Post-translational modifications of GTPases from the Ras superfamily enable them to associate with membrane compartments where they exert their biological activities. However, no protein acting like Rho and Rab dissociation inhibitor (GDI) that regulate the membrane association of Rho and Rab GTPases has been described for Ras and closely related proteins. We report here that the δ subunit of retinal rod phosphodiesterase (PDEδ) is able to interact with prenylated Ras and Rap proteins, and to solubilize them from membranes, independently of their nucleotide-bound (GDP or GTP) state. We show that PDEδ exhibits striking structural similarities with RhoGDI, namely conservation of the Ig-like fold and presence of a series of hydrophobic residues which could act as in RhoGDI to sequester the prenyl group of its target proteins, thereby providing structural support for the biochemical activity of PDEδ. We observe that the overexpression of PDEδ interferes with Ras trafficking and propose that it may play a role in the process that delivers prenylated proteins from endomembranes, once they have undergone proteolysis and carboxymethylation, to the structures that ensure trafficking to their respective resident compartments.

GTPases of the Ras superfamily, including the Ras, Rho/Rac/Cdc42, and Rab families, play a central role in the control of numerous essential biological functions such as proliferation, differentiation, cell morphology, movement, intracellular trafficking, and gene expression (1–3). Most Ras-related proteins are ubiquitously expressed and have been conserved through evolution. They bind GDP and GTP with high affinity and carry an intrinsic GTPase activity. Regulatory proteins such as GEFs or GTPase-activating proteins that, respectively, stimulate their ability to exchange GDP for GTP, or to hydrolyze GDP, enable them to act as molecular switches between their inactive GDP-bound and active GTP-bound forms in response to various extracellular stimuli and exert their biological activities (4).

Following their synthesis as soluble precursors, proteins of the Ras superfamily become associated with intracellular membrane compartments via post-translational modifications of their COOH termini (5). As it was shown for Ras many years ago, the association of these proteins with membranes is essential for their biological activity (6). Proteins of the Ras and Rho/Rac/Cdc42 families contain a CAAX sequence (where C is a Cys, A an aliphatic residue, and X any amino acid) at their COOH-terminal extremity that targets them for their post-translational modifications. The first step consists in prenylation in the cytosol of the Cys of the CAAX motif by a C-15 farnesyl moiety, in the case of Ras and Rap2A, or by a C-20 geranylgeranyl group for most other GTPases (7). Prenylation addresses these proteins to endomembrane compartments (the endoplasmic reticulum and Golgi) (8), which correspond to the intracellular localization of the prenyl CAAX protease that removes the three COOH-terminal AAX residues, and the carboxymethyl transferase that modifies the now COOH-terminal prenyl-cysteine (9–12). Other cellular proteins that carry a COOH-terminal CAAX sequence are processed by the same mechanisms; this group comprises proteins of the nuclear envelope (lamins A and B), the γ subunits of heterotrimeric G proteins, as well as several proteins involved in phototransduction such as rhodopsin kinase, the α subunit of transducin and both α and β catalytic subunits of the cGMP phosphodiesterase from retinal rods and cones (7).

Upstream of the CAAX motif, farnesylated GTPases such as Ras carry a second signal consisting of a stretch of basic residues (as is the case for the K-Ras(4B) protein) or additional cysteines serving as palmitoylation sites (one for N-Ras or two for Ha-Ras) that are necessary for the association of these proteins with the plasma membrane (13). Recent evidence demonstrates that these signals also play a role in the trafficking of newly synthesized Ras proteins to the plasma membrane, as well as in their targeting to membrane subdomains. Palmitoylation addresses Ha- and N-Ras proteins to cholesterol-rich rafts via the exocytotic pathway, whereas K-Ras(4B) proceeds to the non-raft plasma membrane via a yet uncharacterized route (8, 14, 15).

Despite these new advances, the question of how the newly prenylated, and therefore hydrophobic yet still cytosolic, proteins transit in the cytosol to reach membranes remains open. Newly synthesized Rab proteins are escorted to their cognate membrane compartment, after prenylation by Rab-geranylgeranyltransferase, by Rab Escort Protein, a subunit of the enzyme specifically dedicated to this task (16). Rho- and Rab-GDIs, respectively, enable Rho and Rab GTPases to shuttle between membrane compartments through the cytosol due to their ability to solubilize processed proteins from mem-
branes and to maintain them in the cytosol by burying the hydrophobic prenyl groups in a hydrophobic pocket (17, 18). To date, no such activity has been described for GTPases of the Ras subfamily (Ras, Rap, Ral, R-Ras, TC-21). In this study, we show that a protein that was originally identified as the subunit of the eGFP phosphodiesterase from retinal rod cells, or PDEδ (19), is also able to interact with Ras and Rap proteins, and to regulate their association with membranes, similarly to Rho- and RabGDSs.

