Rab GDP Dissociation Inhibitor as a General Regulator for the Membrane Association of Rab Proteins*

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Rab proteins comprise a family of small GTPases that serve a regulatory role in membrane traffic. These proteins are in part cytosolic and in part associated with the membranes of specific exocytic and endocytic organelles. Smg p25A/rab3A GDI, a cytosolic protein which inhibits the dissociation of GDP from smg p25A/rab3A, Sec4p, and rab11, has also been found to prevent association of rab3A with the membrane. In this study, we have used Madin-Darby canine kidney cells permeabilized with the bacterial toxin streptolysin O to test the general activity of rab3A GDI in modulating the membrane association of various small GTP-binding proteins. Rab3A GDP dissociation inhibitor (GDI) removed from the membrane all rab proteins we have tested and inhibited the membrane binding of in vitro translated rab proteins. However, rab3A GDI had a limited effect on the membrane association of a mutant rab5 protein which contained a farnesylated cysteine motif. Finally, we found that, although rab3A GDI resides primarily in the cytosol, it is also associated with compartments of the exocytic and endocytic pathways. Since rab3A GDI can modulate the membrane association of various rab proteins, we propose to rename it rab GDI.

Many genetic and biochemical approaches have now illustrated the implication of small GTPases in the regulation of intracellular vesicular traffic (reviewed in Pfeffer, 1992; Takai et al., 1992). However, the functional mechanism of these proteins still remains unclear. The GTPase cycle of Ypt1p, Sec4p, and rab proteins has been postulated to monitor the specificity of interaction between components of the transport machinery on the vesicle and on the acceptor compartment (Bourne, 1988; Bourne et al., 1990). According to this model, a specific rab protein would be recruited on the donor membrane or on nascent transport vesicles by a specific receptor. This complex would then be recognized by a docking machinery on the target membrane. Hydrolysis of GTP by the rab protein would trigger the fusion of the membrane of the vesicle with that of the acceptor compartment. After GTP hydrolysis, the GTPase would be returned to the donor membrane via the cytosol to perform multiple rounds of vesicular transport.

This hypothesis has originally been supported by the finding that a fraction of these GTPases is present in a soluble cytoplasmic pool.

In a search for regulatory factors for rab proteins, a protein that inhibits the dissociation of GDP but not GTP from rab3A (formerly called smg p25A) was purified from bovine brain cytosol (Sasaki et al., 1990). The cDNA of this factor, rab3A GDI, was subsequently cloned and its primary structure was determined. The protein is composed of 447 amino acids with a calculated $M_t$ of 50,565 (Matsui et al., 1990). Rab3A GDI inhibits the binding to and induces the dissociation from synaptic membranes and vesicles of the GDP-bound form but not of the GTP-bound form of rab3A (Araki et al., 1990). In addition, rab3A GDI has also been found to be active on Sec4p and rab11 (Sasaki et al., 1991; Ueda et al., 1991) and has been shown to form a complex with low molecular weight GTP-binding proteins in the cytosol of insulin-secreting cells (Regazzi et al., 1992). The tissue distribution analysis of rab3A GDI has revealed that it is found in both regulated and constitutive secretory cells (Nonaka et al., 1991).

Rab proteins display an organelle-specific distribution (Pfeffer, 1992). The COOH-terminal regions of these proteins play an important role in the localization process. They include distinct cysteine motifs that are subjected to isoprenylation (Farnsworth et al., 1991; Khoosravi-Far et al., 1991; Kinsella and Maltese, 1992; Peter et al., 1992). While this post-translational modification is essential for membrane association, it does not confer specificity to this process. Other sequences upstream from the cysteine motif determine the association of rab proteins with their specific target membrane (Chavrier et al., 1991). Consistent with the variable location of rab proteins, their COOH-terminal sequences are highly divergent. Additionally, in the case of rab3A, the COOH-terminal region of this protein has been found to be required for the interaction with rab3A GDI (Araki et al., 1991). Owing to the structural divergence in the COOH terminus of rab proteins, these findings raise the question of the specificity of rab3A GDI for the various members of the rab protein family.

In this paper, we have extended the functional studies on rab3A GDI using an in vitro system. First, we show that rab3A GDI removed all endogenous rab proteins we have tested from the membrane of permeabilized cells and inhibited the membrane binding of in vitro translated rab proteins. Second, rab3A GDI could not efficiently interact with a rab5 mutant protein containing a farnesylated cysteine motif. Third, we...
present evidence that rab3A GDI resides primarily in the cytosol, but is also associated with the membrane of various intracellular compartments.

