The delta subunit of rod specific cyclic GMP phosphodiesterase, PDE δ, interacts with the Arf-like protein Arl3 in a GTP specific manner

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Abstract Recently, we have shown that the δ subunit of the cGMP phosphodiesterase (PDE δ) interacts with the retinitis pigmentosa guanine regulator (RPGR). Here, using the two-hybrid system, we identify a member of the Arf-like protein family of Ras-related GTP-binding proteins, Arl3, that interacts with PDE δ. The interaction was verified by fluorescence spectroscopy and co-immunoprecipitation. Arl3 features an unusually low affinity for guanine nucleotides, with a K_D of 24 nM for GDP and 48 μM for GTP. Fluorescence spectroscopy shows that PDE δ binds and specifically stabilizes the GTP-bound form of Arl3 by strongly decreasing the dissociation rate of GTP. Thus, PDE δ is an effector of Arl3 and could provide a novel nucleotide exchange mechanism by which PDE δ stabilizes Arl3 in its active GTP-bound form.

Key words: Phosphodiesterase δ; Retinitis pigmentosa; Spectroscopy

1. Introduction

Retinitis pigmentosa (RP) designates a heterogeneous group of hereditary retinal dystrophies characterized by impaired dark adaptation, progressive visual field defects and severe reduction in visual acuity. X-linked RP (xlRP) is the most severe form of RP. The most common form of xlRP is RP type 3 (RP3) which affects about 70% of xlRP patients and is caused by mutations in the RP guanine regulator gene (*RPGR*). The full length RPGR gene encodes a 90 kDa protein. However, it appears to be spliced in a tissue specific manner and in human and bovine retina, a 52 kDa version is most abundant (A. Wright, unpublished observations). The 52 kDa RPGR protein contains a domain which is homologous to the regulator of chromosome condensation (RCC1), the guanine nucleotide exchange factor of Ran [1,2]. Mouse RPGR has been localized to the Golgi apparatus by immunofluorescence studies, indicating a possible role in vesicular transport [3]. Recently, we have identified the δ subunit of the rod cyclic GMP phosphodiesterase (PDE δ) to interact with the RCC1-like domain (RLD) of RPGR. Mutants of RPGR found in RP3 patients are unable to interact with PDE δ [4].

PDE δ was isolated as the fourth subunit of bovine rod PDE (PDE6) [5]. PDE6 is the effector for the trimeric G protein transducin after its activation by light sensitive rhodopsin molecules. PDE δ was originally shown to bind to the prenylated C-terminus of PDE α,β, which resulted in a translocation of the PDE6 holoenzyme from a membrane to a cytoplasmic fraction [6]. Furthermore, PDE δ has previously been shown to solubilize membrane-bound Rab13 to the cytoplasm [7]. Together, this suggested a possible function for PDE δ similar to the function of GDP dissociation inhibitors (GDIs), which bind to the prenylated proteins to extract them from cellular membranes. To further elucidate the function of PDE δ, we subjected PDE δ to a two-hybrid screen. We identified an Arf-like protein, Arl3, which binds PDE δ in its GTP-bound but not GDP-bound conformation, suggesting a possible role for PDE δ as an effector for Arl3.

2. Material and methods

2.1. Plasmids and strains

PDE δ was cloned as a *Bam*HI fragment into the two-hybrid vector pBTM116 and as a *Bam*HI/*Eco*RI fragment into pVP16, pET28a and Flag-pcDNA3. Restriction sites were generated by PCR using appropriate primers. Arl3, Arl3T31N and Arl3Q71L (mutations were generated according to Picard et al. [8]) were cloned as a *Eco*RI/*Bam*HI fragment into pBTM116, as a *Nco*I/*Hin*dIII fragment into pET21d and as a *Kpn*I/*Bam*HI fragment into pcDNA3. All clonings and mutations were verified by cycle sequencing. *Saccharomyces cerevisiae* reporter strain L40: MATa, his3Δ200, trp1-901, leu2-3,112, ade2, LYS::(lexAop)$_4$-HIS3, URA3::(lexAop)$_8$-lacZ, GAL4, gal80. *E. coli* strains for protein expression: BL21 (DE3) and B834 (DE3) pLysS (Novagen).

