Small Rab GTPases are involved in the regulation of membrane trafficking. They cycle between cytosolic and membrane-bound forms. These membrane association/dissociation are tightly controlled by regulatory proteins. To search for proteins interacting with Rab13, a small GTPase associated with vesicles in fibroblasts and predominantly with tight junctions in epithelial cells, we screened a HeLa two-hybrid cDNA library and isolated a clone encoding a protein of 17.4 kDa. This protein, almost identical to the bovine rod cGMP phosphodiesterase δ subunit, was named human δ-PDE. The δ-PDE binds specifically to Rab13. It exhibits two putative C-terminal sequences necessary for the interaction with PDZ (PSD95,Dlg,ZO-1) domains contained in many proteins localized to specific plasma membrane microdomains. Immunofluorescence microscopic studies revealed that the vesicular stomatitis virus (VSV)-tagged δ-PDE is localized in vesicular structures accumulated near the plasma membrane in epithelial cells. Deletion of the PDZ binding motifs impair VSV-δ-PDE subcellular distribution. Purified recombinant δ-PDE had the capacity to dissociate Rab13 from cellular membranes. Our data support the proposal that δ-PDE, but not GDP dissociation inhibitor, may serve to control the dynamic of the association of Rab13 with cellular membranes.

Rab proteins constitute the largest family (more than 36 members) of the Ras-related small GTPase superfamily. They bind both GDP/GTP and can hydrolyze GTP. Given their specific location in different cellular compartments and their implication in the regulation of specific membrane transport events, the Rab proteins have been proposed to be required for each step of membrane trafficking (1). The Rab activity seems to be necessary for the formation of a complex between vesicle SNAREs and their cognate target SNAREs, which control the docking of transport vesicle with the target membranes (2–4). Recent studies have shown that the Rab protein, Ypt7 is directly involved in docking step of in vitro yeast vacuoles (5). A fruitful approach to understand the mechanism of action of Rab proteins is the identification of their interacting proteins. Several putative effectors interacting with the GTP-bound form of Rab proteins have been identified. Strikingly, these proteins are structurally distinct from each other and are implicated in various cellular processes. This led to the suggestion that Rab proteins control several distinct activities including vesicle movement via kinesin motors (6), actin organization (7), vesicle fusion (8) or kinases (9). To perform their functions, Rab proteins shuttle between a membrane bound and cytosolic distribution. In the cytosol, many Rab proteins have been found complexed to Rab GDP dissociation inhibitor (GDI).1 Rab GDI has the ability to retrieve the GDP-Rab proteins from membranes (10–13). Additional factors such GDI displacement factor are required to mediate the release of GDI from the Rab-GDI complex and the membrane recruitment of Rab proteins (14, 15). This step seems to be coupled by the exchange of GDP for GTP catalyzed by a guanine exchange Factor (16, 17). These factors play an essential role in the association/dissociation of Rab proteins from the membranes and are important elements regulating their multifaceted biological functions.

We have shown that the small GTPase Rab13, associated with vesicles in fibroblasts, is mainly accumulated in tight junctions of polarized epithelial cells. Moreover, this distribution depends upon the integrity of tight junctions which are found in simple epithelia as well as in endothelia. We have proposed that Rab13 may be involved either in the targeting of a subset of membrane proteins destined to the apical or basolateral surfaces and/or in the regulation of the barrier function of tight junctions (18). To elucidate further the function of Rab13, we have searched for proteins able to interact with Rab13. Using a yeast two-hybrid screen and Rab13 wild type, we isolated a cDNA encoding a protein of 17.4 kDa, δ-PDE. We found that this protein interacts with the isoprenylated form of Rab13. Immunofluorescence data suggest that the VSV-G protein-tagged δ-PDE is localized in vesicular structures near the plasma membrane in epithelial cells. We also present evidence that δ-PDE, but not Rab GDI, has the capacity to extract the Rab13 protein from cellular membranes. These findings raise the possibility that this protein is involved in the dissociation and recycling of Rab13 protein from its target membranes.

