

The Glutamic Acid-rich Protein-2 (GARP2) Is a High Affinity Rod Photoreceptor Phosphodiesterase (PDE6)-binding Protein That Modulates Its Catalytic Properties*

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The glutamic acid-rich protein-2 (GARP2) is a splice variant of the β -subunit of the cGMP-gated ion channel of rod photoreceptors. GARP2 is believed to interact with several membrane-associated phototransduction proteins in rod photoreceptors. In this study, we demonstrated that GARP2 is a high affinity PDE6-binding protein and that PDE6 co-purifies with GARP2 during several stages of chromatographic purification. We found that hydrophobic interaction chromatography succeeds in quantitatively separating GARP2 from the PDE6 holoenzyme. Furthermore, the 17-kDa prenyl-binding protein, abundant in retinal cells, selectively released PDE6 (but not GARP2) from rod outer segment membranes, demonstrating the specificity of the interaction between GARP2 and PDE6. Purified GARP2 was able to suppress 80% of the basal activity of the nonactivated, membrane-bound PDE6 holoenzyme at concentrations equivalent to its endogenous concentration in rod outer segment membranes. However, GARP2 was unable to reverse the transducin activation of PDE6 (in contrast to a previous study) nor did it significantly alter catalysis of the fully activated PDE6 catalytic dimer. The high binding affinity of GARP2 for PDE6 and its ability to regulate PDE6 activity in its dark-adapted state suggest a novel role for GARP2 as a regulator of spontaneous activation of rod PDE6, thereby serving to lower rod photoreceptor "dark noise" and allowing these sensory cells to operate at the single photon detection limit.

The visual transduction pathway in vertebrate photoreceptors is remarkable in many respects, including single photon detection capability (in rod photoreceptors), photoresponse kinetics on the millisecond time scale, and the ability to adapt to background illumination levels ranging from very dim illuminance levels (scotopic vision in rods) to bright sunlight (photopic vision in cones) (1). The very first steps in vision occur in the photoreceptor outer segment when photo-isomerized rhodopsin activates the heterotrimeric G-protein transducin, which proceeds to bind to and displace the inhibitory γ -subunit ($P\gamma$)² of the photoreceptor phosphodiesterase (PDE6). Activated PDE6 rapidly lowers the cGMP concentration, resulting in closure of cGMP-gated

channels in the plasma membrane and cell hyperpolarization (2–4). Several feedback mechanisms operate to actively terminate the photoresponse and restore the dark-adapted state, of which regulation of the lifetime of activated transducin is considered rate-limiting (2, 3). Rebinding of $P\gamma$ to the PDE6 catalytic subunits following transducin deactivation returns PDE6 to its nonactivated state and allows cGMP levels to return to their dark-adapted levels.

Electrophysiological evidence supports the hypothesis that factors in addition to transducin deactivation are involved in regulating the lifetime of light-activated PDE6 during light adaptation of rod photoreceptors (5, 6). Several potential feedback mechanisms for modulating activated PDE6 have been proposed (7–9) but have not been explored in sufficient detail to validate their relevance to the phototransduction pathway.

The catalytic activity of PDE6 in its dark-adapted state also must be tightly controlled to prevent any spontaneous activation of PDE6 that would consume metabolic energy unnecessarily and impair the ability of rod cells to reliably detect very dim flashes of light. Physiological measurements of "dark noise" reveal a component that represents spontaneous activation of PDE6 and which is much greater in magnitude in cones than in rods (10–12). Subtle differences in the highly homologous rod and cone isoforms of PDE6 might account for the different dark noise in rods and cones, although this is not evident from biochemical comparisons of purified rod and cone PDE6 (13–16). An alternative possibility is that a rod- or cone-specific PDE6-binding protein suppresses the spontaneous activation of PDE6 by enhancing the affinity of $P\gamma$ at the PDE6 catalytic site.

One candidate protein that might serve to regulate PDE6 in both its nonactivated and activated states is the glutamic acid-rich protein-2 (GARP2), a protein that exists in rod outer segments but is absent in cones (8, 17). GARP2 is a product of alternative splicing of the β -subunit of the rod cGMP-gated ion channel (CNGB1) and contains a unique 8-amino-acid C-terminal extension (8, 17). This 32-kDa protein is unusual in that it has a high content of proline and glutamate residues (17–19).

The functions served by GARP2 in rod outer segments are unknown. Potential binding partners for GARP2 include proteins involved in phototransduction and disk membrane structural integrity (8, 20), but the physiological significance of these interactions is unclear. In one previous study, it was reported that the addition of GARP2 to preparations of PDE6 reversed its activation by transducin, whereas GARP2 had no effect on the nonactivated PDE6 holoenzyme or on the catalytic dimer of PDE6 lacking bound $P\gamma$ (8). It was proposed that GARP2 down-regulation of PDE6 activation in the vicinity of the plasma membrane might conserve metabolic energy during daylight when rod function is saturated.

