Polymerase chain reaction (PCR) methodology and cDNA library screening were used to isolate a cDNA clone encoding a cGMP-binding, cGMP-specific phosphodiesterase (cGB-PDE) from bovine lung. Degenerate oligonucleotides based on cGMP-PDE peptide sequences were used as primers for a PCR reaction with bovine lung cDNA as the template. An 824-base pair PCR product was recovered and used as a probe to screen a bovine lung cDNA library. A 4.5-kilobase pair cDNA clone encoding a full-length cGMP-PDE was isolated. The open reading frame of this cDNA predicted an 875 amino acid (AA), 99,525-Da polypeptide. By Northern analysis, the cGMP-PDE cDNA hybridized to a single lung 6.9-kilobase mRNA. The identity of the cGMP-PDE cDNA was verified by comparison of the deduced AA sequence with several peptide sequences obtained from cGMP-PDE. COS-7 cells transfected with cGMP-PDE cDNA overexpressed cGMP-binding and cGMP-PDE activities characteristic of lung cGB-PDE. The sequence of cGB-PDE contained a segment (AA 578–812) that was homologous to the putative catalytic region conserved among all mammalian PDEs and a segment (AA 142–526) that was homologous to the putative cGMP-binding region of the cGMP-stimulated PDE and the photoreceptor PDEs. As noted also for these PDEs, two internally homologous repeats were contained within the putative cGMP binding region of cGB-PDE. The amino-terminal 142 residues of cGB-PDE showed no significant homology to other PDEs and contained the serine (AA 92) which is phosphorylated by cGMP-dependent protein kinase.

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1 The abbreviations used are: PDEs, 3':5'-cyclic nucleotide phosphodiesterases; cGMP-PDE, cGMP-binding, cGMP-specific phosphodiesterase; cGS-PDE, cGMP-stimulated phosphodiesterase; ROS-PDE, rod outer segment phosphodiesterase; CONE-PDE, cone phosphodiesterase; cAK, cAMP-dependent protein kinase; cGK, cGMP-dependent protein kinase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; ssDNA, single-stranded DNA; MOPS, 3-[N-morpholino]propanesulfonic acid; bp, base pair; kb, kilobase; ORF, open reading frame; C5M-PDEs, calcium/calmodulin-stimulated phosphodiesterases; cGI-PDE, cGMP-inhibited phosphodiesterase; MeOxMeMIX, 3-isobutyl-1-methyl-1-nethoxymethyl-xanthine; IBMX, 3-isobutyl-1-methylxanthine; CAP, E. coli catalytite activator protein; cpm, counts/min; HPLC, high performance liquid chromatography; pfu, plaque-forming unit(s).
cGMP-binding, cGMP-specific Phosphodiesterase

cone phosphodiesterase (CONE-PDE) (9), which is composed of two identical α' subunits and three smaller subunits); and the cGMP-binding, cGMP-specific phosphodiesterase (cGB-PDE), which has been shown to be a homodimer composed of two 93-kDa subunits (10). Comparison of the deduced amino acid sequences of cGS-PDE (11), the α and β subunits of ROS-PDE (12, 13), and the α' subunit of CONE-PDE (14), reveals that each sequence possesses, in addition to a homologous catalytic region located near the carboxyl terminus, a second conserved segment of approximately 340 residues located closer to the amino terminus (5, 13). This additional conserved segment is not present in any non-cGMP-binding PDEs and has been proposed to constitute an allosteric, cGMP-binding region (1, 5). Photoaffinity labeling studies designed to map the catalytic and cGMP-binding sites of cGS-PDE support this hypothesis (3, 15). Full-length amino acid sequence has not been previously reported for cGB-PDE, but it has been predicted that its sequence contains a segment homologous to the putative cGMP-binding regions of the cGMP-PDE and photoreceptor PDEs (5).