MATERIALS AND METHODS

MDCK cells were grown as described before by Babacaloo et al. (1988). Rab3A GDI was purified from bovine brain cytosol (Sasaki et al., 1992). As a positive control, glutathione S-transferase-rab3A GDI fusion protein was obtained by cloning the rab3A GDI cDNA into plasmid pGEX-2T and purifying the fusion protein from transformed Escherichia coli JM109 by using a glutathione-Sepharose 4B column according to the manufacturer's manual. Reduced streptolysin O (SLO) was obtained from Wellcome Diagnostics, Dartford, United Kingdom. The mouse monoclonal antibody against G-20F was a generous gift of T. Kreis (University of Geneva, Switzerland). The fluorescent conjugated secondary antibodies and the horseradish peroxidase-labeled second antibodies for Western blot analysis were obtained from Dianova (Hamburg, Federal Republic of Germany). [1-3H]Farnesyl pyrophosphate (15 Ci/mmol, 98% purity) and [1-3H]geranylgeranyl pyrophosphate (26 Ci/mmol, 99% purity) were purchased from American Radiochemical Corp.

Preparation of Antibodies—Polyclonal antibodies against rab2, rab5, rab7, and rab8 were raised against synthetic COOH-terminal peptides as described (Chavrier et al., 1992). Rabbit anti-rab11 and anti-rab 9 antisera were raised against recombinant bacterially expressed proteins and were then affinity purified (Zerial et al., 1989a; Lombardi et al., 1993). Generation and purification of polyclonal antibodies against a synthetic COOH-terminal GDI-peptide (AESPENMKRRQNDVPGEADQ) was carried out according to Zerial et al. (1992).

Cell Permeabilization with Streptolysin O—MDCK cells grown on plastic dishes were washed once with cold (4°C) PBS. Permeabilization was then achieved by a two-step procedure. First, the toxin was bound to the plasma membrane by incubating cells for 15 min at 4°C with 8 IU/ml of SLO. The excess of toxin was then removed, and the cells were washed once with cold (4°C) intracellular transport (ICT) buffer (78 mM KCl, 50 mM HEPES-KOH, pH 7.0, 4 mM MgCl2, 10 mM EGTA, 8.37 mM CaCl2, 1 mM DTT, Burke and Gerace, 1986). In the permeabilization step, cells were incubated for 15 min at 37°C with ICT buffer. The efficiency of permeabilization was determined by accessibility of antibodies to intracellular antigens (Gravotta et al., 1990) and release of endogenous rab proteins after incubation of permeabilized cells with 0.8 μM of rab3A GDI. Under these conditions, almost 100% of the cells became permeabilized.

Assay for rab3A GDI in Intracellular Translation Products—In vitro translation/transcription experiments, rab cDNAs (Chavrier et al., 1990b) were cloned into pGEM1 plasmid (Promega) under the control of the bacteriophage T7 RNA polymerase promoter, and plasmids were then linearized with suitable restriction enzymes. In order to construct rab5-CVLS, the AccI-HindIII fragment of this polymerase chain reaction product (ICT) buffer (78 mM KCl, 50 mM HEPES-KOH, pH 7.0, 4 mM MgCl2, 10 mM EGTA, 8.37 mM CaCl2, 1 mM DTT, Burke and Gerace, 1986). In the permeabilization step, cells were incubated for 15 min at 37°C with ICT buffer. The efficiency of permeabilization was determined by accessibility of antibodies to intracellular antigens (Gravotta et al., 1990) and release of endogenous rab proteins after incubation of permeabilized cells with 0.8 μM of rab3A GDI. Under these conditions, almost 100% of the cells became permeabilized.

In Vitro Translation/Transcription—For in vitro transcription, 1 μg of linearized rab plasmid DNA was transcribed with 25 units of phage T7 RNA polymerase (NEB) for 1 h at 40°C in a volume of 10 μl. The reaction mixture contained 4 mM GTP of each of the four nucleoside triphosphates, 40 mM Tris-HCl, pH 7.6, 6 mM MgCl2, 2 mM spermidine, 10 mM DTT, and 10 units RNasin (Promega).