Experimental Procedures

Expression Constructs—Full-length human PDEδ cDNA was obtained by screening a Jurkat T cell cDNA library in the yeast two hybrid system with the entire coding sequence of Rap2B as a bait; yeast two-hybrid experiments were performed as described with GTPase baits fused to the COOH terminus of the GALA DNA-binding domain, and prey s (PDEδ and effector controls) expressed from pGAD vectors (20). pRK5-PDEδ, the coding sequence for the human PDEδ protein, was amplified by PCR using Pfu DNA polymerase (Stratagene) with oligonucleotides 5′-ATTTAGGATCC GGCGCCATGC CCAAGGAC GCGGG 3′ and 5′-TACGGTCGAC TCAAACATAG AAAAGTCTCA CT 3′ as forward and reverse amplifiers, respectively. The resulting product was digested with BamHI and 5′-ClONex and inserted into the pET15b expression vector cut with the same restriction enzymes; pPK5myc-PDEδ encoded an epitope recognized by the anti-Myc antibody. His6-RasCt, or pGFP-HRasCtC181SC184S plasmids; 0.5 μg of pRK5-PDEδ and 0.25 μg of pGFP plasmids were used to transfect cells in a 35-mm dish. 13 h after transfection, cells were washed twice with PBS, fixed for 10 min in PBS containing 3% paraformaldehyde and 0.08% glutaraldehyde, treated with 50 mM NH4Cl in PBS for 20 min, washed, and incubated with rat anti-Ras monoclonal antibody Y13-283 at 4 °C. Complexes were recovered with goat anti-rat antibodies bound to protein A-Sepharose, extensively washed, and analyzed by SDS-PAGE followed by fluorography.

Subcellular Localization of GTP-Ras Fusion Proteins—HeLa cells that had been plated on glass cover slips were transfected using LipofectAMINE Plus (Invitrogen) with pPK5-PDEδ or empty pRK5, together with pGFP-HrasCt (15) or from pRK5myn-PDEδ vector cut with the same restriction enzymes; pRK5myc-PDEδ was generated by screening a Jurkat T cell cDNA library in the yeast two-hybrid screen with several GTPases of the Ras superfamily and with Ral family members (RhoA, RhoB, and Rnd1). Analysis of various mutants of these GTPases revealed that PDEδ interacted most strongly with full-length wild type, activated (RasVal12, Rnd1 Val12, and RhoA Val14) or dominant negative (Rap1A Asn17, Rap2B Asn17, and Ras Ala19) forms of the GTPases. In contrast, PDEδ only interacted with those proteins containing an intact COOH terminus, irrespective of the mutations that may also affect their nucleotide binding properties: Table I shows that neither interacted with proteins carrying a COOH-terminal truncation, nor with the Ras mutant Val12→Ser186 where the Cys

Solubilized proteins were recovered by centrifugation at 100,000 × g for 30 min and detected by Western blotting using rabbit affinity purified anti-Rapi (22) and anti-Rap2 (23) antibodies. Mouse monoclonal antibodies directed against Ras, and rabbit polyclonal anti-RalB antibodies, were from Transduction Laboratories.

Overexpression of the PDEδ Proteins—3 × 10^7 HEK293 cells were electroporated with 10 μg of pPK5myc-PDEδ or empty pRK5 vectors. 36 h later, cells were lysed, and membranes were prepared as above. 75 μg of protein from the cytosol and membranes were analyzed by Western blotting using rabbit affinity-purified anti-Rapi (22), anti-Rap2 (23), anti-Ras (24), and mouse monoclonal 9E10 anti-Myc (Roche Molecular Biochemicals) antibodies. When indicated, the cytosol was adjusted to 1% Triton X-114, and the detergent-enriched phase was recovered by centrifugation as described below. Protein was precipitated with trichloroacetic acid and detected by Western blotting.

