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Expression and Mutagenesis of Mouse Rod Photoreceptor cGMP Phosphodiesterase*

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Ning Qin‡ and Wolfgang Baehr‡§¶

From the Departments of ‡Biochemistry and §Ophthalmology, Baylor College of Medicine, Houston, Texas 77030

Using recombinant baculovirus vectors, the three subunits of mouse rod photoreceptor cGMP phosphodiesterase (PDE) ($\alpha\beta\gamma_2$) have been expressed in insect cells. The recombinant α,β subunits accumulate to 5 mg/liter culture, but most (98%) of the expressed polypeptides are insoluble. In the soluble fraction, individually expressed α and β subunits showed insignificant PDE activity, but coexpression (by coinfection) of $\alpha\beta$ subunits elevated PDE activity 7-fold and coexpression of $\alpha\beta\gamma$ up to 15-fold. The soluble expressed holoenzyme associated with ROS membranes under isotonic, but not hypotonic, conditions. The K_m of the soluble holoenzyme was 11–16 μM both for coexpressed $\alpha\beta$ subunits and for $\alpha\beta\gamma$ subunits, similar to the K_m (6–80 μM) of native PDE. Site-directed mutagenesis of cysteine to serine in the C-terminal *CAAX* box of both α and β subunits substantially decreased the protein expression level, abolished posttranslational isoprenylation, and prevented subunit binding to the rod outer segment (ROS) membranes. The mutant holoenzyme, however, showed a cGMP hydrolytic activity comparable with that of the normal recombinant enzyme. These results suggest that both α and β subunits are required for the formation of a functional enzyme and that isoprenylation of the subunits is essential for membrane association and stability of PDE.

Rod photoreceptor cGMP phosphodiesterase (PDE)[1] ($\alpha\beta\gamma_2$) is a key enzyme of the cGMP cascade in vertebrate rod phototransduction (1, 2). In dark adapted photoreceptors, PDE exhibits a low catalytic activity, kept in check by its inhibitory γ subunit (3), hydrolyzing about 50 moles of cGMP/s/mol PDE (4). This "basal" activity adjusts the dark concentration of cytoplasmic cGMP, which is continuously produced by a photoreceptor-specific guanylate cyclase (5), to levels that are sufficient to keep the cGMP-gated cation channels in the plasma membrane open. After light activation of PDE, mediated by the transducin α subunit charged with GTP, the rate of hydrolysis increases to several thousand moles of cGMP/s. This dramatic change causes rapid depletion of cytoplasmic cGMP, closure of plasma membrane channels, and hyperpolarization of the rod cell. In dark and light, most of the PDE is peripherally associated with ROS disk membranes under physiological conditions (4, 6). A lesser soluble form, $\alpha\beta\gamma_2\delta_x$, augmented by an additional small hydrophobic δ subunit of unknown function, has been reported (7).

The three subunits of membrane-associated PDE have been extensively characterized by biochemical analysis and molecular cloning (8–12). The small γ subunit (molecular weight, 10,000, based on cDNA cloning), present in two copies (13, 14) with presumably one each bound to the large subunits α and β, regulates the catalytic activity (3) but does not contribute to membrane association of PDE (15, 16). The two large subunits, α and β (calculated molecular weight, 100,000, based on the predicted sequences), are thought to contain the active site(s) of the enzyme. Molecular cloning showed that α and β have a 70–72% sequence similarity (12, 17) and a duplicated domain structure (18, 19), which includes noncatalytic cGMP binding sites (20, 21), distinct γ binding sites (22), and precursor cysteine/mostly aliphatic residue/aliphatic residue/any residue (*CAAX*) consensus sequences (12, 23) for isoprenylation. The greatest sequence similarity between the α and β subunits is contained in a large domain (~300 amino acid residues) near the C terminus, which is thought to represent the active site of the enzyme. The C-terminal *CAAX* sequence signals multiple posttranslational modifications consisting of isoprenylation of Cys via thioesterification, proteolytic trimming of *AAX*, and carboxymethylation (24). The *X* residues of *CAAX* in the PDE α and β subunits are distinct (Gln and Leu) and specify (25, 26) farnesylation (addition of a C_{15} moiety) in α and geranylgeranylation (C_{20}) in β (23, 27), establishing rod PDE as an enzyme with two distinct *CAAX* sequences and two distinct isoprenoid tails.

To study the functional domains of PDE, the requirements for assembly of subunits to an active enzyme, and the influence of posttranslational *CAAX* box-associated modifications on enzyme activity and stability, we initiated expression of a functional PDE in unicellular systems. As first steps, we previously reported the expression of (inactive) large subunits α and β (23) and the (active) inhibitory γ subunit (28) in bacterial systems. Using the baculovirus system, here we describe the individual expression and coexpression of the three subunits of the mouse rod PDE. We further describe properties of mutant PDE subunits in which the cysteine residue of the C-terminal *CAAX* box was mutated to serine thereby abolishing posttranslational isoprenylation.

MATERIALS AND METHODS

Construction of Transfer Vectors—The transfer vectors pVLA, pVLB, and pVLG (Fig. 1) were constructed by subcloning full-length cDNA fragments encoding mouse α, β, and γ subunits into the pVL1393 transfer vector (Invitrogen). Full-length clones encoding the α, β, and γ subunits were described earlier (23, 28). The pVLA transfer vector for integrating the mouse α subunit cDNA into baculovirus genomic DNA was obtained by ligating a blunt-ended 3.2-kilobase *Eco*RI fragment encoding the PDE α subunit into the *Sma*I site of pVL1393. Similarly, a 2.8-kilobase blunt-ended *Eco*RI DNA fragment encoding mouse β

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¶ The recipient of a Jules and Doris Stein Research to Prevent Blindness Professorship. To whom correspondence should be addressed: Dept. of Ophthalmology, Baylor College of Medicine, 6501 Fannin St., Houston, TX 77030. Tel.: 713-798-5965; Fax: 713-798-5706.

