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Rod photoreceptor cGMP-phosphodiesterase: Analysis of α and β subunits expressed in human kidney cells

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ABSTRACT **The bovine α and murine β subunits of rod-photoreceptor cGMP-phosphodiesterase (PDE_α and PDE_β) were expressed in adenovirus-transformed 293 human embryonic kidney cells. RNA blots from transfected cells showed transcripts of 3.0 and 2.8 kb corresponding to PDE_α and PDE_β, respectively. Protein expression was analyzed by using affinity-purified antibodies against cGMP-PDE on immunoblots and by immunoprecipitation. PDE_α and PDE_β exhibited the expected mobility (and thus apparent molecular size) and had cGMP hydrolytic activity. Reconstitution of the PDE αβ heterodimer with the expressed proteins increased by ≈6-fold the activity of the individual α and β subunits. Addition of expressed β subunit to retinal extracts from 9- to 10-day-old *rd/rd* mice (which have only normal α and γ subunits of rod cGMP-PDE and thus minimal activity) increased enzyme activity by ≈3-fold. Our results therefore demonstrate that photoreceptor-specific cGMP-PDE can be synthesized in human kidney cells with consequent expression of enzymatic activity.**

Rod-photoreceptor cGMP-phosphodiesterase (cGMP-PDE) is one of the key enzymes of the visual phototransduction cascade in the vertebrate retina (1). The holoenzyme is a heterotetrameric complex, consisting of two large catalytic subunits, α (88 kDa; PDE_α) and β (84 kDa; PDE_β), and two identical inhibitory subunits γ (11 kDa) (2, 3). cGMP-PDE activity is stimulated *in vivo* by removal of the inhibitory γ subunits by activated transducin (4). cGMP-PDE can be stimulated *in vitro* by incubation with histones (5) or by limited proteolytic digestion with trypsin (6).

The cDNAs encoding the three subunits of cGMP-PDE from different animal species have been cloned, and their nucleotide and amino acid sequences have been determined (7–10). Sequence homology between the α and β subunits from bovine (9) and murine (11) rod photoreceptors is ≈72% and 74%, respectively. Each of these subunits contains two putative noncatalytic cGMP-binding sites, two γ subunit-binding sites, a putative catalytic domain, and the CAAX motif for isoprenylation (9, 12–15). In rod photoreceptor cells the α and β subunits are farnesylated and geranylgeranylated, respectively, which facilitates their association with the disc membranes. The domain organization of these proteins suggests that they can have independent cGMP-hydrolytic activity.

To carry out studies on the structure and function of cGMP-PDE in normal retinas and in those affected with genetic diseases, each of the biologically active subunits of the enzyme is necessary. Although the separation of the catalytic subunits is possible under denaturing conditions, no reports have been published to date on the isolation of active PDE_α or PDE_β using classical preparative biochemical procedures. Difficulties inherent to these methods can be overcome with the use of recombinant DNA technology, which has been applied in many instances to express large amounts of active proteins in bacterial or mammalian systems. However, previous attempts to express the murine PDE_α and PDE_β in bacteria and yeast have failed to produce proteins with catalytic activity (17).

In this paper we report the successful expression of catalytically active bovine α and murine β subunits of retinal-rod cGMP-PDE in adenovirus-transformed human embryonic kidney cells. We show that the presence of cGMP-PDE in heterodimeric (αβ) form increases its enzymatic activity.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors Containing the cDNAs for α and β Subunits of cGMP-PDE. The steps in preparing the expression constructs used can be inferred from Fig. 1. Recombinant plasmid DNA [clone p13-3.1 (18)] containing the full-length 5′-untranslated and coding regions and 210 bp of the 3′-untranslated region of bovine PDE_α cDNA was cloned into the *Eco*RI site of vector pUC13. The resulting clone was then digested with *Eco*RI and *Nar* I and with *Nar* I and *Sal* I restriction enzymes. The *Eco*RI–*Nar* I fragment (420 bp) and the *Nar* I–*Sal* I fragment (2750 bp), which also contained the restriction enzyme sites from the multiple cloning sequence of pUC13, including *Xba* I, were ligated and cloned between the *Eco*RI and *Sal* I sites of the pBluescript vector, producing pBA3. This cDNA (3201 bp; 858-aa residues) was subcloned from pBA3 into mammalian expression vector pCIS2, by using the restriction enzyme *Xba* I to yield pCISA37 (Fig. 1*a*).

