Binding of the Delta Subunit to Rod Phosphodiesterase Catalytic Subunits Requires Methylated, Prenylated C-Termini of the Catalytic Subunits†

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ABSTRACT: PDE6 (type 6 phosphodiesterase) from rod outer segments consists of two types of catalytic subunits, alpha and beta; two inhibitory gamma subunits; and one or more delta subunits found only on the soluble form of the enzyme. About 70% of the phosphodiesterase activity found in rod outer segments is membrane-bound, and is thought to be anchored to the membrane through C-terminal prenyl groups. The recombinant delta subunit has been shown to solubilize the membrane-bound form of the enzyme. This paper describes the site and mechanism of this interaction in more detail. In isolated rod outer segments, the delta subunit was found exclusively in the soluble fraction, and about 30% of it did not coimmunoprecipitate with the catalytic subunits. The delta subunit that was bound to the catalytic subunits dissociated slowly, with a half-life of about 3.5 h. To determine whether the site of this strong binding was the C-termini of the phosphodiesterase catalytic subunits, peptides corresponding to the C-terminal ends of the alpha and beta subunits were synthesized. Micromolar concentrations of these peptides blocked the phosphodiesterase/delta subunit interaction. Interestingly, this blockade only occurred if the peptides were both prenylated and methylated. These results suggested that a major site of interaction of the delta subunit is the methylated, prenylated C-terminus of the phosphodiesterase catalytic subunits. To determine whether the catalytic subunits of the full-length enzyme are methylated in situ when bound to the delta subunit, we labeled rod outer segments with a tritiated methyl donor. Soluble phosphodiesterase from these rod outer segments was more highly methylated (4.5 ± 0.3-fold) than the membrane-bound phosphodiesterase, suggesting that the delta subunit bound preferentially to the methylated enzyme in the outer segment. Together these results suggest that the delta subunit/phosphodiesterase catalytic subunit interaction may be regulated by the C-terminal methylation of the catalytic subunits.

Photoreceptor cells, also known as rods and cones, are the cells in the retina that directly sense and respond to light. PDE6[1] is an integral part of the phototransduction cascade in rod outer segments. In light, it is activated by transducin to hydrolyze cGMP, thus leading to closure of cGMP-gated channels. About 30% of the PDE isolated from rod outer segments is soluble. Evidence suggests that PDE is solubilized as a result of an interaction between its catalytic and delta subunits. Isolated soluble PDE copurifies with the 17 kDa delta subunit, which is not found on the membrane-bound form of the enzyme (*1*). Membrane-bound PDE has a high affinity for rod outer segment membranes even after repeated isotonic washes, although it can be solubilized by treatment with hypotonic buffers. Nevertheless, under isotonic conditions, the recombinant delta subunit will remove membrane-bound PDE from washed ROS membranes (*2*). These data strongly support the hypothesis that membrane binding of the catalytic subunits of the PDE is controlled by the delta subunit. However, it is not yet known whether the amount of soluble PDE undergoes regulated changes in the photoreceptor.

The catalytic subunits of the PDE, α and *β*, undergo posttranslational C-terminal prenylation. The α subunit has been shown to be farnesylated on the cysteine of the C-terminal motif CCVQ, whereas the *β* subunit has been shown to be geranylgeranylated on the cysteine of CRIL (*3*, *4*), consistent with the specificity rules for protein prenyltransferases (*5*, *6*). Some evidence suggests that the C-termini of the PDE catalytic subunits are required in order for the PDE to remain membrane-bound. Limited proteolysis of the C-termini of the PDE catalytic subunits correlates with loss of membrane binding (*7*, *8*), indicating that the prenyl groups might anchor the enzyme in the membrane. Further work showed that loss of the beta subunit C-terminus alone was sufficient to remove the catalytic subunits from the mem-

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[1] Abbreviations: cGMP, guanosine 3′,5′-cyclic monophosphate; DMF, dimethylformamide; GDI, GDP dissociation inhibitor; GDP, guanosine diphosphate; GST, glutathione-*S*-transferase; GTP, guanosine triphosphate; G-protein, GTP binding protein; HPLC, high-performance liquid chromatography; IPTG, isopropyl *β*-D-thiogalactoside; LB, Luria broth; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PDE6, type 6 3′,5′-cyclic nucleotide phosphodiesterase; ROS, rod outer segment(s); SAM, *S*-adenosylmethionine; Tris, tris(hydroxymethyl)aminomethane.

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brane (*9*). After the catalytic subunits have been proteolyzed with trypsin so that they have lost their C-termini, the catalytic core can no longer interact with the delta subunit (*2*). The exact interaction site of the delta subunit with the catalytic subunits, which catalytic subunit might be involved, and whether posttranslational modifications are important for this interaction have not yet been shown. In light of this, in the experiments described in this paper we test the hypothesis that the delta subunit solubilizes the PDE by binding directly to the C-terminus of one or both catalytic subunits.

