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Colocalization of β-Adrenergic Receptors and Caveolin Within the Plasma Membrane

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Abstract The rapid amplification of β-adrenergic receptor signaling involves the sequential activation of multiple signaling molecules ranging from the receptor to adenylyl cyclase. The prevailing view of the agonist-induced interaction between signaling molecules is based on random collisions between proteins that diffuse freely in the plasma membrane. The recent identification of G protein α- and βγ-subunits in caveolae and their functional interaction with caveolin suggests that caveolae may participate in G protein-coupled signaling. We have investigated the potential interaction of β-adrenergic receptors with caveolin under resting conditions. β1- and β2-adrenergic receptors were recombinantly overexpressed in COS-7 cells. Caveolae were isolated using the detergent-free sucrose gradient centrifugation method. β1- and β2-adrenergic receptors were localized in the same gradient fractions as caveolin, where Gsα- and βγ-subunits were detected as well. Immunofluorescence microscopy demonstrated the colocalization of β-adrenergic receptors with caveolin, indicating a nonrandom distribution of β-adrenergic receptors in the plasma membrane. Using polyhistidine-tagged recombinant proteins, β-adrenergic receptors were copurified with caveolin, suggesting that they were physically bound. Our results suggest that, in addition to clathrin-coated pits, caveolae may act as another plasma membrane microdomain to compartmentalize β-adrenergic receptors. J. Cell. Biochem. 75:64–72, 1999.

Key words: caveolae; caveolin; β-adrenergic receptors; compartmentalization

On a second-to-second basis, β-adrenergic receptors respond to altered levels of norepinephrin released and removed at the synaptic terminals [Ishikawa and Homcy, 1997] leading to the sequential activation of the receptor, G protein, and adenylyl cyclase at the plasma membrane, and the production of the second messenger cAMP. This process culminates in the activation of the cAMP-dependent protein kinase (PKA) [Scott, 1991] which regulates the activity of multiple target molecules by reversible phosphorylation. The current thinking about the ligand-driven, β-adrenergic receptor/G protein/adenylyl cyclase interaction is based on the idea of freely mobile signaling molecules [Orly and Schramm, 1976; Tolkovsky and Levitzki, 1978]. However, there is mounting evidence that transmembrane signaling molecules are more highly organized and compartmentalized within the plasma membrane [for review see Houslay and Milligan, 1997; Neubig, 1994].

Caveolae, nonclathrin-coated plasma membrane vesicles of 50–100 nm in diameter, represent a subcompartment of the plasma membrane that exists in most cell types, although they are extremly abundant in terminally differentiated cells including endothelial cells, adipocytes, fibroblasts, and myocytes [Couet et al., 1997]. Caveolin is a major protein component of caveolae. Three different caveolin genes have been identified so far (caveolin-1, -2, and -3) encoding four different caveolin subtypes that differ in tissue distribution [Couet et al., 1997].

The exact cellular function of caveolae remains controversial, although they have been implicated in transport processes such as transcytosis and potocytosis [Anderson et al., 1992] Montesano et al., 1982]. The recent identifica-

Abbreviations used: FITC, fluorescein isothiocyanate; Mes, 4-morpholinoethanesulfonic acid; NTA, nitrilotriacetic acid; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

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tion of various G protein α- and βγ-subunits and their functional interaction with caveolin suggests that caveolae may participate in transmembrane signaling, in particular, G protein-coupled signaling [for review see Okamoto et al., 1998]. In addition, G protein-modifying bacterial toxins (cholera and tetanus toxin) are localized to caveolae [Montesano et al., 1982]. Accordingly, it has been proposed that caveolae represent a membrane-bound site for concentrating G proteins and related molecules. Using a combination of morphological and biochemical techniques several G protein-coupled receptors, such as muscarinic acetylcholine-, endothelin-, and bradykinin-receptors [Chun et al., 1994; de Weerd and Leeb-Lundberg, 1997; Feron et al., 1997; Haasemann et al., 1998; Raposo et al., 1987], have been localized to caveolae. Previous data on the localization of β-adrenergic receptors in caveolae have been controversial. Some histological studies have shown the localization and internalization of β-adrenergic receptors through caveolae [Dupree et al., 1993; Raposo et al., 1989]. In contrast, other investigators support that β-adrenergic receptors redistribute to clathrin-coated pits upon agonist stimulation [Gagnon et al., 1998; Kallal et al., 1998; von Zastrow and Kobilka, 1992, 1994; Zhang et al., 1996, 1997].