2.2. Two-hybrid screening

We used murine PDE δ in pBTM116 as bait plasmid of the lexA-based two-hybrid system (containing the L40 reporter strain, the pBTM116 and pVP16 vectors and the murine cDNA library fused to pVP16), kindly provided by Stan Hollenberg (Vollum Institute, Portland, OR, USA). The yeast reporter strain L40 was transformed with the PDE δ construct as described [9]. We screened 5×10^7 clones containing a size-selected mouse cDNA library of 9.5–10.5 day embryos cloned in pVP16. After 4 days at 30°C, we obtained 18 Arl3 clones on medium selecting for protein-protein interaction (i.e. THULLy medium which lacks tryptophan, histidine, uracil, leucine and lysine; 25 mM 3-aminotriazole was also added to compensate for basal promotor activity).

2.3. Two-hybrid interaction assay

The reporter strain L40 was transformed with the appropriate constructs. The resulting transformants were grown for 4 days on medium selecting either for the presence of both plasmids (i.e. UTL medium lacking uracil, tryptophan and leucine) or for protein-protein interaction (i.e. THULLy medium)

2.4. Protein expression

5 l LB medium containing ampicillin was inoculated with an overnight culture of Arl3-pET21d-BL21 (DE3). Overnight expression at 30°C was induced at an OD_{600} of 0.7 with 100 μM IPTG. Arl3 protein was isolated from $100\,000\times g$ supernatant in 100 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$ with SourceQ chromatography (Pharmacia). Ammonium sulfate precipitation and S-75 Superdex chromatography in 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$ yielded pure and nucleotide-free protein. PDE δ expression at 28°C in the pET28a-B834 (DE3)-pLysS system was induced with 300 μM IPTG at an OD_{600} of 0.6.

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Cells were harvested after 6 h and the 100 000×*g* supernatant in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl was applied onto a Ni^{2+} NTA column (Qiagen). After imidazole elution, PDE δ was stabilized in buffer with 10% glycerol.

2.5. Transfection and immunoprecipitation

Human 293 kidney cells [10] were cultured in MEM with Glutamax (Gibco) and 10% fetal calf serum and transfected using the calcium-chloride method. To assay protein expression, cells were transfected either with wild-type (WT) Arl3, Arl3T31N, Arl3Q71L in pcDNA3 or with Flag-tagged PDE δ in pcDNA3, respectively. After 48 h, cells were harvested and protein expression was detected by Western blotting using either a polyclonal anti-ARL3 antibody from rabbit (produced by immunization of two rabbits with native Arl3 and subsequent protein G-Sepharose purification of the sera) or a mouse monoclonal anti-Flag antibody (M5, Sigma). For co-immunoprecipitation of the PDE δ-Arl3 complex, cells were co-transfected with the appropriate Arl3 construct and PDE δ. After 48 h, cells were lysed by sonification in lysis buffer (30 mM Tris-HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% NP-40, 2.5% glycerol and protease inhibitor (Boehringer complete)). The lysate was successively incubated with anti-Flag antibody and protein G-Sepharose (Pharmacia) for 1 h. After washing the protein G-Sepharose twice with lysis buffer, protein was detected by Western blotting using anti-ARL3, anti-Flag, anti-rabbit and anti-mouse horseradish peroxidase (HRP) antibody (HRP conjugates from Amersham).

2.6. Fluorescence measurements

All measurements were recorded with a Fluoromax-2 spectrophotometer (Spex Industries, Edison, UK) using *N*-methylanthranil acid-labelled guanine nucleotide (mant guanine nucleotide). Mant fluorescence was excited at 350 nm. All measurements were performed in 50 mM KPO_4, pH 7.4, 2 mM $MgCl_2$ at 30°C. The nucleotide concentration was determined by reverse phase HPLC with a C_{18} column in 100 mM K_2HPO_4/KH_2PO_4, 10 mM tetrabutylammoniumbromide and 20% acetonitrile.