Effects of PDEδ on the Post-translational Processing of Ras—HeLa cells were electroporated as above with 4 μg of pEXV-Hras and 6 μg of pPK5-myPDEδ or pPK5myc vectors. 15 h later, they were incubated for 1 h in Dulbecco’s modified Eagle’s medium without methionine and cysteine supplemented with 5% fetal calf serum and pulse-labeled for 20 min with 0.1 mCi/ml 35S-labeled Protein Labeling Mix (PerkinElmer Life Sciences) in the same medium. Cells were then washed twice with PBS and chased for various amounts of time in complete culture medium. At the indicated times, they were lysed by a 10-min incubation on ice in a buffer containing 25 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 5 mM MgCl2, and 0.1 mM GDP or Gpp(NH)p in the presence of 1 μg of (His)6-PDEδ protein purified from insect cells.

RESULTS

The PDEδ Protein Interacts with GTPases Containing an Intact COOH Terminus—We had previously isolated PDEδ in yeast two-hybrid screens with several GTPases of the Ras superfamily as baits (20, 28). As depicted in Table I, PDEδ interacted with Ras and Rap proteins, but not with Ral (neither RalA nor RalB), and it also interacted with Rho family members RhoA, RhoB, and Rnd1. Analysis of various mutants of these GTPases revealed that PDEδ interacted most strongly with full-length wild type, activated (RasVal12, Rnd1 Val12, and RhoA Val14) or dominant negative (Rap1A Asn17, Rap2B Asn17, and Ras Ala19) forms of the GTPases. In contrast, PDEδ only interacted with those proteins containing an intact COOH terminus, irrespective of the mutations that may also affect their nucleotide binding properties: Table I shows that neither interacted with proteins carrying a COOH-terminal truncation, nor with the Ras mutant Val12→Ser186 where the Cys
Target of farnesylation has been replaced by a Ser.

Solubilization of Ras Family GTPases by PDEδ—Previous reports had shown that purified PDEδ protein was able to solubilize the two α and β catalytic subunits of retinal rod cell cGMP phosphodiesterase, as well as the Rab13 GTPase from membranes in vitro (19, 21); we sought to determine whether PDEδ could exert a similar activity on Ras family GTPases. Indeed, Fig. 1 shows that recombinant PDEδ, purified from a baculovirus/insect cell expression system, extracted a significant proportion of endogenous Rap1 and Rap2, as well as ectopically expressed Ha-Ras proteins from the membranes of HeLa cells. Its activity was not dependent on the nucleotide (GDP- or GTP-bound) state of the GTPases, consistently with previous studies in mammalian cells that had shown that purified PDEδ could exert a similar activity on Ras family GTPases.

Numerous previous studies in mammalian cells have established that at steady state, nearly all of the endogenous Ras and Rap proteins are membrane-bound. We investigated whether the overexpression of PDEδ would affect the membrane association of Ras family proteins in vivo. Fig. 2A shows that the ectopic overexpression of PDEδ in HEK293 cells indeed created a sizeable cytosolic pool of Rap1, Rap2 proteins; a similar effect, albeit to a lesser extent, was also observed with Ras.

Overexpression of PDEδ Does Not Interfere with the Post-translational Processing of Ras Family GTPases—Since our results show that the overexpression of PDEδ affected Rap and Ras localization at steady state, one possibility is that this could have resulted from an interference with the post-translational processing of Ras family GTPases, resulting in the cytosolic accumulation of immature proteins. We evaluated processing by the ability of prenylated Ras and Rap proteins to partition into the detergent-rich phase of the detergent Triton X-114, as well as to migrate faster by SDS-PAGE. GTPases and PDEδ protein were detected by Western blotting with rabbit affinity-purified anti-Rap1, anti-Rap2, and anti-Ras antibodies, and mouse monoclonal antibodies directed against Ras. Lanes labeled T represent the membranes prior to incubation with buffer or PDEδ.

FIG. 1. In vitro solubilization of Ras and Rap GTPases from membranes. Membranes were prepared from untransfected HeLa cells (for the analysis of Rap1, Rap2, and RapB) or cells that had been transfected with a pRK5-HRas expression vector. They were loaded with GDP or Gpp(NH)p and then incubated for 30 min at 30 °C with recombinant (His)6-PDEδ (+ lanes) or buffer alone (− lanes). Solubilized material was then recovered by ultracentrifugation. GTPases were detected by Western blotting with rabbit affinity-purified anti-Rap1 and anti-Rap2 antibodies, as well as mouse monoclonal antibodies directed against Ras. Lanes labeled T represent the membranes prior to incubation with buffer or PDEδ.