While previous studies have predominantly focused on the redistribution of β-adrenergic receptors upon agonist stimulation, little effort was spent on the examination of the subcellular distribution of β-adrenergic receptors in resting cells. We thus investigated in this study whether β-adrenergic receptors are randomly distributed or compartmentalized within the plasma membrane of resting cells. In particular, we examined their localization relative to caveolin.

MATERIALS AND METHODS

Reagents

Cell culture media. Insect Xpress medium was obtained from BioWhittaker Inc. (Walkersville, MD). Dulbecco's modified Eagle's medium (DMEM) was from Mediatech Inc. (Herndon, VA).

Antibodies

Polyclonal antibodies to the β-adrenergic receptor subtypes (β1 and β2) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A polyclonal antibody to Gsα was obtained from Du Pont (Wilmington, DE). The monoclonal antibodies to caveolin-1 (2297) and adaptin β were obtained from Transduction Laboratories (Lexington, KY). Fluorescein isothiocyanate (FITC)-labeled anti-mouse- and Texas Red®-labeled anti-rabbit-immunoglobulin were obtained from Amersham (Arlington Heights, IL).

cDNA clones and plasmids. Caveolin-1 cDNA clone was provided by Dr. M. P. Lisanti. β1- and β2-adrenergic receptor cDNA were from Dr. J. L. Benovic.

Other reagents. Most other reagents were obtained from Sigma (St. Louis, MO). LipofectAMINEô was purchased from Life Technologies (Gaithersburg, MD).

Cell Culture

Spodoptera frugiperda (Sf9) insect cells were grown in Insect Xpress medium containing 6% fetal bovine serum, penicillin (100 µg/ml), and streptomycin (100 µg/ml). COS-7 cells were cultured in DMEM supplemented with 5% fetal bovine serum, penicillin (100µg/ml), and streptomycin (100µg/ml) in a humidified 95% air/5% CO_2 incubator.

Transfection of COS-7 Cells

COS-7 cells were plated in 10 cm dishes at ~60% confluence. For transfection, 16 µl LipofectAMINEô with 800 µl serum- and antibiotic-free DMEM were mixed with 4–8 µg of cDNA (caveolin-1 or β-adrenergic receptors) with 800 µl serum- and antibiotic-free DMEM. After 30 min the LipofectAMINE™/cDNA solution was diluted with 4.8 ml DMEM supplemented with 5% FBS and overlaid onto the cells. After 6 h incubation at 37°C in 5% CO_2 the solution was replaced with complete DMEM culture medium containing 5% FBS and antibiotics. The cells were collected for experiments 48 h after transfection.

Overexpression of β2-Adrenergic Receptors and Caveolin-1 in Insect Cells

A Xho I/Kpn I fragment from the caveolin-1 cDNA containing a myc epitope was as previously described [Scherer et al., 1995]. The β2-adrenergic receptor construct was a generous gift of Dr. J.L. Benovic.

These clones were inserted into the pBluBac vector. The recombinant shuttle vectors were transfected into insect cells using the Bac-N-

Blue transfection kit. The plaques were then purified as previously described [Kawabe et al., 1994]. Forty-eight hours after infection, cells were washed twice with ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.5) and subjected to sucrose gradient centrifugation.

Cell Fractionation by Sucrose Gradient Centrifugation

Caveolin-enriched membrane domains were purified from cultured insect cells and mammalian cells by a previously optimized method [Song et al., 1996]. All steps were carried out at 4°C. Cells were scraped in 2 ml of 500 mM sodium carbonate (pH 11). Homogenization of the suspension with 12 strokes of a Dounce homogenizer was followed by three 10-sec bursts of a Polytron tissue grinder and four 20-sec bursts of a sonicator. The sucrose concentration in cell extracts was adjusted to 45% by the addition of 2 ml of 90% sucrose prepared in MBS (25 mM Mes, pH 6.5; 0.15 M NaCl), and the extracts were placed at the bottom of an ultracentrifuge tube. A 5–35% discontinuous sucrose gradient was formed above (4 ml of 35%/4 ml of 5% sucrose, both prepared in MBS containing 250 mM sodium carbonate), and centrifuged at 39,000 rpm for 16 h at 4°C in a Sorvall TH 641 rotor. A light-scattering band was confined to the 5–35% sucrose interface. From the top of each gradient, a total of 13 fractions (1 ml each) were collected.

