

Regulation of Photoreceptor Phosphodiesterase (PDE6) by Phosphorylation of Its Inhibitory γ Subunit Re-evaluated*

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Phosphorylation of the inhibitory γ subunit ($P\gamma$) of rod cGMP phosphodiesterase (PDE6) has been reported to turn off visual excitation without the requirement for inactivation of the photoreceptor G-protein transducin. We evaluated the significance of $P\gamma$ phosphorylation for PDE6 regulation by preparing $P\gamma$ stoichiometrically phosphorylated at Thr²² or at Thr³⁵. Phosphorylation of $P\gamma$ at either residue caused a minor decrease—not the previously reported increase—in the ability of $P\gamma$ to inhibit catalysis at the active site of purified PDE6 catalytic dimers. Likewise, $P\gamma$ phosphorylation had little effect on its potency to inhibit transducin-activated PDE6 depleted of its endogenous $P\gamma$ subunits. The strength of $P\gamma$ interaction with the regulatory GAF domain of PDE6 was reduced severalfold upon $P\gamma$ phosphorylation at Thr²² (but not Thr³⁵), as judged by allosteric changes in cGMP binding to these noncatalytic sites on the enzyme (Mou, H., and Cote, R. H. (2001) *J. Biol. Chem.* 276, 27527–27534). In contrast, the effects of $P\gamma$ phosphorylation on its interactions with activated transducin were much more pronounced. Phosphorylation of $P\gamma$ at either Thr²² or Thr³⁵ greatly diminished its ability to bind activated transducin, consistent with earlier work. *In situ* phosphorylation of $P\gamma$ by endogenous rod outer segment kinases was enhanced severalfold upon light activation, but only ~10% of the endogenous $P\gamma$ was phosphorylated. This is attributed to $P\gamma$ being a poor substrate for protein kinases when associated with the PDE6 holoenzyme. We conclude that, contrary to previous reports, $P\gamma$ phosphorylation at either Thr²² or Thr³⁵ modestly weakens its direct interactions with PDE6. However, $P\gamma$ phosphorylation subsequent to its dissociation from PDE6 is likely to abolish its binding to activated transducin and may serve to make phosphorylated $P\gamma$ available to regulate other signal transduction pathways (e.g. mitogen-activated protein kinase; Wan, K. F., Sambhi, B. S., Frame, M., Tate, R., and Pyne, N. J. (2001) *J. Biol. Chem.* 276, 37802–37808) in photoreceptor cells.

Rod phototransduction begins when light activates the visual pigment rhodopsin. This leads to GTP-GDP exchange on the heterotrimeric G-protein transducin, causing dissociation

of the activated α_t^* -GTP subunit from the $\beta\gamma$ complex. Activated transducin then binds to and displaces the inhibitory γ subunit ($P\gamma$)¹ of the effector enzyme, cyclic GMP phosphodiesterase (PDE6). PDE6 is a member of a large family of phosphodiesterases that share a conserved catalytic region but vary in substrate specificity, cellular localization, and modes of regulation (1–4). The rod PDE6 holoenzyme is an oligomeric protein consisting of a catalytic dimer of two similar catalytic subunits ($P\alpha\beta$) to which are bound two $P\gamma$ subunits. Activation of PDE6 leads to hydrolysis of cGMP, causing a decrease in cytoplasmic cGMP levels, ultimately resulting in hyperpolarization of the cell due to closure of cGMP-gated ion channels (5).

The 9.7-kDa $P\gamma$ subunit is a multifunctional protein. Its primary function in dark-adapted photoreceptors is to bind tightly to $P\alpha\beta$ to inhibit catalysis of PDE6, thereby maintaining micromolar levels of cGMP in the cytoplasm of the rod outer segment. This is accomplished by direct binding of the C terminus of $P\gamma$ to the active site of $P\alpha\beta$ (6). The central polycationic region of $P\gamma$ provides additional sites of interaction with $P\alpha\beta$ (7). This $P\gamma$ domain is largely responsible for the very high affinity of $P\gamma$ for $P\alpha\beta$, and it allosterically regulates cGMP binding to the GAF domain (a protein domain named for its occurrence in cGMP-regulated PDEs, certain adenylate cyclases, and the bacterial protein Fh1A) on $P\alpha\beta$ (see Ref. 8 and the references therein). During phototransduction, activated transducin binds to the C-terminal domain of $P\gamma$ to relieve the inhibitory constraint of $P\gamma$ at the catalytic site of PDE6 (9–11). The central region of $P\gamma$ also participates in stabilizing interactions of α_t^* -GTP with $P\gamma$ (12, 13). Under conditions where $P\gamma$ affinity to $P\alpha\beta$ is weakened (i.e. in the absence of cGMP binding to the GAF domains), the α_t^* -GTP- $P\gamma$ complex in amphibian ROS can dissociate from PDE6 (14, 15). Dissociation of cGMP and release of $P\gamma$ from the PDE6 holoenzyme also correlate with another function of $P\gamma$, namely, its ability to potentiate the GTPase-accelerating ability of the regulator of G-protein signaling-9 (RGS-9; Refs. 16–18). Finally, there is growing evidence that $P\gamma$ is expressed in nonretinal tissues (19, 20) and can regulate other signaling components besides PDE6, including the closely related cGMP-specific PDE (PDE5) enzyme (21) and the mitogen-activated protein (MAP) kinase (20).

$P\gamma$ has been reported to be a substrate for phosphorylation by several different protein kinases present in rod outer segments (22–27). Two distinct threonine residues in the central polycationic region of $P\gamma$ have been identified as targets for phospho-

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¹ The abbreviations used are: $P\gamma$, 9.7-kDa inhibitory γ subunit of rod PDE6; PDE6, cyclic nucleotide phosphodiesterase from photoreceptor cells (type 6); GTP γ S, guanosine-5'-3-O-(thio)triphosphate; HPLC, high-pressure liquid chromatography; $P\alpha\beta$, catalytic heterodimer of rod PDE6; PKA, protein kinase A; MAP, mitogen-activated protein; ROS, rod outer segment(s); MALDI-TOF, matrix-assisted laser-desorption ionization time-of-flight.

rylation: Thr²² and Thr³⁵. Thr²² is located within a consensus sequence for proline-directed kinases (Pro-X-(Ser/Thr)-Pro), whereas Thr³⁵ is part of a consensus sequence for protein kinase A (PKA) and related kinases (Arg-X-(Ser/Thr)). Phosphorylation at either Thr²² or Thr³⁵ has been reported to increase the inhibitory potency of P γ 2- to 10-fold by increasing its intrinsic binding affinity for purified P $\alpha\beta$ catalytic dimers (24, 28) or for transducin-activated PDE6 attached to ROS membranes (25, 27).

Phosphorylation of P γ within its central polycationic region has also been reported to greatly weaken its binding to α_t^* -GTP (24, 25, 27, 29). These investigations have concluded that P γ phosphorylation can serve to turn off light-activated PDE6 without requiring transducin inactivation by its intrinsic GTPase activity.