A mouse PDE_β cDNA containing the complete coding and 3′-untranslated regions was obtained by using two clones already isolated in our laboratory from normal and *rd* mouse retinal cDNA libraries, MBP1 and 1.5′9, respectively. Except for the silent mutations in codons 108 (ACG → ACA) and 1182 (GTC → GTA) and for the nonsense mutation (TAC → TAA) in codon 347 (19), the nucleotide sequences of 1.5′9 and MBP1 are identical. We replaced the fragment from 1.5′9 cDNA containing the *rd* nonsense mutation (*Bgl* II–*Sca* I) with the corresponding fragment from the normal clone (Fig. 1*b*) by ligating the *Bgl* II–*Sca* I fragment (1638 bp) from MBP1 cDNA with the N-terminal *Eco*RI–*Bgl* II (661 bp) and the C-terminal *Sca* I–*Eco*RI (446 bp) fragments of 1.5′9. The cDNA obtained was subsequently cloned into the *Eco*RI site of pBluescript. The ligation product, pBB1, was digested with *Xba* I and *Xho* I, and a 2860-bp fragment containing the full-length coding and 3′-untranslated regions of the β subunit cDNA (2745 bp; 856 aa) was subcloned between the *Xba* I and *Xho* I restriction sites of the pCIS2 vector to yield pCISB7.

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Abbreviations: PDE, phosphodiesterase; PDE_α and PDE_β, cGMP-PDE α and β subunits, respectively; SV40, simian virus 40; T-antigen, large tumor antigen; CMV, cytomegalovirus.

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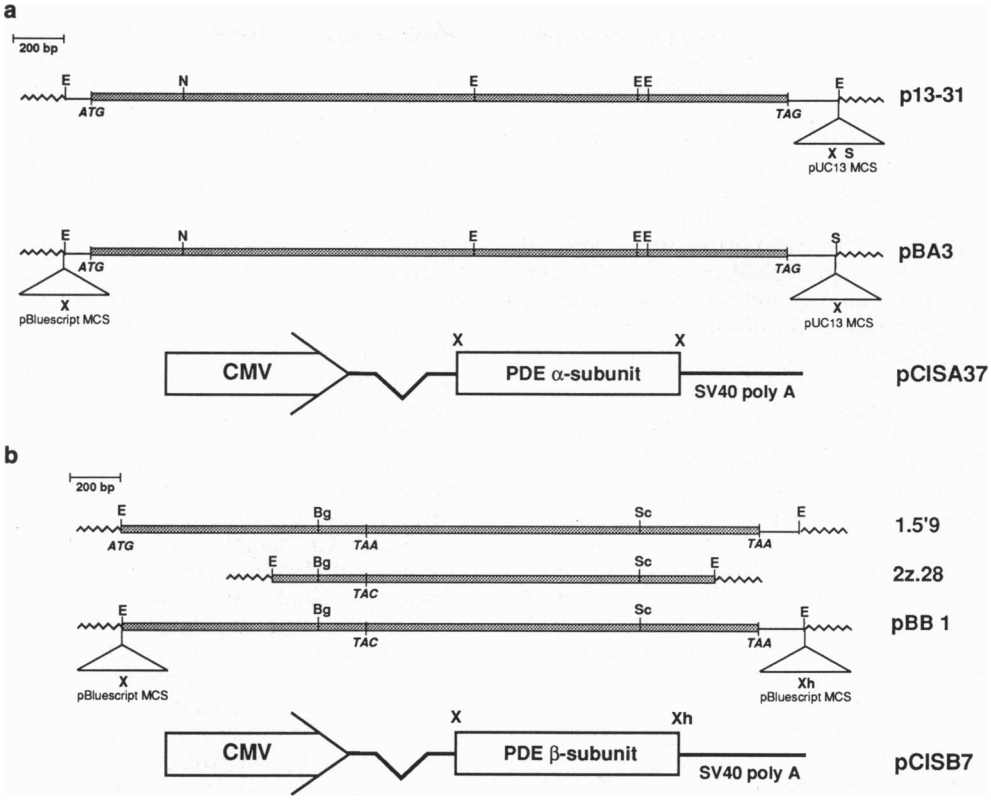

FIG. 1. Expression vectors pCISA37 (*a*) and pCISB7 (*b*). Ori, simian virus 40 (SV40) extrachromosomal replication sequence. E, *Eco*RI; N, *Nar* I; X, *Xba* I; S, *Sal* I; Sc, *Sca* I; Xh, *Xho* I; cross-hatched box, coding region; zigzag line, plasmid DNA; MCS, multiple cloning sequence; CMV, cytomegalovirus immediate-early enhancer and promoter.