In vitro translations were carried out in nuclease-treated reticulocyte lysate according to the manufacturers’ instructions. 1 μg of uncapped RNA was translated at 30°C for 1 h in a reaction volume of 100 μl. To improve the isolation of the proteins, in vitro translations were performed in the presence of 5 mM mevalonic acid (generated by alkaline hydrolysis of mevalonic acid lactone as described by Kita et al. (1980) and were followed by an additional incubation at 37°C for 30 min. Proteins were labeled with [35S]methionine (1 mCi/ml final concentration, Amersham SJ1010) using a methionine-free amino acid mix (Promega).

Isoprenoid Analysis of in Vitro Translated Rab Proteins—1 μg of rab and rab5-CVLS-RNA were in vitro translated for 1 h at 30°C in 100-μl reactions containing [3H]farnesyl pyrophosphate (2 μCi, 15 Ci/mmol) or [3H]geranylgeranyl pyrophosphate (2 μCi, 26 Ci/mmol). Prior to use, the radioactive isoprenoids were dried under a stream of nitrogen in order to opalescent conditions. In order to determine the concentration of isoprenoids, after the incubation the mixture was adjusted to 5 mM MgCl2, 1 mM DTT, and incubated for an additional 60 min at 37°C. The reactions were then ultracentrifuged (1 h, 100,000 g) to generate a cytosol and a membrane fraction. Proportional amounts of membrane and cytosolic fractions were fractionated on SDS-PAGE and analyzed by quantitative Western blotting, using purified rab3A GDI (Sasaki et al., 1992) as a standard. For detection of the protein we used anti-GDI polyclonal antiserum in combination with the enhanced chemiluminescence detection system (Amersham Corp.).

Immunofluorescence Microscopy—MDCK cells were grown on 11-mm round coverslips for 36 h prior to treatment. The cells were washed twice with PBS and then fixed with 3% paraformaldehyde in PBS for 30 min. After fixation the cells were washed with PBS for 10 min and free aldehyde groups were quenched with 50 mM NH4Cl in PBS for 15 min. This was followed by washes with PBS for 10 min and permeabilization by incubating the cells with 0.1% Triton X-100 in PBS for 5 min. After rinsing the coverslips with PBS for 10 min, the cells were incubated with the first antibody in PBS, 5% fetal calf serum for 30 min. The cells were washed with PBS for 15 min and primary antibody binding was visualized with rhodamine-conjugated donkey anti-rabbit or FITC-conjugated donkey anti-mouse antibodies in PBS, 5% fetal calf serum for 20 min. Finally, the cells were washed in PBS for 15 min, and the coverslips were mounted in moviol. For double immunofluorescence, primary antibodies were added together. Confocal fluorescence analysis was performed with the EMBL confocal microscope and the images were photographed with a T-Max 100 ASA film (Kodak).

Internalization of FITC-Transferrin—Canine aprotinin (Sigma) was iron saturated by incubating the meniscus (5%) in the presence of 6 μg of HEPES, pH 7.5, 150 mM NaCl as the elution buffer.
Prior to internalization of FITC-transferrin, the subconfluent MDCK cells were incubated at 37 °C for 1 h in culture medium lacking fetal calf serum. After washing with serum-free medium, cells were continuously labeled by incubation with FITC-transferrin (0.1 mg/ml) for 30 min at 37 °C. Cells were then washed three times with ice-cold PBS and fixed with 3% paraformaldehyde, 1% Triton X-100 in ice and then 15 min at room temperature. For colocalization studies using anti-GDI antibodies, cells were then permeabilized with 0.1% Triton X-100 and immunolabeled as described above.

**RESULTS**

**Effect of Rab3A GDI on the Membrane Association of Exogenous Rab Proteins**—To study the effect of purified rab3A GDI on the membrane association of small GTP-binding proteins, we have used MDCK cells permeabilized with the bacterial toxin SLO. This procedure allows access to intracellular membranes while causing minimal structural damage and permitting vesicular transport (Gravotta et al., 1990; Tan et al., 1992; Pimplikar and Simons, 1993). Selective permeabilization of the plasma membrane was achieved by binding SLO at 4 °C, removing the excess of toxin, and allowing pore formation at 37 °C. We used conditions that allowed permeabilization of almost 100% of the cells (see “Materials and Methods”). We first analyzed the membrane association of small GTP-binding proteins by two-dimensional gel electrophoresis followed by blotting on nitrocellulose and GTP-overlay. As in intact cells, in permeabilized MDCK cells most of the small GTP-binding proteins were detected in the membrane fraction, and only a small fraction was recovered in the cytosol. In contrast, incubation of permeabilized cells in the presence of various concentrations (1 nm-1 µM) of purified rab3A GDI for 30 min at 37 °C removed several small GTP-binding proteins from the membrane (data not shown). Maximum effect was obtained when cells were incubated with 0.8 µM rab3A GDI, in good agreement with the estimated association constant of rab3A and rab3A GDI (Araki et al., 1990). No effect was observed when intact cells were incubated with rab3A GDI.