EXPERIMENTAL PROCEDURES

Two-hybrid Screen.—The yeast two-hybrid system used in this study has been already described (19, 20). Briefly, we have used the plasmid pLEX10 to generate a fusion protein between LexA DNA binding domain and wild type Rab13. LexA Rab13 fusion protein expression was checked by Western blot with anti-Rab13 antibodies. This construct was used to screen a HeLa cDNA library fused to Gal4 activation domain, pGAD (19). Both constructs, LexA Rab13 and pGAD cDNAs, were co-transformed in Saccharomyces cerevisiae L40 strain by the lithium acetate method. Transformants were plated on selective medium lacking tryptophan, leucine, and histidine for selection of His+ colonies. Determination of β-galactosidase activity was performed on patches from selective medium minus Trp/Leu/His plates by filter assay

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1 The abbreviations used are: GDI, GDP dissociation inhibitor; PDE, phosphodiesterase; VSV, vesicular stomatitis virus; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; GTP-γ-S, guanosine 5′-O-(3-thiotriphosphate).

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using 5-bromo-4-chloro-3-indolyl-β-D-galactoside as a substrate. Lib-
library positive clones from transformed yeast were recovered as
described previously (20). All point mutations were introduced by polym-
eter chain reaction and checked by sequencing. Rab6 wild type, T27N,
and Q67L were gifts from Dr. B. Goud (Institut Curie, Paris, France).

Non-denaturing Western Blot Analysis—A filter containing mRNA from human tis-
sues, spleen, thymus, prostate, testis, ovary, small intestine, colon
(mucosal lining), and peripheral blood (leukocytes) were purchased
from CLONTECH (Palo Alto, CA). The human δ-PDE cDNA probe was
synthesized using the random priming procedure (Amersham Pharmace-
Biotech) in the presence of [α-32P]dCTP. Prehybridization and hy-
bridization were performed at 42 °C as described in the GeneScreen
instruction manual (NEN Life Science Products). The filter was washed in 0.1×
SSC, 0.1% SDS at 60 °C and autoradiographed.

In Vitro Interaction of Human δ-PDE with Rab13—The human
δ-PDE cDNA region was inserted into Escherichia coli expression
vector pET-15b and expressed as a histidine-δ-PDE fusion protein. The
protein was then produced and purified on Ni2+-agarose beads by
classical methods. Rab13 and Rab3A cDNAs were cloned in pCR3.1
plasmid (Invitrogen, Carlsbad, CA) under the control of a T7 promoter.

RESULTS

Identification of Rab13 Interacting Proteins—We have used the yeast two-hybrid system to search for proteins interacting
with wild type Rab13. Rab13 wild type in frame with LexA DNA
binding domain (LexA DBD). Lamin was used as a negative
control. The Ras/Raf two-hybrid interaction was used as a positive
control. The co-transformation of either Rab13 wild type DNA binding
domain (DBD) or Rab3Q67L mutant with Gal4 plasmid alone is not
able to activate the His6 reporter gene. B. Rab13 and Rab3A were
translated in vitro in presence of [35S]methionine and incubated with
the histidine-δ-PDE fusion protein immobilized onto Ni2+ beads. After
washing, proteins that had been retained by the beads were subjected
to SDS-PAGE. An autoradiogram is shown. Lanes a, b, Rab13 and Rab3A
translated in vitro. Rab13 appeared as a doublet; the Rab13 lower band
was also tested. An anti-Rab4, and anti-Rab6 antibodies. After blotting,
protein bands were quantified using Image-

Quant software (Molecular Dynamics, Sunnyvale, CA) and analyzed using Sigma Plot (SPSS, Chicago, IL). The experiments with Rab GDI
were performed as indicated above, except that the membranes (100 μg)
were preloaded with 1 mM GDP for 1 h at 30 °C prior to addition of puri-
fied Rab GDI α or β isoforms.