Both of the resulting recombinant plasmids, pCISA37 and pCISB7, were characterized by restriction mapping and sequencing through the junctions used for ligation.

Tissue Culture and Cell Transfection. Adenovirus-transformed 293 human embryonic kidney cells (American Type Culture Collection CRL 1573) were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium, 1:1 (vol/vol), supplemented with 10% horse serum at 37°C in a humidified incubator in 95% air/5% CO_2. Recombinant plasmid DNA (10 μg) was introduced into the cells using the calcium phosphate-mediated transfection procedure, as described by Wigler *et al.* (20) in the presence of 1 μg of SV40 large tumor (T)-antigen expression plasmid DNA pRSVT. After 3–5 hr of exposure to the calcium phosphate–DNA coprecipitate, the cells were briefly treated (30 s) with 15% glycerol to increase transformation efficiency. The cells were harvested 48 hr after transfection for analysis of mRNA expression and newly synthesized protein.

RNA Isolation and Northern Blot Analysis. Total RNA was extracted from transfected cells by the method of Chirgwin *et al.* (21). Ten micrograms of RNA was separated by electrophoresis in a 1.2% agarose gel containing 2.2 M formaldehyde, transferred to Hybond-N^+ (Amersham), crosslinked, and hybridized with radiolabeled PDE_α and PDE_β cDNA probes. After hybridization the RNA blots were washed at a final stringency of 0.3× standard saline/citrate and 0.3% SDS at 60°C. Autoradiographs were obtained by exposing the blots for 1 hr at −80°C to Hyperfilm-MP (Amersham) and Cronex Lightning Plus intensifying screens (DuPont).

Protein Analysis. Cells were homogenized in lysis buffer (10 mM Tris buffer, pH 7.5/0.1 mM $MgCl_2$/1 mM dithiothreitol/100 μM phenylmethylsulfonyl fluoride), centrifuged at 14,000 × *g* for 10 min, and the supernatants were removed and used for protein determination (22), immunoblots, and measurement of cGMP-PDE activity.

Proteins were separated by SDS/PAGE (11.1% acrylamide and 0.6% *N,N'*-methylene-bis-acrylamide, stabilized by 6% glycerol) using a Tris/Tricine buffer system, as described by Schägger and von Jagow (23) and Schägger *et al.* (24). The Tricine gels were run for 23 hr at 32 mA constant current, and the separated proteins were transferred to 0.20-μm nitrocellulose paper (25) for 14 hr at 4°C and a constant voltage of 4 V/cm. The blots were blocked with 5% bovine serum albumin/0.15% Tween 20 in Tris-buffered saline (TBS; 500 mM NaCl/20 mM Tris, pH 7.6) for 2 hr at room temperature.

Immunoblots were incubated overnight with the corresponding dilution of the primary IgG serum fraction against cGMP-PDE in TTBS (0.15% Tween 20 in TBS)/2% bovine serum albumin/0.2% normal goat serum. Immunoblots were visualized by using the amplified alkaline phosphatase protocol (Bio-Rad). All incubations were done at room temperature.

cGMP-PDE activity was measured by the procedure of Farber and Lolley (26).

Preparation of cGMP-PDE Antibodies. Two polyclonal antisera were used. A bovine antiserum against both the rod outer segment PDE_α and PDE_β had been characterized (27). The other antiserum was raised against a 17-mer peptide (αT1) corresponding to aa 599–615 of the murine β subunit. This peptide bears 100% homology with the corresponding sequence of PDE_α.

The IgG fraction of both antisera was separated by protein A-Sepharose chromatography and affinity-purified according to the method of Talian *et al.* (28), as described by Farber *et al.* (27).

Immunoprecipitation. Newly synthesized proteins were biosynthetically labeled with [^{35}S]methionine and immunoprecipitated with affinity-purified antiserum raised against bovine rod cGMP-PDE, as described (27). Labeling was conducted for 3 hr with 100 μCi of L-[^{35}S]methionine per ml

(1 Ci = 37 GBq) in methionine-free medium 199/Hepes buffer (GIBCO/BRL). The cells were then pelleted at 500 × *g* for 3 min, washed once with medium 199, and homogenized in lysis buffer.