cGB-PDE has been purified to homogeneity from rat (16) and bovine lung (16) and has been shown to be present in a variety of tissue and species including rat and human platelets (17), rat spleen (18), guinea pig lung (19), vascular smooth muscle (20), and sea urchin sperm (21). The enzyme exhibits activities (10) and is believed to be a chimeric multidomain protein. This proposal is supported by the finding that DEAE chromatography of a partial a-chymotryptic digest of cGB-PDE separates cGMP-binding fragments from a cGMP hydrolytic fragment (10). In addition to cGMP-binding and hydrolytic domains, cGB-PDE has been shown to contain a single site which can be readily phosphorylated by cGMP-dependent protein kinase (cGK) and, with a lower rate, by CAMP-dependent protein kinase (cAK) (22). The primary amino acid sequences of the phosphorylation site and of the amino-terminal end of the cGMP-binding fragments generated by chymotryptic digestion of cGB-PDE have been reported (10, 22). We report here the cloning of the cDNA and analysis of the deduced amino acid sequence of cGB-PDE. This deduced sequence, in combination with previous biochemical studies of the enzyme, provides new and important insights into the location and structure of individual domains of this enzyme, and into the structural relationship of cGB-PDE to other PDEs. This is an important step in ultimately understanding the mechanisms by which the enzymatic activity of cGB-PDE is regulated.

**EXPERIMENTAL PROCEDURES**

**Methods**

Purification of cGB-PDE and Catalytic Subunit of cAK—cGB-PDE was purified using a previously described method (10) or a modification of that method. For all preparations, processing of bovine lung extract, DEAE-cellulose chromatography, and Blue Sepharose CL-6B chromatography were performed as previously described. Zinc chelate affinity adsorbent chromatography was performed using either an agarose or Sepharose-based gel matrix. The resulting protein pool from the zinc chelation step was processed as previously described or was subjected to a modified purification procedure. If the modified procedure was performed, the protein pool was dialyzed against 20 mM sodium phosphate, pH = 6.8, with 2 mM EDTA and 25 mM β-mercaptoethanol (FMEM), for 2 h, and loaded onto a 10-ml preparative DEAE-Sephaloc column equilibrated in FMEM buffer. The protein was eluted batchwise with 0.5 M NaCl in FMEM, resulting in an approximately 10-15-fold concentration of protein. The concentrated protein sample was loaded onto an 800-ml (2.5 × 154 cm) Sephacryl S-400 gel filtration column equilibrated in 0.1 M NaCl in PEM, and eluted at a flow rate of 1.7 ml/min. The purity of the protein was assessed by Coomassie staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (23). Approximately 0.5–3.0 mg of pure cGB-PDE were obtained per 10 kg of bovine lung.

The catalytic subunit of cAK was purified to homogeneity according to the method of Flockhart and Corbin (24). Limited Amino Acid Sequence Analysis of cGB-PDE—The methods used to obtain the peptide sequences KISASFDRLPR and TSPRDNDGIE/DQ have been described elsewhere (10, 22). Of the sequence-specific peptides REXDANRNYAAVYKVNTM and QSLAAAXVVE were identified during analysis of proteolytic fragments of phosphorylated cGB-PDE. Phosphorylation of cGB-PDE was performed prior to digestion in order to allow for 32P labeling of resultant phosphopeptides, but several non-labeled fragments were also isolated during these studies. For REXDANRNYA-VYKVNTM, approximately 100 μg of purified cGB-PDE was phosphorylated in a reaction mixture containing 9 mM MgCl2, 9 μM [32P]ATP, 10 μg cGMP, and 4.2 μg of purified catalytic subunit of cAK in a final volume of 900 μl. The reaction was incubated for 30 min at 30 °C and stopped by addition of 60 μl of 200 mM EDTA. This solution was incubated for 30 min at 30 °C with 5.7 ml of a 1 mg/ml solution of α-chymotrysin in 10 mM potassium phosphate, pH = 6.8, with 2 mM EDTA (KPE buffer). This limited proteolysis was stopped by addition of 50 μl of 10% SDS and 25 μl of β-mercaptoethanol, and the volume of the sample was reduced to less than 400 μl by boiling. The sample was loaded onto an 8% preparative SDS-polyacrylamide gel (23) in 1× SDS sample buffer (24). Fragments were electrophoretically transferred to Immobilon polyvinylidene difluoride (Millipore), identified by Coomassie Blue staining, and a non-32P-labeled 50-kDa band was excised from the membrane for automated gas-phase amino acid sequencing (25).