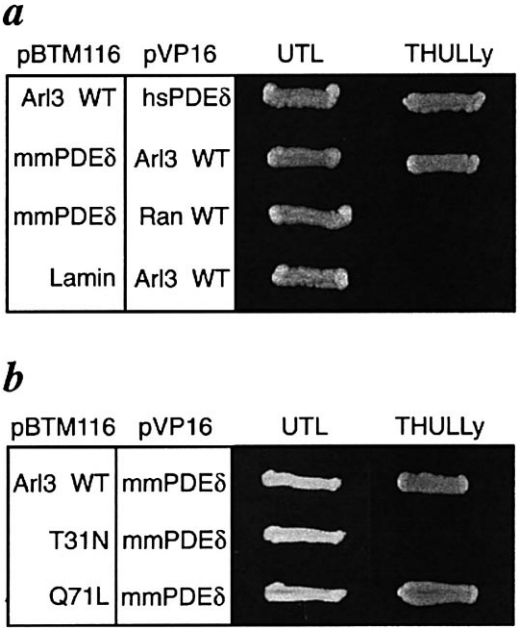

Fig. 1. Interaction of Arl3 with PDE δ in the two-hybrid system. a: The plasmid combinations used for yeast transformation are shown on the left panel, the growth of the appropriate yeast clones on medium selecting either for the presence of both plasmids (UTL) or for protein-protein interaction (THULLy) is shown on the right panel. Arl3 interacts with human and with the 98% identical murine PDE δ in both plasmid combinations. As negative controls, lamin and the small GTPase Ran were used. b: Interaction of WT-Arl3 and its mutants T31N and Q71L with murine PDE δ in the two-hybrid system. Arl3 and Arl3Q71L grew on THULLy medium as indicator for protein-protein interaction whereas Arl3T31N did not interact with PDE δ.

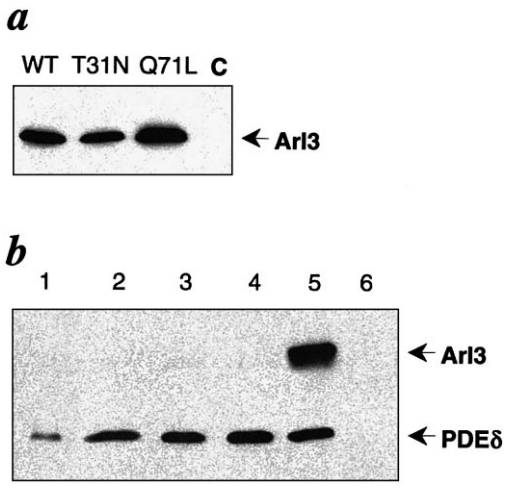

Fig. 2. a: Expression of WT-Arl3 and its mutants Arl3T31N and Q71L in transfected 293 cells. Human kidney 293 cells were transfected with Arl3 in pcDNA3 or its mutants, respectively. 48 h after transfection, the cell lysates were prepared for Western blotting. Proteins were detected by rabbit anti-ARL3 antibody and anti-rabbit HRP-conjugated antibody. The right lane (C) contains lysate from untransfected cells as a control. b: Arl3Q71L binds specifically to PDE δ in transfected 293 kidney cells. Flag-tagged PDE δ was immunoprecipitated from 293 cells transfected with Flag-PDE δ-pcDNA3 and ARL3-pcDNA3 or its mutants, respectively. Lysates were immunoprecipitated using anti-Flag antibodies. Immunoprecipitates were prepared for Western blotting and PDE δ and Arl3 were detected by anti-Flag or anti-ARL3 antibodies, respectively. PDE δ expression in lysates form 293 cells after 48 h (lane 1), PDE δ after immunoprecipitation (lane 2), PDE δ after immunoprecipitation in the presence of WT-Arl3 (lane 3), PDE δ after immunoprecipitation in the presence of Arl3T31N (lane 4) or in the presence of Arl3Q71L (lane 5). Only Arl3Q71L was co-immunoprecipitated with PDE δ. This indicates a GTP specific Arl3-PDE δ interaction under physiological conditions. The lysate in lane 6 was treated like in lane 5 but the antibodies were omitted to account for unspecific binding to protein G-Sepharose.

3. Results

Using PDE δ as a bait in a two-hybrid screen together with a mouse embryo cDNA library, we obtained the Arl3 gene as a potential candidate (accession: GenBank AF143241). The interaction of PDE δ and Arl3 in the two-hybrid system was deemed specific since Arl3 also interacted with PDE δ when present on the bait plasmid but did not interact with lamin. Furthermore, PDE δ did not interact with Ran, another small GTP-binding protein used as a control (Fig. 1a).