FIG. 2. Overexpression of the PDEδ protein in HEK293 cells creates a soluble pool of GTPases. HEK293 cells were transfected with pRK5-myc or pRK5-mycPDEδ constructs. 36 h later, they were lysed and the particulate (Mb) and cytosolic (Cyt) fractions were isolated. A, 75 μg of protein from each fraction were separated by SDS-PAGE. GTPases and PDEδ protein were detected by Western blotting with rabbit affinity-purified anti-Rap1, anti-Rap2, and anti-Ras antibodies and mouse anti-Myc 9E10 antibodies. B, the cytosolic fraction from cells overexpressing or not PDEδ was adjusted to 1% Triton X-114, and material partitioning in the detergent phase was analyzed by Western blotting for the presence of Rap1.
significant way the extent or kinetics of Ha-Ras processing.

PDEδ Creates a Cytosolic Pool of Ras GTPases during Their Trafficking to the Plasma Membrane—We next investigated whether PDEδ might play a role in the trafficking process that leads newly synthesized cytosolic Ras proteins to their final destination, the plasma membrane, by following the subcellular localization of ectopically expressed GFP-Ras fusion proteins; to this end, we used full-length N- and K-Ras(4B) proteins (GFP-Nras and GFP-Kras, respectively), as well as the COOH-terminal region of Ha-Ras fused to the COOH terminus of GFP (GFP-HRasCt). As described previously (8, 15), GFP-Ras fusion proteins initially associated with endomembranes in a perinuclear compartment (not shown). At this step, Ras proteins have undergone farnesylation, proteolytic removal of the three COOH-terminal residues, and carboxymethylation of the COOH-terminal farnesylcysteine (8). After 13 h of expression, GFP-Ras proteins still labeled endomembrane compartments, and staining of the plasma membrane became clearly visible in control cells (Fig. 4, b, f, and j). In contrast, cells overexpressing PDEδ exhibited a strong cytosolic GFP-Ras labeling in addition to the plasma membrane signal (Fig. 4, d, h, and l). It is remarkable that this effect of PDEδ was similar for Ha- and N-Ras proteins that are palmitoylated and transported to the plasma membrane via the exocytotic pathway, as well as for K-Ras(4B) that carries a polybasic sequence and proceeds to the plasma membrane via a yet uncharacterized route.

Since PDEδ was able to act on the nonpalmitoylated K-Ras(4B) protein, we investigated whether it could also act on partially processed nonpalmitoylated Ha- and N-Ras proteins, at some intermediate step of their trafficking. To that end, we analyzed its effects on GFP fused with a mutant of N-Ras in which the cysteine target for palmitoylation had been mutated to serine; such a protein cannot reach the plasma membrane and accumulates in the endoplasmic reticulum as seen by its co-localization with PDI, a marker of this compartment (8) (see Fig. 5, a–c). Fig. 5, d and e, show that the overexpression of PDEδ caused the bulk of this prenylated and nonpalmitoylated GFP-Nras fusion protein to accumulate in the cytosol, despite the fact that the endoplasmic reticulum remained intact (Fig. 5f). A similar result was obtained with a nonpalmitoylated mutant of the COOH-terminal region of Ha-Ras fused to GFP (data not shown).

PDEδ Exhibits Striking Structural Similarities with RhoGDI—This property of PDEδ to extract prenylated Ras family proteins is reminiscent of the well characterized action of RhoGDI and RabGDI proteins on Rho and Rab family GTPases, respectively. We therefore searched whether PDEδ exhibited any similarity with these proteins using the PSI-BLAST program at NCBI. At convergence by iteration 2, similarities were observed with the RhoGDI sequence from fission yeast as well as with its human counterpart (24 and 20% identity over 78 amino acids, respectively, as indicated by the arrows in Fig. 6A). To investigate whether this observed sequence relationship could correspond to structural similarities, we completed the sequence analysis by using information pertaining to secondary structure organization (hydrophobic cluster analysis or HCA (26), not shown) as well as three-dimen-

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**Fig. 3.** *In vivo* kinetics of Ha-Ras processing. HeLa cells were transfected with pEXV-Hras and pRK5-PDEδ or pRK5-myc vectors. 15 h later, neosynthesized proteins were pulse-labeled with [35S]methionine for 20 min. Cells were then chased for indicated times with non-radioactive methionine, lysed in Triton X-114, and fractioned as described under “Experimental Procedures.” Ras GTPases contained in each fraction were immunoprecipitated, analyzed by SDS-PAGE, and visualized by fluorography. Lanes a, aqueous fraction; lanes d, detergent fraction.