In this paper, we have examined the interaction of GARP2 with PDE6 and characterized the effect of GARP2 on PDE6 function. We show that

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² The abbreviations used are: $P\gamma$, inhibitory 10-kDa γ -subunit of PDE6; GARP, glutamic acid-rich protein; PDE6, photoreceptor phosphodiesterase; PrBP/ δ , 17-kDa prenyl-binding protein; GTP γ S, guanosine 5'-3-O-(thio)triphosphate; ROS, rod outer segment; HIC, hydrophobic interaction chromatography; HPLC, high pressure liquid chromatography.

GARP2 binds PDE6 with high affinity and co-purifies with the enzyme through several stages of purification. We have been unable to confirm the previously reported inhibitory effect of GARP2 on transducin-activated PDE6 (8). Instead, we observed that purified, native GARP2 has a strong effect in suppressing the basal activity of PDE6 in its nonactivated state. The implications of GARP2 modulation of basal PDE6 activity in dark-adapted rods are discussed.

EXPERIMENTAL PROCEDURES

Materials—Bovine retinas were purchased from W. L. Lawson, Inc. Chromatography supplies were purchased from G. E. Healthcare and Pierce. Supplies for immunoblotting were purchased from Schleicher & Schuell, Pierce, and Bio-Rad. Chemicals were obtained from Sigma. The bovine recombinant GST-PrBP/ δ fusion protein was a kind gift of Dr. Joe Beavo (University of Washington). Rabbit polyclonal anti-GARP2 antibody to the unique C-terminal sequence of GARP2 (17) was obtained from Affinity Bioreagents (catalog number PA1-728). Chicken and rabbit polyclonal anti-GARP antibodies to bovine sequences common to GARP1, GARP2, and the rod β -subunit CNGB1 were kind gifts of Dr. Steven Pittler (University of Alabama at Birmingham) and Dr. Benjamin Kaupp (Institut für Biologische Informationsverarbeitung, Jülich, Germany). Affinity-purified anti-peptide rabbit polyclonal antibodies directed to the PDE6 GAFb domain (termed NC) and to the C terminus of the P γ -subunit of PDE6 (CT-9710) were produced in our laboratory. The ROS1 monoclonal antibody used for immunoprecipitations (21) was a gift of Dr. Richard Hurwitz (Baylor College of Medicine).

ROS Membrane Isolation and Purification—ROS membranes from bovine retina were prepared as described previously (22). Briefly, ROSs were isolated from frozen bovine retinas on a discontinuous sucrose gradient. ROS membranes were homogenized in an isotonic buffer (10 mM Tris, pH 7.5, 60 mM KCl, 40 mM NaCl, 2 mM MgCl₂, 1 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride) using a glass, handheld homogenizer. The soluble proteins were separated from membranes by centrifugation.

Native GARP2 Purification—GARP2 was isolated from ROS membranes and purified to homogeneity as follows. First, ROS membranes were homogenized in a hypotonic buffer (5 mM Tris, pH 7.5, 0.2 mM MgCl₂, 1 mM dithiothreitol). The soluble proteins were separated from membranes by centrifugation at 100,000 $\times g$ for 45 min. The hypotonic extraction was repeated three times. The pooled hypotonic extract was then adjusted to 500 mM ammonium sulfate and applied to a 15-ml butyl-Sepharose column. The column was washed of unbound proteins using two column volumes of 500 mM ammonium sulfate in 5 mM Tris, pH 7.5, and bound proteins were eluted by a step gradient (400 mM ammonium sulfate, 150 mM ammonium sulfate, and no ammonium sulfate in a solution containing 5 mM Tris, pH 7.5, 1 mM dithiothreitol). The GARP2-containing fractions were pooled, adjusted to 500 mM ammonium sulfate, and rechromatographed on butyl-Sepharose. To concentrate and further purify GARP2 from other contaminating proteins, anion exchange chromatography on Mono Q was used exactly as described for PDE6 purification.

In some instances, the GARP2-containing fractions from the first butyl-Sepharose column were chromatographed on a Mono Q column prior to a final purification using a reversed phase HPLC column (Vydac 214TP54) with a gradient of 0–100% acetonitrile containing 0.1% trifluoroacetic acid. Under these conditions, GARP2 eluted at 48% acetonitrile, P γ was found at 43% acetonitrile, and PDE6 catalytic subunits were undetectable by immunoblot analysis. HPLC-purified GARP2

behaved identically to butyl-Sepharose-purified GARP2 in its effects on PDE6 catalysis.