Arl3 is 45% identical to Arf1 and thought to have an analogous function in intracellular transport like it was suggested for its close relative Arl2 [11]. As the function of guanine nucleotide-binding proteins is mainly determined by their nucleotide-binding characteristics, we first determined the nucleotide affinity of WT-Arl3 and of two mutants, Arl3T31N and Arl3Q71L. In analogy to Ras and other small GTP-binding proteins, the T31N mutant is assumed to be predominantly in its GDP-bound conformation [12], whereas the Q71L mutant is predicted to be in the GTP-bound state as this mutation inactivates GTP hydrolysis [13]. The nucleotide

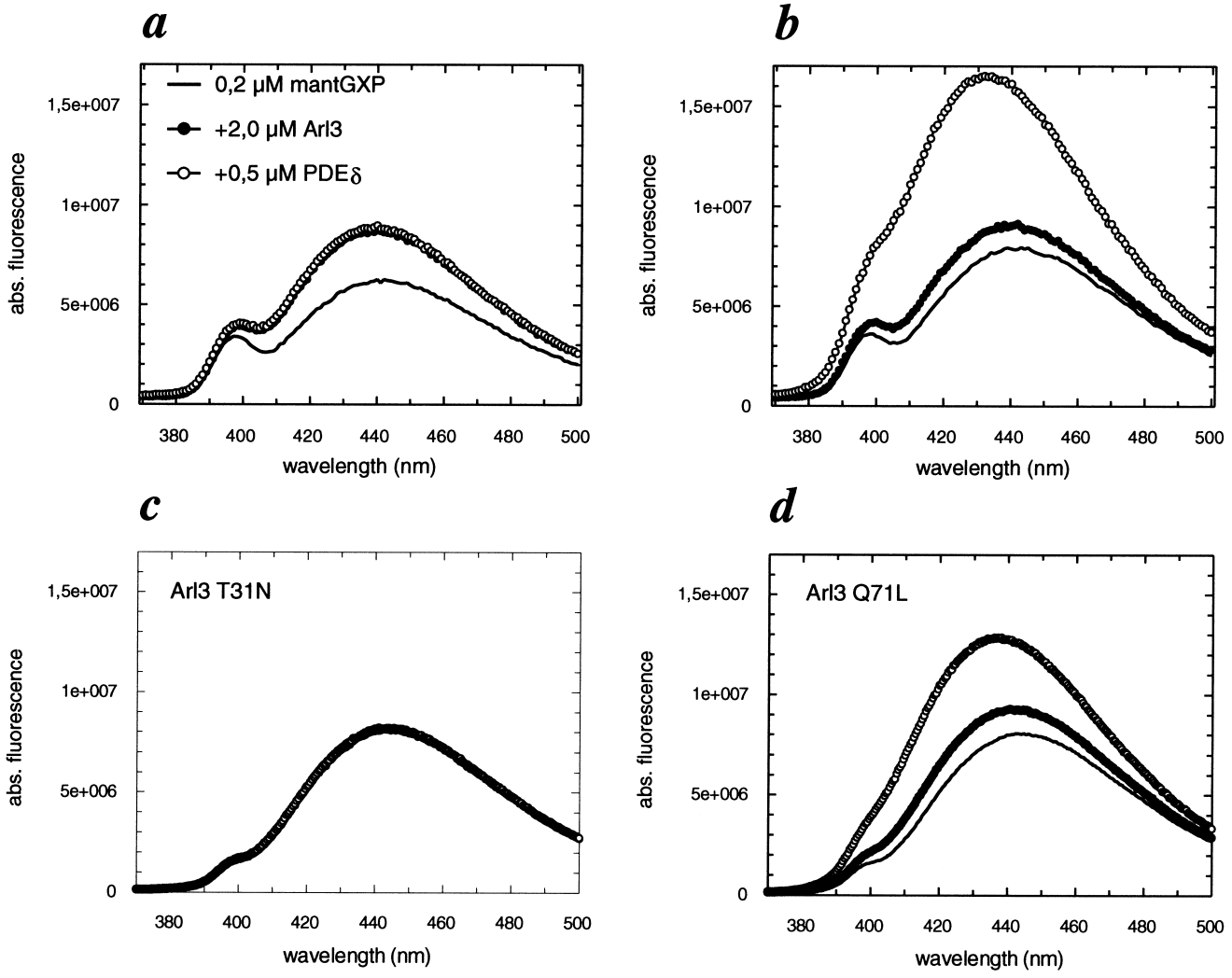

Fig. 3. Change of fluorescence emission spectra of mantGDP (a) and mantGTP (b) upon successive addition of nucleotide-free Arl3 and PDE δ. 2.0 μM nucleotide-free Arl3 protein was added to 0.2 μM mant nucleotide which increased the mant fluorescence emission in both cases. Subsequent addition of 0.5 μM PDE δ increased the fluorescence only in the presence of mantGTP (b). Experiments using Arl3T31N (c) and Arl3Q71L (d) in the presence of mantGTP showed that Arl3Q71l behaves similar to WT-Arl3 whereas Arl3T31N did not bind PDE δ. Results of corresponding experiments using mantGDP were similar to the result in (a).

affinity was determined by fluorescence spectroscopy using mant-labelled guanine nucleotide analogs. First, increasing concentrations of nucleotide-free Arl3 were added to 0.2 μM mantGDP or mantGTP. The time dependent increase of fluorescence was used to determine k_{obs}. k_{obs} was plotted against the Arl3 concentrations to calculate the rate constant of nucleotide association (k_{ass}). Using the equilibrated solution of protein and labelled nucleotide, the rate constant of nucleotide dissociation (k_{diss}) was determined from the decrease of the fluorescence signal after adding a 100-fold excess of unlabelled nucleotide. The nucleotide affinity as K_D was calculated as a ratio of k_{diss} versus k_{ass} (Table 1). The mantGDP affinity of WT-Arl3 was determined to be 24 nM (Table 1), which is in the range of the nucleotide affinity determined for Arf1 [14], a close relative of Arl proteins. The affinity of Arl3T31N, however, is 10-fold and the affinity of Arl3Q71L is 5-fold lower. Most significantly, the affinity for mantGTP is dramatically lower than that for mantGDP. The affinity for mantGTP of WT-Arl3 is 48 μM and that of Arl3Q71L is 35 μM, whereas the mantGTP affinity of Arl3T31N was too low to be measured accurately (Table 1). The decrease in guanine nucleotide affinity caused by the T31N mutation is also found in other small GTP-binding proteins [12] and suggests a similar role for the threonine in the coordination of nucleotide-binding.

