is, a membrane domain having similar characteristics as the detergent-insoluble glycolipid-rich domain in the outer leaflet [30]

may exist in the inner leaflet. Our results both by immunocytochemistry and by fractionation are consistent with the latter view.

Either by our method or by various biochemical methods, G proteins were found 'concentrated in caveolae', but whether the enrichment is 2.5-fold or 20∼-fold should make a difference in assessing the role of caveolae in signal transduction. It remains to be determined whether the concentration and distribution of G proteins change under different physiological conditions and whether the in situ functional states of G proteins in caveolae vs. non-caveolar areas differ. Immunocytochemistry has its own pitfalls and limitations, but the present study implies that reexamination by techniques based on different principles is important for critical evaluation of caveolae and caveola-related molecules.

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