Trichloroacetic acid-precipitable counts were measured, and the protein equivalent to 1.0×10^7 cpm was incubated with affinity-purified antiserum for 14 hr at 4°C in 250 mM NaCl/20 mM Tris, pH 7.6/1 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride/0.1% Tween 20. After precipitation of the immunocomplex with protein A-Sepharose CL-6B (50 μl, packed bed volume per sample), proteins were separated by SDS/PAGE and transferred to nitrocellulose. Blots were then exposed to Hyperfilm-MP.

RESULTS

Features of the Expression Vectors Containing the PDE_α and PDE_β cDNAs. We chose the pCIS2 vector for transient expression studies on the basis of the reported high levels of chloramphenicol acetyltransferase and human growth hormone activities produced by human cytomegalovirus (CMV)-driven vectors in the 293 cell line (29, 30). Expression of subcloned cDNAs or genes in pCIS2 vectors is controlled by the human CMV immediate-early enhancer and promoter regions. Although the PDE_α and PDE_β cDNAs contain the corresponding polyadenylylation signals, these endogenous elements by themselves cannot guarantee polyadenylylation. Therefore, the pCISA37 and pCISB7 vectors (Fig. 1) include the cleavage/polyadenylylation signals derived from SV40, which are extremely efficient for processing mRNA (31).

The pCIS2 expression vectors contain the SV40 origin of DNA replication, which allows replication in the presence of T-antigen (32, 33). For stability and increase in copy number of transfected plasmid DNA and, consequently, to increase the efficiency of the transient expression of PDE_α and PDE_β, 293 cells were cotransfected with a mixture of recombinant plasmid DNA (pCISA37 or pCISB7) and the SV40 T-antigen expression plasmid DNA pRSVT (34).

RNA Analysis. Fig. 2*a* is an autoradiogram of an RNA blot obtained with total RNA from cells harvested 48 hr after transfection with pCIS2 vector alone (lane 1) and pCISA37 (lane 2); Fig. 2*b* is an autoradiogram from cells transfected with pCIS2 (lane 1) and pCISB7 (lane 2); these RNAs were hybridized with radiolabeled bovine PDE_α (Fig. 2*a*) and murine PDE_β (Fig. 2*b*) cDNAs. A single positive band is seen in each autoradiogram corresponding to transcripts of 3.0 and 2.8 kb, respectively. These results indicate that cells transfected by pCISA37 expressed PDE_α and cells transfected by pCISB7 expressed PDE_β. RNA from cells transfected by the pCIS2 plasmid DNA lacked hybridization signal (Fig. 2 *a* and *b*), confirming that human embryonic kidney cells do not express endogenous proteins homologous to rod-specific cGMP-PDE. It should be noted that the cells transfected for this particular experiment expressed more PDE_α than PDE_β mRNA.

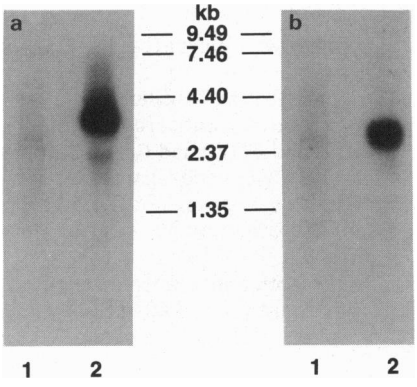

FIG. 2. Autoradiograms of RNA blots prepared with total RNA from transfected 293 cells hybridized with bovine PDE_α cDNA (*a*) and with murine PDE_β cDNA (*b*). Lanes: 1, total RNA from cells transfected with pCIS2; 2 in *a*, total RNA from cells transfected with pCISA37; 2 in *b*, total RNA from cells transfected with pCISB7.