[1] The abbreviations used are: PDE, phosphodiesterase; *CAAX*, precursor cysteine/mostly aliphatic residue/aliphatic residue/any residue; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis.

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subunit from plasmid MPB81 was inserted into pVL1393 to yield the β subunit transfer vector pVLB. For construction of the γ subunit transfer vector, a 280-bp *Bam*HI fragment was inserted into *Bam*HI-digested and -dephosphorylated pVL1393. The correct orientations of the inserts in the transfer vectors pVLA, pVLB, and pVLG were determined by restriction mapping and DNA sequencing (data not shown).

Site-directed Mutagenesis—Site-directed mutagenesis was performed as described by Kunkel *et al.* (29) using a MutaGene kit (Bio-Rad). The primers were the following: Q15, 5'-CAGCTACTGGATGCAAGAGGACTTAGATGC (for mutation of the α subunit) and Q16, 5'-CGGCTTATAGGATACAGGAGGTCGGAGGAC (for mutation of the β subunit). The single mutations in each primer are underlined. The mutant recombinant fragments were released from M13 mp18 vector and subcloned to pVL1393 following the strategy described for construction of pVLA and pVLB. The resulting transfer vectors are named pVLAS and pVLBS (Fig. 1), respectively.

Generation and Purification of Recombinant Viruses—3 × 10^{6} Sf9 cells were cotransfected with 1 μg of wild-type virus and 2 μg of one of the transfer vectors (pVLA, pVLB, and pVLG) by the calcium phosphate precipitation method in a 25 cm² tissue culture flask (T25 flask). At 6 days post-infection, the cotransfection supernatants were diluted 10^1–10^6-fold and amplified twice in 96-well microtiter plates. The supernatant that was the most diluted and contained the highest level of recombinant viruses, as detected by dot blot analysis probed with corresponding cDNA inserts, was used for further purification. The recombinant viruses were purified by three rounds of plaque assay and dot blotting.

Expression of PDE in Sf9 Cells—Sf9 cells grown in monolayer were infected by recombinant viruses with multiplicity of infection of about 10. At 72 h post-infection, the cells were harvested and suspended in lysis buffer (20 mM Tris, pH 8.0, 1 mM dithiothreitol, 2 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of leupeptin, anti-pain, aprotinin, and pepstatin A) to a density of about 1.5 × 10^7 cells/ml. Ten μl of infected cells were directly subjected to SDS-PAGE to analyze protein expression. For coexpression of α and β subunits, a mixture of recombinant pVLA and pVLB at a ratio of 1:1 was used to infect Sf9 cells. Because the expression level of the γ subunit in Sf9 cells was very low, the ratio of recombinant viruses in the mixture for coexpression of α, β, and γ subunits was adjusted to 1:1:4 after titering of the individual viruses.

Isoprenylation—To isoprenylate expressed proteins, 1 × 10^6 Sf9 cells were infected in 6-well plates with different recombinant viruses (pVLA, pVLB, pVLAS, and pVLBS). At 24 h of post-infection, the cells were treated with 10 μM mevinolin for 6 h at room temperature. The media were subsequently replaced by fresh media containing 5 μM mevinolin and 25 μCi/ml of [³H]mevalonolactone (30 Ci/mmol, DuPont NEN). After incubation for another 24 h, the cells were harvested, suspended in 50 μl of NET buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 0.1% Nonidet P-40, 0.25% gelatin, and 0.02% sodium azide). Ten μl were used directly for SDS-PAGE. The rest of the cell suspension was sonicated and centrifuged (15,000 × *g* for 10 min), and the supernatant was used for immunoprecipitation (23) analysis.

Metabolic Radiolabeling of Expressed Proteins—To metabolically label the expressed proteins with [³⁵S]methionine, 6 × 10^5 cells were infected in a 24-well plate by recombinant viruses. At 48 h post-infection, the medium in each well was removed and replaced with 500 μl of methionine-free insect medium (Life Technologies, Inc.) containing 0.5% fetal bovine serum. One h later, the medium was changed again with 200 μl of the same medium containing 10 μCi of [³⁵S]methionine (15 Ci/mmol, Amersham Corp.). After continued incubation for 4 h, the cells were harvested, suspended in 50 μl of lysis buffer, and either subjected to SDS-PAGE or sonicated and used for ROS membrane association.

ROS Membrane Association—The assay for membrane association was performed as described previously (23). Briefly, 20 μl (approximately 20,000 cpm/μl) of [³⁵S]methionine-labeled lysate was incubated under isotonic conditions with 5 μl (2 mg/ml rhodopsin) of depleted ROS membrane on ice for 30 min (total reaction volume was 100 μl). The membrane suspension was pelleted and washed with 1 ml of isotonic buffer three times. Finally, the membrane pellet was subjected to SDS-PAGE and autoradiography.