PDE6 Purification—Purified PDE6 was prepared as described elsewhere (22). Briefly, a hypotonic extract of purified ROS membranes was loaded onto a Mono Q column. The proteins were eluted using a linear gradient from 100 mM NaCl to 1 M NaCl in 5 mM Tris, pH 7.5. The PDE6 peak was collected, concentrated, and further purified on a Superdex 200 gel filtration column using the following buffer: 5 mM Tris, pH 7.5, 300 mM NaCl, 1 mM dithiothreitol, and 3 mM phenylmethylsulfonyl fluoride. The gel filtration column was calibrated using the following: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (43 kDa), ovalbumin (29 kDa), blue dextran for the void volume, and adenosine triphosphate for the included volume.

Immunoprecipitation of PDE6 with the Monoclonal Antibody ROS1—The ROS1 antibody to PDE6 (21) coupled to Sulfolink beads (Pierce) was used for immunoprecipitation of PDE6 and its binding partners. Hypotonic extracts from bovine ROS or Mono Q-purified PDE6 (containing 5–10 pmol of PDE6) were incubated for 2 h at 4 °C with 20 μ l of prewashed ROS1 beads in a total volume of 100 μ l. The samples were centrifuged to separate bound from unbound proteins, and the beads were washed extensively before the proteins were eluted in Laemmli sample buffer. As a control for nonspecific binding of GARP2 to the beads, purified GARP2 was also tested with the ROS1-Sulfolink beads. Portions of the starting material, bound proteins, and unbound proteins were subjected to SDS-PAGE followed by Western blotting for PDE6 (NC antibody) and GARP (chicken anti-GARP antibody).

PrBP/ δ Expression and Purification—Recombinant bovine PrBP/ δ was expressed in the *Escherichia coli* strain BL21 (23). Protein expression was induced by the addition of 1 mM isopropyl- β -D-thiogalactopyranoside to log phase cultures. Bacterial cells were incubated for 1 h at 37 °C, lysed by sonication, and soluble proteins were recovered following centrifugation. GST-PrBP/ δ was purified on a glutathione-agarose column. GST-PrBP/ δ concentration was determined by a colorimetric protein assay (24).

Protein Quantification—The amount of rhodopsin in dark-adapted ROS membranes was determined by difference spectroscopy (25). The PDE6 concentration was routinely determined by measurements of trypsin-activated PDE6 maximum activity (V_{\max}) (26) and knowledge of the turnover number ($k_{\text{cat}} = 5600 \text{ cGMP/s/PDE6}$; $[\text{PDE6}] = V_{\max}/k_{\text{cat}}$) (27). Independent determinations of the ratio of rhodopsin to PDE6 in purified bovine ROS gave a value of 310 ± 20 rhodopsins per PDE6 ($n = 5$), very similar to the values for amphibian ROS of 270 (28) to 330 (29) rhodopsins per PDE.

The amount of purified GARP2 was routinely estimated by immunoblot analysis. Samples of purified GARP2 and known amounts of ROS membranes were resolved on SDS-PAGE and immunoblotted. GARP2 was detected using a GARP2-specific antibody. The intensities of GARP2 immunoreactive bands were determined using Quantiscan (Biosoft) and then compared with GARP2 immunoreactivity in ROS membranes containing known amounts of rhodopsin and PDE6.

SDS-PAGE and Western Blotting—SDS-PAGE was performed by the method of Laemmli (30) in 10, 12, or 15% acrylamide gels. The immunoblotting procedure followed the protocols in Gallagher (31). Note that GARP2 typically migrates at ~ 60 kDa (roughly 2-fold higher than predicted based on its amino acid sequence) and shows size heterogeneity, in accord with previous observations (8, 19). This anomalous behavior was recently explained as being due to GARP2 existing in solution as a natively unfolded protein (32).