**Fig. 4.** *In vivo* trafficking of Ha-, N-, and K-Ras(4B) GTPases. HeLa cells were co-transfected with pEGFP-HRasCt, pEGFP-Nras, or pEGFP-Kras together with pRK5 (-) or pRK5-PDEδ (+) expression vector. 13 h later, cells were fixed, stained for the expression of PDEδ, and observed by indirect immunofluorescence. a, c, e, g, i, and k, PDEδ expression; b and d, GFP-HrasCt; f and h, GFP-Nras; j and l, GFP-Kras.

**Fig. 5.** *In vivo* trafficking of a nonpalmitoylated N-Ras mutant. HeLa cells were co-transfected with pEGFP-NrasC181S and pRK5 (-) or pRK5-PDEδ (+) expression vector. 13 h later, cells were fixed, stained for the expression of PDEδ and the endoplasmic reticulum marker PDI, and observed by indirect immunofluorescence. a and d, PDEδ expression; b and e, GFP-NrasC181S; c and f, PDI.
ional experimental observations available for RhoGDI, such as the structure reported for its complex with the Cdc42 GTPase. GDI proteins are organized in two domains (Fig. 6B), left. The COOH-terminal geranylgeranyl-binding domain folds into an immunoglobulin-like H9252-sandwich whose opposite sheets display a hydrophobic pocket in which the geranylgeranyl moiety of the Cdc42 GTPase inserts; this domain is responsible for the ability of RhoGDI to extract Cdc42 from membranes. An amino-terminal region (green), comprising a helix-loop-helix, is likely to be responsible for the inhibitory effect of RhoGDI on GDP dissociation and GTP hydrolysis by Cdc42 through interactions with the switch I and II regions. A good correspondence was observed between PDEδ and RhoGDI, in particular for the two last strands (H and I) and the loop between them (three conserved aspartic acids) (see Fig. 6A). HCA analysis enabled us to extend the similarity between PDEδ and RhoGDI to the very NH2 terminus of PDEδ, which noticeably corresponds to the NH2-terminal end of the RhoGDI immunoglobulin domain. Accordingly, PDEδ would lack the NH2-terminal regulatory arm characteristic of RhoGDI.

Buried hydrophobic positions of the RhoGDI Ig-like domain, in particular those corresponding to core secondary structures (strands A, D, E, and H in Fig. 6A and B), are conserved in PDEδ, strongly supporting a shared structure for the gera-
nlygeranyl-binding domain of RhoGDI and PDEδ. Moreover, some particular characteristics of the RhoGDI Ig-like fold are also maintained in PDEδ, such as the presence of two additional small strands between strands A and D. Remarkably, most of the amino acids lining the geranylgeranyl-binding pocket of RhoGDI are conserved in PDEδ (stars on Fig. 6A, violet side chains on Fig. 6B). Only amino acids contributing to the external part of the pocket and belonging the NH2 terminus of RhoGDI (Leu11 and Ile14) are absent in PDEδ. Another GTPase-interacting region of RhoGDI is conserved in PDEδ, involving the conserved Asp185 in the H-I loop (Asp147 in PDEδ) whose side chain forms a hydrogen bond with the guanidinium group of Cde42 Arg46 in the Cde42-RhoGDI complex (Fig. 6B).

In light of these relationships, we constructed a model for the three-dimensional structure of PDEδ (Fig. 6B, right). This model proposes that PDEδ should adopt the Ig-like fold of RhoGDI and retain its hydrophobic geranylgeranyl-binding pocket. It predicts that PDEδ should retain the function of the Ig-like domain of RhoGDI, namely binding prenylated GTPases, allowing their release from cellular membranes; in contrast, the absence in PDEδ of a domain corresponding to the NH2-terminal arm of RhoGDI should imply that the action of PDEδ would be independent of the GDP- or GTP-bound state of GTPases, in accordance with our experimental results (see Fig. 1).