Following prenylation on the cysteine of the CaaX motif (a is usually but not necessarily an aliphatic residue, and X can be a variety of different amino acids), -aaX is removed by a prenyl-protein-specific endoprotease, and the α-COOH group of the prenylated cysteine is enzymatically methylated. Methylation is the only step in this modification pathway which is reversible under physiological conditions, and thus may be involved in the regulation of protein/protein interactions. Several studies have investigated the role of methylation in signal transduction and in regulating protein/protein interactions. It has been shown that methylation of ras-related proteins in neutrophils is required in order for these cells to respond to the chemoattractant *N*-formyl-methionyl-leucyl-phenylalanine (*10*). There is also evidence to suggest that methylation of small G-proteins is involved in the release of insulin from beta cells (*11, 12*). Thus far, the molecular mechanism(s) for the effects of methylation on these signal transduction pathways is (are) not known. In addition to effects seen at the level of cellular physiology, other studies show that methylation can affect protein/protein interactions. Methylation of transducin's gamma subunit alters its interaction with some of its effector molecules, most notably phosphoinositide 3-kinase and phosphatidylinositol-specific phospholipase C (*13*), and with its own α and β subunits (*14*). A study using peptides derived from the prenylated small G-protein Rho, which binds to RhoGDI, has shown that methylation greatly increases the affinity of these peptides for RhoGDI (*15*). Direct consequences of methylation-induced changes in protein/protein interactions have not yet been determined in vivo.

Both methyl transferase and methyl ester hydrolase activities have been identified in rod outer segment membranes (*16, 17*). At least a portion of the PDE catalytic subunits in rod outer segments is methylated (*8, 9, 18*), making methylation a feasible mechanism of regulation of the delta subunit/catalytic subunit interaction. Based on these observations, we tested whether methylation could change the affinity of the delta subunit/catalytic subunit interaction.

In this paper, we show that some, but not all, of the endogenous delta subunit in the rod outer segment is bound strongly to the soluble PDE catalytic subunits. We also show that peptides corresponding to the C-termini of the catalytic subunits can block the recombinant delta subunit/PDE interaction. Finally, we show data that support the idea that the interaction between the proteins may be regulated by C-terminal methylation of the PDE catalytic subunits.

EXPERIMENTAL PROCEDURES

Materials. Frozen bovine retinas were purchased from Lawson, Inc. (Lincoln, NE). [^{3}H]SAM and Amplify were purchased from Amersham. Ultrafree filters were purchased from Millipore. Protein A/G Sepharose was purchased from Santa Cruz Biotechnology. Pefabloc and Complete protease inhibitor cocktail tablets were purchased from Boehringer Mannheim. Super Signal West Pico chemiluminescent substrate was purchased from Pierce (Rockford, IL). All other chemicals were purchased from Sigma.

Delta Subunit Expression. Recombinant delta subunit was expressed as a fusion protein with glutathione-*S*-transferase (δ-GST). The coding sequence of the delta subunit was amplified from the cDNA clone 17K-11 (*2*) by PCR using the primers 17KGST1 (5′-GCGCGGATCCATGTCAGCCAAGGACGA-3′) and 17KGST2 (5′-GCGCGGATCCTCAAACGTAGAAAAGC-3′). The amplified DNA was digested and subcloned into the *Bam*HI site of the pGEX-2T vector (Amersham Pharmacia) using standard techniques. The resulting vector produces the delta subunit expressed with an NH_2-terminal GST tag and with a predicted molecular mass of 43 kDa. The sequence was verified by automated sequencing. A few experiments were performed using a construct with a single base pair substitution which caused a change in the amino acid sequence of the protein at amino acid 85 (Q to R). Results with this mutant were indistinguishable from experiments performed with the wild-type sequence. To express the protein, 50 mL overnight cultures of bacteria containing this plasmid were grown at 37 °C in LB + 50 μg/mL ampicillin, and then diluted into 500 mL of LB + 50 μg/mL ampicillin. This was grown at 30 °C for 1.5 h. The expression of the delta subunit was induced with the addition of 0.1 mM IPTG. After 5 more h of growth at 30 °C, the cells were pelleted by centrifugation at 1500*g* for 10 min and frozen (−20 °C). Both growth at 30 °C (as opposed to 37 °C) and freezing after pelleting increased the amount of soluble protein.

Delta Subunit Purification. To purify soluble delta subunit, bacterial pellets were resuspended in 5 mL of 50 mM Tris, pH 7.5, and 1 mg/mL lysozyme. The pellet was incubated on ice for 30 min, with occasional vortexing. Cells were sonicated for 45 1-s pulses on high, and particulate matter was removed by centrifugation at 14000*g* for 10 min. The supernatant was applied to 0.5−1 mL Sepharose beads conjugated to glutathione (Sigma). The column was washed with ∼5 volumes of 50 mM Tris, pH 7.5, and δ-GST eluted (via gravity) with reduced glutathione (10 mM) in 50 mM Tris, pH 7.5. Eluted protein was concentrated using Ultrafree Biomax filters with a molecular mass cutoff of 10 kDa. The flow-through from this concentration step was used as a buffer control when necessary. GST alone was also expressed and purified using the above method. Concentrations of all proteins were determined using the Bradford assay (*19*) with BSA as a standard. δ-GST prepared in this manner was able to solubilize PDE from ROS membranes with the same dose−response as delta subunit without the GST tag purified from Sf9 cells used in the past (data not shown).

