Rac1 and RhoA regulate membrane ruffling and stress fiber formation. Both molecules appear to exert their control from the plasma membrane. In fibroblasts stimulated with platelet-derived growth factor or lysophosphatidic acid, the reorganization of the cytoskeleton begins at specific sites on the cell surface. We now report that endogenous Rac1 and RhoA also have a polarized distribution at the cell surface. Cell fractionation and immunogold labeling show that in quiescent fibroblasts both of these molecules are concentrated in caveolae, which are plasma membrane domains that are associated with actin-rich regions of the cell. Treatment of these cells with platelet-derived growth factor stimulated the recruitment of additional Rac1 and RhoA to caveolae fractions, while lysophosphatidic acid only caused the recruitment of RhoA. We could reconstitute the recruitment of RhoA using either whole cell lysates or purified caveolae. Surprisingly, pretreatment of the lysates with exoenzyme C3 shifted both resident and recruited RhoA from caveolae to noncaveolae membranes. The shift in location was not caused by inactivation of the RhoA effector domain. Moreover, chimeric proteins containing the C-terminal consensus site for Rac1 and RhoA prenylation were constitutively targeted to caveolae fractions. These results suggest that the polarized distribution of Rho family proteins at the cell surface involves an initial targeting of the protein to caveolae and a mechanism for retaining it at this site.

The Rho family of small GTPases, which include Rac1, Cdc42, and RhoA, regulate the rearrangement of actin cytoskeleton when cells are exposed to growth factors and cytokines (1). A remarkable feature of these proteins is the ability of each family member to control a specific organizational state of the actin cytoskeleton in response to a common stimulus. For example, stimulation of fibroblasts with epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) activates Ras, Rac1, and then RhoA, suggesting that a G-protein cascade might be involved (2, 3). The activated Rac1 promotes ruffling of the plasma membrane, while the activated RhoA regulates the formation of stress fibers and focal adhesion sites. Lysophosphatidic acid (LPA), by contrast, activates RhoA but not Rac1, so stress fiber formation occurs but membrane ruffling does not (3). Stress fiber formation is also much faster in response to LPA than PDGF, suggesting that the timing of RhoA activation is an important control point for cytoskeleton reorganization.

Rho family G-proteins function as molecular switches that promote the activity of effector molecules in response to exchanging GDP for GTP. As with the Ras family of G-proteins, multiple molecular interactions regulate the nucleotide state of the Rho family members, including GDP-GTP exchange factors that facilitate GTP loading and GTPase-activating proteins that stimulate GTP hydrolysis (4). The exchange process for the Rho family members, however, has an additional layer of regulation. Most GDP-bound RhoA, and probably Rac1 (5, 6), is in a complex with RhoGDI (7). Dissociation from RhoGDI is a prerequisite for nucleotide exchange, and this step correlates with translocation of Rac1 and RhoA to the plasma membrane (8). The control of membrane recruitment may involve interactions between cytosolic ERM proteins (ezrin-radixin-moesin), transmembrane proteins containing positively charged sequences such as CD44 (8, 9), and RhoGDI (6).

In fibroblasts stimulated with PDGF or LPA, the reorganization of the cytoskeleton begins at specific sites on the cell surface. We were interested in knowing if membrane-bound Rac1 and RhoA also exhibited a polarized distribution in these cells. We now show that in quiescent fibroblasts endogenous Rac1 and RhoA are both concentrated in caveolae, which are plasma membrane domains that are clustered in regions of the cell surface rich in actin filaments. Components of the protein machinery thought to be involved in recruiting these proteins to the cell surface were also enriched in these domains. Moreover, incubation of these cells in the presence of growth factors or cytokines that stimulate cytoskeleton reorganization caused additional amounts of both regulatory proteins to appear in caveolae. Ribosylation of RhoA, by contrast, caused it to relocate to noncaveolae membrane, suggesting that separate mechanisms exist for recruiting Rho family proteins to caveolae and for retaining them at this site.