Immunoblotting

Gradient fractions were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). After transfer, the membranes were blocked in Tris-buffered saline (TBS) supplemented with 5% nonfat dry milk and 0.05% Tween 20 and subjected to immunoblotting. Bound primary antibodies were visualized using Amersham (Arlington Heights, IL) chemiluminescence Western blotting detection reagents.

FACS® Analysis

Flow cytometry was performed to quantify co-expression of caveolin-1 and β1-adrenergic receptors. Cotransfected COS-7 cells were incubated for 1 h at room temperature with a mouse monoclonal antibody directed against caveolin-1 and a polyclonal rabbit antibody to β1-adrenergic receptors. After washing in PBS the cells were incubated with FITC-labeled anti-mouse- and Texas Red®-linked anti-rabbit-immunoglobulin, and then subjected to FACS® analysis (FACSCalibur, Becton Dickinson, San Jose, CA). Red and green fluorescence was recorded respectively by the FL1 and FL2 channels.

Immunofluorescent Microscopy

Cotransfected COS-7 cells were rinsed three times in PBS and fixed with 4% paraformaldehyde in PBS. The fixed cells were rinsed with PBS and treated 10 min with 100 mM NH_4Cl. Cells were permeabilized with 0.1% Triton X-100 for 10 min at room temperature and washed four times with PBS. After 30 min incubation in PBS supplemented with 5% bovine serum albumin and 50 µg/ml each of normal sheep and donkey serum, cells were exposed 60 min to a monoclonal antibody directed against caveolin-1 and a polyclonal antibody to β1-adrenergic receptors (1:100). Immunofluorescent staining was performed for 60 min with FITC-labeled anti-mouse- and Texas Red®-linked anti-rabbit-immunoglobulin. Cells were washed three times with PBS between incubations. Coverslips were mounted onto slides with antifade (Oncor, Gaithersburg, MD) and observed under a Nikon eclipse E800 fluorescent microscope (Melville, NY).

Affinity Chromatography

The caveolin-enriched fraction (fraction 5) from Sf9 insect cells overexpressing a polyhistidin epitope-tagged form of caveolin-1 and β2-adrenergic receptors was adjusted to pH 8 using 1 M Mes buffer, followed by incubation with Ni-NTA-agarose resin (Qiagen, Chatsworth, CA) for 6 h at 4°C [Song et al., 1996]. The Ni-NTA-agarose resin was allowed to settle by gravity (5 min on ice) and washed twice with Tris-buffered saline (TBS; 10 mM Tris, pH 8 and 0.15 M NaCl) and then three times with TBS containing 30 mM imidazole (5 min each). Finally, bound proteins were eluted with TBS containing 200 mM imidazole for 1 h at 4°C. Washes and eluates were subjected to immunoblot analysis.

RESULTS

Caveolin and β-Adrenergic Receptors Are Colocalized After Subcellular Fractionation

Caveolin-enriched membrane fractions were obtained from cultured COS-7 cells using the detergent-free purification method [Song et al., 1996]. A single light-scattering band corresponding to a low-density complex was observed, mainly in fractions 5 and 6 of these gradients, as previously reported with other cell lines [Sargiacomo et al., 1993]. Caveolin-1 migrated with a molecular mass of 24 kDa and was detected only in these fractions (5 and 6; Fig. 1A). Compared with other gradient fractions, these complexes excluded ~97% of the plasma membrane marker alkaline phosphodiesterase (data not shown) as previously demonstrated [Lisanti et al., 1994]. An exogenous, recombinant caveolin-1 was similarly concentrated in fractions 5 and 6 and was detected as double bands (caveolin-1-α and -β) in COS-7 cells as previously described (Fig. 1B) [Scherer et al., 1995]. Thus mammalian cells may be useful to investigate the subcellular distribution of both endogenous and recombinant proteins relative to caveolin.

After overexpressing either β1- or β2-adrenergic receptors in COS-7 cells, caveolin-enriched membrane fractions were similarly purified. Both β1- and β2-adrenergic receptors were located to these caveolin-enriched membrane