The finding that rab3A GDI removed several small GTP-binding proteins from the membrane of SLO-permeabilized MDCK cells prompted us to examine the membrane association of various Rab proteins under the same experimental conditions. We studied the effect of rab3A GDI on endogenous rab2, rab5, rab7, rab8, rab9, and rab11 proteins by quantitative immunoblot analysis using specific affinity-purified antibodies. As shown in Fig. 1, in permeabilized MDCK cells incubated in the absence of rab3A GDI, these proteins were predominantly membrane associated as shown previously (Chavrier et al., 1990a; Goud et al., 1990; van der Sluijs et al., 1991). In contrast, after incubation with 0.8 µM purified rab3A GDI all these Rab proteins were removed from the membrane and could be recovered in the cytosolic fraction. Furthermore, the concentration of endogenous rab3A GDI was estimated to be ~1.2 µM in the cytosol of MDCK cells (see “Materials and Methods”) and ~2 µM in the cytosol of neuron bodies and synaptosomes (Nonaka et al., 1991). Thus, in our in vitro system rab3A GDI was active at its physiological concentration. These results indicate that rab3A GDI is active on several members of the Rab protein family.

In order to examine whether Rab proteins would remain complexed with rab3A GDI upon extraction from the membrane, we incubated SLO-permeabilized MDCK cells with recombinant glutathione S-transferase-rab3A GDI and then adsorbed the fusion protein onto glutathione sepharose. Similar to rab3A GDI purified from bovine brain, the recombinant glutathione S-transferase-rab3A GDI was able to remove rab5 from the membrane (Fig. 2). Moreover, rab5 was found to be bound to glutathione S-transferase-rab3A GDI and was recovered on the Sepharose beads. These data suggest that Rab proteins remain complexed with rab3A GDI upon removal from the membrane.

**Effect of Rab3A GDI on the Membrane Association of Exogenous Rab Proteins**—If rab3A GDI is able to extract endogenous Rab proteins from the membrane, it should also be able to prevent the membrane association of exogenous Rab proteins. To address this question, we established an in vitro assay which measures binding of Rab proteins to the membrane of SLO-permeabilized MDCK cells. Since rabbit reticulocyte lysate supports the post-translational modifications of Ras and ras-related proteins that are required for membrane association (Hancock et al., 1991; Kinsella and Maltese, 1992), we used [35S]methionine-labeled in vitro translated rab proteins. We first investigated the biochemical properties of membrane binding of in vitro translated rab5 (Fig. 3). As noted previously (Peter et al., 1992), occasionally the rab5 protein appeared as a doublet when analyzed by SDS-PAGE. SLO-permeabilized and unpermeabilized MDCK cells were incubated with in vitro translated [35S]methionine-labeled rab5 for 30 min at 37 °C, then extensively washed and analyzed by SDS-PAGE and subsequent fluorography. Binding of rab5 was detected only in permeabilized cells indicating that it was due to specific association with the cytosolic face of cellular membranes (Fig. 3a). We estimated that 75 pg of in vitro translated rab5 associated with 1 x 10^5 permeabilized MDCK cells (as calculated from the incorporated [35S]methionine). Binding was completely abolished at 4 °C, indicating that membrane association is a temperature-dependent process (Fig. 3b). Furthermore, isoprenylation of rab5 was required for membrane association. Association of rab5 with the membrane was inhibited when in vitro translation was carried out in the presence of an excess of recombinant rab5 purified from E. coli (Fig. 3c), which competes for isoprenylation (Peter et al., 1992). However, addition of recombinant rab5 after in vitro translation did not affect membrane binding, ruling out a nonspecific inhibitory effect of the recombinant protein on membrane association.

In vitro translated rab5 was then incubated with permeabilized cells, the cells were then homogenized, and a high speed membrane fraction was prepared. The fact that we could hardly detect any rab5 in the supernatant suggested that the protein was strongly membrane associated (Fig. 3d). The protein was resistant to extraction of the membranes with high salt, urea, and high pH but was removed upon extraction with Triton X-100, indicating a tight binding to membranes via hydrophobic interactions. These results show that in vitro translated membrane-associated rab5 exhibits the same biochemical properties as endogenous rab proteins (Goud et al., 1990).