**Experimental Procedures**

**Materials**

Dulbecco’s modified Eagle’s medium, fetal bovine serum, iodoxanol, penicillin, and streptomycin were from Life Technologies, Inc. EGF and exoenzyme C3 were from Calbiochem. PDGFββ was from Upstate Biotechnology, Inc. (Lake Placid, NY). Rac1 pAb IgG and RhoA mAb IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The c-Raf, RhoGEF, moesin, ROCK1, and ezrin mAbs were from Transduction Laboratories (Lexington, KY). Myc mAb was prepared in the laboratory from the 9E10 hybridoma. Myc-tagged RhoA (V14A39) in the murine leukemia virus pL1 vector was a gift of Richard Treisman. Myc-tagged Rac1 (A37/L61) in the pRK5 vector was a gift of Alan Hall. CD44 mAb IgG was from Chemicon (Temecula, CA). Horseradish peroxidase-conjugated goat anti-mouse and rabbit IgG...
were from Cappel (Durham, NC). Enhanced green fluorescent protein vector pEGFP C1 was from CLONTECH (Palo Alto, CA). Prestained molecular weight markers, 125I-Protein A and ECL reagents were from Amersham Pharmacia Biotech. PVDF membranes were from Millipore Corp. All chemicals for SDS-polyacrylamide gel electrophoresis were from Bio-Rad. All other chemicals were from Sigma.

Methods

Cell Culture—Rat-1B cells and normal human fibroblasts were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 1 mM glutamate, 100 mg/ml penicillin, and 100 mg/ml streptomycin for 24–40 h.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis—Protein concentrations were determined by the method of Bradford (Bio-Rad) using bovine serum albumin as a standard. Samples were resolved in SDS-polyacrylamide gel electrophoresis sample buffer (20 mM Tris, pH 8, 0.5% (v/v) SDS, 10% (v/v) glycerol, 0.05% (w/v) bromphenol blue), loaded on a 4% stacking gel, and separated using a 5–17% linear gradient gel. Proteins were transferred to PVDF membrane by electroblotting at 20 V for 2 h on ice. After transfer, immunoblots were blocked with 5% (w/v) milk plus 1% nonfat dry milk for 1 h at room temperature. Membranes were then washed with buffer A plus 0.2% milk once for 15 min and twice for 5 min at room temperature. The membranes were incubated with the appropriate horseradish peroxidase-conjugated goat anti-lg antibody (0.1 mg/ml) in buffer A plus 1% milk for 1 h. The membranes were then washed 1 × 15 min and 4 × 5 min with buffer A containing 0.2% milk. Staining was detected using enhanced chemiluminescence (ECL). Apparent molecular masses were estimated using prestained molecular weight markers (broad range Rainbow).

Preparation of Cell Lysates—Two methods were used to prepare cell lysates. In method 1, cells were washed in ice-cold buffer B (20 mM Tricine, pH 7.8, 1 mM EDTA, and 250 mM sucrose) and collected by scraping in the same buffer. Cells were lysed by a Dounce homogenizer in buffer B in the presence of 1 mg/ml leupeptin, 1 mg/ml peptatin A, 1 mg/ml benzamidine, and 100 mg/ml phenylmethylsulfonyl fluoride. A postnuclear supernatant fraction was prepared by spinning the lysate at 900 × g for 10 min. In method 2, cells were washed twice in ice-cold phosphate-buffered saline, scraped in buffer C (50 mM Hepes, pH 7.5, 50 mM NaCl, 1 mM MgCl2, 2 mM EDTA, 0.5 mM Na3VO4, 10 mM NaF, 1 mM dithiothreitol, 1 mM peptatin A, 1 mg/ml leupeptin, 1 mg/ml benzamidine, and 100 mg/ml phenylmethylsulfonyl fluoride), collected by centrifugation, and resuspended in buffer C. Cells were then lysed by passage through a 27-gauge needle five times.