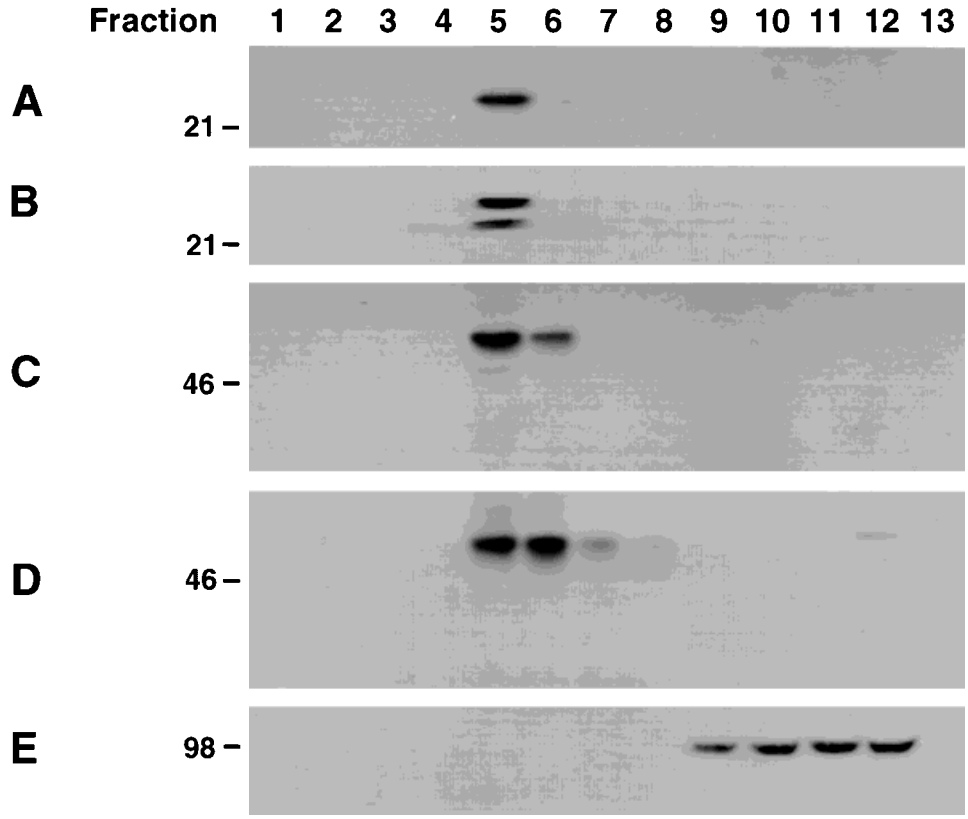

Fig. 1. Detection of endogenous and recombinant proteins after subcellular fractionation of COS-7 cells. Caveolin-enriched membrane fractions were obtained from cultured COS-7 cells using the sucrose density gradient centrifugation. One-ml aliquots were collected from the top of the gradient and subjected to SDS-PAGE. Fractions 1–8 represent the 5–35% sucrose layer, fractions 9–12 are the 45% sucrose layer, and fraction 13 is the unsoluble pellet. Endogenous caveolin-1 in COS-7 cells was detected by immunoblotting with an antibody raised against the C-terminal portion of caveolin-1. Caveolin-1 migrated on SDS-PAGE with a molecular mass of 24 kDa and was localized to fractions 5 and 6 (**A**). Caveolin-1 was recombinantly overexpressed in COS-7 cells and was detected on SDS-PAGE as double bands (1-α and -β; **B**). Recombinant caveolin-1 contained a myc epitope tag and migrated slightly slower than endogenous caveolin-1. Note that the exposure time of the film was much shorter than that in the above (A). COS-7 cells overexpressing either α1- or β2-adrenergic receptors were homogenized and subjected to subcellular fractionation. Fractions 1–13 of sucrose gradients were analyzed by immunoblotting with isoform-specific antibodies to α1- and β2-adrenergic receptors. Note that β1- (**C**) and β2-adrenergic receptors (**D**) were localized to the caveolin-enriched membrane fractions. In contrast, adaptin β was excluded from the caveolin-enriched membrane fractions and remained in fractions 9–12 (**E**).

fractions (Fig. 1C,D). In contrast to the conventional idea of the "freely mobile receptors within the plasma membrane," these findings suggest that the distribution of β-adrenergic receptors is confined to a small fraction of the plasma membrane under resting conditions. Conversely, adaptin β, which has been implicated in the formation of clathrin-coated pits [Kirchhausen et al., 1989], was completely excluded from the caveolin-enriched membrane fractions and detected only in the bottom-loaded layer of the sucrose gradient fractions (Fig. 1E).