PDE Assay—The PDE assay was performed as described previously by counting GMP_{formed} (4) with minor modifications. Briefly, crude extracts from infected Sf9 cells or partially purified expressed PDE was incubated with 25 μM [8-³H]cGMP (14.9 Ci/mmol, Amersham Corp.), 50 mM Tris, pH 8.0, 5 mM $MgCl_2$, and 1 mM dithiothreitol in a total volume of 100 μl at 37 °C. For trypsin activation of PDE, 1 μl of trypsin (5 mg/ml) was added before incubation and quenched after 30 s at room temperature by addition of 10-fold excess of soybean trypsin inhibitor (Sigma). cGMP hydrolysis was stopped by incubation of the assay mixture at 95 °C for 1 min. The mixture was centrifuged (15,000 × *g*, 5 min), an aliquot of the supernatant was spotted on a 4 × 4-cm polyethyleneimine cellulose (Brinkman) TLC sheet, and the sheet was washed with 0.012 M LiCl (30). The [8-³H]GMP bound to the polyethyleneimine cellulose was eluted with 2 ml of elution buffer (20 mM Tris, pH 7.5, 700 mM $MgCl_2$) for 2–4 h. Finally, 5 ml of scintillation mixture were added, and the mixture was counted. To ensure the absence of 5'-nucleotidase activity in Sf9 cell lysates, assays were performed under identical conditions in which [8-³H]cGMP was replaced by [8-³H]GMP (Moravek Biochemicals, Brea, CA). The 5'-nucleotidase activity was found to be insignificant. For determination of K_m, the substrate cGMP concentration ranged from 0.005 to 1 mM cGMP.

SDS-Gel Electrophoresis and Western Blots—SDS-PAGE was performed in 15% acrylamide/0.08% bisacrylamide gels as described previously (4). After electrophoresis, the proteins were transferred to nitrocellulose at 300 mA for 2 h. The blots were processed as described previously (23). The Western blot (Fig. 2*B*) was performed with the same samples applied to Fig. 2*A* by using a mixture of bovine PDE-specific polyclonal antibody MOE (Dr. B. K-K. Fung, UCLA) and γ subunit-specific polyclonal antibody (Dr. T. G. Wensel, Baylor College of Medicine) at a ratio of about 1:100 to enhance the γ subunit signal. Neither antibodies cross-react with any of the Sf9 cell proteins (Fig. 2*B*, *lanes 1* and *2*).

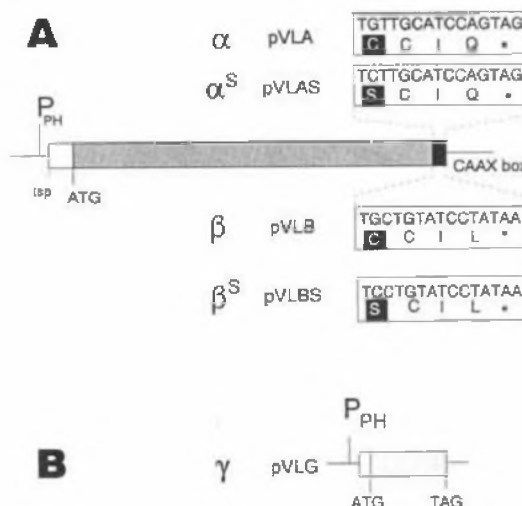

FIG. 1. **Baculovirus transfer constructs of normal and mutant PDE subunits.** *A*, schematic representation of the chimeric cDNA constructs for expression of PDE α and β subunits. The polyhedrin promoter is depicted as P_{PH}, the translated region as a *shaded box*, and the C-terminal *CAAX* motif as a *black box*. pVLA and pVLB are constructs of the normal PDE α and β subunits, respectively. pVLAS and pVLBS are the mutant constructs. The respective *CAAX* box sequences are enlarged, the C of *CAAX* and C to S mutation are *boxed*. The constructs are not drawn to scale. *B*, schematic representation of the γ subunit construct pVLG.

RESULTS

Recombinant Virus Constructs—pVLA and pVLB (Fig. 1*A*) contain the complete coding sequences of the mouse PDE α (2577 bp) and β (2568 bp) subunits, respectively, and pVLG (Fig. 1*B*) contains that of mouse γ (261 bp). According to cDNA sequences, the unprocessed α and β polypeptides are 859 and 856 amino acid residues in length, and each contains a C-terminal *CAAX* box signaling posttranslational isoprenylation (31). We previously used the coding sequences for expression of unprocessed α and β fusion polypeptides (23) and a biologically active mouse γ subunit (28) in bacteria. In addition to the coding sequences, pVLA and pVLB contain 45 or 37 bp 5'-untranslated sequences deriving from the respective mouse PDE α and β subunit genes. pVLG (Fig. 1*B*) contains no endogenous 5'-untranslated region. The transcription start points are those of the invertebrate polyhedrin gene (32). The endogenous ATG of the polyhedrin gene was mutated so that nonfu-

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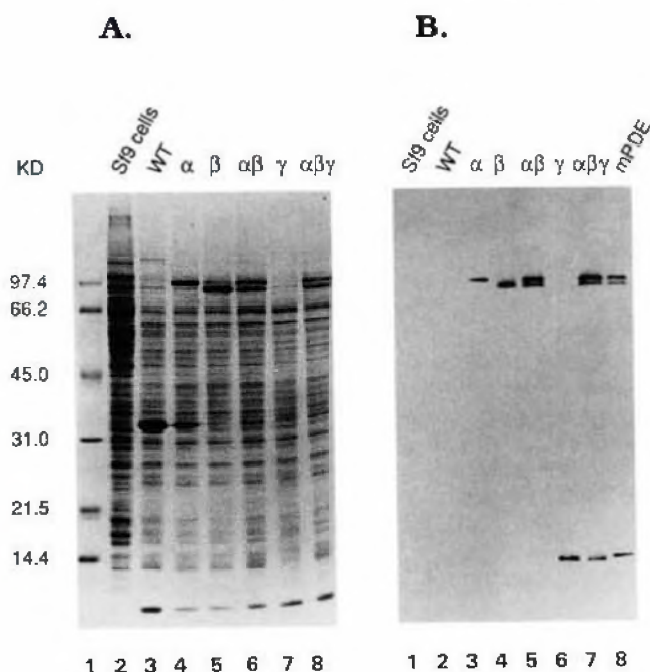