Protein Expression. Protein expression was analyzed by using both immunoblots and immunoprecipitation. Proteins transferred to nitrocellulose paper after separation on polyacrylamide gels were probed with affinity-purified peptide antiserum (αT1) raised against a murine PDE_β sequence. Fig. 3 shows a representative immunoblot. The expressed cGMP-PDE proteins exhibited the expected mobility. In lane 1, the α and β subunits from bovine rod outer segments can be clearly discerned. In lanes 2 and 3, the antibodies recognized only the PDE_β, the gene product resulting from pCISB7 expression in the adenovirus-transformed human embryonic kidney cells. Similarly, in lanes 4 and 5, only PDE_α is detected by the antibodies as the protein expressed by pCISA37. The samples in lanes 3 and 5 were additionally treated with Triton X-100 to ensure extraction of all membrane-associated proteins, because the subcellular localization of the expressed subunits is unknown. Lane 6 contains proteins of the 293 cells transfected with pCIS2 vector alone and shows a protein of larger molecular size than PDE_α or PDE_β that cross-reacted intensely with the antibodies. This 92-kDa protein is also seen in the lanes containing the expressed PDE_α and PDE_β, and it may be a component of the kidney cells that has some homologous sequence to both enzyme subunits. All other secondary bands stained on the immunoblot are due to cross-reactivity with the biotin–streptavidin reagent used for amplifying the signals.

Immunoprecipitation allows the detection of small amounts of proteins at high specificity. We used this method to confirm that the protein bands stained on the immunoblots of 84 and 88 kDa indeed corresponded to PDE_α and PDE_β. Biosynthetically ^{35}S-labeled proteins (5.9×10^4 trichloroacetic acid-precipitable cpm/μg of protein from extracts of cells

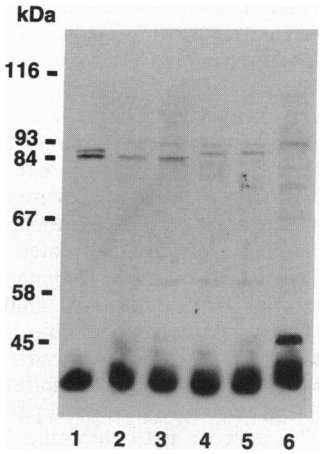

FIG. 3. Immunoblot containing the protein extracts of pCIS2-, pCISA37-, and pCISB7-transfected cells probed with affinity-purified peptide antiserum (1:1000 dilution). Lanes: 1, purified bovine rod PDE (10 μg of protein); 2, hypotonic extract of pCISB7-transfected cells; and 3, Triton X-100 extract of pCISB7-transfected cells; 4, hypotonic extract of pCISA37-transfected cells; and 5, Triton X-100 extract of pCISA37-transfected cells; 6, Triton X-100 extract of pCIS2-transfected cells. Lanes 2–6 contain 100 μg of total protein. Molecular size markers are phosphorylase *a* (93 kDa), bovine serum albumin (67 kDa), and ovalbumin (45 kDa). β-Galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), and pyruvate kinase (58 kDa) are prestained molecular size markers from Sigma. Large bands at the bottom correspond to the pyronin Y tracking dye used to define sample lanes.

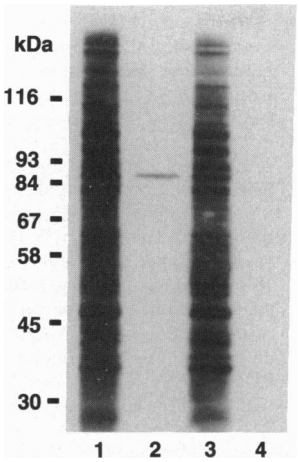

FIG. 4. Autoradiogram of [^{35}S]methionine-labeled proteins of human embryonic kidney cells transfected with expression constructs containing PDE_α and PDE_β, immunoprecipitated with affinity-purified antiserum against bovine rod cGMP-PDE and separated by SDS/PAGE. Lanes: 1 and 3, all proteins from extracts of pCISA37- and pCISB7-transfected cells, respectively; 2 and 4, proteins immunoprecipitated from pCISA37- and pCISB7-transfected cells, respectively. Molecular size markers are the same as for Fig. 3, except that carbonic anhydrase (30 kDa) is included.

transfected with pCISA37, and 5.5×10^4 cpm/μg of protein from extracts of cells transfected with pCISB7) were precipitated by affinity-purified antiserum raised to bovine rod PDE_α and PDE_β. An autoradiogram of the labeled proteins separated by SDS/PAGE shows quite distinctly the expressed PDE_α (Fig. 4, lane 2) and PDE_β (Fig. 4, lane 4). The proteins from the cell extracts before immunoprecipitation are shown in lanes 1 and 3. Intensity of the immunoprecipitated bands is directly proportional to amount of protein biosynthesis (27). In this experiment, more α than β subunit was precipitated, which agrees with the different mRNA levels detected for each subunit transcribed.