The peptide sequence QSLAAAXVVE was obtained as follows: approximately 200 μg of purified cGB-PDE was added to 10 mM MgCl2, 10 μM [32P]ATP, 100 μg cGMP, and 1 μg/ml purified catalytic subunit of cAK (24) in a final volume of 1.4 ml. The reaction was incubated for 30 min at 30 °C and was terminated by addition of 180 μl of 0.2 M EDTA. Nine μl of 1 mg/ml Staphylococcal aureus V8 protease followed by KPE buffer were added and incubation was continued at 30 °C. Proteolysis was stopped by addition of 88 μl of 10% SDS and 45 μl of β-mercaptoethanol. The sample was boiled, and the digestion products were separated by electrophoresis on a preparative 10% SDS-polyacrylamide gel (23), run at 25 mAmps for 4.5 h. Proteins were electrophoretically transferred to Immobilon and stained, and a non-32P-labeled 28-kDa protein band was excised from the membrane for automated gas-phase amino acid sequencing (25).

Purified cGB-PDE was previously found to be resistant to amino-terminal amino acid sequencing (10). However, the peptide sequence AGPGSARKPQKW was obtained by direct Edman degradations of a proteolytic digest of 100 mg cGB-PDE dissolved in 50% formic acid. These results imply that partial proteolysis occurred 3 residues from the amino terminus.) Sequencing was performed on an Applied Biosystems Model 470A gas-phase sequencer equipped with an on-line model 120A phenylthiohydantoin-peptide analyzer. This sequence was also obtained from cGB-PDE electrophoretically transferred to polycrylamide gel (23), run at 25 mAmps for 4.5 h. Proteins were electrophoretically transferred to Immobilon and stained, and a non-32P-labeled 50-kDa band was excised from the membrane for automated gas-phase amino acid sequencing (25).

The sequence GTRMNVAXFAERVHVT was obtained from a peptide purified after digestion of cGB-PDE with Achromobacter protease I. The digest of approximately 20 μg of cGB-PDE was performed in 200 μl of 2 M urea, 50 mM Tris-HCl, pH 8.5, using a...
protease/substrate ratio of 1:50, for 12 h at 37 °C, followed by reduction and pyridylethylolation as described previously (26). The resultant peptide mixture was purified by reversed-phase HPLC on a RP-300, C8 column (2.1 × 100 mm (Pierce Chemical Co.)) using a Hewlett-Packard model 1090 HPLC system.