**DISCUSSION**

In this manuscript, we show that the δ subunit of cGMP phosphodiesterase from retinal rod cells, PDEδ, which is ubiquitously expressed (19, 21), can act in vitro as well as in vivo to solubilize Ras as well as the closely related Rap proteins from the membranes of mammalian cells. Proteins able to exert such an activity on GTPases from the Rho and Rab families, RhoGDI (18) and RabGDI (17) respectively, have already been identified. However this is the first time that such an activity has been described to act in vitro as well as in vivo on Ras and Rap proteins.

The sequence of PDEδ is highly conserved among mammals, since the bovine, canine, and murine proteins only differ from the human one by one nonconservative (T68A) and zero to three conservative changes; a search for homologous proteins in invertebrate species revealed the existence of orthologues in *Drosophila melanogaster* and *Caenorhabditis elegans*, respectively, sharing 61 and 69% identity with human PDEδ (Fig. 7A, upper panel). Such a high evolutionary conservation strongly suggests that the function of PDEδ has been conserved as well. Searches for the conservation of sequence and structural features of PDEδ with other proteins revealed striking similarities with the established structure of RhoGDI, as visualized in its complex with the Cde42 GTPase (18). This allowed us to build a model for the three-dimensional structure of human PDEδ suggesting that it may also adopt a general Ig-like fold very similar to that of RhoGDI. More importantly, our model suggests that the pocket lined with hydrophobic residues in RhoGDI that binds the geranylgeranyl group of Cde42 is likely to be conserved in PDEδ, hence providing structural support for our functional data showing that PDEδ is able to extract prenylated proteins such as Ras family GTPases from membranes.

A further search for proteins closely related to PDEδ revealed significant similarities with the UNC-119/RG4 group of proteins from mammals, zebrafish, *C. elegans*, and *D. melanogaster* (Fig. 7A, lower panel). These proteins, of yet unknown function, were identified on the basis of their high expression in the *C. elegans* nervous system as well as in mammalian photoreceptor cells (29, 30). Besides an unrelated NH2-terminal region of 50–90 residues (depending on the species), they exhibited significant sequence similarities with PDEδ (47% similarity, 22% identity for the human proteins), extending through to their COOH termini. Alignment of UNC119/RG4, PDEδ, and RhoGDI of human origin revealed that the structural features conserved between PDEδ and RhoGDI, namely the elements responsible for their Ig-like fold and the presence of hydrophobic residues at conserved positions, which could line the surface of a prenyl-binding pocket, are also conserved in UNC119/RG4 (Fig. 7B). Hence we propose that UNC119/RG4, PDEδ, and RhoGDI define a novel family of proteins conserved through evolution, that interact with prenylated proteins, and whose function is to regulate their association with membranes.

One major difference between PDEδ and RhoGDI is that the latter exhibits a strong functional preference for GDP-bound Rho GTPases (31, 32), whereas PDEδ is able to solubilize both the GDP- and GTP-bound form of Ras and Rap. Such a distinction may have important implications for the physiological processes involving the function of PDEδ, such as possibly the trafficking of prenylated proteins. The NH2-terminal domain of RhoGDI, by interacting with switch I and switch II regions of the Cde42 GTPase, is responsible for this selectivity, as well as the inhibition of GDP dissociation and GTPase-activating protein-stimulated GTPase activity (18). The fact that there is no equivalent region in PDEδ supports our data that it extracts GTPases from membranes independently of the nucleotide (GDP or GTP) bound and predicts that PDEδ should neither affect the rate of nucleotide binding and dissociation, nor the GTPase activity of Ras family proteins. UNC-119/RG4 proteins present an NH2-terminal domain, upstream of the Ig-like core, that exhibits the same length and low content in hydrophobic residues as RhoGDI (not shown). By analogy, it is tempting to propose that this region could play a role in the selectivity of UNC119/RG4 proteins for their prenylated targets.

Another distinctive feature between PDEδ and RhoGDI is the large size and sequence divergence of the loop connecting β-sheets B and C (Fig. 6, A and B). This region is highly exposed in both structures and could be involved in their interactions with other nonprenylated proteins. Indeed, PDEδ has been reported to interact with the retinis pigmentosa GTPase regulator (33) and the Arf-like protein Arl3 (34). However the role of such interactions, and possible positive or negative interference with the membrane-extracting ability of PDEδ, are presently unknown.