Peptide Synthesis. All peptides used in this study were prepared using the previously published general method for peptides with or without prenyl groups and C-terminal methyl esters (*20*). All peptides were purified to apparent homogeneity by HPLC on a C18 reverse-phase column, and their structures were confirmed by electrospray ionization mass spectrometry (not shown). Sequences, modifications, and names of these peptides are shown in Table 1. Stock solutions of peptides (20 mM) were made in DMF and diluted in experiments to the stated concentrations.

Table 1: Structures of Peptides Used in These Studies (See Experimental Procedures for Details)

catalytic subunit	peptide length (amino acids)	amino acid sequence	prenyl group[a]	functional group of C-terminal cysteine[b]	name of peptide
α	13	GKQPGGGPASKSC	f	Me	α-13-f-Me
α	13	GKQPGGGPASKSC	f	OH	α-13-f-OH
α	6	PASKSC	f	Me	α-6-f-Me
α	6	PASKSC	np	Me	α-6-np-Me
β	6	PRSSTC	gg	Me	*β*-6-gg-Me
β	6	PRSSTC	np	Me	*β*-6-np-Me
none	1	C	gg	Me	cys-gg-Me

[a] f, farnesyl; gg, geranylgeranyl; np, not prenylated. [b] Me, methyl ester; OH, acid.

Preparation of ROS Membranes and Retinal Extracts from Frozen Retinas [Modification of Papermaster and Dreyer, 1974 (21)]. To purify ROS for solubilization experiments, all procedures were performed in complete darkness with the aid of an infrared viewer or under dim red light. Fifty frozen bovine retinas in a light-tight container were thawed overnight at 4 °C. These were added to 50 mL of ROS buffer (20 mM MOPS, 2 mM $MgCl_2$, 60 mM KCl, 30 mM NaCl, pH 7.2) containing 47.5% sucrose (w/v) and 1 mg/mL Pefabloc. This mixture was vigorously stirred on a magnetic stir plate for 10 min to shear the ROS from the rest of the retina. The retinal homogenate was spun at 1500*g* for 2 h at 4 °C, and then the supernatant was diluted with 1.5 volumes of ROS buffer and centrifuged at 4 °C for 1 h at 16000*g* to pellet the ROS. The supernate was removed and the pellet resuspended in 10 mL of 1.105 density ROS buffer + sucrose (274 g of sucrose/L). This was layered onto a noncontinuous sucrose gradient containing 1.135 density ROS buffer (354 g of sucrose/L) and 1.115 density ROS buffer (300 g of sucrose/L). Gradients were spun at 100000*g* in a swinging-bucket rotor at 4 °C for 30 min. The ROS migrate as a thick band at the 1.135/1.115 interface. This band was removed with a plastic pipet, diluted with 1 volume of ROS buffer, and respun at 16000*g* for 10 min to repellet, the supernatant was removed, and the ROS were stored at −70 °C. The concentration of protein was determined using the Bradford assay. To estimate the concentration of rhodopsin in these samples, we assumed that 80% of the total protein in the ROS was rhodopsin.

Retinal extracts for methylation and localization experiments were prepared in room light. Ten frozen retinas were thawed and placed into 10 mL of ROS buffer + 47.5% (w/v) sucrose containing one Complete protease inhibitor tablet. The mixture was shaken vigorously for 1 min to dissociate ROS. The retinal homogenate was spun at 4 °C at 1500*g* for 5 min to pellet non-ROS retinal tissue. The supernatant from this step contains ROS and soluble ROS proteins and is referred to as "retinal extract".

Purification of soluble PDE was performed using immunoaffinity chromatography as described (*1, 22*).

PDE Assay. Enzymatic activity of PDE was quantified as previously described (*22*−*24*). Briefly, samples containing PDE6 were incubated with trypsin (0.02 mg/mL final concentration) in 10× assay buffer (200 mM Tris, pH 7.5, 100 mM $MgCl_2$, 5 mg/mL BSA) for 4 min on ice to activate the PDE. Proteolysis was stopped by the addition of soybean trypsin inhibitor to a final concentration of 0.125 mg/mL. cGMP hydrolysis was measured by addition of cGMP to 1 mM and incubation of the reaction at 30 °C for 10 min. At 7 min, snake venom was added to hydrolyze the phosphate group of GMP. At 10 min, the reaction was stopped by addition of SDS to a final concentration of 2%. An equal volume of developing reagent [0.4 N H_2SO_4, 0.2% ammonium molybdate (w/v)] was added, and reactions were incubated at 30 °C for 30 min. Free phosphate was quantified by measuring the absorbance at 650 nM.