Because of the potential importance of transducin-independent pathways for terminating and adapting the biochemical cascade of phototransduction in rod photoreceptors, we chose to examine in detail how P γ phosphorylation might modulate PDE6 activity. We used purified PKA and MAP kinase to phosphorylate P γ *in vitro* at Thr³⁵ and Thr²², respectively, with well-defined stoichiometries of 1 phosphate/P γ . Under conditions where the interaction of P γ with PDE6 can be precisely determined, we find relatively minor effects of P γ phosphorylation on its interactions with the catalytic and GAF domains of P $\alpha\beta$. Likewise, we see little effect of phosphorylation on the ability of P γ to re-inhibit transducin-activated PDE6 bound to ROS membranes. We offer explanations for the discrepancy between our results and earlier reports in the literature demonstrating large changes in P γ binding to PDE6 upon phosphorylation. However, we are able to confirm previous results showing that P γ phosphorylation reduces the ability of P γ to interact with activated transducin. Finally, we present evidence that P γ is a poor substrate for phosphorylation by protein kinases when complexed with the PDE6 holoenzyme and that the small light-induced increase in P γ phosphorylation in ROS is unlikely to directly regulate either transducin or PDE6 during phototransduction.

EXPERIMENTAL PROCEDURES

Materials—Bovine retinas were purchased from W. L. Lawson, Inc., and frogs (*Rana catesbeiana*) were obtained from Niles Biologicals. [³H]cGMP and [³⁵S]GTP γ S were purchased from PerkinElmer Life Sciences, and [γ -³²P]ATP was from ICN Biochemicals. Filtration and ultrafiltration products were from Millipore. Scintillation fluid (Ultima Gold) was from Packard Instrument Co. Zaprinast was a gift of Rhone-Poulenc Rorer (Dagenham, United Kingdom). BCA protein assay reagent was from Pierce. All other chemicals were obtained from Sigma. Superdex 200 and Mono-Q columns were from Amersham Biosciences, Inc., and the C18 reversed phase column (300A, 22 \times 250 mm) was from Vydac. PKA, MAP kinase/extracellular signal-regulated kinase 2, and P-81 phosphocellulose membranes were from Upstate Biotechnology. The P γ mutants used in this study were kind gifts of Drs. N. O. Artemyev (P γ T22C and P γ T35C) and N. P. Skiba (P γ 1-45C).

Preparation of Frog ROS, P γ -depleted ROS Membranes, and Frog P $\alpha\beta$ —Intact frog ROS were purified on a discontinuous Percoll density gradient as described previously (30). Purified ROS were homogenized at 4 °C in ROS buffer containing 77 mM KCl, 35 mM NaCl, 1.0 mM CaCl₂, 2.0 mM MgCl₂, 10 mM HEPES, pH 7.5, 1 mM EGTA, 1 mM dithiothreitol, 1 mM 4-(2-aminoethyl)benzene-sulfonyl fluoride, 5 μ M leupeptin, and 1 μ g/ml pepstatin. Homogenized ROS were incubated in the dark at room temperature for 30 min to deplete endogenous nucleotides. The rhodopsin concentration was determined by difference spectroscopy (31). The concentration of PDE6 was determined either by measuring the maximum cGMP hydrolytic activity of trypsin-activated PDE6 using the turnover number (k_{cat}) of 7550 mol cGMP hydrolyzed/s/mol PDE6 (15) or by determining the ability of PDE6 to bind 2 mol cGMP/mol PDE6 (30). P γ -depleted frog ROS membranes were prepared as described previously (30) by incubating nucleotide-depleted, light-exposed ROS homogenates with GTP γ S (~1 GTP γ S/rhodopsin) at 4 °C for 1 h. After centrifugation (110,000 \times g for 2 min), the membranes

were resuspended with GTP γ S and incubated for \geq 2 h at 4 °C. After the second centrifugation, the P γ -depleted ROS membranes retained 25–40% of the original P γ content.

Purified frog P $\alpha\beta$ was prepared by limited trypsinization of ROS membranes with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, followed by the addition of a 6-fold molar excess of soybean trypsin inhibitor (30). The time course of frog PDE6 activation was empirically determined in each experiment to minimize exposure to the protease.

Purification and Quantitation of P γ and P γ Mutants—The recombinant bovine rod full-length P γ and P γ mutants were expressed using the pET11a expression plasmid in the host strain *Escherichia coli* BL21 DE3. The mutants P γ T22C and P γ T35C both had a substitution of Ser at Cys⁶⁸ of the bovine rod P γ sequence, in addition to a Cys substitution for the naturally occurring Thr at positions 22 or 35, respectively (32). The P γ 1-45C mutant was truncated at position 45, and a C-terminal Cys was introduced (13). Expression and purification conditions were identical to those described previously (33).

The concentrations of purified P γ , P γ T22C, P γ T35C, and P γ 1-45C were measured spectrophotometrically at 277 nm with an extinction coefficient of 7550 cm⁻¹M⁻¹ (30) or with a colorimetric protein assay using bovine serum albumin as a standard (34). The inhibitory activity of each full-length P γ preparation was verified to agree to \leq 20% with the spectrophotometric estimate of the P γ concentration by measuring inhibition of trypsin-activated PDE6 (3–5 nM) (see Fig. 1; Ref. 30).

P γ Peptide Synthesis and Purification—P γ 18-41 and two singly phosphorylated peptides (P γ 18-41(22P) and P γ 18-41(35P)) were synthesized at the Protein Facility in the Department of Biochemistry and Molecular Biology at the University of New Hampshire. Cleavage of peptides from the resin and protecting groups was accomplished with anhydrous hydrofluoric acid. The peptides were first purified with cation exchange chromatography on CM-Sephadex C25, which was equilibrated with 1 mM NH₄HCO₃, pH 8.0, and eluted with a linear 0–1.0 M NH₄HCO₃ gradient. After ion exchange chromatography, the peptides were further purified with a preparative C18 reverse-phase column (300A, 22 \times 250 mm) using a linear gradient of 30–70% acetonitrile in 0.1% trifluoroacetic acid. The dried peptides were resuspended in 10 mM Tris, pH 7.5, and the concentration was measured by the BCA protein assay. Other synthetic peptides reported in this study have been described previously (8).

Preparation of Bovine Rod PDE6 Holoenzyme and P $\alpha\beta$ —Membrane-associated bovine rod PDE6 was prepared from frozen bovine retinas and purified by using the ion exchange, immunoaffinity, and gel filtration chromatography procedures described previously (33). Bovine P $\alpha\beta$ was prepared by limited trypsin proteolysis, and P γ fragments were removed by Mono-Q chromatography or Superdex 200 gel filtration chromatography (33).

Phosphorylation of P γ and Purified Bovine PDE6 Holoenzyme by PKA or MAP Kinase—Phosphorylation assays were performed in a phosphorylation reaction buffer containing 20 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.2, 25 mM β -glycerol phosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 5 mM EGTA, 15 mM MgCl₂, and 100 μ M ATP (with or without 1.0 μ Ci/ μ l [γ -³²P]ATP). Phosphorylation was initiated at room temperature by adding PKA (50 ng kinase/ μ g protein) or MAP kinase (100 ng kinase/ μ g protein) to the reaction mixture containing P γ , P γ peptides, or purified bovine PDE6 holoenzyme. Phosphorylation by [γ -³²P]ATP was quantified by spotting samples onto P-81 phosphocellulose membranes (35). Once stoichiometric incorporation was reached, the kinase activity was quenched by heating the samples at 70 °C for 5 min. Incorporation of phosphate into P γ was confirmed by matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry at the Molecular Biology Core Facilities (Dana Farber Cancer Institute) with a Perceptive Biosystems Voyager DE-STR mass spectrometer operated at 20,000 V. Collection of data was performed in the positive ion mode utilizing α -cyano-4-hydroxy-trans-cinnamic acid as the matrix.