RhoGDI and PDEδ exhibit a very different spectrum of activity: while RhoGDI is only active on GTPases of the Rho family, PDEδ is able to exert its membrane extraction activity on a wide variety of proteins, all of which are prenylated on their COOH-terminal extremity (Table II). Indeed, PDEδ has previously been shown to be able to solubilize the α and β catalytic subunits from retinal rod cGMP phosphodiesterase (19), which are, respectively, modified by the C15 farnesyl and C20 geranylgeranyl isoprenoids, as well as the geranylgeranylated Ras-related Rab13 protein (21). We additionally established that PDEδ is active on Ras and Rap proteins of the Ras family and that it is also active on the Rho family protein RhoA (data not shown). All of these proteins, including the Rab family protein Rab13, carry a COOH-terminal CAAX sequence that directs them to be modified by a single farnesyl or geranylgeranyl group; they are further processed by proteolysis of the three COOH-terminal residues, and carbamethylation of the prenylcytsteine, modifications that have been shown to be necessary for binding to PDEδ in vitro (35). In contrast, Rab4 and Rab6, which, respectively, exhibit COOH-terminal CGC and CSC sequences and are modified by two geranylgeranyl groups, are not targets of PDEδ (21). Surprisingly, we show that Rab proteins (Rala and RabB), which are closely related to
Ras, cannot interact with PDE\textsubscript{6} in the yeast two-hybrid system (Table I) and that RalB is not extracted from membranes \textit{in vitro} by recombinant PDE\textsubscript{6}/H9254 (Fig. 1). Their lack of interaction with PDE\textsubscript{6}/H9254 could indicate that they are not processed \textit{in vivo} like other CAAX-containing proteins; this is, however, unlikely since their COOH-terminal sequences (CCIL for RalA and CCLL for RalB) are identical or closely related to those of the catalytic subunits of retinal rod cGMP phosphodiesterase (CCIQ and CCIL, respectively, see Table II), which are indeed extracted from membranes by PDE\textsubscript{6}/H9254 (19). Alternatively, it is possible that the COOH-terminal region of Ral could adopt a conformation that prevents its interaction with PDE\textsubscript{6}/H9254; this point could be investigated experimentally by using Ras/Ral chimeric proteins.

Upstream from the CAAX sequence, some GTPases that are susceptible to the action of PDE\textsubscript{6} such as K-Ras(4B), Rap1A, and RhoA carry a polybasic sequence that has been shown in the case of K-Ras(4B) to address the newly synthesized protein via a yet undescribed trafficking pathway toward the nonraft plasma membrane (8, 15). Others, such as Ha-Ras, N-Ras, Rap2 (A and B), and RhoB carry one or two palmitoylation sites upstream from the prenylation site (see Table II). Using mutants, we have shown that PDE\textsubscript{6}/H9254 is active on the nonpalmitoylated forms of Ha- and N-Ras proteins. However, since palmitoylation, in contrast with prenylation, is not a stable modification in cells and exhibits a mean turnover time of less than 30 min (36), a significant proportion of cellular Ha-Ras, N-Ras, and Rap2 proteins should be depalmitoylated at steady state. It is therefore conceivable that in our experiments, PDE\textsubscript{6}/H9254 only extracted nonpalmitoylated Ras and Rap2 proteins. We attempted to address this question directly by comparing the ability of recombinant PDE\textsubscript{6} to extract wild-type and nonpalmitoylated mutants of Ha- and N-Ras fused to GFP from the membranes of transfected HeLa cells; however, due to the relative instability of the mutant proteins, we were unable to perform comparative experiments. In the case of RhoGDI, the reported structure for the geranylgeranyl-binding pocket is unlikely to accommodate a nearby palmitate group, and a re-
PDEδ Regulates Membrane Association of Ras and Rap

Prenylated cysteines are in red, palmitoylated cysteines in green and underlined, and polybasic sequences in blue and underlined. F, farnesy1; GG, geranylgeranyl; palm, palmitoylation sites; memb extraction, extraction from membranes; overexpr, experiment performed with ectopically expressed GTPase; GFP, microscopic analysis of GFP fusion proteins; WB, Western blot assessment of membrane extraction by purified PDEδ endog, in vitro extraction from membranes of endogenous GTPases by recombinant PDEδ (Fig. 1); nd, not done.