Immunoblots. Samples were prepared for immunoblot analysis by addition of 6× Laemmli sample buffer. Samples containing ROS were not boiled. After running these samples on 12% or 15% gels, the gels were blotted onto nitrocellulose. Nitrocellulose was blocked with 5% milk in TBST [20 mM Tris, pH 8, 140 mM NaCl, 0.05% (v/v) Tween 20]. Delta subunit immunoreactivity was measured using the 3223J antibody, a rabbit polyclonal antibody, at 1:10 000 dilution. PDE catalytic subunit immunoreactivity was measured using the PDE6 cat pAb (*2*), a rabbit polyclonal antibody, at 1:3000 dilution. Both antibodies were detected by incubation with a horseradish peroxidase linked goat anti-rabbit secondary antibody. The secondary antibody was visualized with Super Substrate West Pico chemiluminescent substrate. For quantitation, exposed film was scanned and analyzed using NIH Image 1.61.

Immunoprecipitation. Extracts from retinal homogenates were prepared as described in the figure legends. ROS-1 antibody, a mouse monoclonal antibody (*25*), was purified from mouse ascites fluid using Staph-A Sepharose. Purified antibody (1 mg/mL) was incubated with 25% protein A/G Sepharose in ROS buffer + 0.5 mg/mL BSA for at least an hour at 4 °C. The ROS-1-conjugated Sepharose was washed twice with ROS buffer containing 0.5 mg/mL BSA, and resuspended to its original volume. Resin (20 μL) was incubated with each sample to be immunoprecipitated for at least an hour.

Solubilization Assays. Purified recombinant δ-GST (2 μM) was incubated with the indicated amounts of peptides in ROS buffer for several hours or overnight at 4 °C. ROS membranes from frozen retinas were washed 3 times with ROS buffer, resuspended in ROS buffer + 1 mg/mL Pefabloc, added to the delta subunit/peptide mixture (final concentration of ROS = 2 mg of protein/mL in 35 μL), and incubated overnight at 4 °C. The next day, ROS membranes were pelleted by centrifugation at 4 °C for 10 min at 14000*g*, and the supernatant was separated from the pellet. Supernatants were respun for 5 min to pellet any membranes that may have remained in the supernatant. Pellets were resuspended in 35 μL of ROS buffer. The PDE activity in both the supernatant and the pellet was measured using the PDE activity assay described above, and results are presented as a percent of the total activity in each reaction that remains in the supernatant.