Caveolin and β-Adrenergic Receptors Are Immunocytochemically Colocalized

The colocalization of caveolin-1 and β1-adrenergic receptors in resting cells was examined by immunofluorescence microscopy. The efficiency of cotransfection experiments was monitored by FACS® analysis (Fig. 2A). We observed that cotransfected COS-7 cells expressed increased levels of caveolin-1 and β1-adrenergic receptors, indicating that a subpopulation of the cells overexpressed both proteins. Figure 2B shows that after cotransfection, caveolin-1 and β1-adrenergic receptors appeared identically in discrete micropatches mainly along the plasma membrane, indicating the colocalization of both recombinantly expressed proteins within the same subcompartment of the cell. A whole cell radioligand binding assay using a hydrophilic ligand demonstrated that the receptor was located largely on the cell surface with a smaller proportion in the cytoplasm, which was in agreement with the immunocytochemical findings (data not shown).

G Proteins Are Diffusely Distributed

Previous studies [Chang et al., 1994; Li et al., 1995] have agreed that G protein α-subunits are present in, but not limited to, caveolin-enriched plasma membrane fractions. In accordance with these results, Gsα was located in both caveolin-enriched plasma membrane fractions and the bottom-loaded sucrose layer (Fig. 3A). Gβγ was predominantly detected in caveolin-enriched fractions (Fig. 3B), which was similar to β-adrenergic receptors. This diffuse distribution of G protein α-subunits relative to β-adrenergic receptors may reflect the large stoichiometric excess of G proteins over other transmembrane signaling molecules [Post et al., 1995] or differences in the state of activation as previously proposed [Okamoto et al., 1998].

β-Adrenergic Receptors Were Copurified With Caveolin

To examine whether caveolin and β-adrenergic receptors are physically bound, we performed copurification experiments using Sf9 cells coexpressing β2-adrenergic receptors and a polyhistidine epitope-tagged form of caveolin-1. The caveolin-enriched fractions from the insect cells were incubated with Ni-NTA agarose resin, to which the polyhistidine epitope-tagged caveolin-1 was bound. Washing the resin with TBS twice and then three times with TBS/imidazole eliminated nonspecific binding of the receptor. In the presence of a polyhistidine epitope-tagged form of caveolin-1, the receptor was retained on the resin and detected in the final eluate (Fig. 4, right). In the absence of a polyhistidine epitope-tagged form of caveolin-1, however, the receptor was readily washed off from the resin (Fig 4, left). Thus, caveolin can bind to β-adrenergic receptors with a high affinity.

DISCUSSION

Heterotrimeric G proteins have been shown to be present in, but not limited to, the same membrane fraction as caveolin [Chang et al., 1994; Couet et al., 1997; Li et al., 1995]. Using cell fractionation techniques we further demonstrated that β-adrenergic receptors were colocalized to the same membrane fraction as caveolin, where G protein α- and βγ-subunits were present. Immunofluorescence microscopy confirmed this colocalization under resting conditions. Furthermore, caveolin was copurified with β-adrenergic receptors, suggesting that the two molecules (β-adrenergic receptors and caveolin) can directly interact with each other. It is well known that β-adrenergic receptors maintain the high affinty state for the heterotrimeric G proteins (Gα and Gβγ) forming a receptor-G protein complex under resting conditions [for review see Neer, 1995]. Thus, it is reasonable to assume that β-adrenergic receptors and the G protein heterotrimers reside within the same microdomain of the plasma membrane in resting cells. Indeed, our data demonstrated that β-adrenergic receptors are located to the same microdomain as Gsα and Gβγ (fractions 5 and 6).