Having established conditions to obtain binding of exogenous rab5 to membranes, we then examined whether the binding of endogenous Rab, rho, and ras proteins would also be regulated by rab3A GDI. In the absence of rab3A GDI, in vitro translated rab1a, rab4b, rab5, rab7, and rab10 as well as rhoA and N-ras bound to permeabilized MDCK cells (Fig. 4). When the in vitro translated proteins were incubated with permeabilized cells in the presence of rab3A GDI, membrane association of rab proteins was completely inhibited. In contrast, binding of rhoA and N-ras was not affected by this treatment.

These data indicate that purified rab3A GDI inhibits binding of exogenous Rab proteins to the membrane of permeabilized MDCK cells, whereas it is not active on members of the rho and ras families of small GTP-binding proteins.
FIG. 1. Removal of endogenous rab proteins by rab3A GDI from the membranes of SLO-permeabilized MDCK cells. MDCK cells were permeabilized with SLO and incubated for 30 min at 37 °C without (−) or with (+) 0.8 μM rab3A GDI. The released cytosol (C) and the cellular membranes (M) were resolved on SDS-PAGE and transferred to nitrocellulose. The distribution of the rab proteins was determined by Western blot analysis using affinity purified antibodies. A faster migrating mipratine cysterisk. This band could well correspond to a degradation product of rab9 anti-rab9 antibodies is indicated by an asterisk. The cytosolic proteins released from the cells were washed twice with ICT buffer, boiled in SDS sample buffer, and the proteins from membranes and beads were analyzed by SDS-PAGE and immunoblotting. Anti-rab5 antibodies were used to detect rab5.

Specificity of Isoprenylation for the Interaction between Rab5 and Rab3A GDI—Previous studies have shown that rab3A GDI can interact with rab3A only in the presence of an intact post-translationally modified COOH terminus (Araki et al., 1991). The geranylgeranyl moiety but not the methyl group is essential for this interaction (Musia et al., 1992). However, it is not known whether the structurally distinct cysteine motifs of rab proteins and/or their corresponding isoprenoid modification play a specific role in this process. Therefore, we replaced the cysteine motif of rab5 (CCSN) which is modified by one or two geranylgeranyl isoprenoids (Kinsella and Maltese, 1992), with the CAAX-motif of H-ras (CVLS), modified by one or two geranylgeranyl isoprenoids (Kinsella and Maltese, 1992). Thus, the rab5 mutant protein displayed low membrane binding but localized together with human transferrin receptor (not shown). This indicates that at least a small percentage of the mutant protein is correctly targeted to the early endosomes in vivo. In SLO-permeabilized MDCK cells, membrane association of the rab5-CVLS mutant was 10 times less efficient (7.5 pg/1 × 10⁶ cells) than that of the wild type rab5 protein. Thus, replacement of the COOH-terminal cysteine motif of rab5 with a farnesylated CAAX box drastically reduced membrane association. We then examined whether rab3A GDI would affect the membrane association of [35S]methionine-labeled rab5-CVLS mutant protein. Unfortunately, membrane association of the [3H]geranylgeranyl- or [3H]farnesyI-labeled proteins could not be detected due to their low specific activity. While membrane binding of rab5 was completely abolished in the presence of rab3A GDI, attachment of the mutant protein to the membrane was reduced only by 40% (Fig. 5b). Thus, the rab5-CVLS mutant protein is partially resistant to the activity of rab3A GDI. This effect is consistent with extraction of a geranylgeranylated fraction of rab5-CVLS from the membrane by rab3A GDI. This explanation is in agreement with the finding that rab8, which contains a CAAX box of the mono-geranylgeranyl type, was also found to be removed from the membrane by rab3A GDI (Fig. 1).

These data suggest that rab3A GDI requires the specific presence of a geranylgeranylated COOH terminus to effi-
postnuclear supernatant in 24-well dishes were permeabilized with SLO and incubated with rab5, rab3A, and unpermeabilized pl supplemented with 2 µg of ICT buffer, 0.005% Triton X-100 containing 100 µl rab5 reticulocyte lysate), then washed, and homogenized in ICT buffer at 4 °C. A cytosol fraction to continuous sucrose density gradient tested whether rab5 can be found complexed with rab3A GDI of exogenous rab, rho, and ras proteins.