Production of an Initial cDNA Clone by the Polymerase Chain Reaction (PCR) for the oligonucleotides corresponding to the amino acid sequences NYMYAQYV and FDNDEG(E/D)Q2 (obtained from purified cGB-PDE as described above) were synthesized using an Applied Biosystems Model 380A DNA Synthesizer. After ethanol precipitation, oligonucleotides were combined at 400 μM each in a PCR containing 50 ng of first strand cDNA, 200 μM dNTP, 10 μM 5′-deoxynucleotide triphosphates, and 2 units of Taq polymerase. The initial denaturation step was carried out at 94 °C for 5 min, followed by 30 cycles of a 1-min denaturation step at 94 °C, a 2-min annealing step at 50 °C, and a 2-min extension step at 72 °C. PCR was performed using a Hybaid Thermal Reactor. The resulting PCR product (referred to as the “PCR clone”) was purified by electrophoresis on a 1% low melting point agarose gel run in 40 mM Tris acetate, 2 mM EDTA, and isolated using the Gene Clean DNA purification kit according to the manufacturer’s protocol. The PCR product (20 ng) was ligated into the EcoRI site of the pBluescript SK(+) vector to obtain cDNA library vectors and inserted into E. coli JM103. For the construction and screening of the cDNA library, plasmid constructs were referred to as pCDM8-cGB-PDE. The DNA was purified by phenol-chloroform extraction and ethanol precipitation, followed by digestion with T7 gene 6 exonuclease (32). For the latter procedure, 30 μg of plasmid DNA containing the cDNA insertion was digested with either Apal or Smal. The digested DNA was purified by phenol/formaldehyde extraction and ethanol precipitation, followed by digestion with T7 gene 6 exonuclease (75 units/μg) in a solution containing 50 mM Tris-HCl, pH 8, 20 mM KCl, 5 mM MgCl2, and 10 mM β-mercaptoethanol in a final volume of 20 μl. The resulting ssDNA was purified by phenol-chloroform extraction and ethanol precipitation, and quantitated by measuring A260, resulting in a typical yield of 10–15 μg of ssDNA. Sequencing of the entire cGB-8 cDNA clone was completed for both strands.

Phosphodiesterase Activity—Phosphodiesterase activity was measured using a modification of the assay procedure described for the cGS-PDE (6). Incubation mixtures contained 40 mM MOPS, pH 7, 0.8 mM EGTA, 15 mM magnesium acetate, 2 mg/ml bovine serum albumin, 20 μM cGMP or cAMP, [3H]cGMP or [3H]cAMP (100,000–200,000 cpm/μg), and 10–40 μl of cell extract in a final volume of 250 μl. For the PDE inhibition studies, 25 μl of the indicated PDE inhibitor, diluted to the appropriate concentration, was added to the reaction mixture. For each PDE inhibitor tested, two types of control experiments were performed: 1) an inhibition profile was obtained using purified bovine lung cGB-PDE diluted into homogenization buffer. This control was used to ensure that the phosphodiesterase activity was not affected by the additions of inhibitors used in these studies produced similar patterns of
inhibition as had been previously reported for purified cGB-PDE (10); 2) a PDE inhibition profile was obtained using purified bovine lung cGB-PDE diluted in 10–40 μl of extract from mock-transfected COS-cells. This control was used to determine if any factors present in COS cell extracts affected the rate of cGMP hydrolysis or efficacy of the PDE inhibitors. Reaction mixtures were incubated for 10 min at 30 °C, and stopped by boiling. Next, 10 μl of 10 mg/ml Crotaulus atrox venom was added followed by a 10-min incubation at 30 °C.

Nucleotide products were separated from unreacted nucleotides as described (6). In all studies, less than 15% of the total [3H]cyclic nucleotide was hydrolyzed during the reaction.

The cGMP-binding assay, modified from the previously described assay (10), was conducted in a total volume of 80 μl; 60 μl of cell extract (see above) was combined with 20 μl of a binding mixture such that the final concentrations of the components of the mixture were 5 μM CAMP, 10 μM 8-bromo-cGMP, and 1.0 or 2.5 μM [γ-32P]ATP. The cAMP and 8-bromo-cGMP were blocked to any [3H]cGMP binding attributable to cAK and cGK, respectively. Binding was measured in the absence and presence of 0.2 mM IBMX, as indicated. Excess unlabelled cGMP or cAMP, diluted in water to the appropriate concentration, was added when indicated. The reaction was initiated by the addition of the cell extract, followed by incubation for 60 min at 0 °C. Reaction mixtures were filtered as described (10). Assay blanks were determined by parallel incubations with homogenization buffer replacing cell extracts, or with a 100-fold excess of unlabelled cGMP. Similar results were obtained with both methods. Total protein was determined by the method of Bradford (34) using bovine serum albumin as the standard.