FIG. 2. **SDS-PAGE and Western blot analysis of proteins expressed in Sf9 cells.** *A*, SDS-PAGE. *Lane 1*, protein standards (Bio-Rad). The relative mobilities are shown on the *left*. *Lane 2*, mock-infected Sf9 cells. *Lane 3*, Sf9 cells infected with wild-type (*WT*) virus (AcNPV). *Lanes 4*, *5*, and *7*, Sf9 cells infected with pVLA, pVLB, and pVLG recombinant viruses, respectively. *Lanes 6* and *8*, Sf9 cells coinfected with pVLA/pVLB and pVLA/pVLB/pVLG recombinant viruses, respectively. *B*, Western blot of *panel A*, except that *lane 1* has been omitted. *Lane 8*, native mouse rod PDE holoenzyme (*mPDE*) as a comparison.

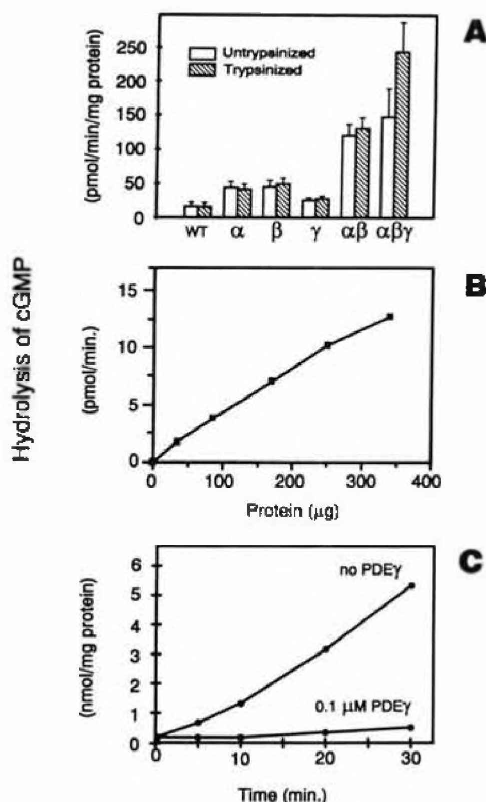

FIG. 3. **Characterization of cGMP hydrolytic activity of individually expressed and coexpressed PDE subunits in Sf9 cells.** *A*, PDE activity in the 30,000 × *g* supernatant of Sf9 cells infected by various recombinant viruses. α, β, γ represent activity in singly infected cell lysates, αβ and αβγ represent activity in co-infected lysates. The *white boxes* symbolize untrypsinized samples and the *cross-hatched boxes*, trypsinized samples. The values are the mean of three independent assays. *B*, PDE activity of coexpressed αβγ subunits *versus* protein concentration. *C*, PDE activity of coexpressed αβ subunits in the presence and absence of PDEγ subunit (0.1 μM) expressed in bacteria (28). *WT*, wild type.

sion proteins are generated. The mutant vectors pVLAS and pVLBS are identical to their corresponding parent vectors except that the triplets TGT (pVLA) and TGC (pVLB) were altered to TCT and TCC (Fig. 1*A*). The mutations change the codons for cysteine to serine in the C-terminal *CAAX* box, effectively preventing posttranslational isoprenylation.

Expression of Mouse Rod PDE Subunits—To demonstrate expression of the three subunits of PDE, Sf9 cells were infected with recombinant pVLA, pVLB, and pVLG viruses. To examine expression levels and to optimize expression conditions for each subunit, we performed a post-infection time course study by SDS-PAGE/Western blotting (not shown). All subunits started to express at about 24 h post-infection and reached maximal expression levels at about 72 h. SDS-PAGE analysis (Fig. 2) of Sf9 lysates showed that cells infected by pVLA or pVLB recombinant viruses produced large amounts of 88- and 84-kDa polypeptides (*lanes 4* and *5*), which were absent in mock-infected cells or cells infected by wild-type virus (*lanes 2* and *3*). As expected, the mobilities of the expressed subunits were identical to those of the native subunits, and the polypeptides were antigenic to PDE-specific antibodies (Fig. 2*B*, *lanes 4* and *5*). The expressed α and β subunits accumulated to about 5 mg/liter cell culture. Most of the expressed subunits, however, were insoluble in the aqueous buffers of moderate ionic strength. Only about 1–2% of the total expressed subunits were soluble in hypotonic (low salt) solution, a condition that disrupts membrane association of native PDE (4). A 10-fold reduction of the amount of recombinant viruses pVLA and pVLB lowered the yield of expressed subunits but did not influence the amount of soluble material or cGMP hydrolytic activity. The expression level of the γ subunit was very low compared with α and β subunits, possibly due to the lack of a 5′-untranslated region or to protease sensitivity of the γ polypeptide. The PDEγ polypeptide was not visible on stained SDS gels (Fig. 2*A*, *lanes 7* and *8*) but could be detected with a PDEγ-specific polyclonal antibody (Fig. 2*B*, *lanes 6–8*) after Western blotting. Coexpression of αβ and αβγ subunits could be achieved by coinfection of insect cells with pVLA, pVLB, and pVLG recombinant viruses. Coexpression apparently does not interfere with expression of the individual subunits (Fig. 2*A*, *lanes 6* and *8*, and Fig. 2*B*, *lanes 5* and *7*). The expressed polypeptides were stable; only minor degradation products could be detected after prolonged exposure of the Western blots.