Rab3A GDI Is Found Both in the Cytosol and on the Membrane of MDCK Cells—Having shown that purified rab3A GDI can remove rab proteins from the membrane, we next tested whether rab5 can be found complexed with rab3A GDI in the cytoplasm of MDCK cells. To this end, we subjected a cytosol fraction to continuous sucrose density gradient ultracentrifugation as previously described (Araki et al., 1990). Western blot analysis of the fractions using affinity-purified anti-rab5 and anti-rab3A GDI antibodies indicated that endogenous rab5 cosedimented with rab3A GDI with an apparent M, of 80,000 (not shown). These results suggest that, similar to rab3A (Araki et al., 1990; Regazzi et al., 1992), rab5 exist in a 1:1 complex with rab3A GDI in the cytosol of MDCK cells.

![Fig. 3. Binding properties of in vitro translated rab5 to membranes of SLO-permeabilized MDCK cells.](image)

MDCK cells grown in 24-well dishes were permeabilized with SLO and incubated with in vitro translated [35S]methionine-labeled rab5 for 30 min at 37 °C (150 µl of ICT buffer, 0.005% Triton X-100 containing 4 µl of rab5 reticulocyte lysate). The cells were then extensively washed for 1 h at 4 °C, boiled in SDS sample buffer and analyzed by SDS-PAGE and fluorography. a, binding of in vitro translated rab5 to SLO-permeabilized (+) and unpermeabilized (-) cells; b, binding of in vitro translated rab5 at 37 °C and 4 °C to SLO-permeabilized cells; c, membrane binding of rab5, in vitro translated under conditions that allow (+) or prevent (-) isoprenylation. (+, left side) rab5 in vitro translated in the absence of recombinant rab5 from E. coli; (-) rab5 in vitro translated in the presence of 2 µg of E. coli rab5; (+, right side) in vitro translated rab5 supplemented with 2 µg of E. coli rab5 added after in vitro translation; d, treatment of membrane-bound in vitro translated rab5 with high salt, urea, high pH, and Triton X-100. Permeabilized MDCK cells from a 10-cm plastic dish were incubated with in vitro translated rab5 (3 ml of ICT buffer, 0.005% Triton X-100 containing 100 µl rab5 reticulocyte lysate), then washed, and homogenized in ICT buffer at 4 °C. A postnuclear supernatant (PNS) was prepared (10 min, 800 x g) which was then centrifuged at 100,000 x g for 1 h to generate a soluble fraction (S) and a membrane fraction (MB). The fractions were analyzed by SDS-PAGE and fluorography. Aliquots of the membrane fraction were resuspended in 1 M NaCl, 6 M urea, 0.2 M sodium carbonate, pH 11, in water, or in 1% Triton X-100 and incubated for 30 min on ice. After centrifugation for 1 h at 100,000 x g, the supernatants and pellets were boiled in SDS sample buffer and analyzed by SDS-PAGE and fluorography.

![Fig. 4. Effect of rab3A GDI on the membrane association of exogenous rab, rho, and ras proteins.](image)

MDCK cells grown in 24-well dishes were permeabilized with SLO and incubated for 30 min at 37 °C with in vitro translated [3H]farnesyl pyrophosphate in 150 µl of ICT buffer, 0.005% Triton X-100). Incubations were carried out in the absence (-) or presence (+) of 0.5 µM rab3A GDI. The cells were then washed for 1 h at 4 °C, boiled in SDS sample buffer, and analyzed by SDS-PAGE and fluorography. cently interact with this protein.

Rab3A GDI was purified from bovine brain cytosol (Sasaki et al., 1990) and was mostly found in the cytosol fraction of rat cerebrum and of insulin-secreting cells (Nonaka et al., 1991; Regazzi et al., 1992). Given the effect of rab3A GDI on the membrane association of rab proteins in MDCK cells, we next asked whether a fraction of total rab3A GDI could be detected in a membrane-bound form. First, we performed Western blot analysis with anti-rab3A GDI antibodies on membrane and cytosol fractions from MDCK cells (Fig. 6). While the majority of rab3A GDI was detected in the cytosol, 20% of total rab3A GDI was found in the membrane fraction. The interaction of rab3A GDI with the membrane was not