To investigate the nucleotide specificity of the interaction,

Table 1
Nucleotide affinities (K_D) of WT-Arl3, Arl3T31N and Arl3Q71L determined by fluorescence measurements using mant-labelled nucleotides

	k_{diss} (s^{-1})	k_{ass} (s^{-1} μM^{-1})	K_D (nM)
MantGDP			
WT-Arl3	0.00017	0.00704	24
T31N	0.00030	0.00116	260
Q71L	0.00011	0.00101	109
MantGTP			
WT-Arl3	0.0351	0.00073	48
T31N	n.d.	n.d.	n.d.
Q71L	0.0454	0.00129	35

n.d., not detectable.

we used Arl3T31N and Arl3Q71L in the two-hybrid system. The two-hybrid interaction assay showed (Fig. 1b) that WT-Arl3 and Arl3Q71L both interacted with PDE δ, whereas the Arl3T31N mutant showed no growth on THULLy medium, indicating no or a very weak interaction. This result suggested that PDE δ binds specifically to Arl3-GTP. Essentially the same result was obtained using recombinant GST-Arl3 and PDE δ in a pull-down experiment (data not shown).

Arl3 and PDE δ are expressed in kidney tissue [6,15]. Therefore, we used human 293 kidney cells to verify the Arl3-PDE δ interaction under physiological conditions. Transfecting 293 cells with pcDNA3-Arl3, pcDNA3-Arl3T31N or pcDNA3-Arl3Q71L resulted in roughly the same expression as detected by Western blotting using anti-ARL3 antiserum (Fig. 2a). To show protein interaction in a co-immunoprecipitation experiment, 293 cells were transfected with pcDNA3-Flag-PDE δ together with either pcDNA3-Arl3, pcDNA3-Arl3T31N or pcDNA3-Arl3Q71L, respectively. PDE δ was immunoprecipitated via the anti-Flag antibody and the immunoprecipitant was subsequently subjected to Western blotting. Using the anti-ARL3 antiserum, only Arl3Q71L could be detected as a co-immunoprecipitant (Fig. 2b). As ArlQ71L is the GTP-bound and therefore the constitutively active form of Arl3, this result further supported the idea that PDE δ is an effector protein of Arl3 which is defined as a protein that interacts specifically with the GTP-bound form of Arl3. This is partially in contrast to the result of the two-hybrid experiment where both WT-Arl3 and Arl3Q71L interacted with PDE δ. It is likely that an Arl3 specific GTPase activating protein (GAP) converts WT-Arl3 to the GDP-bound form in the mammalian cell system. In yeast cells, however, this GAP is probably absent and thus, Arl3 remains in the GTP-bound form during the two-hybrid experiment. The absence of WT-Arl3 in the co-immunoprecipitation experiments indicates that WT-Arl in 293 cells is mostly in the GDP-bound form despite an estimated 20–30-fold higher intracellular concentration of GTP. However, we cannot exclude the possibility that preparing the cells for the immunoprecipitation caused the conversion of Arl3-GTP to Arl3-GDP.