Regulation of PDE6 by GARP2

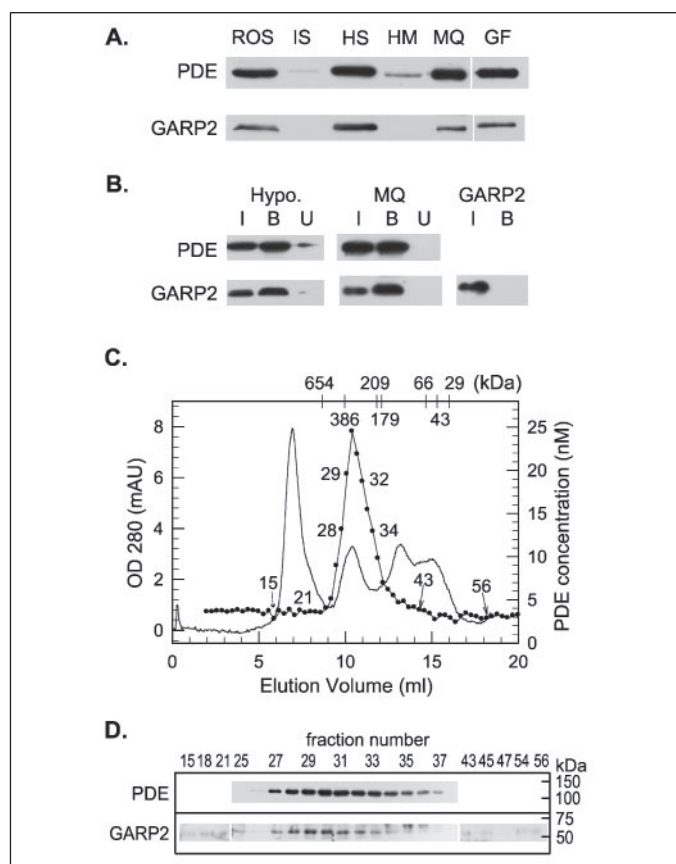


FIGURE 1. GARP2 is a high affinity PDE6-binding protein. *A*, immunoblot of PDE6 samples during purification. Purified ROS membranes (ROS), hypotonic extract of ROS membranes (HS), Mono Q-purified PDE6 (MQ), and gel filtration purified PDE6 (GF) lanes were each loaded with 1 pmol of PDE6. For the isotonic supernatant (IS), a volume equivalent to the original ROS homogenate was loaded. For the ROS membranes following hypotonic extraction (HM), an amount of rhodopsin equivalent to the ROS sample was loaded. The blots were probed with the PDE6 NC antibody and the GARP2-specific antibody. *B*, co-immunoprecipitation of PDE6 with GARP2. Hypotonically extracted PDE6 (Hypo.), Mono Q-purified PDE6 (MQ) or purified GARP2 were incubated with ROS1 antibody attached to Sulfolink beads (see "Experimental Procedures"). Samples representing 10% of the total PDE6 sample (input, I), 50% of bound proteins (B), and 10% of the unbound proteins (U) were run on 15% SDS-PAGE, and immunoblots were examined with antibodies specific for PDE6 and GARP2. As a control for nonspecific binding of GARP2 to the beads, immunoprecipitation was also carried out on PDE6-free-purified GARP2, and samples representing 10% of the input and 50% of the pellet were analyzed. *C*, a hypotonic extract of ROS membrane proteins (200 nM PDE) was loaded on a Superdex 200 column. The protein absorbance profile (continuous line) and the PDE6 activity (filled circles) are shown. The top x-axis shows the molecular mass of protein standards run on the column. Numbers adjacent to data points refer to fractions used for immunoblot analysis. OD, optical density; AU, absorbance units. *D*, immunoblots of a fixed volume of the indicated column fractions were probed with PDE6- and GARP2-specific antibodies.

RESULTS AND DISCUSSION

GARP2 Is a High Affinity PDE6-binding Protein—Because there is uncertainty about the proteins with which GARP2 interacts in rod photoreceptors (8, 20), we first re-examined whether PDE6 interacts in a specific manner with GARP2. Following homogenization of purified ROS from bovine retina and removal of soluble proteins with isotonic washes, PDE6 and GARP2 both remained associated with ROS disk membranes (Fig. 1A). Release of PDE6 from ROS membranes by exposure to a hypotonic buffer also caused the release of almost all detectable GARP2 (Fig. 1A). Subsequent purification of PDE6 by anion-exchange chromatography on a Mono Q column resulted in co-elution of PDE6 and GARP2 at 400 mM NaCl. Gel filtration chromatography of the Mono Q-purified GARP2-PDE6 also failed to separate GARP2 from PDE6 (Fig. 1A). The results in Fig. 1A conclusively demonstrate that

PDE6 and GARP2 are both associated with ROS membranes, are co-eluted by exposure to a hypotonic buffer, and co-purify by two different chromatographic procedures.

To directly show that GARP2 is associated with PDE6, we immunoprecipitated PDE6 with the ROS1 antibody (21) coupled to Sulfolink beads. Fig. 1B shows that unpurified PDE6 obtained from hypotonic extraction of ROS membranes was pulled down in a complex with GARP2 and that very little GARP2 remained unbound under these conditions. Mono Q-purified PDE6 was also immunoprecipitated in tight association with GARP2, whereas purified GARP2 failed to bind to the ROS1 antibody in the absence of PDE6 (Fig. 1B).

To estimate whether a significant amount of the PDE6 exists free of bound GARP2, we performed gel filtration chromatography on proteins solubilized from dark-adapted ROS membranes with a hypotonic buffer. A single peak of PDE6 hydrolytic activity (Fig. 1C) and immunoreactivity (Fig. 1D) was observed at an apparent molecular mass of 330 kDa. (The higher than predicted molecular mass for the PDE6-GARP2 complex by gel filtration chromatography may be due to its asymmetric shape (14).) Qualitatively, the observed ratio of PDE6 and GARP2 immunoreactivity did not vary in the fractions containing PDE6, indicating that there is not a large fraction of the total PDE6 that exists free of bound GARP2. Further, only small amounts of GARP2 immunoreactivity could be detected at an apparent molecular mass of ~30–60 kDa (Fig. 1D). This result indicated that PDE6 is tightly associated with GARP2 and that there is no evidence for a significant amount of unbound GARP2 or PDE6.