PDE Activity. In all experiments, a basal level of cGMP-PDE activity always appeared in cell extracts transfected with pCIS2 plasmid alone. The measured activity had a mean value of 0.4 nmol of cGMP hydrolyzed per min·mg of protein. cGMP-PDE activity in the pCISA37-transfected cells was 3.8-fold higher and in the pCISB7-transfected cells was 3.0-fold higher than the basal level (Fig. 5*a*). The higher specific activity observed for PDE_α may be from the relative higher level of expression of the protein and of the mRNA encoding it in the cell extracts used, as seen in the autoradiogram of immunoprecipitated proteins (Fig. 4) and of RNA blots (Fig. 2).

We also measured cGMP-PDE activity of the proteins newly synthesized in the human embryonic kidney cells that were immunoprecipitated by the affinity-purified antisera. A baseline level of activity was obtained by incubating cell extracts with preimmune serum. cGMP-PDE activity in the immunoprecipitates was defined as net cpm above baseline. cGMP-PDE activity for the α and β subunits was detected in the immunoprecipitated extracts from cells transfected with pCISA37 and pCISB7. No activity was observed in the cells transfected with pCIS2 alone (Fig. 5*b*).

The catalytic core of the enzyme was reconstituted by incubating the expressed α and β subunits (in equal amounts of total protein) overnight at 10°C. cGMP-PDE activity of the recombined α and β subunits was ≈6-fold higher than that of the individual subunits (Fig. 6*a*). Increased cGMP-PDE activity (≈3-fold) was also observed when the expressed β subunit was incubated with supernatant from 9- to 10-day-old *rd/rd* mouse retina that contains only the normal α and γ subunits of the rod enzyme (Fig. 6*b*).

DISCUSSION

Our results demonstrate that the α and β subunits of rod-specific cGMP-PDE can be synthesized in human kidney cells with consequent expression of enzymatic activity. That each individual subunit exhibits catalytic activity is not surprising because there is evidence of independent catalytic activity in the subunits of cGMP-stimulated PDE from bovine heart and adrenal cortex (35). This fact also supports the idea that rod-specific cGMP-PDE may be present and functional not only as the $\alpha\beta\gamma\gamma$ heterotetramer, but also as $\alpha\alpha\gamma\gamma$ and $\beta\beta\gamma\gamma$ complexes; PDEs of other tissues have this type of composition. For example, the catalytic subunit of cone cGMP-PDE exists as a homodimer, $\alpha'\alpha'$ (36), and the catalytic and allosteric domains of cGMP-stimulated PDE from bovine heart and adrenal cortex are also homodimeric (35, 37). However, most previous studies on rod cGMP-PDE could only show the existence of the $\alpha\beta\gamma\gamma$ form.

It is difficult to understand the advantage to the photoreceptor cell of having two different genes, α and β, expressed in three different protein complexes to carry out identical functions. We could speculate that the presence and function of cGMP-PDE as an $\alpha\beta\gamma\gamma$ complex in the rod photoreceptor may occur because the $\alpha\beta$ heterodimer is structurally more stable than the $\alpha\alpha$ and $\beta\beta$ homodimers and/or because proper tertiary structure and thus maximal PDE activity is obtained only with the $\alpha\beta$ heterodimeric form. Results of our experiments attempting to reconstitute the catalytic core of cGMP-PDE agree with this hypothesis. Several additional findings also support it. (*i*) Previous studies on the *rd* mouse (an animal model of retinal degeneration)