Isolation of Caveolae—Caveolae were isolated by the method of Smart et al. (10). Cell lysates were prepared by the indicated method and layered over 23 ml of ice-cold 30% Percoll in buffer D (20 mM Tricine, pH 7.8, 1 mM EDTA) plus 250 mM sucrose. After centrifugation at 85,000 × g for 30 min, the cytosol and plasma membrane fractions were collected. The plasma membrane fraction was briefly sonicated (6 × 50-J bursts at 10 watts each), mixed with buffer E (50% (v/v) iodoxil in buffer D plus 40 mM sucrose) to a final iodoxil concentration of 23%, and overlaid with a 6-ml, linear (10–20%) gradient of 20 mM Tris, pH 7.6, 137 mM NaCl) plus 1% nonfat dry milk for 1 h at room temperature. Membranes were then washed with buffer A plus 0.2% milk for 15 min and twice for 5 min at room temperature. The membranes were then ligated into the pEGFP C1 vector using the EcoRI and XhoI restriction enzymes. The expression vectors for both Rac1 and RhoA were added to the carboxyl end of enhanced green fluorescence protein (EGFP) by ligating the appropriate coding sequences in frame to the C-terminal end of the EGFP using the pEGFP C1 vector cloning kit (Clontech). Two methods were used to prepare cell lysates. In the first method, caveolae were prepared from 50-100-mm dishes of serum-starved Rat-1B fibroblasts using method 2. The lysates were divided into 1-ml portions and incubated separately in the presence of 1 mM GDP, 1 mM GTP, 100 μM GTPγS, or no nucleotide for 30 min at the indicated temperature. In some cases, these mixtures were incubated in the presence or absence of exoenzyme C3 (5 μM/ml) plus 130 μM NADH for 10 min at 30 °C prior to the addition of nucleotide. Cytosol and caveola fractions were then prepared by standard methods. In the second method, cytosol, plasma membrane, noncaveolae membrane, and caveola were isolated from cell lysates prepared by method 2. Cytosol (250 μg) was mixed with 10 μg of purified plasma membrane, noncaveolae membrane, or caveola in buffer F (phosphate-buffered saline plus 0.8% bovine serum albumin, pH 7.6), washed for 30 min with buffer F, and then gold-conjugated to either anti-mouse or anti-rabbit IgG for 60 min in buffer F and washed with buffer F for 30 min. For double labeling, membranes were incubated in the following sequence: the primary antibody (either 5 μg/ml pAb anti-caveolin-1 IgG or 15 μg/ml mAb anti-RhoA IgG for 60 min in buffer F; phosphorylated saline plus 0.8% bovine serum albumin, pH 7.6), washed for 30 min with buffer F, and then gold-conjugated to either anti-mouse or anti-rabbit IgG for 60 min in buffer F and washed with buffer F for 30 min. After washing, membranes were incubated with the following sequence: the first indicated antibody (either 15 μg/ml mAb anti-RhoA IgG or 5 μg/ml pAb anti-RhoA IgG for 60 min in buffer F) plus 1% wheat germ agglutinin, 50 μg/ml streptomycin for 24–40 h.

Immunogold Labeling—Plasma membranes from normal human fibroblasts were attached to poly-L-lysine-coated formvar (11) and immuno-gold-labeled with antibodies to RhoA, caveolin-1, or clathrin using either a single labeling or a double labeling protocol. For single antibody labeling, membranes were incubated in the following sequence: the primary antibody (either 5 μg/ml pAb anti-caveolin-1 IgG or 15 μg/ml mAb anti-RhoA IgG for 60 min in buffer F; phosphorylated saline plus 0.8% bovine serum albumin, pH 7.6), washed for 30 min with buffer F, and then gold-conjugated to either anti-mouse or anti-rabbit IgG for 60 min in buffer F and washed with buffer F for 30 min. For double labeling, membranes were incubated in the following sequence: the first indicated antibody (either 15 μg/ml mAb anti-RhoA IgG or 5 μg/ml pAb anti-RhoA IgG for 60 min in buffer F) plus 1% wheat germ agglutinin, 50 μg/ml streptomycin for 24–40 h.