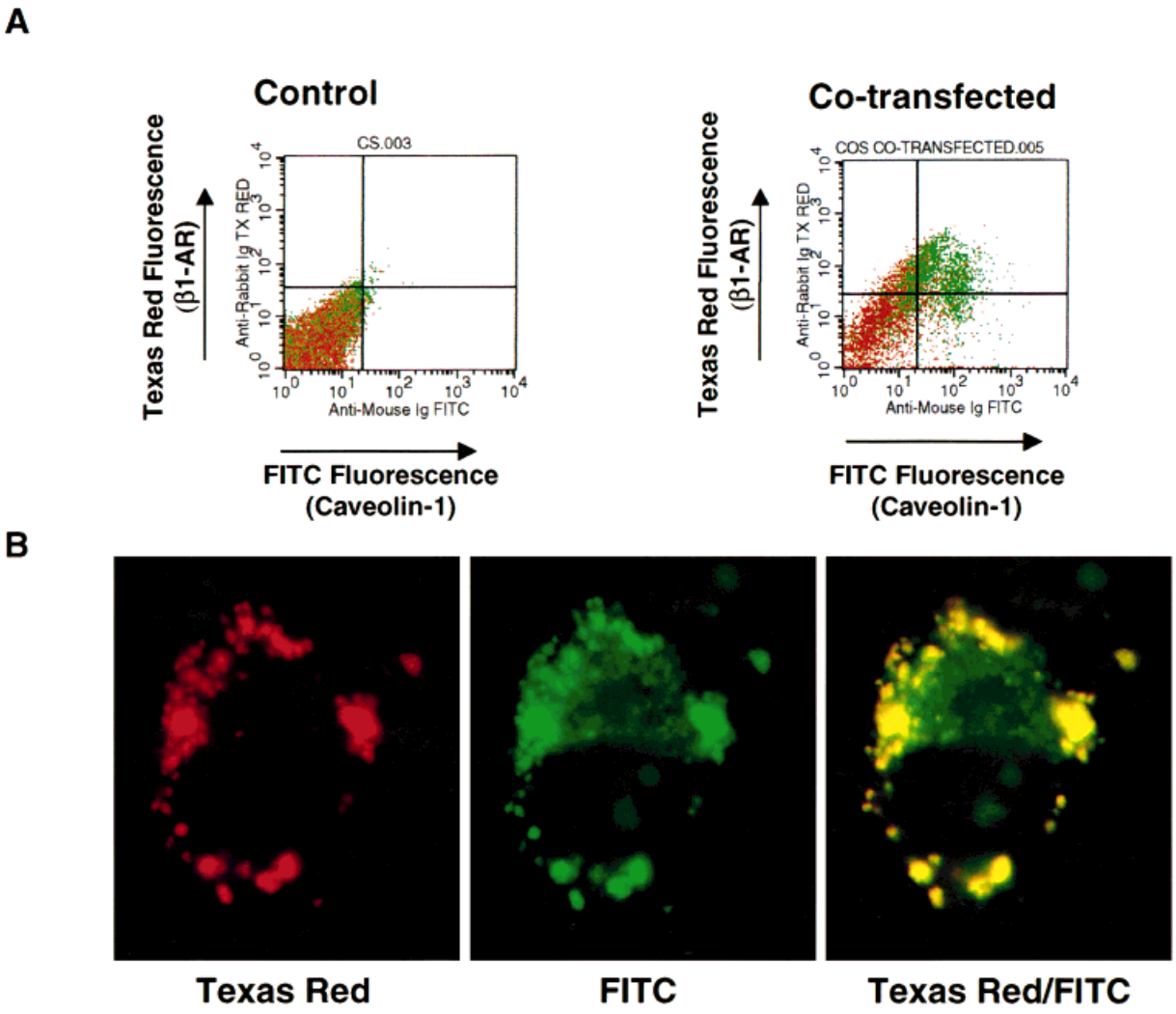

Fig. 2. Immunofluorescent analysis of COS-7 cells overexpressing β1-adrenergic receptors and caveolin-1. COS-7 cells were co-transfected with the cDNA of caveolin-1 and β1-adrenergic receptors, followed by incubation with a mouse monoclonal antibody to caveolin-1 and a rabbit polyclonal antibody to β1-adrenergic receptors. Immunostaining was performed using FITC-labeled anti-mouse- (caveolin-1) and Texas Red®-linked anti-rabbit-immunoglobulin (β1-adrenergic receptors). FACS® analysis was performed on a Becton Dickinson FACSCalibur (**A**). According to light scatter signals, two populations of COS-7 cells appeared (green and red dots). The green population showed much greater fluorescent intensity in both FL1 (for Texas Red®) and FL2 (for FITC) channels. **Left:** Results from mock-transfected control cells. **Right:** Results from caveolin-1 and β1-adrenergic receptor cotransfected cells. Note that a considerable proportion of the cotransfected cells expressed both caveolin-1 and β1-adrenergic receptors (green dots). After fixation, the distribution of β1-adrenergic receptors and caveolin-1 was visualized by immunofluorescent staining (**B**) using Texas Red® (left: β1-adrenergic receptors, red signal), and FITC (middle, caveolin-1, green signal) labeled immunoglobulins, or a combination of both images (right, yellow signal). A representative result is shown.