The nucleotide specificity of the Arl3-PDE δ interaction was further analyzed by fluorescence spectroscopy. As observed earlier, adding nucleotide-free Arl3 to mantGDP increased the mant emission signal by 40%, whereas the addition to mantGTP increased the emission signal by only 10% (Fig. 3). The subsequent addition of PDE δ doubled the mantGTP emission signal while there was no change in the mantGDP emission signal. This clearly proved that PDE δ specifically binds to Arl3 in its GTP-bound form but not to its GDP-bound form. The same result was obtained using ARL3Q71L, whereas Arl3T31N showed no increase of fluorescence in the presence of mantGTP and PDE δ (Fig. 3), most likely because the nucleotide affinity of ArlT31N was too low.

Since the affinity of Arl3 for GTP was extremely low, we hypothesized that the binding of PDE δ would have to increase the binding affinity of Arl3 for GTP in order to account for the efficient binding of the two proteins. To determine how PDE δ effects the binding affinity of Arl3 for GTP, 0.4 μM mantGTP was incubated together with a 5-fold excess of nucleotide-free Arl3 and increasing amounts of PDE δ (Fig. 4). Increasing amounts of PDE δ lead to an enhanced nucleotide-binding of Arl3 as measured by the time dependent increase in fluorescence. Here, the increase of the fluorescence

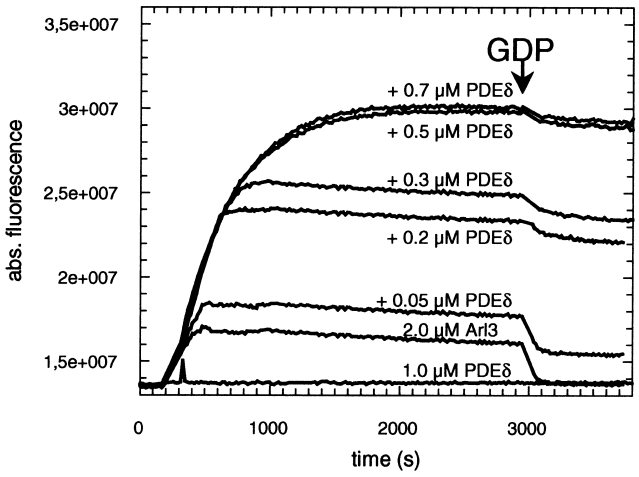

Fig. 4. PDE δ increases the mantGTP affinity of Arl3. The increase in mant emission was measured over time in the presence of 0.4 μM mantGTP together with 1.0 μM PDE δ or together with 2.0 μM Arl3 (lower two curves). Next, increasing amounts of PDE δ (0.05–0.70 μM) and 2.0 μM nucleotide-free Arl3 were added to 0.4 μM mantGTP (following upper curves). The addition of a 250-fold excess of unlabelled GDP after 3000 s (arrow) replaced the mantGTP in the absence of PDE δ, whereas the addition of PDE δ stabilized the mantGTP-Arl3 complex.

signal is caused by two independent steps, the binding of mantGTP to Arl3 and the binding of PDE δ to the Arl3-mantGTP complex. The fluorescence reaches its maximum close to an equimolar ratio of nucleotide and PDE δ (about 0.5 μM), indicating the formation of a ternary mantGTP-Arl3-PDE δ complex with a 1:1:1 stoichiometry. In this complex, mantGTP was bound so tightly to Arl3 that even a 250-fold excess of unlabelled GDP could not replace mantGTP. This effect was not observed when a large excess of GDP was added to mantGDP-ARL3 in the presence of PDE δ. The dissociation rate constant for mantGDP was determined to be 1.1×10^{-4} s^{-1}, regardless of whether PDE δ was present or not (data not shown). PDE δ binding did not alter the mantGTP association rate since the apparent rates (k_{obs}) of all binding reactions were nearly identical (Fig. 4). From these observations, we conclude that PDE δ increases the affinity of Arl3 for GTP by several orders of magnitude and does so by decreasing the nucleotide dissociation rate which qualifies PDE δ as a GTP specific guanine nucleotide dissociation inhibitor.