To assess whether GARP2 binding to PDE6 might be an artifact of the initial hypotonic extraction of ROS membrane proteins, we also solubilized PDE6 and GARP2 from dark-adapted ROS membranes with 1% Triton X-100. After removing the ROS membranes by centrifugation and immunoprecipitation of the detergent-solubilized PDE6 with the ROS1 antibody, we detected both PDE6 and GARP2 in the immunoprecipitates; control samples with beads lacking the ROS1 antibody failed to pull down either protein (data not shown).

Together, these results demonstrate that most of the GARP2 in ROS co-purifies with PDE6 through several stages of purification. The fact that GARP2 remained bound to PDE6 after repeated washing of the ROS1 immune complex (Fig. 1B) and that little unbound GARP2 was observed during gel filtration chromatography (Fig. 1D) demonstrates that GARP2 binds PDE6 with high affinity.

GARP2 Content in Rod Photoreceptors—If GARP2 is to regulate PDE6 activity during phototransduction, it would need to be present in ROS in molar equivalence to PDE6. A previous study by Kaupp and colleagues (8) has suggested that GARP2 is actually ~3-fold more abundant than PDE6 (1 GARP2 per 100 rhodopsins). Another GARP2-interacting protein, peripherin, is believed to bind only 10% of the total GARP2 in ROS (20). Our observation that practically all GARP2 in bovine ROS co-purified with PDE6 (Fig. 1) suggested that GARP2 was not significantly more abundant than PDE6 in ROS.

To directly address the question of the GARP2 content in ROS, we purified native GARP2 from bovine ROS, as described in the next section, and compared the immunoreactivity of known amounts of GARP2 to that of intact ROS whose rhodopsin and PDE6 concentration were measured. The precision of our measurements were hampered by uncertainties in the concentration of purified GARP2 used for quantitative immunoblots, because the poor staining of GARP2 by Coomassie and other protein stains limited our ability to assess its purity on SDS-PAGE (see next section). Taking this into consideration, we estimate that there are 1–2 GARP2 molecules per PDE6 holoenzyme in bovine ROS (data not shown). This value agrees well with two other reports (8,

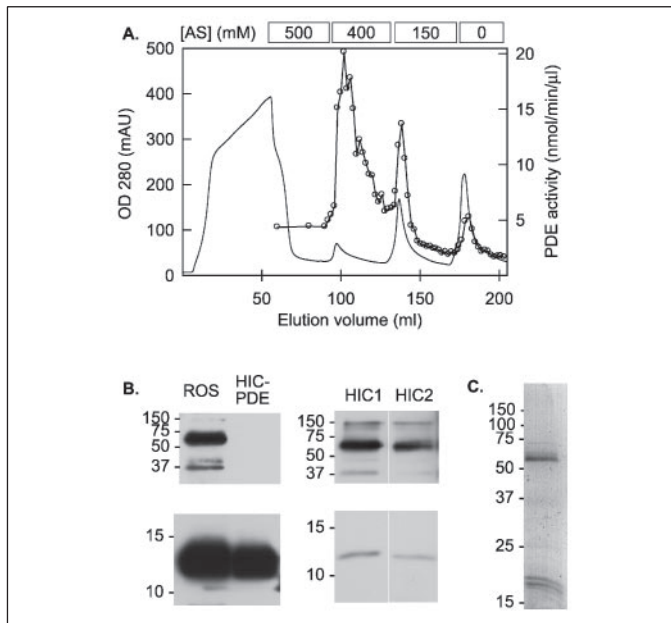


FIGURE 2. Separation of GARP2 from PDE6 by butyl-Sepharose chromatography. A, a hypotonic extract (containing 2.2 nmol of PDE6) was adjusted to a 500 mM ammonium sulfate concentration before being loaded on a 15-ml butyl-Sepharose column. The ammonium sulfate concentration was lowered in steps as indicated at the top of the graph and verified by conductivity measurements. The protein absorbance (continuous line) and PDE6 activity profile (filled circles) were determined. B, immunoblots showing the starting material (ROS membranes; 0.5 pmol of PDE), the PDE6 peak from butyl-Sepharose chromatography (0.5 pmol of PDE6), and equivalent volumes of GARP2 following one or two purifications of the GARP2 peak eluting at zero ammonium sulfate concentration. Immunoblots were probed with the rabbit anti-GARP antibody (upper panels) and the anti-P γ -subunit antibody CT-9710 (lower panels). C, HPLC-purified GARP2 (5 μ g of protein) was loaded onto a 10% polyacrylamide gel and the separated proteins stained with Coomassie dye. The position and size (in kDa) of protein standards are indicated.