cGMP Hydrolytic Activity of Expressed PDE Subunits—PDE activity of expressed subunits was assayed by using hypotonic lysates of infected cells (Fig. 3*A*). The hydrolysis of cGMP in Sf9 cells infected by pVLA or pVLB or pVLG recombinant viruses individually was very low (25–50 pmol/min/mg protein), only about 2-fold higher than the background activity of the cells infected by wild-type virus (17 pmol/min/mg protein). In contrast, when Sf9 cells were coinfected with either pVLA/pVLB or pVLA/pVLB/pVLG recombinant viruses, hydrolysis of cGMP activities increased about 7–9-fold (120–150 pmol/min/mg protein). Limited trypsin treatment of lysates coexpressing α, β, and γ subunits further increased PDE activity to about 15-fold (250 pmol/min/mg protein) above background but did not influence the activity of the coexpressed α and β subunits or individually expressed α or β subunits. The hydrolysis of cGMP of coexpressed PDE subunits was dependent on the protein concentration (Fig. 3*B*). Kinetic analysis showed that the K_m of coexpressed mouse PDEαβ and trypsin-treated PDEαβγ sub-

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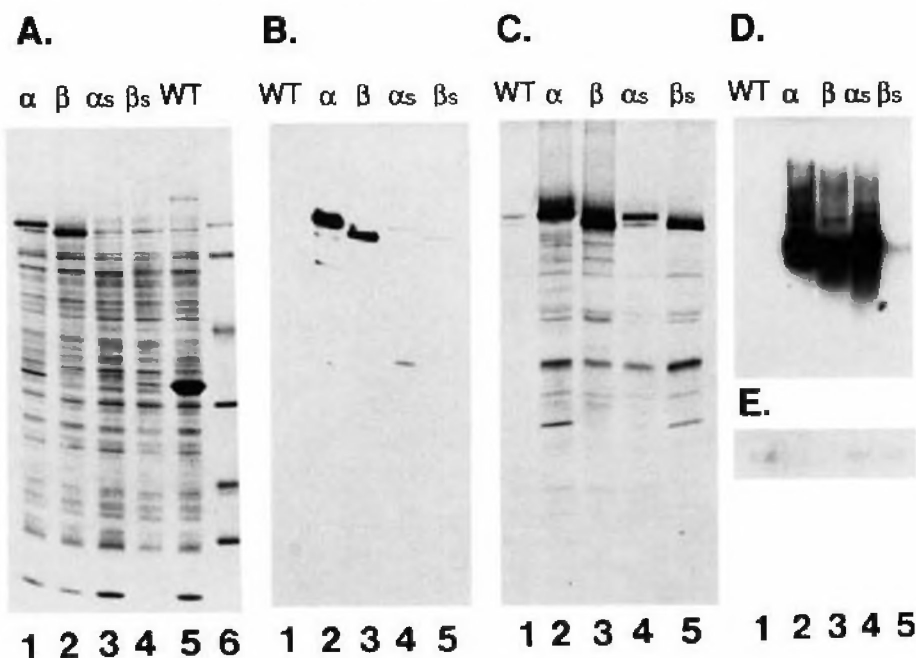

FIG. 4. **Analysis of normal and mutant α and β subunits expressed in Sf9 cells.** *A*, SDS-PAGE of normal and mutant subunits, 72 h post-infection. An aliquot of lysates infected by pVLA virus (*lane 1*), by pVLB virus (*lane 2*), by mutant pVLAS virus (*lane 3*), by pVLBS virus (*lane 4*), and by wild-type (*WT*) baculovirus AcPNV (*lane 5*) are shown. *Lane 6*, protein molecular weight standards are as in Fig. 1. *B*, Western blot of lysates prepared as in *panel A*, probed with polyclonal antibody MOE. Sf9 cells were infected by wild-type virus AcPNV (*lane 1*), by pVLA virus (*lane 2*), by pVLB virus (*lane 3*), by pVLAS virus (*lane 4*), and by pVLBS virus (*lane 5*). *C*, autoradiography of proteins contained in Sf9 lysates after metabolic labeling with [^{35}S]methionine. *D*, Northern blot of RNA isolated from Sf9 cultures infected by normal and mutant viruses. The blot was probed with nick-translated cDNA encoding α and β subunits. The *lane* order in *C* and *D* is as in *B*. *E*, the same blot stripped and reprobed with β-actin cDNA as internal control.

units was about 11–14 μM, very similar to the K_m of native rod PDE (6–80 μM). Under identical conditions, a K_m of an endogenous PDE could not be determined in uninfected Sf9 cells or Sf9 cells infected by wild-type virus. Addition of excess of PDEγ subunit expressed in bacteria (GK-PDEγ) suppressed the activity (Fig. 3*C*). This inhibition, thought to be very specific for rod PDE, completely mimics inhibition of trypsin-activated native PDE by γ.