![Fig. 5. Role of the rab5 COOH terminus for the interaction with rab3A GDI.](image)

a, wild type rab5 (CCSN) and rab5-CVLS mutant protein were in vitro translated in reticulocyte lysate in the presence of either [3H]farnesyl pyrophosphate ([3H]-FPP) or [3H]geranylgeranyl pyrophosphate ([3H]-GGPP). Labeled proteins were immunoprecipitated with polyclonal anti-rab5 antibodies and analyzed by SDS-PAGE and fluorography. b, MDCK cells grown in 24-well dishes were permeabilized with SLO and incubated with in vitro translated [35S]methionine-labeled wild type rab5 and rab5-CVLS mutant proteins for 30 min at 37 °C (4 µl of reticulocyte lysate in 150 µl of ICT buffer, 0.005% Triton X-100). Incubation was performed in the absence (-) or presence (+) of 0.8 µM rab3A GDI. The cells were then washed for 1 h at 4 °C and boiled in SDS sample buffer prior to SDS-PAGE and fluorography.
then separated by ultracentrifugation into membrane (MB) and cytosolic (CYT) fractions. An aliquot of the pelleted membranes was then resuspended in ICT buffer containing 1 M NaCl and incubated for 30 min on ice. Incubation was followed by ultracentrifugation to obtain a membrane pellet (MB) and a supernatant (S) containing the salt-extracted proteins. The samples were resolved on SDS-PAGE and Western blot analysis was performed using anti-GDI polyclonal antiserum. Purified rab3A GDI (GDI) was used for quantitative Western blotting.

very strong, since the protein could be removed by 1 M NaCl salt wash. Second, we determined the membrane localization of rab3A GDI in MDCK cells by confocal immunofluorescence microscopy (Fig. 7, A and C). Rab3A GDI showed a diffuse cytoplasmic staining as expected for a cytosolic protein. However, most strikingly, rab3A GDI exhibited a vesicular staining pattern spread throughout the cytoplasm. Double immunofluorescence microscopy revealed partial colocalization of rab3A GDI with β-COP (Fig. 7B), a coat protein associated with the Golgi apparatus (Duden et al., 1991). Rab3A GDI was also found to be associated with endocytic structures labeled by internalization of canine FITC-transferasin for 30 min at 37 °C (Fig. 7D).

These results demonstrate that rab3A GDI is not restricted to the cytoplasm but is also associated, in part with organelles of the exocytic and endocytic pathways in MDCK cells.

DISCUSSION

Rab3A GDI, a protein which inhibits the dissociation of GDP from smg p25A/rab3A, Sec4p, and rab11, has been also found to prevent association of rab3A with the membrane (Araki et al., 1990; Sasaki et al., 1991; Ueda et al., 1991). The COOH-terminal region of rab3A is required for the interaction with rab3A GDI (Araki et al., 1991). Since this region displays the highest sequence variability among all rab proteins it became important to determine whether rab3A GDI can interact with distinct or with any member of the rab proteins family. We have used a permeabilized cell system to test the activity of rab3A GDI in modulating the membrane association of various small GTP-binding proteins. By two-dimensional gel electrophoresis and GTP overlay, we observed that incubation of SLO-permeabilized MDCK cells with purified rab3A GDI induced the dissociation of several GTP-binding proteins from the membrane. Further analysis suggested that these proteins are members of the rab family. We found that rab3A GDI removes all rab proteins we have tested from the membrane and prevents the membrane association of exogenous rab proteins. Therefore, since rab3A GDI acts as a general regulatory component for various rab proteins, we propose to rename it rab GDI.

Association of rab proteins with their corresponding organelles is a multi-step process involving post-translational modifications and recognition of targeting sequences (Chavrier et al., 1991). Truncated rab proteins lacking the COOH-terminal cysteine motif are unable to associate with cellular membranes and remain functionally inert (Chavrier et al., 1991; Khosravi-Far et al., 1991; Bucci et al., 1992; Lombardi et al., 1993). Rab proteins have been shown to acquire geranylgeranyl groups on the terminal cysteines which also become methyl-esterified (Farnsworth et al., 1991; Khosravi-Far et al., 1991; Kinsella and Maltese, 1992; Peter et al., 1992). Conversion of the geranylgeranylated cysteine motif of rab5 to -CVLS, which is a substrate for farnesylation, dramatically reduced binding of the mutant protein to the membrane of permeabilized MDCK cells. These results suggest that the higher hydrophobicity of the geranylgeranylated group, compared to farnesol, is required to confer the efficient binding of rab5 to the membrane. While the addition of one or two 20-carbon isoprenoid groups increases the hydrophobicity of rab proteins allowing them to become tightly membrane associated, this process can be reversed by rab GDI. Previous studies have shown that the geranylgeranylated moiety but not the methyl group of rab3A is essential for the interaction with rab GDI (Musha et al., 1992).