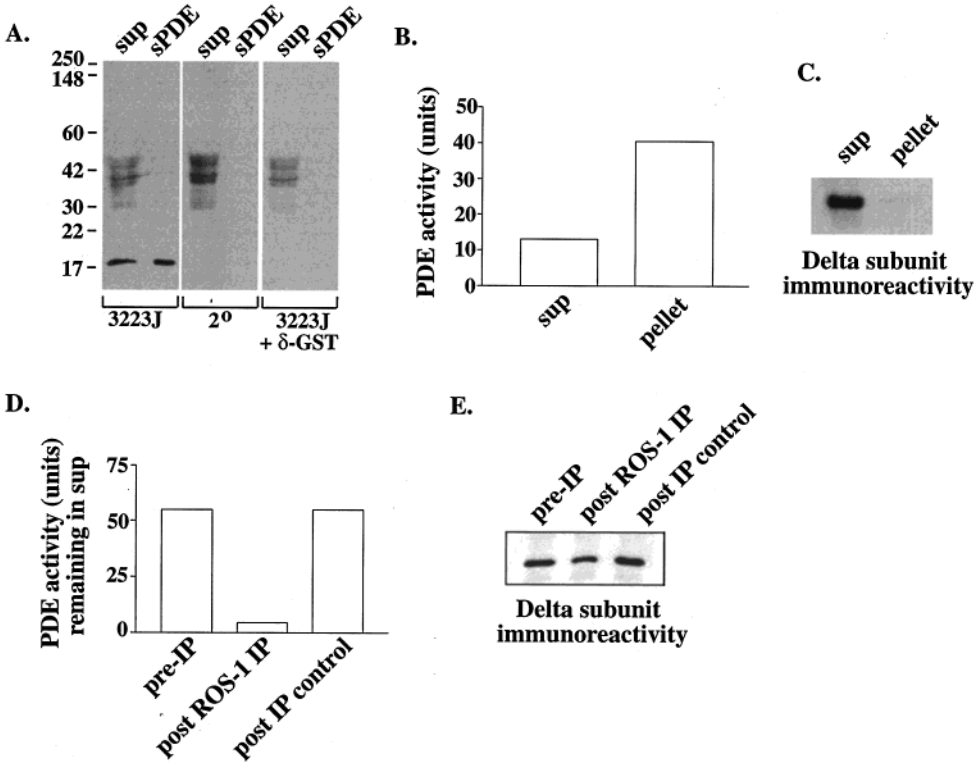

FIGURE 1: Distribution of PDE and delta subunit within the rod outer segment. An aliquot of the extract was diluted 1:1 with ROS buffer and spun in an airfuge at room temperature at 100000*g* for 10 min to separate the membranes from the soluble proteins. The supernatant was removed and the pellet resuspended in a volume equal to the supernatant. (A) Specificity of the anti-delta subunit antibody, 3223J. Immunoblots were performed on 20 μL of retinal extract supernatants (sup) and 150 ng of immunopurified soluble PDE (PDEs). Lanes 1 and 2 were immunoblotted with 3223J diluted 1:10 000. Lanes 3 and 4 were immunoblotted with secondary antibody only and show that the 30−50 kDa bands are nonspecific secondary antibody reactivity. Lanes 5 and 6 were blotted with 1:10 000 3223J preincubated with 15 μg/mL δ-GST. (B and C) Aliquots of the supernatant and resuspended pellet were assayed for PDE activity (B) or analyzed by immunoblot for delta subunit immunoreactivity (C). An aliquot of the supernatant was then immunodepleted of PDE activity using Protein A/G Sepharose beads bound to the ROS-1 antibody. PDE activity (D) and delta subunit immunoreactivity (E) in the supernatant were measured before and after this treatment. Post-IP control was performed using Protein A/G beads without ROS-1 antibody to control for the dilution of the supernatant by addition of the beads as well as for nonspecific binding to the beads. Figures are representative of experiments performed at least 3 times with different retinal preparations.

Methylation of ROS Proteins and Autoradiography. Aliquots (100−150 μL) of retinal extracts (20 mg of protein/mL) in ROS buffer were incubated with ^{3}H-SAM (0.03 mCi/mL, 450 nM) at 30 °C for 1−3 h. The extract was then fractionated as desired, and aliquots of the labeled samples were run on 12% SDS−PAGE gels. The gels were stained with Coomassie blue and soaked in Amplify for fluorography. Gels were dried and exposed to film at −80 °C for 3−7 days. Bands containing tritiated PDE were cut out, and the amount of tritium was quantified by counting in a Tri-Carb liquid scintillation counter (Packard).

RESULTS

The delta subunit of PDE6 has been shown to directly interact with the catalytic subunits of the PDE (*2*). We wanted to know whether the delta subunit in rod outer segments constitutively binds all available PDE catalytic subunits, or whether binding might be a regulated event. To determine whether there was excess PDE or delta subunit in rod outer segments, we separated retinal extract into supernatant and membrane-bound fractions. About 75% of the PDE activity was found in the membrane fraction, consistent with previously reported results (Figure 1, panel B). However, delta subunit immunoreactivity was found exclusively in the soluble fraction (Figure 1, panel C). Therefore, not all of the PDE catalytic subunits were bound to the delta subunit. To determine whether the delta subunit in the supernatant was all bound to PDE, we depleted the PDE catalytic subunits in the soluble fraction by immunoprecipitation (Figure 1, panels D and E). Although nearly all of the PDE activity was removed from the supernatant by this treatment (90.7% ± 2.4% in three experiments; Figure 1D shows representative data), much of the delta subunit immunoreactivity remained in the supernatant (31% ± 5% in three experiments; Figure 1E). Therefore, in the isolated rod outer segment, these proteins are not completely bound to one another.

Incomplete binding of the delta subunit to the PDE catalytic subunits can be explained by four possibilities. First, the delta subunit could be in a cellular compartment separate from the PDE so it cannot interact with the PDE. We think that this is unlikely as both proteins localize to the outer segment and both proteins seem to be freely accessible by buffers without any special homogenization of the ROS. Second, the delta subunit may be bound to some other protein that prevents it from interacting with the PDE catalytic subunits. Third, the interaction between the two proteins may be regulated by some dynamic process. Finally, the interaction between the two proteins may be relatively weak. This final possibility seemed unlikely since previous work sug-

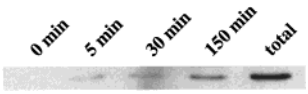

FIGURE 2: Delta subunit is tightly bound to the PDE catalytic subunits. 100 nM purified soluble PDE (with endogenous delta subunit bound) was incubated with 9 μM δ-GST (43 kDa). The PDE was then immunoprecipitated with Staph A/G-ROS-1 beads. Endogenous delta subunit moving off of the PDE was measured by immunoblot of endogenous 17 kDa delta subunit in aliquots of the supernatant at the indicated time points. This experiment gave us the same result whether the immunoprecipitation was performed before or after the incubation with δ-GST, indicating that the ROS-1 antibody does not interfere with the exchange of the delta subunit.

gested that the delta subunit has a high (nanomolar) affinity for the catalytic subunits. Nevertheless, to test this possibility, soluble PDE with endogenous delta subunit (17 kDa) bound was immunoprecipitated and incubated with excess recombinant δ-GST (43 kDa) (Figure 2). A small amount of exchange of the endogenous delta subunit into the soluble fraction occurred; however, even after 2.5 h, only 36% of the delta subunit had exchanged. Therefore, it appeared that the binding of the delta subunit to the PDE catalytic subunits was relatively long-lasting. Together with the observation that there is an excess of delta subunit over PDE in the soluble fraction but an excess of PDE catalytic subunits of delta subunit in the membrane fraction of ROS, this suggests that either the interaction between the proteins might be regulated, the proteins may be bound to other proteins, or both.