32) and indicates that GARP2 is present in rod photoreceptors in sufficient amounts to bind all of the PDE6.

Purification of Native GARP2 Free of Contamination with PDE6—To study the effects of GARP2 on PDE6 catalytic activity, we needed to purify GARP2 free of PDE6 subunits. We discovered that the association of GARP2 with PDE6 could be disrupted using high concentrations of ammonium sulfate. This permitted separation of the two proteins by hydrophobic interaction chromatography (HIC).

A hypotonic extract containing PDE6 and GARP2 was mixed with 500 mM ammonium sulfate, and the sample was applied to a butyl-Sepharose column. A decreasing, discontinuous ammonium sulfate gradient permitted the separation of PDE6 (at higher ammonium sulfate concentrations) from the GARP2 (which eluted only when ammonium sulfate was omitted from the buffer) (Fig. 2A). Examination of the PDE6 peak by immunoblot analysis revealed no detectable GARP2 (Fig. 2B, HIC-PDE). The disruption of GARP2 binding to PDE6 by ammonium sulfate suggested that hydrophobic domains in GARP2 (which contains 27% hydrophobic amino acids) may be important in promoting its binding to PDE6 catalytic subunits.

The GARP2 peak eluting from the butyl-Sepharose column in the absence of ammonium sulfate still contained traces of the inhibitory P γ -subunit immunoreactivity at \sim 12 kDa as well as a faint band of GARP1 immunoreactivity at \sim 130 kDa (Fig. 2B, HIC1). By exposing the partially purified GARP2 sample to 500 mM ammonium sulfate and running the sample on butyl-Sepharose again, most of the residual P γ was removed from the GARP2 (Fig. 2B, HIC2). Alternatively, the GARP2-enriched fractions from the butyl-Sepharose could be completely separated from PDE6 subunits by reversed phase HPLC.

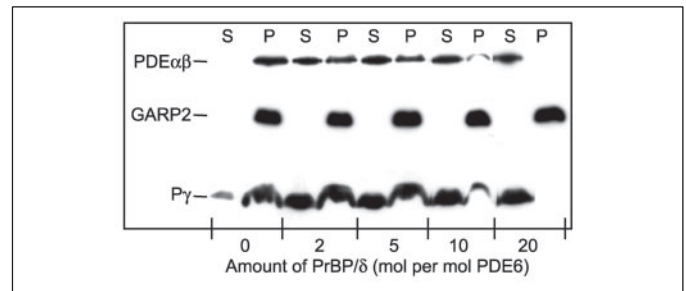


FIGURE 3. PrBP/ δ selectively solubilizes PDE6 from ROS membranes, whereas GARP2 remains on the membranes, free of PDE6 subunits. Purified ROS membranes were incubated overnight at 4 $^{\circ}$ C with the indicated amounts of GST-PrBP/ δ (relative to the PDE6 subunit concentration). Membranes were centrifuged and soluble (S) and membrane (P) fractions were analyzed by immunoblotting using a GARP2-specific antibody, the PDE6 NC antibody, and the P γ CT-9710 antibody.

On Coomassie-stained gels of purified GARP2, >50% of the total staining was observed at \sim 60 kDa, corresponding to GARP2 (Fig. 2C). No detectable protein was observed at molecular masses corresponding to PDE6 catalytic or inhibitory subunits. The GARP2 purity was likely to be much higher, because this glutamate-rich protein binds Coomassie protein stain very poorly relative to other proteins. It is therefore unlikely that the effects of GARP2 on PDE6 activity reported in this paper could be ascribed to a contaminating protein in our purified GARP2.

The 17-kDa Prenyl-binding Protein (PrBP/ δ) Releases PDE6, but Not GARP2, from ROS Membranes—The 17-kDa prenyl-binding protein (PrBP/ δ), originally described as the δ -subunit of PDE6 (33), is able to solubilize membrane-associated rod PDE6 *in vitro* (23, 34) by binding to the hydrophobic prenyl groups attached to the C termini of the PDE6 catalytic subunits (35). Because hydrophobic interactions may stabilize GARP2-PDE6 interactions (see previous section), we wondered whether PrBP/ δ binding to PDE6 would solubilize the enzyme as a complex with GARP2 or, alternatively, compete with GARP2 for binding to a hydrophobic region on PDE6. Fig. 3 shows an experiment in which increasing amounts of PrBP/ δ were added to ROS membranes (containing bound PDE6 and GARP2) and the solubilization of PDE6 monitored by centrifugal separation of bound and soluble PDE6. Although PDE6 catalytic and P γ -subunits were released from ROS membranes by PrBP/ δ in a concentration-dependent manner, GARP2 remained completely associated with ROS membranes (Fig. 3).