Proteolytic digestion of phosphorylated, recombinant bovine P γ (100 μ g) with endoproteinase Lys C (1:40, w/w) or trypsin (1:50, w/w) was carried out at room temperature for 12 h in 100 mM Tris HCl, pH 8.5. Fragments were then separated by reversed-phase HPLC using a 0–100% linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Fractions were assayed for radioactivity and assigned to their corresponding peaks on the HPLC chromatogram. Nonradioactive samples treated in an identical manner were also analyzed by MALDI-TOF. The molecular ion masses of the peptide fragments generated by proteolytic digestion were matched with values obtained by theoretical digestion patterns generated by PeptideMass (36).

Phosphorylation of P γ Present in ROS—Frog ROS homogenates (50

μM rhodopsin) were incubated in ROS buffer containing $100 \mu\text{M}$ [γ - ^{32}P]ATP under nonactivated (dark-adapted, no GTP γS) or activated (light-exposed, $100 \mu\text{M}$ GTP γS) conditions in the presence of 25 mM β -glycerol phosphate and 1 mM sodium orthovanadate. Soluble P γ was separated from ROS membranes by first centrifuging the homogenate (3 min at $110,000 \times g$). The supernatant portion was assayed for P γ content, whereas the ROS membrane pellet was resuspended in 40% acetonitrile/ 0.1% trifluoroacetic acid. After heating at 80°C for 5 min , the resuspended membrane fraction was centrifuged again. Samples were injected onto an analytical C-4 reversed-phase HPLC column, and proteins were resolved with a 20 – 100% acetonitrile/ 0.1% trifluoroacetic acid linear gradient. The column fractions were assayed for radioactivity and P γ concentration (based on the ability of P γ to inhibit P $\alpha\beta$). The stoichiometry of phosphorylation was calculated based on the amount of ^{32}P incorporation relative to the total P γ in the sample. To confirm that P γ was the only phosphoprotein in these fractions, the fractions were lyophilized, resuspended in SDS-PAGE sample buffer, and electrophoresed on 15% acrylamide gels (37). After transfer onto nitrocellulose membranes, autoradiography was performed for 12 – 24 h at -80°C . In some cases, the membrane was rehydrated and processed for immunoblot analysis of P γ as described previously (30).

Analytical Procedures—PDE6 hydrolytic activity was measured by either colorimetric or radiotracer assays as described recently (30). The equilibrium binding and kinetics of [^3H]cGMP association with PDE6 were measured with a filter binding assay exactly as described previously (30). Binding of [^{35}S]GTP γS to activated transducin was performed as described previously (38). All experiments were performed at least three times. Fitting of the data was performed using nonlinear regression analysis (Sigmaplot; SPSS, Inc.), using the curve-fitting function described in the figure legends.

RESULTS AND DISCUSSION

P γ Is Phosphorylated by PKA at Thr 35 and by MAP Kinase at Thr 22 —To determine the effect of P γ phosphorylation on PDE6 activation and recovery, we first needed to prepare *in vitro* phosphorylated P γ at defined sites. Using the catalytic subunit of PKA, we were able to incorporate $1.0 \pm 0.2 \text{ mol}$ of phosphate into recombinant, purified P γ . We saw no evidence for a second site of phosphorylation, even with extensive incubations with the kinase (Fig. 1A). MALDI-TOF analysis of full-length P γ phosphorylated with PKA yielded a single species with an average molecular ion mass of $9750 \pm 1.7 \text{ Da}$, a shift of 80 Da (the mass of a single phosphate group) compared with nonphosphorylated P γ ($9669 \pm 1.0 \text{ Da}$).

The site of incorporation of phosphate into P γ by PKA was unequivocally determined to be Thr 35 based on the following results: (a) incorporation of ^{32}P into P γ was greatly reduced when Thr 35 was substituted with cysteine, whereas stoichiometric P γ phosphorylation was observed when Thr 22 was substituted with cysteine (Fig. 1A); (b) a synthetic peptide comprising amino acids 21–46 of P γ was able to incorporate ^{32}P , whereas central region peptides lacking Thr 35 or its neighboring residues were unable to be phosphorylated by PKA (data not shown); (c) proteolytic digestion of full-length P γ with endoprotease Lys C generates a peptide fragment consisting of residues 32–39 (QRQTRQFK; predicted molecular ion mass of 1091.7 Da), and when the proteolytic fragments of PKA-phosphorylated P γ were analyzed, we observed an 80 -Da shift in molecular ion mass (1171.5 Da) of this peptide; and (d) digestion of full-length P γ with trypsin also reveals an 80 -Da shift (688.4 to 768.4 Da) in the peptide containing residues 32–36 (QRQTR). We conclude that PKA catalyzes the stoichiometric incorporation of phosphate only into Thr 35 under our experimental conditions, in agreement with the findings of Xu *et al.* (24).

To prepare P γ phosphorylated at Thr 22 , we used MAP kinase because its substrate specificity (Pro-X-(Thr/Ser)-Pro; Ref. 39) is identical to the residues surrounding Thr 22 (Pro 20 -Val 21 -Thr 22 -Pro 23). Under the conditions shown in Fig. 1B, $1.0 \pm 0.2 \text{ mol}$ of phosphate was incorporated into P γ . MALDI-TOF analysis of MAP kinase-phosphorylated P γ confirmed stoichiomet-

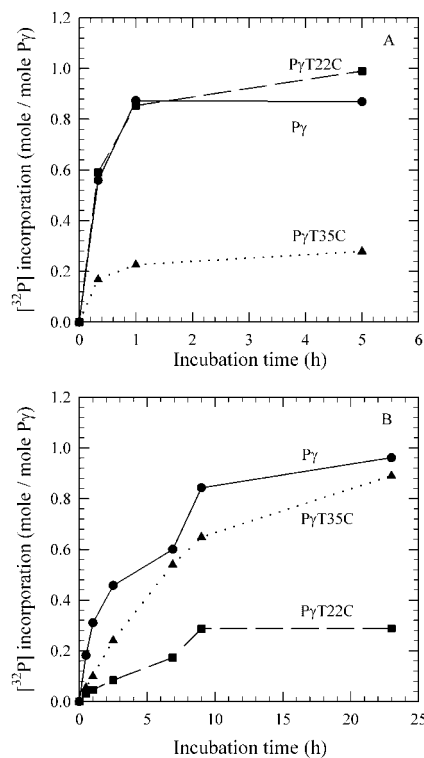


FIG. 1. Phosphorylation of P γ and P γ mutants by PKA or MAP kinase. Purified P γ (●), P γ T22C (■), or P γ T35C (▲) at $1 \mu\text{M}$ concentration was phosphorylated with 50 ng PKA/ μg protein (A) or 100 ng MAP kinase/ μg protein (B) in phosphorylation reaction buffer. $100 \mu\text{M}$ [γ - ^{32}P]ATP was added, and aliquots were removed at various times and assayed for ^{32}P incorporation (see “Experimental Procedures”).

ric incorporation of phosphate, with an average molecular ion mass of $9750 \pm 1.2 \text{ Da}$ under these conditions.²

The site of P γ phosphorylation catalyzed by MAP kinase was ascertained to be Thr 22 based on the following observations: (a) the P γ mutant in which Thr 22 was replaced with cysteine failed to incorporate significant amounts of ^{32}P compared with wild-type P γ or P γ substituted at Thr 35 with a cysteine residue (Fig. 1B); (b) synthetic peptides consisting of amino acid residues 10–30 or 15–26 of P γ can both be phosphorylated by MAP kinase (but at a reduced rate), whereas synthetic peptides lacking amino acids 20–23 failed to incorporate ^{32}P (data not shown); (c) MALDI-TOF analysis of endoprotease Lys C fragments of P γ showed that the fragment containing amino acid residues 8–25 (AEIRSATRVMGGPVTTPRK) increased in molecular ion mass by 80 Da (1927.3 to 2007.3 Da) upon P γ phosphorylation by MAP kinase; (d) trypsin-proteolyzed P γ also showed an 80 -Da increase (913.5 to 993.5 Da) of the peptide fragment containing amino acid residues 16–24 (RVMGGPVTTPR). We conclude that MAP kinase directs stoichiometric incorporation of phosphate into Thr 22 under our *in vitro* conditions, consistent with results obtained with another proline-directed kinase, cyclin-dependent kinase 5 (26).