One mechanism by which regulation could occur is posttranslational modification of one or both proteins. To determine potential sites of regulation, we needed to define the delta subunit/PDE catalytic subunit interaction site. Proteolysis studies (*2*, *8*, *9*) suggested that the C-termini of the α and β catalytic subunits might be the site of interaction. To more directly test this hypothesis, peptides that correspond to the C-termini of the PDE's catalytic subunits were synthesized (Table 1). In vivo, the PDE catalytic subunits can be both prenylated and methylated on their C-terminal cysteine, so these modifications were included in some of the peptides. The solubilization assay described under Experimental Procedures was used to determine whether the peptides could interfere with the delta subunit's ability to solubilize the PDE. Twenty amino acid peptides showed that fully modified peptides could block the interaction (data not shown). To further narrow down which area of the catalytic subunit C-termini was interacting with the delta subunit, peptides of 13 and 6 amino acids were used. Data in Figures 3 and 4 show the percent of the total PDE activity that is found in the supernatant after these incubations.

Prenylated and methylated peptides corresponding to both the alpha and beta subunits blocked the ability of δ-GST to solubilize the PDE with EC_{50}s in the low micromolar range (Figure 3 and Table 2). The true affinity may be even higher as these experiments were done in the presence of 2 μM δ-GST, so the EC_{50}s may represent titration of the delta subunit rather than a true expression of binding affinity. Since both the alpha and the beta peptides could block the effect, the endogenous delta subunit may bind to either or both of the catalytic subunits of the PDE. Peptides of each length tested were able to block the solublization of the PDE by the delta subunit (Table 2).

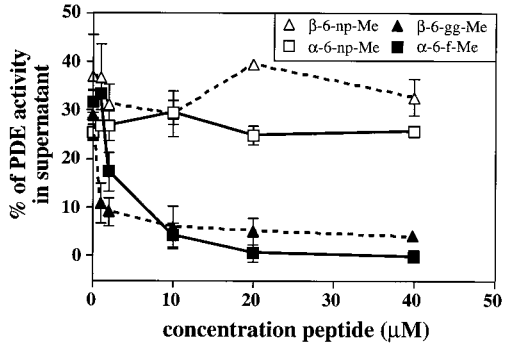

FIGURE 3: Prenylated peptides corresponding to the C-termini of the alpha and beta subunits of PDE6 can prevent the delta subunit from making the catalytic subunits soluble. Purified δ-GST (2 μM in final incubation) was incubated with peptides at the indicated concentrations, and solubilization assays were performed as described under Experimental Procedures. Peptides were incubated with δ-GST before the addition of membranes to decrease the possibility of nonspecific peptide binding to ROS membranes. The percent of the total PDE activity which remains in the supernatant after incubation at each point, minus the amount of activity in the supernatant without any added delta subunit, is shown. Solid triangles, β-6-gg-Me; solid squares, α-6-f-Me; open squares, α-6-np-Me; open triangles, β-6-np-Me. Data shown are representative of at least 3 experiments; EC_{50} values for all experiments are shown in Table 2. Total PDE activity in supernatant + pellet remained relatively constant throughout each assay, showing that the delta subunit is not inhibiting the membrane-bound PDE or activating the soluble PDE.

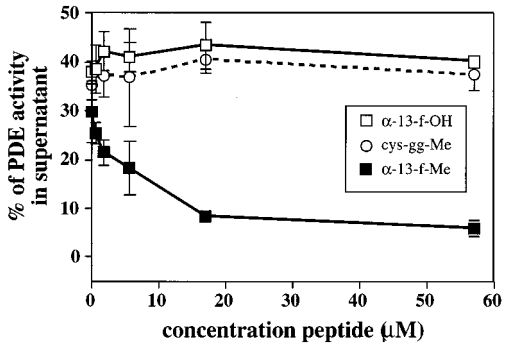

FIGURE 4: Peptides must be methylated to block the interaction between the delta subunit and the catalytic subunits. Experiments were performed as in Figure 3. Solid squares, α-13-f-Me; open squares, α-13-f-OH; open circles, cys-gg-Me.

Table 2: Ability of Peptides To Interfere with the Solubilization of PDE6 by the Delta Subunit

name of peptide	EC_{50} (μM)	no. of experiments
α-13-f-Me	5.6 ± 2.9	5
α-13-f-OH	nr[a]	3
α-6-f-Me	8.3 ± 5.9	4
α-6-np-Me	nr	3
β-6-gg-Me	1.4 ± 0.8	5
β-6-np-Me	nr	3
cys-gg-Me	nr	5

[a] nr: no reduction in delta subunit activity seen at highest concentration tested (57 μM).