Expression Studies—Either the consensus site for prenylation (CAAX) or the CAAX box plus the adjacent polybasic region (CAAXPB) for both Rac1 and RhoA was added to the carboxyl end of enhanced green fluorescent protein (EGFP) by ligating the appropriate coding sequences in frame to the C-terminal end of the EGFP using the pEGFP C1 vector cloning kit (Clontech). Two methods were used to prepare cell lysates. In the first method, caveolae were prepared from 50-100-mm dishes of serum-starved Rat-1B fibroblasts using method 2. The lysates were divided into 1-ml portions and incubated separately in the presence of 1 mM GDP, 1 mM GTP, 100 μM GTPγS, or no nucleotide for 30 min at the indicated temperature. In some cases, these mixtures were incubated in the presence or absence of exoenzyme C3 (5 μM/ml) plus 130 μM NADH for 10 min at 30 °C prior to the addition of nucleotide. Cytosol and caveola fractions were then prepared by standard methods. In the second method, cytosol, plasma membrane, noncaveolae membrane, and caveola were isolated from cell lysates prepared by method 2. Cytosol (250 μg) was mixed with 10 μg of purified plasma membrane, noncaveolae membrane, or caveola in buffer F (phosphate-buffered saline plus 0.8% bovine serum albumin, pH 7.6), washed for 30 min with buffer F, and then gold-conjugated to either anti-mouse or anti-rabbit IgG for 60 min in buffer F and washed with buffer F for 30 min. After washing, membranes were incubated with the following sequence: the first indicated antibody (either 15 μg/ml mAb anti-RhoA IgG or 5 μg/ml pAb anti-RhoA IgG for 60 min in buffer F) plus 1% wheat germ agglutinin, 50 μg/ml streptomycin for 24–40 h.

RESULTS

Rac1 and RhoA Are Dynamically Associated with Caveolae—The cellular distribution of Rac1 and RhoA in quiescent Rat-1B fibroblasts was determined as a base line for further investigation. Equal amounts of protein (5 μg/lane) from the postnuclear supernatant fraction, cytosol, plasma membrane, noncaveolae membrane, and caveola were separated by gel electrophoresis and immunoblotted with specific antibodies (Fig. 1). Relative to the plasma membrane, Rac1, RhoA, and caveolin-1 (Cav1) were highly enriched in the caveola fraction. Rac1 and RhoA appeared to be more enriched in the caveola fraction than in the other membranes.
cytosol. Using quantitative immunoblotting, we determined that Rac1 and RhoA were enriched ~23-fold in the caveola fraction relative to the plasma membrane. We did not detect any RhoGDI in the caveola fraction even after loading up to 20 μg of protein in each lane (data not shown). Therefore, endogenous Rho family proteins appear to be constitutively concentrated in caveolae of quiescent fibroblasts. The absence of RhoGDI in the caveola fraction suggests the Rac1 and RhoA in this fraction are either available for activation or already activated.

To confirm that the Rho family proteins in the caveola fraction were associated with invaginated caveolae, we used immunogold to co-localize RhoA with caveolin-1 on the inner surface of isolated plasma membranes attached to formvar grids (Fig. 2). One set of membranes from normal human fibroblasts was labeled directly with either RhoA mAb (A) or caveolin-1 pAb (C). Another set was double labeled with either RhoA mAb (5-nm gold) plus caveolin-1 pAb (15-nm gold) (B) or clathrin mAb (5-nm gold) plus caveolin-1 pAb (15-nm gold) (D). As indicated by the localization of caveolin-1 pAb gold (C, large gold particles), invaginated caveolae in these air-dried preparations appear as pear-shaped protuberances that at higher magnification (A and B, insets) seem to be outlined by a bilayer structure. Virtually all of these structures were decorated with multiple, 15-nm gold particles when the membranes were probed with anti-caveolin-1 IgG (C). Similarly, many of these same domains labeled with 5-nm gold particles when RhoA mAb was used (A, arrowheads and inset). The surrounding membranes were nearly devoid of gold particles (A and C). When the membranes were double-labeled using 5-nm (RhoA) and 15-nm (caveolin-1) gold particles, both sizes of particles were clustered in association with invaginated caveolae (B, inset). Occasional clusters of RhoA-gold that were not associated with caveolin-1 pAb gold were also seen. As a test for co-localization specificity, we co-localized clathrin (5 nm) and caveolin-1 (15 nm) using clathrin mAb and found that each was associated with their respective domains but not with each other (D).