Mutagenesis of the C-terminal CAAX Box of PDEα and PDEβ—To further study the biological significance of the *CAAX* box-associated isoprenylation, we mutated the Cys residue of the *CAAX* box to Ser in both α and β subunits. The transfer vectors used for expression of mutant α and β subunits (α^S and β^S) were purified as described for pVLA and pVLB. The point mutations caused a dramatic decrease in the steady-state expression levels of α^S and β^S subunits. The subunits were not visible on stained SDS-PAGE (Fig. 4*A*, *lanes 3* and *4*) but could be detected after Western blotting (Fig. 4*B*, *lanes 4* and *5*). To gain insight into the mechanisms that lower the expression levels of α^S and β^S subunits, we analyzed the stability of the mutant polypeptides after metabolic labeling with [^{35}S]methionine (Fig. 4*C*) and the levels of RNA transcripts of the mutant genes by Northern blotting (Fig. 4*D*). When Sf9 cells were labeled for 3 h, the ratios of α^S/α and β^S/β were higher than that at steady state (72 h), as detected by Western blotting (Fig. 4*B*). Shorter time of metabolic labeling could further increase the ratio but never reached the expression levels of α and β (data not shown). Polypeptides of lower molecular weight in *lanes 4* and *5* (Fig. 4*B*) indicate that the expressed mutant proteins may be less stable than the normal proteins. A Northern blot (Fig. 4*D*) showed that the mRNA levels of α, β, and α^S were very similar, but those of β^S (*lane 5*) were drastically lower. The control β-actin message level of the β^S lysate (Fig. 4*E*, *lane 5*) was almost the same as that of the wild type and α^S lysates (Fig. 4*E*, *lanes 1* and *4*) and higher than those of α and β samples (Fig. 4*E*, *lanes 2* and *3*). These results indicate that there are at least two different mechanisms to decrease α^S and β^S expression levels. The first mechanism reduces the mRNA levels (β^S), and the second may decrease the stability of the mutant gene products.

To compare the cGMP hydrolytic activity of coexpressed normal and mutant α and β subunits in all possible combinations, we adjusted the amount of recombinant viruses used for infection of Sf9 cells so that the corresponding expression levels are almost identical in the soluble fraction. After normalization of the expression levels by Western blotting and densitometer scanning, the cGMP hydrolytic activity in lysates was found to be nearly identical (Fig. 5). The kinetic analysis showed that the K_m of the different combinations of coexpressed subunits was about 11–16 μM. These results indicate that posttranslational isoprenylation is not essential for cGMP hydrolytic activity.

Isoprenylation and ROS Membrane Association—Rod PDE α and β subunits are isoprenylated by protein prenyl transferases, which specifically transfer farnesyl and geranylgeranyl moieties, respectively. To confirm that the expressed α and β subunits also undergo this kind of posttranslational modification, we incubated the Sf9 cells infected with pVLA/pVLB with the isoprenoid precursor [^{3}H]mevalonolactone in the presence of mevinolin (lovastatin), which inhibits 3β-hydroxy-3-methylglutaryl-coenzyme A reductase (33). In the presence of the inhibitor, synthesis of endogenous mevalonic acid is greatly reduced. Addition of exogenous [^{3}H]mevalonic acid leads to formation of radioactively labeled isoprenylpyrophosphates and isoprenylation of expressed α and β subunits. As shown in Fig. 6, the normal α and β subunits could be labeled (*lanes 4* and *5*), but mutant α^S and β^S subunits could not be labeled (*lanes 2* and *3*). Immunoprecipitation by a rod PDE-specific antibody (MOE) of labeled Sf9 lysates unambiguously identified the labeled ~90-kDa polypeptides as PDE α and β subunits (Fig. 6, *lanes 6* and *7*).

To examine the importance of posttranslational isoprenylation of α and β subunits for ROS membrane association, we metabolically labeled infected Sf9 cells with [^{35}S]methionine, then incubated the soluble fraction of lysates containing almost equal amounts of labeled normal and mutant α and β subunits with depleted ROS membranes under conditions that promote membrane association (Fig. 7). As shown in *lanes 2* and *3*, the normal α and β subunits could tightly associate with depleted ROS membranes. In contrast, the mutant α^S and β^S subunits could not, or only very weakly, associate with depleted ROS membranes under the same conditions (*lanes 4* and *5*). Because isoprenylation precedes proteolytic trimming of *AAX* and carboxymethylation, we assume that α^S and β^S are unmodified at

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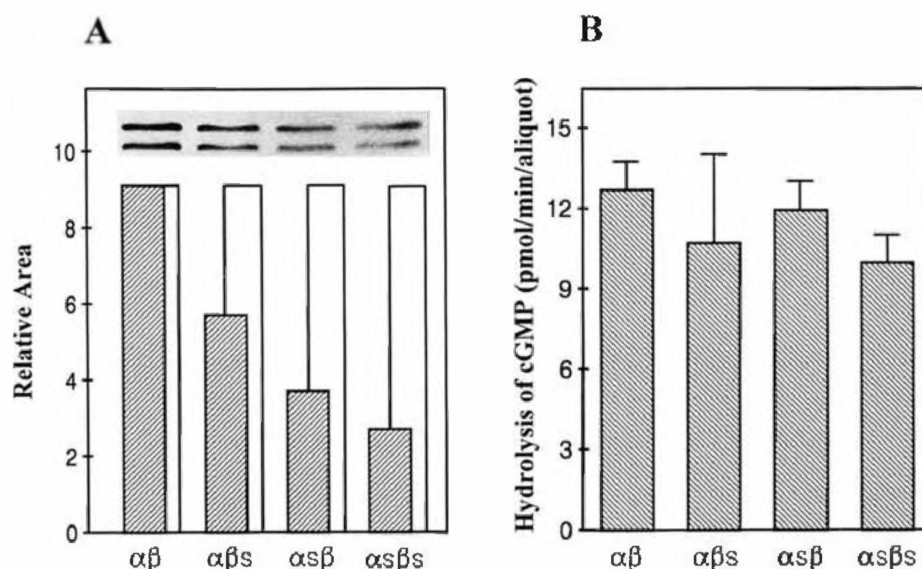

FIG. 5. **Comparison of cGMP activity in Sf9 cells co-infected with normal, mutant, and a combination of normal and mutant recombinant viruses.** *A*, normalization of expressed normal and mutant PDE subunits. *Cross-hatched bars*, peak areas of the PDE subunits indicated at the *bottom*, as determined by semi-quantitative densitometry. *White bars*, normalized peak areas. The *inset* shows the original Western blot and PDE subunit expression levels used for scanning. *B*, the bars depict cGMP hydrolytic activity of the various combinations of expression of PDE subunits after normalization. The *error bars* are the mean of three independent measurements.