To further investigate the structural requirements for the δ-GST/PDE peptide interaction, we tested whether prenylation and methylation of the peptides were necessary for this interaction to occur. Both farnesylated (alpha) and geranylgeranylated (beta) peptides blocked the interaction at similar concentrations (Figure 3 and Table 2); however,

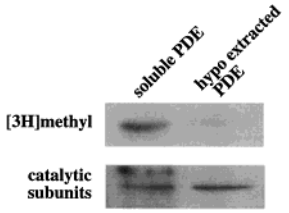

FIGURE 5: Soluble PDE incorporates more methyl group than membrane-bound PDE. Retinal extract (20 mg of protein/mL) was incubated at 30 °C with [^{3}H]SAM (0.03 mCi/mL; 450 nM) for 1 h. Membranes were separated from soluble proteins by centrifugation at 14000*g* for 10 min. The supernatant was removed. Membranes were washed once with ROS buffer, and then PDE was extracted with hypotonic buffer. Aliquots of soluble and hypotonically extracted proteins were immediately placed into SDS−PAGE sample buffer. Other aliquots were assayed for PDE activity (data not shown). Samples containing equal amounts of PDE activity were loaded onto 12% SDS−PAGE gels and subjected to electrophoresis. Immunoblot analysis (bottom) confirmed that approximately the same amount of catalytic subunit is present in each lane. Autoradiography showed that soluble PDE contained more methyl group (top).

nonprenylated methylated peptides were unable to interfere with the interaction at concentrations up to 57 μM. Interestingly, methylation, which is a potentially reversible modification, was also required, as peptides which were prenylated but not methylated did not interfere with the reaction at concentrations up to 57 μM (Figure 4, Table 2).

We considered the possibility that the delta subunit might be binding primarily to the prenylated cysteine methyl ester. In this case, it would be expected that the delta subunit might be able to interact with any protein with a prenylated and methylated cysteine. To test this, we tried to block the δ-GST/catalytic subunit interaction with geranylgeranylated cysteine methyl ester. This did not block the interaction (Figure 4). This result showed that more structure beyond the prenylated cysteine methyl ester termini of the peptides was required for δ-GST binding. Some amino acid sequence in addition to the modified cysteine was required, although it may be that any peptide backbone, and not a specific peptide sequence, is required. To test for sequence specificity, a prenylated, methylated peptide unrelated to the PDE sequences was used to try and block the interaction. This peptide was the 11 amino acid a-factor from yeast. When this peptide was used at 68 μM, it blocked the interaction between the delta subunit and the catalytic subunits (data not shown). However, the degree of block was variable—in three experiments, inhibition of δ-GST-dependent solublization of PDE catalytic subunits ranged from 27% to 60%. Therefore, a methylated, prenylated peptide unrelated to the PDE can block the δ-GST/PDE interaction, but with a higher apparent effective concentration than the peptides which are based on the PDE sequence.

The results from the peptide studies suggest that the interaction between the delta subunit and the PDE catalytic subunits might be regulated by methylation. In this case, soluble PDE should be more extensively methylated than membrane-bound PDE. This was tested by labeling proteins in ROS with [^{3}H]SAM, the methyl donor for prenyl protein-specific methyltransferase. When the same amounts of PDE activity from the soluble and hypotonically extracted fractions of ROS were loaded on a gel, the soluble form of the PDE had incorporated more tritium (4.5 $\pm$ 0.3-fold in 3 experiments; Figure 5 shows representative data). The possibility that soluble PDE underwent more rapid demethylation/methylation cycling than membrane-bound PDE seems unlikely since both the methyltransferase and methylesterase enzymes are found in the membrane fraction of the rod outer segments. It is also unlikely that the hypotonically extracted form of the enzyme underwent C-terminal proteolysis, as the samples were placed in denaturing sample buffer immediately after the PDE was extracted from the membrane, and the immunoblot showed no evidence of degradation of the hypotonically extracted PDE.

DISCUSSION

The delta subunit was originally identified as a protein that copurifies with the soluble form of PDE6 (*1*). Further work established that recombinant purified delta subunit could solubilize membrane-bound PDE6 (*2*). This biochemical effect of the delta subunit, as well as its amino acid structure, is well conserved among species as diverse as *C. elegans*, mouse, and human (*26*, *27*), implying that the functional consequences of the delta subunit's actions may be important.

Previous work showed that the C-termini of the alpha and beta subunits, which are posttranslationally modified with prenyl and methyl groups, are involved in the anchoring of the PDE to the membrane. We have shown that the interaction between the delta subunit and the catalytic subunits of the PDE can be interrupted by incubation of the delta subunit with prenylated, methylated peptides corresponding to the C-termini of the catalytic subunits. The requirement for the prenyl groups points out the importance of these groups not only for regulating the interaction of proteins with membranes, but also for acting as a molecular "handle" which other proteins can grasp to form protein/protein interactions.