RESULTS

Identification of Rab13 Interacting Proteins—We have used the yeast two-hybrid system to search for proteins interacting
with wild type Rab13. Rab13 wild type in frame with LexA DNA
binding domain was used to screen 3 × 106 clones from a
HeLa cDNA library fused to a Gal4 activation domain. Several
cDNAs activating the expression of both the selection marker

FIG. 1. Interaction of human δ-PDE protein with Rab proteins. A. Analysis by yeast two-hybrid system. An L40 yeast strain was co-
transformed with two plasmids, one producing a cDNA clone encoding
δ-PDE as a fusion protein with Gal4 activating domain (Gal4 AD), the
second expressing different Rab or Rab mutant proteins as a fusion with
LexA DNA binding domain (LexA DBD). Lamin was used as a negative
control. The Ras/Raf two-hybrid interaction was used as a positive
control. The co-transformation of either Rab13 wild type DNA binding
domain (DBD) or Rab13Q67L mutant with Gal4 plasmid alone is not
able to activate the His6 reporter gene. B. Rab13 and Rab3A were
translated in vitro in presence of [35S]methionine and incubated with
the histidine-δ-PDE fusion protein immobilized onto Ni2+ beads. After
washing, proteins that had been retained by the beads were subjected
to SDS-PAGE. An autoradiogram is shown. Lanes a, b, Rab13 and Rab3A
translated in vitro. Rab13 appeared as a doublet; the Rab13 lower band
was also tested. An anti-Rab4, and anti-Rab6 antibodies. After blotting,
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Quant software (Molecular Dynamics, Sunnyvale, CA) and analyzed using Sigma Plot (SPSS, Chicago, IL). The experiments with Rab GDI
were performed as indicated above, except that the membranes (100 μg)
were preloaded with 1 mM GDP for 1 h at 30 °C prior to addition of puri-
fied Rab GDI α or β isoforms.
Hiss, and the LacZ reporter gene were isolated. A second round of screening yielded eight clones that interact with Rab13 wild type, but not with lamin used as a negative control. The analysis of these clones revealed that they encoded the same protein. The largest cDNA clone obtained by the two-hybrid screen was sequenced. It encoded a protein of 150 amino acids with a predicted molecular mass of 17.4 kDa. This protein, 98% identical to the bovine δ-PDE (24), was named human δ-PDE. We then checked the specificity of the interaction of δ-PDE and found that it did not interact, in the yeast two-hybrid assay, with Rab8 wild type, Rab6 wild type, or T27N or Q72L mutants. Based on sequence similarity with Ras proteins, two Rab13 mutants, T22N and Q67L, expected to lock the protein in either GDP or GTP state, respectively, were tested for their ability to interact with Rab13. Mutants interacted with δ-PDE. In addition, Rab13CSLG, a Rab13 mutant lacking the C-terminal CAAX box required for its geranylgeranylation (25), is not able to interact with δ-PDE. This indicated that the isoprenylation of Rab13 is required for its interaction with δ-PDE (Fig. 1A). Further two-hybrid dissections demonstrated that the 40 N-terminal residues of δ-PDE are important for its interaction with Rab13 (not shown).

To confirm that the human δ-PDE interacts with Rab13, we also studied it by in vitro binding assay. Since rabbit reticulocyte lysate supports the post-translational geranylgeranylation of Rab proteins that is required for membrane association (13, 21), we used [35S]methionine-labeled in vitro translated Rab13. The δ-PDE was expressed as a His-δ-PDE fusion protein. The protein was purified, immobilized on Ni²⁺ beads, and incubated with [35S]methionine-labeled Rab13 or Rab3A. Rab13, but not Rab3A, protein bound to δ-PDE immobilized on Ni²⁺ beads. The in vitro translated Rab13 appeared as a doublet of 25 and 16 kDa. Both protein bands fractionated into the detergent phase with Triton X-114, suggesting that they are isoprenylated (not shown). Therefore, the truncated form of Rab13 might result from a degradation or could be generated from an internal initiation site at methionine 82. We observed that both bands interact with δ-PDE, the shorter band binds more efficiently. Rab13 did not bind to Ni²⁺ beads alone (Fig. 1B). These results confirm those of the two-hybrid assay.

When used as a hybridization probe in Northern blot, the cDNA clone detects one transcript of 1 kilobase expressed in several tissues such as spleen, prostate gland, testis, ovary, small intestine, colon, and peripheral blood (Fig. 2).