The amount of Rac1 and RhoA in the caveola fraction changed when we stimulated cells with growth factors and cytokines (Fig. 3). Immunoblots of caveola fractions from cells incubated in the presence of PDGF for various times (PDGF) showed that the concentration of Rac1 increased within 1–3 min and remained at this level for the remainder of the incubation. RhoA, by contrast, increased much more slowly, reaching a maximum at 60 min. The rapid recruitment of Rac1 followed by a slower appearance of RhoA in the caveola fraction matched the respective time frame for the assembly of membrane ruffles (Rac1-dependent) and stress fibers (RhoA-dependent) (2). When we treated cells with LPA, which stimulates stress fiber formation within 10 min but has no effect on membrane ruffling (3), RhoA was rapidly recruited to the caveola fraction (LPA, Fig. 3) while the concentration of Rac1 remained relatively unchanged. In contrast to the differential behavior of Rac1 and RhoA, both PDGF and LPA caused the same amount of Raf-1 to be recruited to the caveola fraction, and with similar kinetics (12). Activation of adenylyl cyclase with 10 μM forskolin, which blocks Raf-1 recruitment to caveolae (12), had no effect on EGF-stimulated recruitment of Rac1 and RhoA (data not shown). None of the treatments we tested had any effect on the amount of caveolin-1 in the caveola fraction (Cav1).

Recruitment of RhoA to Caveolae—ERM proteins have been implicated in the membrane recruitment (6) and activation (13) of Rho family proteins. We used immunoblotting to determine if these proteins were present in caveola fractions (Fig. 4). Both moesin and ezrin were highly enriched in fibroblast caveolae.

![Image of immunogold co-localization of RhoA and caveolin-1](image-url)
lae fraction compared with the plasma membrane (PM) and cytosol (CYT) fractions. Immunofluorescence also showed that caveolin-1 co-localized with both ezrin and moesin (data not shown). ERM proteins form a complex in vitro with a plasma membrane hyaluronic acid binding protein called CD44 (8). Immunoblotting showed that the 85-kDa form of CD44 is highly enriched in human fibroblast caveolae relative to noncaveolae membrane and cytosol fractions.

The traffic of Rho proteins through caveolae may be necessary for their activation. Several studies have shown that GTPγS will stimulate the recruitment of Rho proteins to the membrane fraction of cell lysates (14) and induce cytoskeleton reorganization in permeabilized cells (13). To determine if GTPγS would stimulate the recruitment of Rho proteins to caveolae, we made cell lysates and added either GDP, GTP, GTPγS, or buffer. The lysates were then incubated at either 4 or 30 °C for 30 min (Fig. 5) before membranes were separated from cytosol and used to prepare caveolae and noncaveolae fractions. Immunoblotting either cytosol (cytosol) or caveolae (caveolae membrane) fractions showed that the basal amount of RhoA in both fractions was unaffected by either 30 °C incubation (compare lanes 1 and 2 and lanes 6 and 7), GDP (lanes 3 and 8), or GTP (lanes 4 and 9). The addition of GTPγS, by contrast, caused a marked increase in the amount of RhoA in the caveolae fraction (compare lanes 6 and 10). Under the same conditions, RhoA was no longer detectable in the cytosol fraction (lane 5). Therefore, GTPγS appears to stimulate the movement of cytosolic RhoA to caveolae. The targeting was specific because we did not detect any increase in the concentration of RhoA in noncaveolae membrane (data not shown). Therefore, the caveolae fraction contains functional machinery for recruiting RhoA.