4. Discussion

Proteins of the Arf-like family share sequence homology with Arf proteins but they lack the typical cofactor activity in cholera toxin-catalyzed ADP ribosylation of $G_{\alpha s}$ and the ability to rescue a lethal $arf1^{-}/arf2^{-}$ double mutant in *S. cerevisiae* [16]. Arl proteins have been localized to the Golgi apparatus and like their closest relatives, the Arf proteins, they are assumed to function in intracellular transport [11]. We have isolated Arl3 in a two-hybrid screen using the δ subunit of the cGMP specific rod phosphodiesterase as a bait. The initial two-hybrid result was verified by co-immunoprecipitation and fluorescence spectroscopy, which shows conclusively that PDE δ binds and stabilizes Arl3-GTP by decreasing the nucleotide dissociation. Therefore, PDE δ is not

only the first effector protein for Arl3 identified but also a GTP specific GDI. The GDI effect of PDE δ is not unusual for effector proteins and was also observed for the effector of Ras, Raf kinase [17].

The 48 μM affinity of Arl3 for GTP is comparatively low and 2000-fold lower than for GDP. This suggests that in vivo, Arl3 exists preferentially in the GDP-bound state despite the fact that the intracellular GTP concentration is significantly higher than the GDP concentration. However, the GTP stabilizing effect of PDE δ binding to Arl3 shows that the nucleotide-bound state is subject to regulation by other factors. In specifically stabilizing the GTP-bound state of Arl3, PDE δ could serve as a 'passive' nucleotide exchange factor whose presence results in an activation of Arl3. GDP to GTP exchange initiates membrane anchoring of Arf1 [18] and subsequently vesicle formation. As PDE δ is localized at intracellular membranes [7]. This would suggest a role for Arl3 in intracellular transport similar to Arf proteins.

PDE δ was originally identified as the forth subunit of the rod cyclic guanine nucleotide phosphodiesterase [5]. Initially, PDE δ was suggested to regulate the membrane localization of the PDE holoenzyme [6] by binding to its prenylated subunits. This function of PDE δ was further supported by its ability to bind to Rab13, a small GTP-binding protein which also possesses a C-terminal consensus motif for geranyl-geranylation. However, our finding that RPGR [4] and Arl3 bind PDE δ in the absence of any post-translational modifications demonstrates that PDE δ binding is not prenylation dependent. Furthermore, since PDE δ binds to many proteins other than the subunits of the PDE6 holoenzyme, it appears that PDE δ is not a genuine subunit of the PDE holoenzyme but merely an associated protein.

The function of RPGR and therefore the cause of RP3 is still unknown. The observation that the RPGR-binding protein PDE δ interacts with small GTP-binding proteins of the Rab and Arl family indirectly puts RPGR in the functional context of vesicular trafficking.

Since the N-terminal region of RPGR is homologous to RCC1, the RLD of RPGR is likely to have a structure similar to the β propeller of RCC1 [2]. Previously, another protein with multiple RLDs, p532 [19], has been shown to interact with proteins of the Rab and Arf family of small GTP-binding proteins, supporting a possible role of RPGR in intracellular transport. It is interesting to note that another important player in intracellular transport, the Rab escort protein 1 (REP1), is involved in a different form of retinal dystrophy named choroideremia [20]. The binding of REP1 to Rab proteins has a GDI effect and interestingly, PDE δ binding has a similar effect on Arl3. However, whereas PDE δ interacts preferentially with the GTP-bound form of Arl3, REP1 prefers the GDP-bound form of Rab proteins [21]. Together, these observations support our hypothesis that RPGR, PDE δ and Arl proteins regulate intracellular vesicle transport, which we will test further in appropriate in vitro transport systems.

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