**Structural Features of Human δ-PDE**—The sequence of human δ-PDE was compared with protein sequences in the databases using BLAST and FASTA software (Fig. 3). The human δ-PDE protein shares 70% identity with a Caenorhabditis elegans putative protein (C27H5; accession no. g540267), suggesting that it may be highly conserved during evolution. The C terminus of the δ-PDE contains putative SRV and FYV sequence motifs described as necessary and sufficient for the interaction with PDZ domains present in many proteins of synaptic, septate, and tight junctions (PSD95, Dlg, ZO-1). PDZ domains are involved in submembranous protein networks and in the recruitment of proteins to specific plasma membrane microdomains (26, 27). The amino acid sequence of δ-PDE is consistent with a molecule that may interact with membrane proteins.

**Human δ-PDE, but Not Rab GDI, Dissociates Rab13 from Cellular Membranes**—The bovine δ-PDE has been shown to solubilize membrane-bound retinal rod phosphodiesterase α and β subunits (24). We tested whether purified human δ-PDE had the capacity to retrieve Rab13 from membranes. Total cellular membranes were incubated with increasing amounts of purified recombinant human δ-PDE and centrifuged to separate membranes from supernatant. The supernatant fractions were then analyzed by blotting with anti-Rab13 antibodies to determine the effect of δ-PDE on membrane-bound Rab13. Rab4 and Rab6 distribution were also examined in the same fractions to test the functional specificity of δ-PDE. As shown in Fig. 4, in the absence of δ-PDE, Rab13 remained predominantly membrane-associated. Importantly, upon addition of δ-PDE, Rab13 was extracted from cellular membranes. A large amount, 50–60%, of Rab13 could be recovered in the supernatant when membranes were preincubated with an excess of GDP and GTP prior to addition of δ-PDE (Fig. 4B). Furthermore, the solubilization of Rab13 occurred in a dose-dependent manner with increasing amounts of purified δ-PDE. In contrast, Rab4 and Rab6 were not released from the membranes.

![Fig. 2. δ-PDE mRNA expression in human tissues. The filter was purchased from CLONTECH (Palo Alto, CA). 3 μg/lane mRNA, from spleen, thymus, prostate, testis, ovary, small intestine (sm int), colon, and peripheral (per) blood were probed with human α2β2 mRNA (accession no. g540267). Identical amino acids are highlighted in reverse type.](image-url)
upon addition of δ-PDE (Fig. 4B), suggesting that the effect of δ-PDE is specific for Rab13.

The δ-PDE solubilization property resembled, to some extent, that of Rab GDI. Several Rab GDI isoforms with no remarkable functional differences have been identified. All have been shown able to dissociate many geranylgeranyl GDP bound forms of Rab proteins from membranes (10–13, 28). We have shown that of Rab GDI. Several Rab GDI isoforms with no remarkable functional differences have been identified. All have been shown able to dissociate many geranylgeranyl GDP bound forms of Rab proteins from membranes (10–13, 28). We have shown that of Rab GDI. Several Rab GDI isoforms with no remarkable functional differences have been identified. All have been shown able to dissociate many geranylgeranyl GDP bound forms of Rab proteins from membranes (10–13, 28).

We next asked whether a fraction of Rab13 remains in the membrane fraction after prior addition of the purified Rab GDI isoform (not shown). These results indicate that δ-PDE, but not Rab GDI, is capable of dissociating Rab13 from the membranes.

Subcellular Distribution of Human VSV-δ-PDE—Given the effect of δ-PDE on the membrane association of Rab13 protein, we next asked whether a fraction of δ-PDE could be detected as a membrane-bound form. We transiently overproduced the human δ-PDE tagged with the VSV-G protein epitope at the N terminus (VSV-δ-PDE) in the epithelial cell line LLC-PK1. We performed a Western blot analysis on cytosol and membrane fractions from the transfected LLC-PK1 cells. While the majority of the VSV-δ-PDE is found in the cytosol, 12% of total VSV-δ-PDE is detected in the membrane fraction (Fig. 5). We then determined the membrane localization of the VSV-δ-PDE by immunofluorescence microscopy. Subconfluent VSV-δ-PDE-transfected LLC-PK1 cells were fixed with paraformaldehyde, permeabilized, and stained with anti-VSV antibody. The subcellular distribution of VSV-δ-PDE was analyzed by confocal laser microscopy. The cells that highly overproduced VSV-δ-PDE exhibited a patchy staining pattern outlining the periphery of the cells (Fig. 6A). Our immunofluorescence observations suggest that VSV-δ-PDE may be localized in vesicular structures in close contact with the lateral plasma membrane domains of these epithelial cells.