The low levels of phosphorylation seen with P γ T35C phosphorylated with PKA or P γ T22C phosphorylated with MAP kinase (Fig. 1) may reflect autophosphorylation of the added kinases or, alternatively, phosphorylation of other proteins

² With higher enzyme concentrations or longer incubations, $>1 \text{ mol}$ phosphate/mol P γ was detected by MALDI-TOF and ^{32}P incorporation. The site of this second phosphorylation event has yet to be determined. All MAP kinase-phosphorylated P γ preparations used in this study were phosphorylated under the conditions described for Fig. 1 (0.7 – 1.1 mol phosphate/mol P γ), and the site of phosphorylation was identified as exclusively Thr 22 .

contaminating our purified P γ mutant preparations. These phosphorylations are not relevant to the functional studies of P γ phosphorylation. It is very unlikely that a second site of phosphorylation on P γ is responsible for the observed phosphate incorporation because mass spectrometric analysis of trypsin- or Lys C-proteolyzed P γ failed to detect other peptides undergoing a mass shift after phosphorylation of P γ under the conditions described for Fig. 1.

Phosphorylation of P γ at Thr²² or Thr³⁵ Has Little Effect on the Inhibition of P $\alpha\beta$ —Previous studies reporting enhanced inhibition of PDE6 by phosphorylated P γ (24, 25, 27, 28, 40) generally failed to consider that the very high intrinsic P γ binding affinity for P $\alpha\beta$ ($K_D \leq$ picomolar concentrations) should result in titration behavior except at very low enzyme concentrations (see Ref. 8 for discussion). Furthermore, with one exception (28), the existence of two classes of P γ binding sites on P $\alpha\beta$ (8, 33, 41) was also overlooked in investigating the effects of P γ phosphorylation on PDE6 regulation.

To re-examine whether phosphorylation alters the affinity of P γ to its two distinct binding sites on PDE6, we first attempted to reproduce published results reporting a 3- to 5-fold increased ability of P γ phosphorylated at Thr³⁵ to inhibit purified bovine P $\alpha\beta$ (24). Using the same conditions described in Fig. 9 of Xu *et al.* (24), namely 180 ng/tube bovine P $\alpha\beta$, 4 mM cGMP, and a 10-min incubation, we found that >90% of the cGMP was degraded in the absence of added P γ , thereby precluding initial rate measurements. When we obtained initial velocity measurements at early times (<10 s) where <20% of the cGMP had been hydrolyzed, we observed stoichiometric inhibition of P $\alpha\beta$ activity by P γ regardless of the state of phosphorylation (data not shown). This contrasts with the observation of Xu *et al.* (24) that P γ phosphorylated at Thr³⁵ inhibited catalysis stoichiometrically, whereas nonphosphorylated P γ required a 5-fold excess of P γ relative to P $\alpha\beta$ to fully inhibit the enzyme. In eight different preparations of nonphosphorylated and phosphorylated P γ , we found that the concentration of P γ required to inhibit 50% of the hydrolytic activity of 1–5 nM P $\alpha\beta$ did not deviate from stoichiometric inhibition (nonphosphorylated P γ , $IC_{50} = 1.1 \pm 0.2$ P γ /P $\alpha\beta$; Thr²²-phosphorylated P γ , $IC_{50} = 1.0 \pm 0.2$ P γ /P $\alpha\beta$; Thr³⁵-phosphorylated P γ , $IC_{50} = 1.1 \pm 0.2$ P γ /P $\alpha\beta$). This well-documented linear relationship between added P γ and the extent of inhibition is characteristic of a titration phenomenon when the P $\alpha\beta$ concentration greatly exceeds the K_D for P γ binding (8, 42, 43).

To determine the effect of Thr²² or Thr³⁵ phosphorylation of P γ on its inhibition of P $\alpha\beta$, it was therefore necessary to work at picomolar enzyme concentrations. Using 1 pM frog P $\alpha\beta$ and the phosphorylated P γ preparations described in the previous section, we were able to measure the ability of P γ to inhibit cGMP hydrolysis under equilibrium binding conditions (Fig. 2A). A two-site model was required for each P γ condition to fit the binding data, consistent with our previous results with P γ binding to bovine P $\alpha\beta$ (8). P γ binding heterogeneity was not abolished upon phosphorylation at either threonine position, as had been reported previously (28). For all three P γ s tested, one P γ binding site displayed a very high affinity ($K_{D,1} \leq 0.4$ pM) that did not vary with phosphorylation. The extremely high affinity of this P γ binding site makes it highly improbable that this site is subject to regulation during phototransduction.

The second, lower affinity class of P γ binding sites on P $\alpha\beta$ showed a small but significant decrease in binding affinity upon phosphorylation of P γ at Thr³⁵ ($K_{D,2} = 1.3$ pM) or Thr²² ($K_{D,2} = 2.5$ pM), compared with identically treated, nonphosphorylated P γ ($K_{D,2} = 0.8$ pM; Fig. 2A). To rule out a species difference, we also tested purified bovine P $\alpha\beta$ at 1 pM concentration and found identical behavior to frog P $\alpha\beta$ (data not