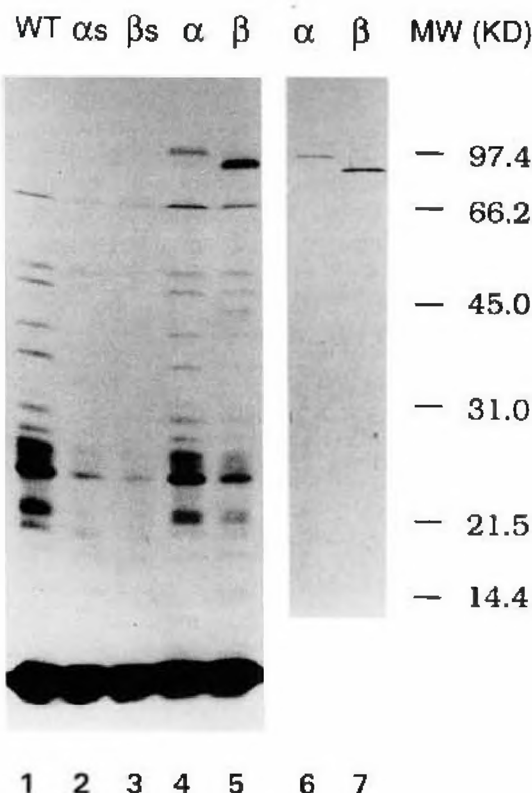

FIG. 6. **Isoprenylation of normal and mutant α and β subunits.** Autoradiography of proteins expressed in Sf9 cells after infection by different recombinant baculoviruses and labeled metabolically in the presence of [^{3}H]mevalonic acid. *Lane 1*, Sf9 cells infected by wild-type (*WT*) virus AcPNV, *lane 2*, by pVLAS virus, *lane 3*, by pVLBS virus, *lane 4*, by pVLA virus, *lane 5*, by pVLB virus. *Lanes 6* and *7* show polypeptides, which were immunoprecipitated from labeled lysates shown in *lanes 4* and *5* with PDE-specific polyclonal antibody MOE.

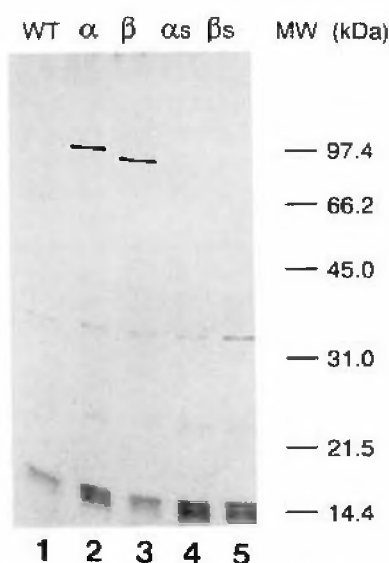

FIG. 7. **Membrane association of normal and mutant α and β subunits with depleted ROS membranes.** Autoradiography of metabolically labeled proteins expressed in infected Sf9 cells. Before loading, the lysates were exposed to depleted ROS membranes under isotonic conditions which promote binding and then were extensively washed (see "Materials and Methods"). *Lane 1*, Sf9 cells infected with wild-type (*WT*) virus, *lane 2*, by pVLA virus, *lane 3*, by pVLB virus, *lane 4*, by pVLAS virus, and *lane 5*, by pVLBS virus. The normal and mutant recombinant PDE concentrations were adjusted before exposure to membranes as similarly described in Fig. 5.

their C termini. The result confirms that the farnesyl and geranylgeranyl moieties, which are covalently attached to the C-terminal Cys of α and β subunits, serve as anchors for ROS membrane association.

DISCUSSION

At the onset of this investigation, the baculovirus/insect cell system appeared to be most suitable for expression of large amounts of PDE subunits, because it overexpresses eucaryotic genes and carries out posttranslational modifications such as *CAAX* box-associated isoprenylation (34), the major known modifications of the PDE α and β subunits (27). Moreover, the baculovirus system has been successfully used for coexpression of a variety of enzymatically active enzymes, among them the single subunit bovine opsin (35), bovine brain adenylate cyclase (36), bovine phosphatidylinositol 3-kinase (37), the multisubunit ($\alpha_2\beta_2$) human prolyl 4-hydroxylase (38), and the invertebrate casein kinase (39). We found that rod PDE subunits were overproduced in Sf9 cells, but most of the expressed material was insoluble in aqueous buffers and catalytically inactive. The insoluble inclusion-like material could not be renatured after treatment with detergents or urea, reminiscent of properties of bacterially expressed PDE subunits (23). 1–2% of the total expressed PDE, however, was soluble in a hypotonic buffer system, which is known to solubilize native PDE by disrupting membrane association, was biologically active, and with re-

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spect to its biochemical properties, was indistinguishable from native PDE.