Our results further suggest that efficient interaction between rab5 and rab GDI requires the presence of one or two geranylgeranyl groups on the cysteine motif. Rab GDI only partially affected membrane association of the rab5-CVLS mutant protein. Since mono-geranylgeranylated rab8 was shown to be efficiently removed by rab GDI from the membrane it is likely that the partial effect of rab GDI on the membrane association of the rab5 mutant protein is due to poor interaction with the farnesylated rather than with the mono-geranylgeranylated rab5 mutant protein. These data indicate for the first time that the COOH-terminal cysteine motif together with its corresponding isoprenoid group play a specific role in the regulation of membrane association of rab proteins. However, geranylgeranylation is not sufficient per se for interaction with rab GDI, since membrane association of geranylgeranylated rhoA proteins is not affected by rab GDI. This implies that rab GDI binds both to the geranylgeranyl group and to sequences upstream the cysteine motif.

Rab proteins are thought to shuttle between their specific membrane compartments and the cytoplasm in a guanine nucleotide-dependent manner. According to the proposed model, membrane association would depend on a GDP/GTP exchange protein which would induce GDP release and GTP binding (Bourne, 1988; Pfeffer, 1992). GTP hydrolysis would be required to trigger fusion of the membrane of the vesicle with the membrane of the acceptor compartment. Subsequently, the inactive GDP-bound rab protein would return to the donor membrane via a soluble intermediate, where it would be recharged with GTP to initiate a new round of transport. This model implies the presence of factors that regulate the association of rab proteins with the membrane as well as their release into the cytosol. It is conceivable that the observed activity of rab GDI may reflect a physiological role in regulating the reversible transition of rab proteins from their specific membrane compartment to the cytosol. Three observations are consistent with this explanation. First, rab5 remains complexed with rab GDI upon removal from the membrane. Second, rab GDI has been found complexed with rab3A and other small GTP-binding proteins in the cytosol of insulin-secreting cells (Regazzi et al., 1992). Our data further suggest that rab GDI is complexed with cytosolic rab5. Third, we detect by Western blotting and immunofluorescence a significant fraction of rab GDI associated with various intracellular membrane compartments in MDCK cells. Two different possibilities that are not mutually exclusive could account for the membrane association of rab GDI. A fraction of rab GDI could be in complex with rab proteins in the process of associating with the membrane. In addition, a significant pool of rab GDI could be associated at sites of vesicle docking and fusion with the target membrane to effi-

**FIG. 6. Western blot analysis of the subcellular distribution of rab3A GDI in MDCK cells.** MDCK cells were homogenized and then separated by ultracentrifugation into membrane (MB) and cytosolic (CYT) fractions. An aliquot of the pelleted membranes was then resuspended in ICT buffer containing 1 M NaCl and incubated for 30 min on ice. Incubation was followed by ultracentrifugation to obtain a membrane pellet (MB) and a supernatant (S) containing the salt-extracted proteins. The samples were resolved on SDS-PAGE and Western blot analysis was performed using anti-GDI polyclonal antiserum. Purified rab3A GDI (GDI) was used for quantitative Western blotting.
FIG. 7. Confocal fluorescence microscopy localization of rab3A GDI in MDCK cells. A and B, MDCK cells were fixed and then permeabilized with Triton X-100. Cells were double-labeled for indirect immunofluorescence using affinity purified rabbit anti-GDI antibody and mouse monoclonal antibodies against β-COP. After washing, cells were incubated with rhodamine-labeled donkey anti-rabbit IgG to label the bound anti-GDI IgG (A) and with fluorescein-labeled donkey anti-mouse IgG to label bound anti-β-COP IgG (B). C and D, MDCK cells were incubated at 37°C for 30 min with FITC-labeled canine transferrin. After fixation, cells were permeabilized with Triton X-100 and incubated with affinity purified rabbit anti-GDI antibody. For labelling the bound anti-GDI IgG (C) cells were incubated with rhodamine-labeled donkey anti-rabbit IgG. D, cells were photographed with fluorescein optics showing FITC-transferrin distribution. Bar, 10 μm.

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these proteins belong. Incubation of a subcellular fraction with rab GDI could offer a direct approach for the identification of rab proteins present in a subcellular preparation. The same approach could also be attempted for the identification of rho proteins. Finally, the in vitro assay we have established will hopefully allow us to study the effects accompanying the removal of rab proteins from the membrane on the regulation of intracellular traffic.

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