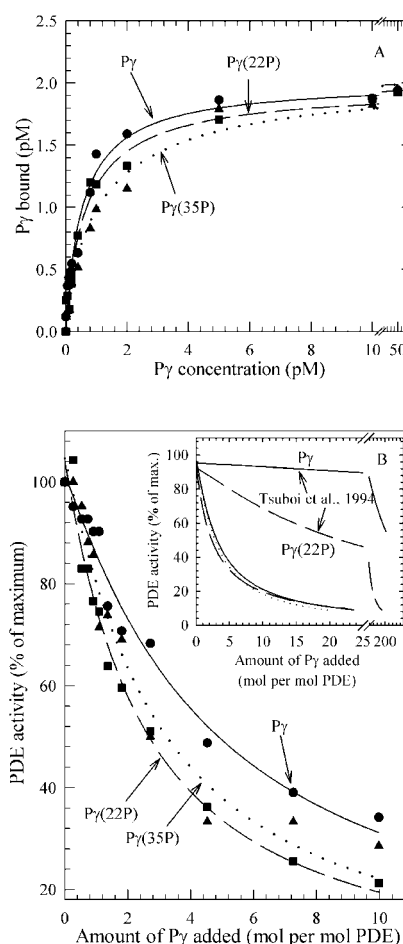


FIG. 2. Affinity of P γ for P $\alpha\beta$ catalytic dimers or for P γ -depleted PDE6 is not greatly affected by phosphorylation at Thr²² or Thr³⁵. A, frog P $\alpha\beta$ was prepared by limited trypsin proteolysis (see “Experimental Procedures”) and diluted to 1 pM final concentration. P $\alpha\beta$ was then incubated with P γ (●), P γ phosphorylated by PKA (P γ (35P), ▲), or P γ phosphorylated with MAP kinase (P γ (22P), ■) for 10 min. The extent of P γ binding was determined by inhibition of catalysis after the addition of 100 μ M cGMP. The data (averaged from three experiments with S.E. < 15%) were fit to double hyperbolic functions, assuming two equal classes of binding sites (8). The higher affinity class of sites displayed titration behavior with apparent K_D values of ≤ 0.4 pM in all three cases. The lower affinity class of sites had K_D values of 0.8 (P γ , solid line), 1.2 (P γ (22P), dashed line), and 2.5 pM (P γ (35P), dotted line). B, P γ -depleted ROS membranes (72% P γ removal) were prepared as described under “Experimental Procedures” and diluted to 130 pM final P $\alpha\beta$ concentration before the addition of P γ (●), P γ (35P) (▲), or P γ (22P) (■). Inhibition of hydrolysis of 1 mM cGMP was determined under conditions where <30% substrate was hydrolyzed. Inset, comparison of data averaged from five separate experiments for P γ (solid line; $IC_{50} = 3.3$ P γ /P $\alpha\beta$), P γ (22P) (dashed line; $IC_{50} = 2.6$ P γ /P $\alpha\beta$), and P γ (35P) (dotted line; $IC_{50} = 3.1$ P γ /P $\alpha\beta$) with the published results of Tsuboi *et al.* (25), Fig. 9. Their P $\alpha\beta$ concentration was estimated from the hydrolytic rate in the absence of added P γ , using a $k_{cat} = 7000$ s⁻¹ (15).

shown). Thus, under conditions where changes in P γ binding affinity to P $\alpha\beta$ can be quantitated, phosphorylation of Thr²² or Thr³⁵ of P γ decreases rather than increases the intrinsic binding affinity of P γ to its lower affinity binding site on P $\alpha\beta$.

The Ability of P γ -depleted PDE6 or Transducin-activated PDE6 to Be Inhibited by P γ Is Not Significantly Altered by P γ Phosphorylation—We also studied PDE6 that had been activated by depletion of the endogenous P γ but was still attached to ROS membranes. Removal of most of the bound P γ is carried out by allowing persistently activated transducin (α_q^* -GTP γ S) to bind to P γ and solubilize it from P $\alpha\beta$ when the noncatalytic cGMP binding sites of PDE6 are unoccupied (14, 15). When we tested the ability of 100 pM P γ -depleted frog PDE6 to be inhibited

ited by nonphosphorylated or phosphorylated P γ , we found that the apparent inhibitory potency was slightly increased in some experiments upon phosphorylation at Thr²² or Thr³⁵ (Fig. 2B). However, when results from five experiments were averaged (Fig. 2B, inset), no statistically significant difference in P γ inhibitory potency could be discerned upon phosphorylation at Thr²² or at Thr³⁵. This contrasts with the reported 10- to 15-fold enhancement of inhibitory activity of P γ upon P γ phosphorylation by cyclin-dependent kinase 5 at Thr²² (25, 27). From the large molar excess of P γ needed to inhibit P γ -depleted PDE in Tsuboi *et al.* (Fig. 9 of Ref. 25, replotted in Fig. 2B, inset), we suspect that inaccuracies in the determination of the P γ concentration and/or different biological activities for nonphosphorylated and phosphorylated P γ preparations may account for the differences in inhibitory potency they report.

We examined the possibility that unfractionated ROS homogenates might reveal an effect of P γ phosphorylation on PDE6 regulation that was overlooked in studying P $\alpha\beta$ or P γ -depleted PDE6. However, when we added increasing amounts of Thr²²- or Thr³⁵-phosphorylated P γ to light-activated frog ROS homogenates incubated with GTP γ S, the amount of phosphorylated P γ needed (P γ (22P), IC₅₀ = 8 P γ /PDE6; P γ (35P), IC₅₀ = 7 P γ /PDE6) was the same as that for nonphosphorylated P γ (IC₅₀ = 7 P γ /PDE6). The fact that P γ -depleted PDE6 and transducin-activated PDE6 require greater than stoichiometric amounts of P γ to inhibit catalysis is likely due to the binding of α_t^* -GTP γ S to P γ in these two preparations (15).

In summary, under a variety of conditions where P γ binding affinity to P $\alpha\beta$ can be accurately determined, we find no support for the hypothesis that phosphorylation of P γ results in an increased ability of P γ to directly inhibit catalysis of PDE6. We therefore turned to other mechanisms by which P γ phosphorylation might indirectly regulate PDE6 function.

Phosphorylation of P γ at Thr²² Lowers the Ability of the Central Region of P γ to Bind to and Regulate the GAF Domain of PDE6—The two sites of phosphorylation we studied reside in the polycationic central region of the P γ molecule that is known to bind P $\alpha\beta$ with high affinity and to restore high-affinity cGMP binding to a low-affinity class of binding sites on bovine rod PDE6 (7, 8, 44). We therefore synthetically prepared nonphosphorylated and phosphothreonine-containing peptides corresponding to amino acids 18–41 of the bovine rod P γ sequence to test the effects of a phosphate group at Thr²² or Thr³⁵. After incubating P $\alpha\beta$ with [³H]cGMP and increasing amounts of the synthetic peptides P γ 18–41, P γ 18–41(22P), or P γ 18–41(35P), we observed that all three peptides restored high-affinity cGMP binding to one GAF domain of PDE6. In the absence of P γ or central region peptides, P $\alpha\beta$ is able to bind 1 cGMP/P $\alpha\beta$ (8, 33). To detect differences in the ability of these P γ peptides to stabilize cGMP binding to this site, we compared the rates of cGMP exchange after the addition of unlabeled cGMP (“cold chase”). Overall, the central region peptides all stabilized cGMP binding 10- to 20-fold compared with P $\alpha\beta$ (Fig. 3A). Introduction of a phosphate group at Thr²² reduced the central region peptide’s affinity for P $\alpha\beta$ by ~2-fold, whereas phosphorylation at Thr³⁵ stabilized cGMP binding by about 2-fold compared with P γ 18–41 (Fig. 3A).

We also examined the ability of these central region P γ peptides to compete with and displace full-length P γ bound to the PDE6 holoenzyme, thereby causing cGMP hydrolysis at the active site. This competition experiment took advantage of the fact that the N-terminal half of P γ binds to P $\alpha\beta$ with a 50-fold higher affinity than its C-terminal binding domain but is unable to inhibit catalysis (8). We found that phosphorylation at Thr²², but not at Thr³⁵, reduced the ability of P γ 18–41 to compete with endogenous P γ bound to PDE6 holoenzyme (Fig.