Previous data on the localization of β-adrenergic receptors in the plasma membrane of resting cells have been contradictory. Some immunohistochemical studies [Raposo et al., 1989; Saffitz and Liggett, 1992], including a most recent one using green-fluorescent protein-tagged β-adrenergic receptors [Kallal et al., 1998] have suggested that unstimulated β-adrenergic receptors are diffusely distributed on the cell surface. In contrast, others have observed a punctate, nonrandom immunofluorescence staining of β-adrenergic receptors throughout the cell surface [Wang et al., 1989; Zemcik and Strader, 1988]. Our data support the latter; however, differences in cell lines and their conditions examined in these studies may need to be considered. The subcellular localization and sequestration of β-adrenergic recep-

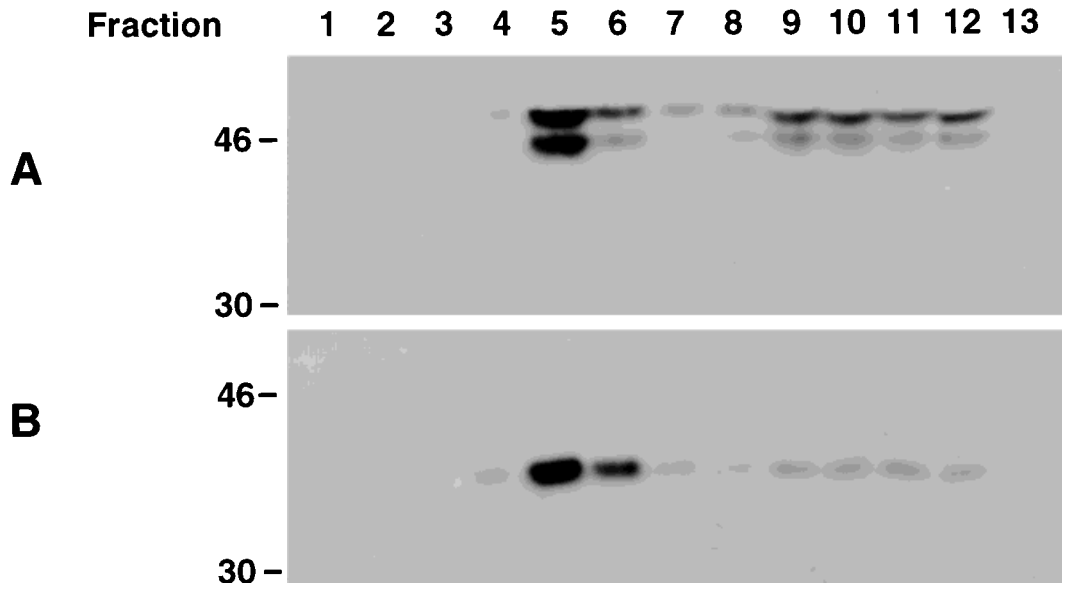

Fig. 3. Subcellular distribution of endogenous G protein α- and Gβγ- subunits. The subcellular distribution of endogenous Gsα in COS-7 cells (**A**) was detected by immunoblotting after detergent-free fractionation. Note that both Gsα subtypes (45 and 52 kDa) were detected in fraction 5 and 6 as well as in 9–12. To detect endogenous Gβγ-subunits (**B**), an antibody directed against Gβ was utilized as Gβ- and γ-subunits are tightly attached to each other and form a functional monomer. Endogenous Gβγ-subunits were detected predominantly in fraction 5 and 6 of the sucrose gradient.

tors upon agonist treatment has been controversial as well. Immunocytochemical analysis of the cellular localization of β-adrenergic receptors demonstrated an agonist-induced internalization via non-clathrin-coated vesicles [Raposo et al., 1989]. The colocalization of agonist-stimulated β-adrenergic receptors with caveolin has prompted the hypothesis that these receptors can utilize a sequestration pathway through caveolae [Dupree et al., 1993]. Contrary to this hypothesis, others have demonstrated the involvement of clathrin-coated pits after the redistribution of β-adrenergic receptors upon agonist stimulation [Gagnon et al., 1998; Kallal et al., 1998; von Zastrow and Kobilka, 1992, 1994; Zhang et al., 1996, 1997]. We did not examine this issue in the current study since we first wanted to clarify the distribution of the receptor under resting conditions. Nevertheless, it is important to note that our data do not deny the functional role of clathrin-coated vesicles for the agonist-promoted internalization of β-adrenergic receptors since we do not know whether all the receptors are bound to caveolin under any conditions.