The ability of PrBP/ δ to disrupt GARP2-PDE6 interactions was not restricted to PDE6 associated with ROS membranes. If PDE6 and GARP2 were first released from ROS membranes by hypotonic extraction and then PrBP/ δ was added, some of the GARP2 that normally co-migrated with PDE6 at \sim 300 kDa during gel filtration chromatography was now observed eluting at an apparent molecular mass of \sim 60 kDa (data not shown). The disruption of GARP2-PDE6 interactions by PrBP/ δ suggests that one site of interaction may be at the hydrophobic isoprenyl groups at the C termini of PDE6 catalytic subunits. It is possible that this same binding interface may be disrupted during hydrophobic interaction chromatography.

Purified, Native GARP2 Suppresses Basal PDE6 Catalytic Activity but Does Not Inhibit Activated PDE6—It has been previously reported (8) that GARP2 potently inhibited PDE6 hydrolytic activity when purified PDE6 was activated by transducin in solution; in contrast, trypsin-activated PDE6 (lacking P γ) or nonactivated enzyme ($\alpha\beta\gamma\gamma$) were not greatly affected by GARP2. However, Korschen *et al.* (8) used a recombinant GARP2 fusion protein for these experiments, and unpublished results from the same group have called into question the ability of native GARP2 to inhibit transducin-activated PDE6 (36). Furthermore,

Regulation of PDE6 by GARP2

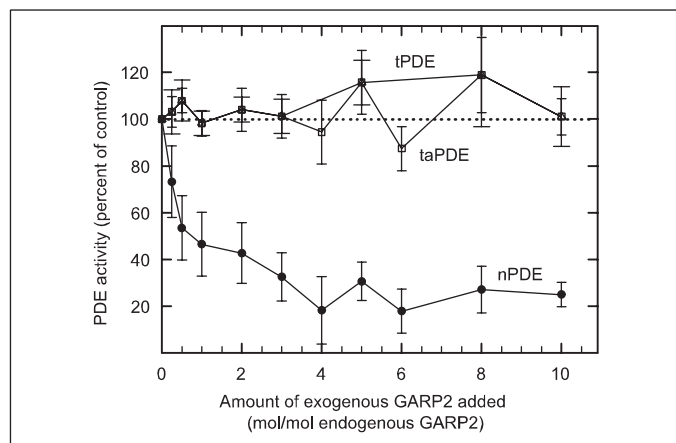


FIGURE 4. GARP2 suppresses nonactivated PDE6 activity but is ineffective with activated PDE6. Bovine ROS homogenates (10 nM PDE6) were either trypsin-activated (tpPDE), transducin-activated by the addition of 10 μ M GTP γ S (taPDE), or maintained in a nonactivated state (nPDE), as described under "Experimental Procedures." Purified GARP2 was added to each sample in an amount referenced to the amount of endogenous GARP2 present in these ROS membranes (see "Experimental Procedures"). For each experimental condition, catalytic activity was normalized to the PDE6 activity in the absence of exogenous GARP2 (defined as zero on the x-axis): 87 ± 19 ($n = 9$), 398 ± 105 ($n = 5$), and 4260 ± 680 ($n = 4$) cGMP hydrolyzed/PDE6/s for nonactivated PDE, transducin-activated PDE, and trypsinized PDE, respectively.

transducin activates PDE6 more effectively when both proteins are associated with the disk membrane compared with activation in solution (37, 38). Therefore, we chose to examine whether native, purified GARP2 exerted an effect on nonactivated or activated PDE6 under more physiological conditions in which PDE6 remains associated with the disk membrane.

As seen in Fig. 4, the addition of purified, native GARP2 to nonactivated PDE6 attached to ROS membranes (which contain endogenous GARP2) inhibited the basal rate of PDE6 activity by 80%. The suppression of PDE6 activity by GARP2 was maximal when an amount of purified GARP2 was added equal to its endogenous level in ROS (as determined by quantitative immunoblot analysis). A similar result was obtained with purified PDE6 that had been extracted from ROS membranes and chromatographically purified (data not shown).

In contrast, no significant effect of GARP2 on either transducin-activated PDE6 (attached to ROS membranes) or trypsin-activated PDE6 was seen (Fig. 4). Even following the addition of a 10-fold excess of purified GARP2 relative to its endogenous concentration in ROS membranes, the PDE6 activity of both transducin- and trypsin-activated PDE6 remained within 20% of its activity in the absence of GARP2. Although the results in Fig. 4 for trypsin-activated PDE6 are in accord with those of Korschen *et al.* (8), we failed to observe the inhibitory effect of GARP2 on transducin-activated PDE6 that they reported.