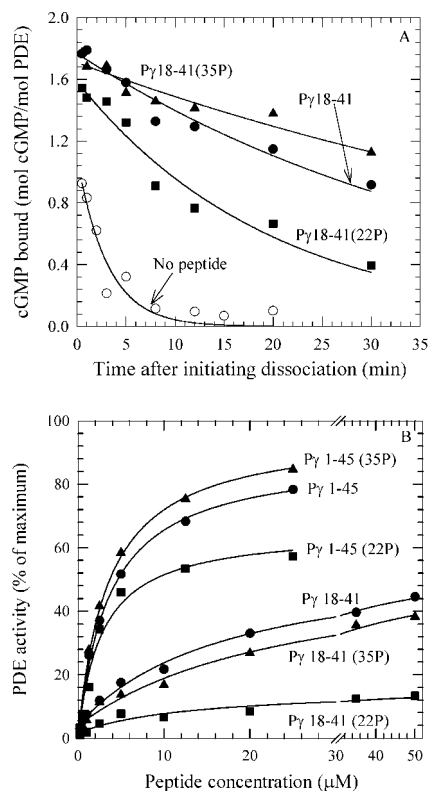


FIG. 3. Phosphorylation at Thr²² reduces the ability of the central region of P γ to interact with P $\alpha\beta$. A, 5 nM bovine P $\alpha\beta$ was incubated with 0.6 μ M [³H]cGMP and 200 μ M zaprinast (\circ), and then 20 μ M P γ 18–41 (\bullet), P γ 18–41(22P) (\blacksquare), or P γ 18–41(35P) (\blacktriangle) was added. Dissociation of bound [³H]cGMP was initiated at time 0 with 2 mM cold cGMP, and portions were filtered at the indicated times. The curves represent a single exponential decay with rate constants of 0.323 min⁻¹ (no peptide), 0.024 min⁻¹ (P γ 18–41), 0.052 min⁻¹ (P γ 18–41(22P)), and 0.014 min⁻¹ (P γ 18–41(35P)). B, bovine PDE6 holoenzyme (5 nM) was incubated with increasing concentrations of P γ peptides to compete with endogenous, bound P γ , and the extent of PDE6 activation was determined. In the absence of P γ peptide, the basal PDE6 activity was 60 cGMP hydrolyzed/PDE6/s, and the fully activated rate was 5600 cGMP/PDE6/s.

3B). Increasing the length of the P γ peptide (P γ 1–45) and enzymatically phosphorylating Thr²² or Thr³⁵ confirmed the reduced affinity of the Thr²²-phosphorylated protein to bind PDE6 (Fig. 3B).

We conclude from Fig. 3 that phosphorylation at Thr²² somewhat weakens the intrinsic affinity of the central region of P γ for PDE6, whereas phosphorylation at Thr³⁵ may have a slight stabilizing effect on P γ binding to PDE6. Thus, the 3-fold decrease in the ability of full-length P γ phosphorylated at Thr²² to inhibit catalysis (Fig. 2A) can be accounted for by a comparable loss in binding affinity of the central region of P γ to its binding sites on P $\alpha\beta$. In summary, we find no evidence that either of the two major sites of interaction of P γ with P $\alpha\beta$ undergoes an increase in its affinity for P $\alpha\beta$, as claimed previously.

Phosphorylation of P γ at Thr²² or Thr³⁵ Greatly Reduces Its Interaction with Transducin α -Subunit—It is well documented that P γ can bind to transducin with high affinity based on its co-purification with transducin α -subunit (14), direct binding measurements (12, 45), and its ability to alter the binding and hydrolysis of guanine nucleotides (16, 46). We studied P γ - α_t^* -GTP γ S interactions based on the ability of nonphosphorylated P γ to bind to transducin with high affinity and block GTP γ S binding to the α -subunit (46) in part because previous studies used this approach to document loss of P γ interactions with transducin after phosphorylation or ADP-ribosylation of P γ (24, 40, 47).

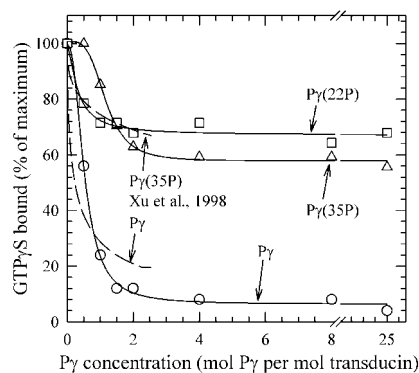


FIG. 4. $P\gamma$ phosphorylation disrupts its interaction with activated transducin. Light-exposed frog ROS homogenates ($5 \mu\text{M}$ rhodopsin) were incubated with $P\gamma$ (○), $P\gamma(22\text{P})$ (□), or $P\gamma(35\text{P})$ (△) before the addition of $1 \mu\text{M}$ [^{35}S]GTP γS . After a 1-h incubation, samples were analyzed for GTP γS binding with a filter binding assay. *Data points and solid lines* represent one of four similar experiments. The *dashed lines* are replotted from Fig. 10B of Xu *et al.* (24) using $P\gamma$ -depleted PDE with nonphosphorylated (*lower curve*) or Thr 35 -phosphorylated (*upper curve*) $P\gamma$.

When we incubated light-exposed frog ROS homogenates with nonphosphorylated or phosphorylated $P\gamma$ and then assayed the ability of [^{35}S]GTP γS to bind to the transducin α -subunit, we found that $P\gamma$ phosphorylated at either Thr 22 or Thr 35 was much less effective in blocking GTP γS binding to transducin compared with nonphosphorylated $P\gamma$ (Fig. 4). Nonphosphorylated $P\gamma$ was able to inhibit GTP γS binding in a stoichiometric manner, with 2 $P\gamma$ /transducin blocking 90% of GTP γS binding. Approximately 40% of the total GTP γS binding could be inhibited by low concentrations of phosphorylated $P\gamma$, but thereafter, phosphorylated $P\gamma$ failed to further block GTP γS binding. The reason for the partial inhibition of GTP γS binding by Thr 35 -phosphorylated $P\gamma$ is not understood. Our results with nonphosphorylated and Thr 35 -phosphorylated $P\gamma$ are in complete agreement with the data of Xu *et al.* (Ref. 24; see *dashed lines* in Fig. 4). For the case of Thr 22 -phosphorylated $P\gamma$, our results differ from the complete lack of inhibition of GTP γS binding reported by Tsuboi *et al.* (40), but in this case, the difference may be ascribed to different experimental conditions for the GTP γS binding assay.

We conclude that phosphorylation of $P\gamma$ in its central polycationic region destabilizes its interaction with the α -subunit of transducin, whereas there is, at most, a modest effect of $P\gamma$ phosphorylation directly on the PDE6 catalytic dimer. Earlier claims that $P\gamma$ enhanced its inhibitory potency when phosphorylated probably confounded direct effects of $P\gamma$ at the active site of PDE6 with the substantial reduction in binding affinity of phosphorylated $P\gamma$ to activated transducin present in the PDE6 preparations used in these previous studies.