Table 1. GTPγS Stimulates Movement of RhoA from Cytosol to Caveolae

| Cytosol | CM | Fraction |
|---------|----|---------|
| GDP | GTP | GDP | GTP | GDP | GTP |
| 4 | 30 | 30 | 30 | 4 | 30 | 30 | 30 |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

Retention of RhoA in Caveolae—To determine if the effector domain of RhoA might be involved in its localization to caveolae (Fig. 6), we looked at the effects of exoenzyme C3. This enzyme ADP-ribosylates asparagine 41 in one of the two effector regions within RhoA (15) and blocks its ability to control cytoskeleton reorganization (3). Cell lysates were prepared and incubated in the presence or absence of C3 for 10 min at 30 °C before the addition of GDP, GTP, or GTPγS for an additional 30 min at 30 °C. The cell lysates were then fractionated into cytosol (CYT, 50 μg/lane), plasma membrane (PM, 20 μg/lane), noncaveolae membrane (NCM, 20 μg/lane), and caveolae membrane (CM, 5 μg/lane) and processed for immunoblotting. Regardless of whether or not GDP was present (compare lanes 1 and 2), immunoblots detected RhoA in the cytosol, plasma membrane, and caveolae but not the noncaveolae fractions (note that the noncaveolae lane was loaded with 4 times more protein than the caveolae lane). The addition of GTP had no effect on the amount of RhoA in any of the fractions (compare lanes 1 and 4). By contrast, GTPγS stimulated an increase in the concentration of RhoA in isolated plasma membranes (compare lanes 4 and 5). Fractionation of this membrane showed that the concentration of RhoA was specifically increased in the caveolae fraction (compare lanes 4 and 5). When we preincubated the lysate with C3 before adding GDP, the molecular weight of the cytosolic RhoA shifted (lane 3), indicating ribosylation, and the amount of RhoA in the caveolae fraction markedly declined (compare lanes 2 and 3). Unexpectedly, C3 caused RhoA to appear in the noncaveolae fraction in the presence of GTPγS instead of the caveolae fraction (compare lanes 5 and 6). The total amount of RhoA translocated to the plasma membrane was similar (compare lanes 5 and 6), consistent with the observation that C3 ADP-ribosylation does not affect nucleotide binding or exchange (15). These results suggest that the effector domain plays a role in the localization of RhoA to caveolae.
We used immunogold labeling to see if C3 affected the level of RhoA in invaginated caveolae (Table I). Membranes attached to a formvar grid were incubated in the presence or absence of C3 for 30 min at 30 °C. At the end of the incubation, the membranes were processed for the immunogold localization of RhoA. Samples were quantified by standard methods. In two separate experiments, we found that C3 reduced the number of gold particles per caveolae by 47 and 32%.

We noted that the residual RhoA in the caveola fraction following C3 treatment appeared not to be ribosylated (Fig. 6, lanes 3 and 6) and that C3 caused a decline in the amount of endogenous RhoA in caveolae (Fig. 6, lane 3). Since these treatments were carried out prior to sonication, the plasma membrane was intact during the C3 treatment. Thus, C3 could influence RhoA localization either by inducing recruitment of RhoA to noncaveolae membrane or by promoting the migration of RhoA from caveolae to noncaveolae membranes. To distinguish between recruitment and retention in caveolae, the recruitment step was analyzed using isolated membrane fractions (Fig. 7). Noncaveolae membrane or caveolae membrane (10 μg) was mixed with cytosol (250 μg of protein) in the presence of GTPγS and incubated for various times at 30 °C (A). Membrane and supernatant fractions were prepared and analyzed by immunoblotting (10 μg/lane). Initially, RhoA was present in caveolae and supernatant fractions (lane 1) but absent from noncaveolae membrane (lane 5). After the addition of GTPγS, however, there was a rapid increase in the concentration of RhoA in the caveolae fraction and a corresponding decline in the amount in the supernatant fraction. Some RhoA was also recruited to noncaveolae membranes under these conditions (lanes 5–8). The 30-min incubation time was used to determine if C3 affected the recruitment of RhoA (B). In the absence of C3, GTPγS, but not GTP, stimulated recruitment to caveolae (compare lanes 7 and 8). After C3 treatment, GTPγS still caused approximately the same amount of ribosylated RhoA to appear in caveolae (compare lanes 8 and 9). There was some recruitment to noncaveolae membrane, which also was not affected by C3 (lanes 4–6). Therefore, ribosylation does not appear to block the initial targeting to caveolae but instead affects the ability of RhoA to remain in this domain once it has arrived.