The δ-PDE C terminus displays FYV and SRV motifs necessary for the interaction with PDZ domains contained in many proteins of intercellular junctions (29, 30). Consequently, the C-terminal region was suspected to be required for VSV-δ-PDE membrane localization. Thus, we examined, by immunofluorescence, whether VSV-δ-PDE lacking the last C-terminal 7 residues encompassing FYV and SRV sequences (VSV-δ-PDE1–143) could localize to vesicular structures near the plasma membrane. VSV-δ-PDE1–143 transfected LLC-PK1 cells were stained for VSV-δ-PDE1–143. In contrast to the tagged wild type protein, the VSV-δ-PDE1–143 labeling appeared diffusely distributed in the cytoplasm. Strikingly, no patchy staining outlining the cell borders could be detected (Fig. 6B). These data strongly suggested that FYV and SRV motifs are required for the localization of the VSV-δ-PDE.
DISCUSSION

Using the yeast two-hybrid screen, we isolated a cDNA clone encoding a human δ-PDE protein that is able to bind to the small GTPase Rab13 and not to Rab6 or Rab8. Further dissections of this two-hybrid interaction revealed that the C-terminal CAAX (CSLG) box is required for δ-PDE-Rab13 interaction. The Rab13 CSLG motif has been shown to be necessary for the post-translational geranylgeranylation of the protein (25). We then analyzed the binding of Rab13 to immobilized δ-PDE, and found that Rab13 bound to δ-PDE. Thus, there appears to be a specific interaction of δ-PDE with Rab13. However, our results do not exclude the possible interaction of δ-PDE with Rab13 via bridging proteins such as PDEα and β subunits that could be present in yeast as well as in the reticulocyte lysate.

A computer homology search revealed that the amino acid sequence of the human δ-PDE was almost identical to that of bovine δ-PDE protein. The bovine δ-PDE protein was first found as a protein that co-purified with the rod-soluble PDEα and β subunits in the retina. However, this protein has no regulatory effect on the activity of the rod PDE involved in phototransduction in the retina (24). Moreover, in contrast to the rod PDE, δ-PDE mRNA expression was detected in large variety of tissues. These observations strengthen the idea that δ-PDE may interact with other proteins than rod PDE (24) (this work). It is striking to notice that δ-PDE is highly conserved from C. elegans to human (75% identity), suggesting a conserved function during evolution.

Many of the PDZ domain-containing proteins are localized at specific regions of cell-cell contacts. In general, the PDZ domains interact preferentially with C-terminal hydrophobic peptide sequences including S/TXV, YYV, and FYV (31). The C-terminal region of δ-PDE contains SRV and FYV sequences that conform to the PDZ binding motifs. This suggests that δ-PDE is a candidate for the interaction with PDZ domain-containing proteins. Deletion of the RVGLFYV C terminus amino acid sequence abolishes the distribution of the VSV-δ-PDE-containing vesicles along the plasma membrane. This indicates that the sequence encompassing SRV and FYV motifs play a key role in targeting the VSV-δ-PDE to a peripheral vesicular compartment. We have determined the intracellular distribution of the δ-PDE protein through transient transfection of epitope-tagged constructs. However, to reduce the possibility of mislocalization due to overexpression, we based our conclusions on cells that express a relatively low level of the ectopically expressed VSV-δ-PDE. A specific antibody recognizing the δ-PDE will be needed to precisely define the subcellular location of the endogenous protein.