$P\gamma$ Is a Poor Substrate for Phosphorylation When Bound to Nonactivated PDE6 but Shows a Small Light-induced Increase in Phosphorylation upon Transducin Activation—Although purified $P\gamma$ is a good substrate for *in vitro* phosphorylation by a number of protein kinases, there is limited evidence on the ability of $P\gamma$ to be phosphorylated *in situ* in ROS. We first examined whether $P\gamma$ associated with PDE6 was capable of being phosphorylated as readily as free $P\gamma$. Fig. 5A shows that neither MAP kinase nor PKA was able to phosphorylate its cognate threonine phosphorylation sites when bound to $P\alpha\beta$; the same concentration of free $P\gamma$ was readily phosphorylated under identical conditions. This result agrees with and extends previous work showing that protein kinase C (28), a phosphatidylinositol-stimulated kinase (22), and cyclin-dependent kinase 5 (26, 40) poorly phosphorylate $P\gamma$ when complexed with $P\alpha\beta$. It is noteworthy that $P\gamma$ complexed with activated trans-

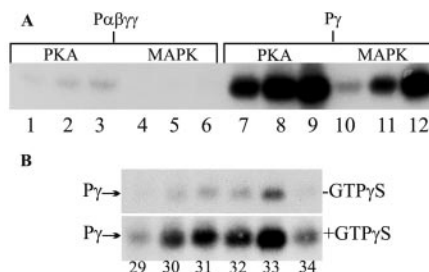


FIG. 5. $P\gamma$ bound to PDE6 is a poor substrate for phosphorylation, but transducin activation enhances its ability to be phosphorylated. *A*, purified bovine PDE6 holoenzyme (100 nM) or free $P\gamma$ (200 nM) was phosphorylated in the presence of PKA (*lanes 1–3* and *7–9*) or MAP kinase (*lanes 4–6* and *10–12*) for 10, 30, or 120 min, and samples were analyzed by SDS-PAGE and autoradiography (see “Experimental Procedures”). *B*, frog ROS homogenates ($50 \mu\text{M}$ rhodopsin) containing phosphatase inhibitors (but no exogenous protein kinases) were incubated for 5 h with $100 \mu\text{M}$ [γ - ^{32}P]ATP either in the dark ($-\text{GTP}\gamma\text{S}$) or exposed to light with $100 \mu\text{M}$ GTP γS added ($+\text{GTP}\gamma\text{S}$). $P\gamma$ was then extracted and purified by HPLC, and the $P\gamma$ -containing HPLC fractions (*numbered* below the lanes) were analyzed by SDS-PAGE and autoradiography (see “Experimental Procedures”).

ducin is a good substrate for phosphorylation by cyclin-dependent kinase 5 (40) but not for phosphorylation by PKA or the phosphatidylinositol-stimulated kinase (22, 24). These results led us to hypothesize that $P\gamma$ phosphorylation would occur *in vivo* only when $P\gamma$ dissociates from PDE6, either as a free $P\gamma$ subunit or as a complex with transducin α -subunit.

We therefore tested whether transducin activation of PDE6 in frog ROS homogenates would expose phosphorylation sites on $P\gamma$ to the action of endogenous ROS protein kinases. We incubated dark-adapted or light-activated (illumination plus GTP γS) frog ROS homogenates with [γ - ^{32}P]ATP (along with phosphatase inhibitors) and then purified the endogenous $P\gamma$ to determine the extent of phosphorylation (see “Experimental Procedures”). Under nonactivated conditions, only $3.1 \pm 1.4\%$ ($n = 3$) of the total $P\gamma$ in ROS was phosphorylated after incubation with [γ - ^{32}P]ATP (Fig. 5B). This low level of phosphate incorporation was expected based on *in vitro* results because $P\gamma$ remains in a complex with $P\alpha\beta$ under these experimental conditions (15). For transducin-activated samples, the level of phosphate incorporation into $P\gamma$ increased to $11.7 \pm 1.3\%$ of the total $P\gamma$. The failure to observe stoichiometric incorporation of phosphate into $P\gamma$ cannot be accounted for by $P\gamma$ bound to $P\alpha\beta$ because most $P\gamma$ is released from membrane-bound PDE6 under these conditions (see Fig. 4 of Ref. 15). The addition of exogenous PKA or MAP kinase did not enhance $P\gamma$ phosphorylation *in situ* under dark-adapted or transducin-activated conditions (data not shown), indicating that the potential phosphorylation sites on $P\gamma$ (including Thr 22 and Thr 35) are not very accessible to protein kinases. We speculate that the 4-fold increase in $P\gamma$ phosphorylation upon light activation of ROS may reflect a small percentage of $P\gamma$ molecules that transiently dissociate from α_t -GTP γS or other binding proteins and are thereby able to be phosphorylated in their unbound state.

Conclusions—This study clarifies the existing literature on regulation of PDE6 by $P\gamma$ phosphorylation and places severe constraints on the role of $P\gamma$ phosphorylation in the visual transduction pathway in rod photoreceptors. First, we performed a series of experiments with PDE6 under conditions where the active subunit concentrations, catalytic properties, and affinity for cGMP binding to the GAF domains are precisely defined (8, 15, 33, 48), and we show that $P\gamma$ phosphorylation at Thr 22 or Thr 35 does not greatly enhance $P\gamma$ binding to $P\alpha\beta$. Rather, we observe a modest decrease in $P\gamma$ binding affinity to $P\alpha\beta$ that is reflected both at the catalytic site (Fig. 2) and at the GAF domains (Fig. 3), with Thr 22 phosphorylation

generally having a more significant destabilizing influence. Second, we showed that phosphorylation in the central polycationic region of P γ disrupted the ability of P γ to interact with transducin, as judged by the ability of nonphosphorylated P γ , but not Thr²²- or Thr³⁵-phosphorylated P γ , to block GTP γ S binding to the α -subunit of activated transducin. In this respect, our results agree qualitatively with those of Yamazaki and colleagues (24, 40). Finally, we demonstrate that free P γ can be stoichiometrically phosphorylated at its respective consensus target sites by PKA or by MAP kinase (Fig. 1) but that P γ is present in ROS homogenates in a state that precludes stoichiometric phosphorylation under all conditions we tested (Fig. 5).

The inability of ROS to phosphorylate P γ at stoichiometric levels in this study and a previous study (27) makes it very unlikely that the extent or lifetime of the PDE6 catalytic dimer can be directly regulated by this posttranslational modification of P γ . Likewise, the fact that the transducin concentration in ROS (49, 50) exceeds the P γ concentration by ~15-fold (30) casts doubt on any mechanism in which P γ phosphorylation regulates transducin directly.

The observation that P γ phosphorylation causes its dissociation from the α_t^* -GTP γ S-P γ complex suggests that the phosphorylated P γ might act as an intermediate in a signaling pathway distinct from the classical visual transduction pathway. One intriguing candidate is MAP kinase, in view of the recent report that P γ can regulate MAP kinase in a phosphorylation-dependent manner (20). The presence of MAP kinase in ROS³ (51) lends further support to this idea. The ability of P γ to interact with several cell signaling proteins other than PDE6 (arrestin (52), PDE5 (21, 53), and dynamin II (20)) and the presence of an SH3 recognition site that overlaps the MAP kinase consensus sequence at Thr²² of P γ lead us to speculate that P γ phosphorylation may serve to bridge the classical phototransduction pathway (rhodopsin \rightarrow transducin \rightarrow PDE6) with other signaling pathways involved in light desensitization or circadian rhythms in photoreceptor cells.