Ribosylated RhoA may migrate out of caveolae because it is unable to interact with one or more resident effector molecules. Therefore, we looked at the localization of RhoA (V14/A39) and Rac1 (L61/A37), which bear mutations that reduce the activity of their effector domains. Rat-1 cells were transiently transfected with Myc-tagged versions of these two proteins, and the various fractions were separated (Fig. 8A). Equal protein loads of each fraction were separated and immunoblotted with the a Myc pAb. Both mutant proteins were concentrated in the caveolae fraction to the same degree as their endogenous counterparts (compare with Fig. 1).

Another possibility is that C3 shifts RhoA effector molecules to the noncaveolae membrane, taking the caveolae RhoA with it. A likely effector to be involved in stress fiber formation is ROCK (16), so we looked at the effect of C3 on the distribution...
FIG. 8. Localization of RhoA and Rac1 to caveolae does not depend on effector interactions. A, Rat-1 cells were transiently transfected either with Myc-tagged RhoA (V14/A39) or Rac1 (L61/A37) cDNA. Cells were grown to confluence and fractionated into postnuclear supernatant (PNS), cytosol (CYT), plasma membrane (PM), noncaveolae membrane (NCM), or caveolae membrane (CM). Samples were separated and immunoblotted with a Myc mAb. B, cell lysates were prepared from Rat-1 cells and incubated in the presence of either buffer, GTP S or C3 plus GTP S, using the same conditions as described in the legend to Fig. 4. The lysates were fractionated, and each fraction was processed for immunoblotting with either a ROK a mAb (lanes 4–6) or a RhoA mAb (lanes 1–3).

of this protein (Fig. 8B). Cell lysates were prepared and incubated in the presence of buffer alone (lanes 1 and 4), GTP S (lanes 2 and 5), or GTP S plus C3 (lanes 3 and 6) for 30 min at 30 °C. At the end of the incubation, the plasma membrane was separated from the cytosol and fractionated into caveolae and noncaveolae membranes. Each sample was then immunoblotted with either anti-RhoA (lanes 1–3) or anti-ROKa (lanes 4–6) mAb. C3 shifted recruited RhoA from caveolae (lane 2) to noncaveolae (lane 3) membrane, just as was seen in Fig. 5. ROKa was enriched in the caveolae fraction, but neither C3 nor GTP S had any effect on the distribution of the protein. Therefore, the C3-dependent shift of RhoA to the noncaveolae fraction appears not to involve an interaction with this effector molecule.

These experiments suggest that RhoA is initially targeted to caveolae but can migrate from this domain unless retained. The retention mechanism appears to be inactivated by ribosylation. Since lipid modifications such as GPI or fatty acids will target proteins to caveolae (17), we were interested to know if prenylation has a role in Rho protein localization. We constructed two sets of chimeric proteins using EGFP for the core part of the protein. One set contained the C-terminal CAAX box of either RhoA or Rac1, while the other set had the respective CAAX boxes plus the corresponding adjacent polybasic domains. Cells were transiently transfected with the cDNA coding for these constructs or with the EGFP alone (Fig. 9). Cells expressing these proteins were then fractionated and immunoblotted with green fluorescent protein mAb. All of the unmodified EGFP was in the cytosol fraction (CYT). By contrast, proteins containing either the CAAX box (lanes 2 and 3) or the CAAX box plus the polybasic domain (lanes 4 and 5) were all highly enriched in the caveolae fraction (CM). Therefore, the simple addition of a prenyl group is sufficient to concentrate a protein in this membrane domain.