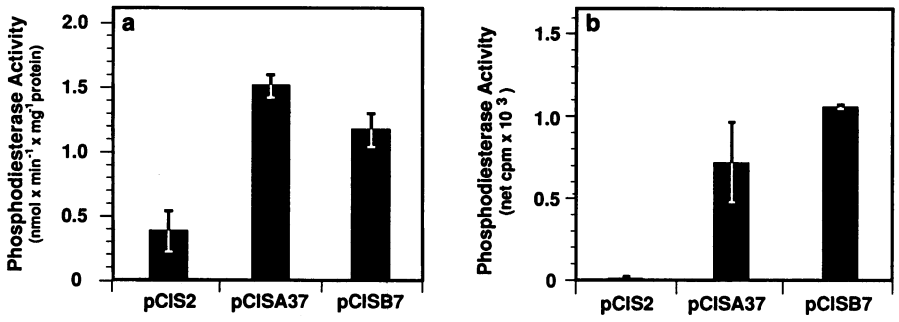

FIG. 5. (*a*) cGMP-PDE activity of extracts from pCIS2-, pCISA37-, and pCISB7-transfected cells. The assay was conducted with 100 μM cGMP as substrate at 37°C and ≈100 μg of total protein. (*b*) cGMP-PDE activity of proteins from pCIS2-, pCISA37-, and pCISB7-transfected cells immunoprecipitated with affinity-purified antiserum raised to bovine α,β cGMP-PDE. Fifty micromolar cGMP was used as substrate at 37°C and a 30-μl vol of the protein A-Sepharose immunoprecipitate. Net cpm were obtained by subtracting nonspecific activity (in cpm) precipitated by preimmune serum and protein A-Sepharose from counts precipitated by the cGMP-PDE-specific serum. Results are from typical experiments conducted in triplicate (±SD).

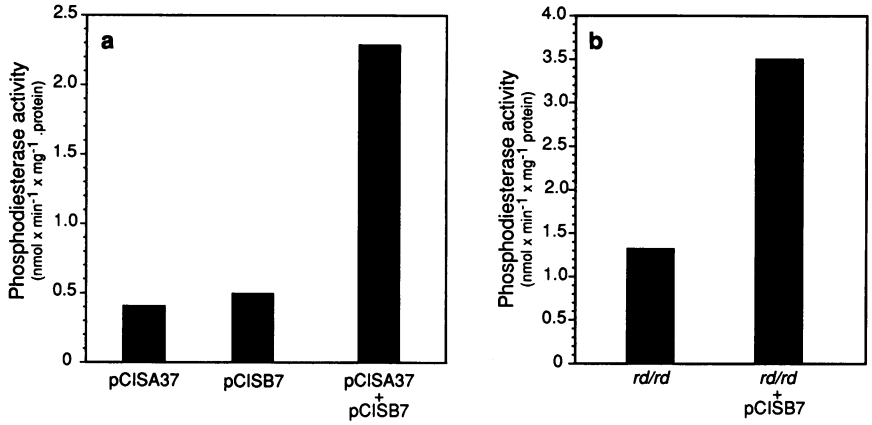

FIG. 6. (*a*) cGMP-PDE activity of expressed α and β subunits and possibly reconstituted $\alpha\beta$ heterodimer. Equal amounts of protein (200 μg) from pCISA37- and pCISB7-transfected cell extracts were combined and incubated overnight at 10°C. Aliquots were removed to determine protein concentration and cGMP-PDE activity. Results are from a typical experiment conducted in quadruplicate. (*b*) Expressed β subunit (200 μg of protein from pCISB7-transfected cells) was incubated overnight at 10°C with 9- to 10-day-old *rd/rd* mouse retinal extract (40 μg of protein from the supernatant fraction of the retinal homogenate). Aliquots were removed to determine protein concentration and cGMP-PDE activity. Results are from an experiment conducted in quadruplicate.

have shown that cGMP-PDE activity is deficient in its rod visual cells, which are the target of the *rd* mutation, before onset of the disease (38). The deficient activity has been shown to result from a defect in the gene encoding the β subunit of the enzyme (10, 39). Thus, on the basis of the data discussed here, the minimal levels of cGMP hydrolysis measured early in development in the *rd* retina (26) seem to reflect lack of the $\alpha\beta\gamma\gamma$ complex (40) and could be due to the activity of PDE_α alone, which is not encoded by the *rd* gene. The increased enzyme activity seen after the expressed β subunit complexes with the cGMP-PDE of 9- to 10-day-old *rd* mouse retinal extract supports this possibility. (*ii*) Other studies on the *rd* enzyme indicate that it is not activatable by histones (40) or trypsin (unpublished data), which removes the inhibitory γ subunits from the core holoenzyme in normal rod photoreceptors (6). Therefore, the $\alpha\beta$ heterodimer may be further needed for kinetic control of the enzyme by the γ subunits.

In contrast with our results on the synthesis of PDE_α and PDE_β by human kidney cells, expression of the same enzyme components in bacteria and in yeast (16, 17) has yielded inactive products. Possibly the *in vivo* expression of catalytically active cGMP-PDE subunits of the vertebrate rod visual cell may require proteins and/or other factors that are present in mammalian cells and absent from bacteria and yeast.

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