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REFERENCES

- Manganiello, V. C., and Degerman, E. (1999) *Thromb. Haemostasis* **82**, 407–411
- Conti, M. (2000) *Mol. Endocrinol.* **14**, 1317–1327
- Francis, S. H., Turko, I. V., and Corbin, J. D. (2000) *Prog. Nucleic Acid Res. Mol. Biol.* **65**, 1–52
- Soderling, S. H., and Beavo, J. A. (2000) *Curr. Opin. Cell Biol.* **12**, 174–179
- Pugh, E. N., Jr., and Lamb, T. D. (2000) in *Molecular Mechanisms in Visual Transduction* (Stavenga, D. G., Pugh, E. N., Jr., and Pugh, E. N., Jr., ed), Elsevier Science B. V., New York
- Granovsky, A. E., Natochin, M., and Artemyev, N. O. (1997) *J. Biol. Chem.* **272**, 11686–11689
- Artemyev, N. O., and Hamm, H. E. (1992) *Biochem. J.* **283**, 273–279
- Mou, H., and Cote, R. H. (2001) *J. Biol. Chem.* **276**, 27527–27534
- Lipkin, V. M., Dumler, I. L., Muradov, K. G., Artemyev, N. O., and Etingof, R. N. (1988) *FEBS Lett.* **234**, 287–290
- Brown, R. L. (1992) *Biochemistry* **31**, 5918–5925
- Slep, K. C., Kercher, M. A., He, W., Cowan, C. W., Wensel, T. G., and Sigler, P. B. (2001) *Nature* **409**, 1071–1077
- Artemyev, N. O., Rarick, H. M., Mills, J. S., Skiba, N. P., and Hamm, H. E. (1992) *J. Biol. Chem.* **267**, 25067–25072
- Skiba, N. P., Bae, H., and Hamm, H. E. (1996) *J. Biol. Chem.* **271**, 413–424
- Yamazaki, A., Hayashi, F., Tatsumi, M., Bitensky, M. W., and George, J. S. (1990) *J. Biol. Chem.* **265**, 11539–11548
- Norton, A. W., D'Amours, M. R., Grazio, H. J., Hebert, T. L., and Cote, R. H. (2000) *J. Biol. Chem.* **275**, 38611–38619
- Arshavsky, V. Y., and Bownds, M. D. (1992) *Nature* **357**, 416–417
- He, W., Cowan, C. W., and Wensel, T. G. (1998) *Neuron* **20**, 95–102
- Calvert, P. D., Ho, T. W., LeFebvre, Y. M., and Arshavsky, V. Y. (1998) *J. Gen. Physiol.* **111**, 39–51
- Tate, R. J., Lochhead, A., Brzeski, H., Arshavsky, V., and Pyne, N. J. (1998) *Cell Biochem. Biophys.* **29**, 133–144
- Wan, K. F., Sambli, B. S., Frame, M., Tate, R., and Pyne, N. J. (2001) *J. Biol. Chem.* **276**, 37802–37808
- Lochhead, A., Nekrasova, E., Arshavsky, V. Y., and Pyne, N. J. (1997) *J. Biol. Chem.* **272**, 18397–18403
- Hayashi, F., Lin, G. Y., Matsumoto, H., and Yamazaki, A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 4333–4337
- Udovichenko, I. P., Cunnick, J., Gonzales, K., and Takemoto, D. J. (1993) *Biochem. J.* **295**, 49–55
- Xu, L. X., Tanaka, Y., Bondarenko, V. A., Matsuura, I., Matsumoto, H., Yamazaki, A., and Hayashi, F. (1998) *Biochemistry* **37**, 6205–6213
- Tsuboi, S., Matsumoto, H., Jackson, K. W., Tsujimoto, K., Williams, T., and Yamazaki, A. (1994) *J. Biol. Chem.* **269**, 15016–15023
- Matsuura, I., Bondarenko, V. A., Maeda, T., Kachi, S., Yamazaki, M., Usukura, J., Hayashi, F., and Yamazaki, A. (2000) *J. Biol. Chem.* **275**, 32950–32957
- Hayashi, F., Matsuura, I., Kachi, S., Maeda, T., Yamamoto, M., Fujii, Y., Liu, H., Yamazaki, M., Usukura, J., and Yamazaki, A. (2000) *J. Biol. Chem.* **275**, 32958–32965
- Udovichenko, I. P., Cunnick, J., Gonzalez, K., and Takemoto, D. J. (1994) *J. Biol. Chem.* **269**, 9850–9856
- Udovichenko, I. P., Cunnick, J., Gonzalez, K., Yakhnin, A., and Takemoto, D. J. (1996) *Biochem. J.* **317**, 291–295
- Cote, R. H. (2000) *Methods Enzymol.* **315**, 646–672
- Bownds, J., Gordon-Walker, A., Gaide Huguenin, A. C., and Robinson, W. (1971) *J. Gen. Physiol.* **58**, 225–237
- Granovsky, A. E., McEntaffer, R., and Artemyev, N. O. (1998) *Cell Biochem. Biophys.* **28**, 115–133
- Mou, H., Grazio, H. J., Cook, T. A., Beavo, J. A., and Cote, R. H. (1999) *J. Biol. Chem.* **274**, 18813–18820
- Smith, P. K., Krohn, R. L., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goetze, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76–85
- Roskoski, R., Jr. (1983) *Methods Enzymol.* **99**, 3–6
- Wilkins, M. R., Lindskog, I., Gasteiger, E., Baird, A., Sanchez, J. C., Hochstrasser, D. F., and Appel, R. D. (1997) *Electrophoresis* **18**, 403–408
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Fung, B. K. K., Hurley, J. B., and Stryer, L. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 152–156
- Stokoe, D., Campbell, D. G., Nakielny, S., Hidaka, H., Leevers, S. J., Marshall, C., and Cohen, P. (1992) *EMBO J.* **11**, 3985–3994
- Tsuboi, S., Matsumoto, H., and Yamazaki, A. (1994) *J. Biol. Chem.* **269**, 15024–15029
- Berger, A. L., Cerione, R. A., and Erickson, J. W. (1999) *Biochemistry* **38**, 1293–1299
- Fung, B. K. K., Young, J. H., Yamane, H. K., and Griswold-Prenner, I. (1990) *Biochemistry* **29**, 2657–2664
- Hamilton, S. E., Prusti, R. K., Bentley, J. K., Beavo, J. A., and Hurley, J. B. (1993) *FEBS Lett.* **318**, 157–161
- Takemoto, D. J., Hurt, D., Oppert, B., and Cunnick, J. (1992) *Biochem. J.* **281**, 637–643
- Otto-Bruc, A., Antonny, B., Vuong, T. M., Chardin, P., and Chabre, M. (1993) *Biochemistry* **32**, 8636–8645
- Yamazaki, A., Yamazaki, M., Tsuboi, S., Kishigami, A., Umbarger, K. O., Hutson, L. D., Madland, W. T., and Hayashi, F. (1993) *J. Biol. Chem.* **268**, 8899–8907
- Bondarenko, V. A., Desai, M., Dua, S., Yamazaki, M., Amin, R. H., Yousif, K. K., Kinumi, T., Ohashi, M., Komori, N., Matsumoto, H., Jackson, K. W., Hayashi, F., Usukura, J., Lipkin, V. M., and Yamazaki, A. (1997) *J. Biol. Chem.* **272**, 15856–15864
- D'Amours, M. R., and Cote, R. H. (1999) *Biochem. J.* **340**, 863–869
- Hamm, H. E., and Bownds, M. D. (1986) *Biochemistry* **25**, 4512–4523
- Gray-Keller, M. P., Biernbaum, M. S., and Bownds, M. D. (1990) *J. Biol. Chem.* **265**, 15323–15332
- Harada, Y., Sanada, K., and Fukada, Y. (2000) *J. Biol. Chem.* **275**, 37078–37085
- Qin, N., and Baehr, W. (1993) *FEBS Lett.* **321**, 6–10
- Frame, M., Wan, K. F., Tate, R., Vandenabeele, P., and Pyne, N. J. (2001) *Cell. Signal.* **13**, 735–741

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