| targets | C-terminal sequences | prenyl | palm | two hybrid interaction | memb extraction overexpr | endog |
|---------|----------------------|--------|------|------------------------|------------------------|-------|
| H-Ras  | NPPDSEGPCGMSCKVL       | F      | 2    | +                      | GFP+WB                 |       |
| N-Ras  | NSSDDGTCGMCGLPCCVNN     | F      | 1    | +                      | GFP                    | 4c    |
| K-Ras(4B)| SKDGKKKSSKSKTVL         | F      | -    | nd                     | GFP                    | 4c    |
| Rap1A  | RKTPVEKKPKKSSCLL        | GG     | -    | +                      |                        |       |
| Rap1B  | RKTPVPGAKRRKSSQLL       | GG     | -    | nd                     |                        |       |
| Rap2A  | YAQQPDKDPPCCSACTQ       | F      | 2b   | +                      | WB                     | 4c    |
| Rap2B  | YAAQSDNGECSCAVL         | GG     | 2b   | +                      |                        |       |
| Ra1A   | KKKRKLAKRTRECCIL        | GG + GG ?  | -    | -                      |                        |       |
| Ra1B   | GKKSSNREEPKRECCIL       | GG + GG ?  | -    | -                      |                        |       |
| Rap2A  | YAQQPDKDPPCCSACTQ       | F      | -    | +                      |                        |       |
| Rap2B  | YAAQSDNGECSCAVL         | GG     | -    | nd                     |                        |       |
| Rnd1   | ŁQKRYSGQGNGCCVL         | F or GG | 2b   | +                      |                        |       |
| Rab6B  | SKLEPQEPVPVSEGSC       | GG + GG | -    | -                      |                        |       |
| Rab13  | STDLaTEDNRNSKLG         | GG     | +    | -                      |                        | 4d    |
| PDEδα  | GNPSPGGATSSCCTIQ        | F      | -    | nd                     | 4e                      |       |
| PDEδβ  | TEICNGGGPPSSCCTIL       | GG     | -    | nd                     | 4e                      |       |

*a In the case of Ra1A and Ra1B, the possibility that both cysteines of the CCX motif could be palmitoylated is hypothetical.

*b Palmitoylation of both cysteines has been demonstrated for Ha-Ras and remains hypothetical for Rap2 (A and B) as well as for RhoB and Rnd1.

*c Due the absence of sensitive and specific antibodies that discriminate between simultaneously expressed Ras and Rap isoforms, biochemical extraction of the endogenous protein from membranes by recombinant PDEδ in vitro may concern one or more isoforms.

*d This work and Ref. 21.

*e From Ref. 19.

cent report establishes that a palmitoylation site inserted into the COOH-terminal sequence of RhoA blocks RhoGDI binding (32). Given its structural similarities with RhoGDI, it is possible that PDEδ is only active on the nonpalmitoylated form of Ras family proteins.

The effects of PDEδ overexpression on Ras trafficking provide some insight into the possible physiological role of this ubiquitously expressed protein. Contrarily to the case of Rho and Rab proteins, there is no significant cytosolic pool of Ras and Rap proteins, which indicates that the main function of PDEδ is probably not to regulate the level of membrane bound GTPases available for signal transduction. Following their synthesis as cytosolic precursors and prenylation in the cytosol, Ras proteins associate with endomembranes such as the endoplasmic reticulum and Golgi which constitute the site of their proteolysis and carboxymethylation (8). One possibility is that PDEδ binds the newly prenylated proteins in the cytosol and escorts them to the endomembranes. This is, however, unlikely, since PDEδ only binds prenylated proteins after they have undergone proteolysis and carboxymethylation (35). Moreover, a recent report suggests that the prenylated Rab acceptor protein PRA1, which interacts and co-localizes with Ha-Ras and RhoA proteins in the Golgi compartment (37) could actually fulfill that role, i.e. escort newly prenylated proteins from the cytosol to endomembranes. Observation of later trafficking steps shows the overexpression of PDEδ induces the formation of an important cytosolic pool of Ras proteins, but that some Ras proteins nevertheless reach the plasma membrane under those conditions (see Fig. 4). We have shown that the same effect of PDEδ overexpression is observed with all three Ras proteins, despite their distinct trafficking pathways from endomembrane compartments to the plasma membrane (15). We therefore surmise that PDEδ should intervene at some common step along this pathway and propose that it may play a role in the process that delivers prenylated proteins from endomembranes, once they have undergone proteolysis and carboxymethylation, to the structures that ensure trafficking to their respective resident compartments.

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