To more sensitively test whether GARP2 influences the ability of transducin to activate PDE6, we first supplemented light-exposed ROS membranes with either GARP2 or nothing and then added increasing amounts of GTP γ S to persistently activate transducin in a concentration-dependent manner. Fig. 5 shows that, in the presence of GARP2 (at a concentration 2-fold greater than required to maximally suppress PDE6 basal activity), the ability of transducin to activate PDE6 was only slightly impaired; the small decrease in activation at any given GTP γ S concentration was not statistically significant. Once sufficient GTP γ S was added to activate all of the transducin present (the transducin:PDE6 ratio in bovine ROS being 30:1), adding more GTP γ S had no further effect on PDE6 activation in the absence or presence of GARP2.

This result shows that the activity-lowering effect of GARP2 on the nonactivated PDE6 holoenzyme is distinct from the molecular events by

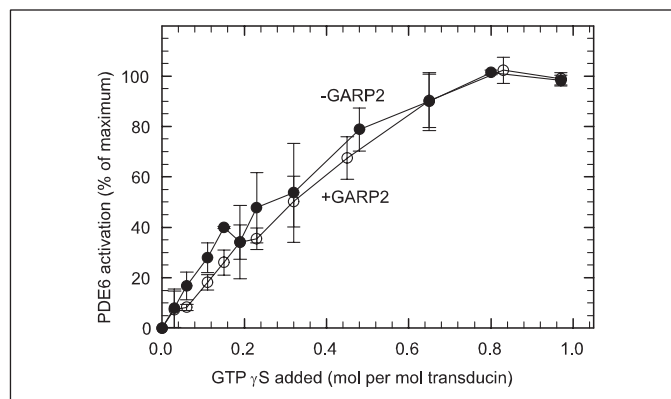


FIGURE 5. Inability of GARP2 to reduce activation of PDE6 by transducin. ROS homogenates (1.5 nM PDE6) were first incubated with either a 2-fold excess of GARP2 relative to its endogenous concentration (+GARP2) or buffer (-GARP2). The ROS membranes were exposed to light, and then the indicated amount of GTP γ S was added to persistently activate transducin. The PDE6 activity is normalized to the extent of activation by saturating amounts of GTP γ S compared with the nonactivated PDE6 rate (150 or 110 cGMP/PDE6/s in the absence or presence of GARP2, respectively). The data represent the mean \pm S.E. ($n = 3$).

which the activated α -subunit of transducin binds to PDE6 and displaces its P γ -subunit, thereby causing light activation of PDE6.

Proposed Physiological Role for GARP2 Regulation of PDE6 in Rod Photoreceptors—In this study, we have shown that GARP2 is a high affinity PDE6 regulatory protein capable of suppressing the basal activity of nonactivated PDE6 but with negligible effects on transducin-activated PDE6. The exclusive localization of GARP2 to rod outer segments (8, 17) suggests that this protein may regulate rod PDE6 in a way that helps distinguish the rod and cone phototransduction pathways. One feature that differentiates rods from cones is the amplitude of fluctuations in the dark current (dark noise) in the photoreceptor outer segment (10, 11). The low dark noise of rods permits reliable signaling at the single photon level (39), whereas cones require several photons to generate a detectable signal (40).

Because the rates of PDE6 activation/inactivation determine the characteristics of photoreceptor dark noise (12), GARP2 is an attractive candidate for regulating rod PDE6 to lower its spontaneous activation. This study suggests that the binding of GARP2 to nonactivated rod PDE6 will lower its catalytic activity, most likely by enhancing the affinity of P γ for the active site of the enzyme. The observed localization of GARP2 to the rim of the disk membrane in ROS (8, 17) may serve as a mechanism to reduce spontaneous PDE6 activation and thereby minimize fluctuations in cGMP concentrations in the vicinity of the cGMP-gated ion channel.

The lack of an effect of GARP2 on transducin-activated PDE6 is also consistent with the need for rod PDE6 to be rapidly and stoichiometrically activated upon binding of activated transducin. The single photon sensitivity of rod photoreceptors would likely be impaired if GARP2 were to reduce the efficiency of PDE6 activation by transducin. In summary, our results support a role for GARP2 in maintaining a very low spontaneous activation of PDE6 without interfering with the efficiency of the visual excitation pathway in rod photoreceptors in response to photic stimuli. Reports that GARP2 is associated with the disk rim protein peripherin (20, 32) leave open the intriguing possibility that GARP2 might preferentially regulate PDE6 in the vicinity of the cGMP-gated channel, where cGMP metabolic flux might be most stringently controlled.

Summary—This work has demonstrated that GARP2 is a novel PDE6 interacting protein that is capable of regulating the basal activity of the PDE6 holoenzyme in rod outer segments. The high affinity with which

it binds nonactivated PDE6 suppresses catalytic activity without adversely affecting the ability of transducin to activate PDE6. This novel regulatory mechanism may be of fundamental importance in establishing the high signal-to-noise ratio needed for single photon detection in rod photoreceptors. Future studies will be directed toward determining the molecular mechanism of GARP2 interaction with the catalytic and/or inhibitory subunits of dark-adapted and light-activated PDE6.

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