Singly expressed soluble α and β subunits appear to be stable but show little, if any, cGMP hydrolytic activity. Coexpressed $\alpha\beta$ or $\alpha\beta\gamma$ subunits, in contrast, exhibit a significant increase in PDE activity, which rises up to 15-fold above background of insect cell lysates. Moreover, this activity can be inhibited by PDEγ, a rod PDE-specific inhibitor (3), indicating that the expressed subunits form a functional $\alpha\beta$ core enzyme. Coexpressed mutant PDE subunits ($\alpha^S\beta^S$), in which the two cysteines of the *CAAX* boxes were altered to serine preventing C-terminal processing, or coexpressed combinations of normal and mutant subunits ($\alpha^S\beta$, $\alpha\beta^S$) display a PDE activity that is comparable with the activity of the coexpressed normal enzyme. These results suggest that in the case of PDE, isoprenoid tails most likely do not mediate α-β subunit interactions, as has recently been proposed for interaction of farnesylated transducin $\beta\gamma$-subunits with transducin-α (40) or with rhodopsin (41). The finding that C-terminal mutations do not influence PDE activity is consistent with the location of the putative active site further upstream and the presence of catalytic activity in native PDE in which short C-terminal fragments were removed in α and β by limited trypsinolysis (16, 42).

Another consequence of the *CAAX* mutations in recombinant PDE is the inability of the mutant subunits to bind to ROS disk membranes, consistent with the abolishment of membrane binding of trypsinized PDE (15, 16, 43) and in agreement with loss of membrane interaction (and loss of biological activity) in *CAAX* mutant p21 proteins of the *ras* family (44–46). The inability of mutant PDE to associate with membranes supports the notion that the hydrophobic tail provided by the C-terminal posttranslational modification serves as an anchor that may be immersed into the disk membrane of photoreceptor outer segments, thus mediating peripheral membrane association, one of the hallmarks of native PDE. Hydrophobic receptors, which would interact with the isoprenoid tails, are currently under investigation in several laboratories (47). The hydrophobic δ subunit of soluble rod PDE (7) exhibiting a mobility of 15 kDa may in fact represent such a receptor.

The *CAAX* mutations further caused a significant decrease in RNA transcripts produced from the transfer vector pVLB but not of pVLA. While nonsense mutations (translation termination mutants) affect frequently by an unknown feed-back mechanism the stability of their mRNAs (48–50), missense mutations in general do not (50, 51). In this context, reduction of β^S RNA levels in Sf9 cells infected by pVLB is rather surprising. On the translational level, we observed a strong decrease of expression of both mutant subunits, most likely due to instability of the modified polypeptides. Both α^S and β^S, however, accumulate to a detectable level after Western blotting. The instability of α^S and β^S is in contrast to corresponding *CAAX* mutants of the *ras* family in which the expression levels of mutant and wild-type are indistinguishable (44–46).

Almost complete lack of activity of individually expressed α and β subunits is somewhat surprising, because both subunits contain an approximately 300 residue domain, which is supposed to contain the active site. This domain is highly conserved in related rod and cone cGMP PDEs of various vertebrate species (52) and mammalian cAMP PDEs (53) and present in cyclic nucleotide phosphodiesterases like *dunce* cAMP PDE in *Drosophila melanogaster* (54) or in PDE2 from *Saccharomyces cerevisiae* (55). Several single subunit calmodulin-stimulated, rolipram-sensitive, cGMP-inhibited, or dunce-like high affinity cAMP phosphodiesterases (56–61) have been expressed in various unicellular systems including insect cells and shown to be biologically active. Recent experiments in which N- and C-terminal portions of a cAMP PDE were deleted showed that indeed the active site resides in the 300 residue conserved domain and that two residues (Thr and His) are essential for cGMP hydrolytic activity (58).

Absence of activity, however, of the expressed rod PDEα subunit is consistent with the lack of PDE activity in animal mutants (*rd* mouse, *rcd1* Irish setter) in which expression of the β subunit is disabled by a null mutation in the β subunit gene (49, 62). In the *rd* mouse, a nonsense mutation in exon 7 of the β subunit gene truncates the β subunit, essentially removing the catalytic domain and the *CAAX* box motif from the expressed truncated β subunit. In the *rcd1* Irish setter, a premature stop codon in exon 21 leads to a much shorter truncation, which does not affect the putative catalytic domain. In both animal mutants, truncated β subunits (40 kDa in *rd*, 85 kDa in *rcd1*) appear to be unstable, because they do not accumulate to a detectable level, and a functional PDE is not produced leading to a rapidly progressing photoreceptor degeneration. Rescue of the mutation in the *rd* mouse and restoration of a functional PDE by expression of an intact β subunit in photoreceptors of transgenic mice (63) strongly suggest that expression of both large subunits, correctly processed by farnesylation and geranylgeranylation at their C terminus, may be necessary for assembly of a functional rod PDE enzyme. Recently, the biological importance of posttranslational geranylgeranylation has been further emphasized by linking a defect in a Rab geranylgeranyl transferase subunit to human choroideremia, an *X*-linked disease of the heterogeneous retinitis pigmentosa type (64), in which both the retina and the choroid degenerate.

In summary, we have shown that singly expressed subunits show little enzymatic activity although each carries the putative catalytic domain characteristic of other single subunit PDEs. However, an enzymatically active rod PDE can be expressed when both large subunits are coexpressed in insect cells. We further showed that isoprenylation and associated modifications of the Cys residue of the precursor *CAAX* domain are responsible for membrane association, one of the important features of native PDE.

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