In order to generate and maintain surface polarity, epithelial cells have to target proteins and lipids to both apical and basolateral membranes. Indeed, it has been proposed that the junctional complexes may contain the machinery required for docking and fusion of vesicles (32). Moreover, it has been suggested that several small GTPases, such as Rab13, Rab8, and Rab3B may be involved in the delivery of transport vesicles to the plasma membrane or to specialized plasma membrane mi-

![Image](image1.png)

**FIG. 5.** Immunoblot analysis of the subcellular distribution of the VSV-δ-PDE in LLC-PK1 cells. Cells transiently transfected with VSV-δ-PDE were homogenized and separated by ultracentrifugation into membrane and cytosol fractions. Equal amounts (20 μg) of each fraction were resolved on SDS-PAGE and analyzed by Western blot with anti-VSV monoclonal antibody. The blot was quantified as indicated under “Experimental Procedures.”

**FIG. 6.** Confocal immunofluorescence microscopy showing the distribution of the VSV-G tagged δ-PDE protein in epithelial LLC-PK1 cells. The VSV-G epitope tag was inserted at the N terminus of δ-PDE. A, VSV-δ-PDE protein or B, VSV-δ-PDE truncated at Arg144 (VSV-δ-PDE1–143) were transiently overproduced in the epithelial cell line LLC-PK1 by transfection. 16 h later, cells were processed for immunofluorescence as indicated under “Experimental Procedures.” A and B, VSV-δ-PDE immunoreactivity was detected using the mouse monoclonal anti-VSV antibody and Cy2-conjugated goat anti-mouse IgG antibodies. Six horizontal sections (taken at 0.8 mm from the base of the cells) were obtained to generate the image disclosing the distribution of VSV-δ-PDE. Note that, in one transfected cell, VSV-δ-PDE is so overexpressed that cytosolic staining is also seen. Bar, 10 μm.
Interaction of Rod cGMP Phosphodiesterase δ Subunit with Rab13

As proposed by Mayer and Wickner (1997), Rab proteins associate with transport vesicle from the donor membrane, the vesicle moves to, docks, and fuses with the acceptor membrane. Upon GTP hydrolysis, Rab proteins are recycled back to the donor compartment via a cytosolic intermediate. The model requires the presence of multiple factors that regulate the association of Rab proteins with the membrane as well as their release into the cytosol. So far, it has been shown that Rab GDI may account for the recycling of Rab proteins to the cytosol. Intriguingly, Rab GDI is not able to retrieve Rab13 from the membranes. We tested whether Rab13 can be recovered in the supernatant upon addition of Rab GDI. The results presented indicate that a large fraction (50–60%) of Rab13 can be recovered in the supernatant upon addition of Rab GDI. The release of Rab13 into the supernatant was dependent upon the concentration of Rab GDI. About 50% of the Rab13 could not be removed from the membranes due probably to the association of a fraction of membranous Rab13 with other molecules that prevent the interaction with Rab GDI. It is also possible that only a fraction of membranous Rab13 is accessible in our assay. The Rab GDI was not able to solubilize Rab4 and Rab6 from the membranes, confirming the specific binding of Rab GDI to Rab13. Thus, Rab GDI, dissociating Rab13 bound to GTP or GDP, may allow transit of Rab13 between the physically separated effector and activator.

δ-PDE has also been shown to solubilize the membrane-bound rod PDE. Similarly to Rab13, δ-PDE/PDE interaction requires isoprenylation and/or a C-terminal domain of either or both PDE α and β subunits. Thus, it has been suggested that δ-PDE may recognize the isoprenylated C terminus to bind to the target proteins (24). However, the C-terminal regions of Rab13 and PDE α and β subunits are divergent. Therefore, we propose that the binding of δ-PDE to its targets requires (i) targeting of δ-PDE to a specialized membrane microdomain through interaction with membrane proteins, and (ii) recognition of the isoprenylated C terminus. Taken together, our results show that δ-PDE may dissociate the Rab13 protein from the membranes, allowing its release into the cytosol. They also suggest that the mechanisms controlling the dissociation of Rab proteins from the membranes can be diverse and may imply the interference of distinct proteins.

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