FIG. 9. Prenylation is sufficient to localize a protein to caveolae. Cells, transiently expressing EGFP, EGFP-CLVL, EGFP-CLLL, EGFP-RGKX, or EGFP-KKRX, were harvested and fractionated as described. Samples (5 μg) of postnuclear supernatant (PNS), cytosol (CYT), plasma membrane (PM), noncaveolae membrane (NCM), and caveolae membrane (CM) were processed for immunoblotting using a green fluorescent protein mAb.

DISCUSSION

Previous studies have established that Rho family proteins are recruited to the plasma membrane when cells are stimulated with growth factors and cytokines (5, 14). We have used a combination of in vitro and in vivo techniques to look at the distribution of endogenous Rac1 and RhoA in quiescent and stimulated cells. Immunoblotting showed that RhoA and Rac1 were constitutively enriched (23–fold) in the caveolae fractions, while immunogold labeling showed that RhoA was associated with invaginated caveolae. The concentration of both proteins in noncaveolae membranes was markedly lower. When membrane ruffling was stimulated, additional Rac1 appeared in the caveolae fraction. Activation of stress fiber formation, by contrast, caused the recruitment of RhoA to caveolae. The recruitment step could be reconstituted in vitro using GTP S. Surprisingly, exoenzyme C3 caused the caveolae-associated RhoA to move laterally to noncaveolae membrane without affecting the initial recruitment step.

Two Requirements for Localization of Rac1 and RhoA to Caveolae—ERM proteins may control the translocation of Rac1 and RhoA to caveolae. These proteins appear to be involved in the activation of Rac1 and RhoA, since they are required for GTP S-dependent stimulation of membrane ruffling and stress fiber formation in permeabilized cells (13). They may do so by binding RhoGDI, releasing the inhibition of GDP-GTP exchange and facilitating the insertion of the RhoA or Rac1 into the membrane (6, 18). The co-localization of ezrin and moesin with caveolin-1 implies that caveolae are a primary membrane site of insertion and that most of the Rac1 and RhoA in caveolae may be in an activated state.

Previous immunofluorescence studies have shown that CD44 and ERM co-localize after cells are extracted with Triton X-100 and that GTP S stimulates the binding of purified ERM proteins to Triton X-100-insoluble segments of membrane containing CD44 (6, 8). These results suggest that CD44 works cooperatively with ERM to recruit RhoA-RhoGDI to membrane regions that are linked to the cytoskeleton. However, caveolae are also Triton X-100-insoluble (17), and no attempt was made in these studies to remove caveolae from the fraction. This raises the possibility that the interactions between CD44 and ERM detected in those studies were actually occurring in Triton X-100-insoluble caveolae rather than in membrane linked to the cytoskeleton. In support of this interpretation, ezrin has previously been found to be enriched in Triton X-100 insoluble caveolae fractions (19); immunoprecipitation assays have shown that RhoA interacts with caveolin-1 (20); immunoblots
There is a growing body of evidence that the multiple molecular interactions required for cells to process information received from their environment occurs in specific membrane domains such as caveolae, focal adhesion sites, or sites of cell-cell contact. Previous work has shown that in quiescent cells EGFR and PDGF receptors (12, 26), along with Ras (12), SOS, Grb2, the regulatory subunit of phosphatidylinositol 3-kinase (12, 26, 27), and phosphatidylinositol 4,5-diphosphate (22), are concentrated in caveolae. Briefly incubating these cells in the presence of either EGFR or PDGF increases the tyrosine phosphorylation of multiple substrates in caveolae (12, 26) and activates a resident population of mitogen-activated protein kinase (27). Identification of caveolae as a site where Rac1 and RhoA are initially recruited, therefore, implies that the critical molecular interactions required for linking growth factor receptors to cytoskeletal reorganization can take place in this domain.

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