Although we did not examined the subcellular distribution of agonist-stimulated β-adrenergic receptors, various G protein-coupled receptors appear preferentially to enter a clathrin-*in*dependent pathway for endocytosis. Recent observations demonstrated the association of B2 bradykinin- [de Weerd and Leeb-Lundberg, 1997; Haasemann et al., 1998] and m2 muscarinic acetylcholine receptors [Feron et al., 1997; Pals-Rylaarsdam et al., 1997; Raposo et al., 1987] with caveolae in a ligand-dependent fashion while the endothelin receptor subtype A resides in caveolae under resting conditions as well as after agonist-binding [Chun et al., 1994]. The fact that G protein-coupled receptors utilize clathrin-dependent and -independent endocytic pathways indicates a diversification of receptor sequestration. Furthermore the specific mechanisms involved in receptor sequestration may differ among distinct cellular phenotypes.

Cytochemical experiments have suggested that adenylyl cyclase activity is associated with structures resembling caveolae [Wagner et al., 1972]. Most recently, Huang et al. [1997] demonstrated the highest specific adenylyl cyclase activity in caveolin-enriched plasma membrane fractions after detergent-free cell fractionation. Thus, it appears that molecules involved in the β-adrenergic signaling cascade are not randomly distributed, but are compartmentalized within the same microdomain of the plasma membrane. The robust nature of our findings is

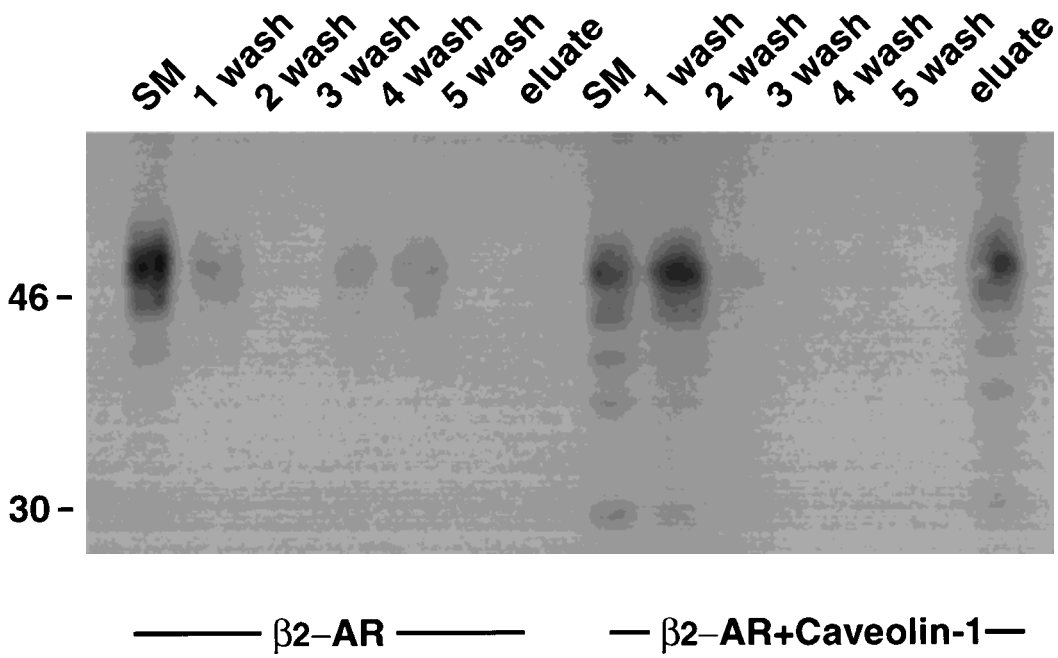

Fig. 4. Copurification of β2-adrenergic with caveolin. Caveolin-1 was co-expressed with β2-adrenergic receptors in Sf9 cells. Caveolin-enriched membrane fractions were incubated with Ni-NTA-agarose resin, which binds to polyhistidine epitope tagged-proteins. After washing, bound proteins were eluted and subjected to SDS-PAGE, followed by immunoblotting. SM (starting material), fraction 5 of sucrose gradient before incubation with the resin; RM (remaining material), supernatant after 6 h incubation with the resin; wash 1 and wash 2, wash with TBS; wash 3-wash 5, wash with TBS containing 30 mM imidazole; eluate, final elution from the resin with TBS containing 200 mM imidazole. Results from Sf9 cells overexpressing β2-adrenergic receptors alone (left) or both β2-adrenergic receptors and a polyhistidine-tagged caveolin-1 (right) are shown. Note that β2-adrenergic receptors were detected in the final eluate only in the cells coexpressing caveolin-1 with β2-adrenergic receptors.

to suggest that the interaction of β-adrenergic receptors and G proteins, even under resting conditions, appears to be more highly organized than previously thought.

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