Methylation, unlike prenylation, is a potentially reversible reaction (*28*), and both methyl transferase (*18*, *29*) and methyl ester hydrolase (*17*) activities have been identified in ROS membranes. Although mechanisms of regulation for these enzymes have not yet been identified, it is possible that methylation could regulate the interaction between the delta subunit and the PDE. We show here that the soluble form of the PDE, which is bound to the delta subunit, incorporates more labeled methyl group than the membrane-bound form. This suggests that methylation may contribute to the regulation of the delta subunit/PDE catalytic subunit interaction. Unfortunately, we were unable to perform experiments comparing the affinity of the delta subunit for methylated versus unmethylated PDE, as treatment of the PDE with methyl esterase led to loss of PDE activity. This loss of activity could have been due to proteolysis, as extensive proteolyis of PDE6 results in loss of catalytic activity. It may also have been due to an unknown inhibitory factor in the esterase preparation. It is unlikely that demethylation alone affects PDE catalytic activity, since trypsin-treated PDE, which has lost its methylated C-terminus, shows no reduction (actually an increase) in activity.

If methylation does regulate the fraction of PDE found in membranes, one might wonder why the catalytic subunits which remain on the membrane in the presence of endogenous delta subunit during the purification of ROS can then be solubilized by the addition of excess exogenous δ-GST.

Several potential explanations for this observation exist. The excess delta subunit in the supernatant may be bound to another protein or modified so that it is unavailable to bind to the PDE, thus leaving methylated PDE on the membrane. In fact, prolonged exposure of autoradiographs does show some methylated PDE remaining on the membrane. Another possibility is that while methylation of the holoenzyme may change the affinity for the delta subunit for the catalytic subunits, addition of excess δ-GST may lead to interaction of unmethylated PDE with δ-GST. Despite these caveats, our data do suggest that the methylation of the catalytic subunits is a requirement for the high-affinity protein/protein interaction, although it may not be sufficient, and other factors may also be necessary. Further work is needed to evaluate each of these possibilities.

Addition of the delta subunit to permeablized ROS results in a reduction of PDE activity in response to light (T.A.C., submitted for publication). It is therefore possible that methylation of the C-termini of the catalytic subunits, alone or in concert with other factors, could regulate PDE activity in the intact rod. Although further experiments are needed to rigorously test this hypothesis, it is an intriguing possibility.

Solubilization of PDE catalytic subunits by the delta subunit is similar to the effect of GDI (guanine nucleotide dissociation inhibitor) proteins acting on small G-proteins. The GDIs bind to various small G-proteins and make them soluble, thus allowing the G-proteins to move from one subcellular membrane to another. The interaction between the two proteins requires that the G-protein be prenylated (*30*) and is regulated by the GTP binding state of the G-protein. The crystal structure of RhoGDI in complex with Cdc42, a member of the Rho family, has been solved (*31*). The geranylgeranyl tail of Cdc42 is bound in a hydrophobic cleft between two beta sheets in the 16 kDa immunoglobin-like domain of the 45 kDa RhoGDI. The GDI is known to remove Cdc42 from the membrane with a two-step mechanism thought to correspond to binding of GDI to Cdc42 (fast step) and subsequent movement of the prenyl tail from the membrane to the hydrophobic binding pocket of GDI (slow step) (*32*).

Interestingly, the results of our peptide work are similar to recent results from Mondal et al. (*15*). This group found that methylation of prenylated Rho peptides can greatly increase the affinity of RhoGDI for the peptides. These peptide interactions also had affinities in the low micromolar range. It is also interesting to note that the prenyl binding domain of RhoGDI is similar in size to the delta subunit, and that there is 18% amino acid identity between the delta subunit and the prenyl binding domain of RhoGDI (data not shown). Given these similarities, it is possible that the delta subunit may have similar secondary structure to RhoGDI and therefore may also have a similar mechanism of action. These structural similarities extend the analogy between the delta subunit in the PDE system and GDIs in the small G-protein system.

The data presented provide evidence that the prenylated, methylated C-terminus of at least one catalytic subunit is required for the high-affinity delta subunit/catalytic subunit interaction. However, this does not rule out an interaction of the delta subunit with other parts of the PDE or with other proteins. Other surfaces of interaction could increase the affinity of the delta subunit for the catalytic subunits, or allow the delta subunit to affect other aspects of PDE6 function. It is interesting to note that the delta subunit can interact with the nonprenylated N-terminus of the retinal protein RPGR (*33*), which is homologous to a guanine nucleotide exchange factor for a small G-protein, and with the nonprenylated protein Arl1 in a GTP-dependent manner (*34*). This suggests possible additional roles for the delta subunit as a linker between prenylated and nonprenylated proteins, and as a modulator of the activity of small GTP binding proteins.

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