Sequence Analyses—DNA sequence analysis was performed using the DNA INSPECTOR program from Textco, SwissProt and GenEmbl databases (February, 1992) were searched using the FASTA (35) program supplied with the Genetics Computer Group (GCG) Software packages, and regions of homology between two sequences were located with the COMPARE (36) program. The DOT PLOT dot matrix program was used to plot the results of the COMPARE program. The DOT PLOT parameters used in these studies were window = 30 and stringency = 15. Pairwise sequence alignments were computed using the CLUSTAL (distributed by the National Biomedical Research Foundation (37) and BESTFIT (38) (included in the GCG package) programs. The ALIGN program calculates a maximum alignment score for a pair of real sequences and the distribution of maximum scores for random permutations of the two sequences. The alignment score is the number of standard deviations by which the maximum score for the real sequences exceeds the average maximum score for the random permutations. The probability of a score ≥5 standard deviations occurring by chance is 2.87 x 10-3.

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The following strategy was utilized in specifically defining the boundaries of the putative catalytic and cGMP-binding regions of cGB-PDE. First, dot matrix analysis was used to compare the sequence of cGB-PDE to each of the other individual PDE sequences shown in Fig. 5. The results of this analysis provided a rough estimate of the location of the catalytic region, conserved among all PDEs, and the noncatalytic cGMP-binding region, conserved only in cGB-PDE, cG5-PDE, and the photoreceptor-PDEs. Next, a multiple sequence alignment including the entire sequence of all nine PDEs described in Fig. 5 was performed (not shown) and used as a guideline in rationally assigning boundary residues. The amino- and carboxyl-terminal boundaries of the catalytic region were arbitrarily assigned to the first and last residues, respectively, which were identical in at least three of the five cGMP-binding PDEs shown in Fig. 6. The amino-terminal boundary of the cGMP-binding region (Leu41 in cGB-PDE) was preceded by a 14-residue gap introduced by the amino acid sequence (not shown) of the third clone (cGB-PDE) was followed by a 20-residue gap introduced by the alignment program.

Dot matrix analysis of the deduced cGB-PDE sequence was used to approximate the location of the internally homologous repeats within the sequence. Multiple sequence alignments of the putative catalytic domains of nine PDEs region spanning residues 190-330 and 351-526 of cGB-PDE was performed. The amino-terminal boundaries of the repeats were arbitrarily assigned to residues Glu235 (repeat a) and Glu410 (repeat b) because these residues were preceded by a 4-residue gap introduced by the alignment program. Similarly, the carboxyl-terminal boundary residues, Glu241 (repeat a), and Glu476 (repeat b) were followed by a 2-residue gap introduced by the alignment program, and a region of only 11% sequence identity.
appeared to be identical to cGB-8 by restriction map analysis (data not shown).

The cGB-G-8 cDNA clone was 4474 bp in length and contained a large ORF of 2625 bp (Fig. 1). A translation initiation site (ATG) was identified at position 99–101 in the nucleotide sequence. It was preceded by an in-frame stop codon, and the surrounding bases were compatible with the Kozak consensus initiation site for eukaryotic mRNAs (40). The stop codon, TAG, was located at position 2724–2726, and was followed by 1748 bp of 3′- untranslated sequence. The sequence of cGB-8 did not contain a transcription termination consensus sequence (41) or a poly(A) tail; therefore, the clone did not represent the entire 3′ sequence of the corresponding mRNA. This was consistent with the fact that in Northern analysis of bovine lung polyadenylated RNA, a 32P-labeled cGB-PDE cDNA hybridized to a single 6.9-kb species (Fig. 2).

The ORF of the cGB-8 cDNA encoded an 875-AA polypeptide with a calculated molecular mass of 99,525 daltons. This was in good agreement with the molecular mass of a cGB-PDE monomeric subunit, 93 kDa, estimated by SDS-PAGE analysis of purified protein (10). Also, the AA sequences of six peptides obtained from proteolytic digests of purified bovine lung cGB-PDE (boxed in Fig. 1) corresponded exactly to the AA sequence encoded by cGB-8, thus providing strong evidence that cGB-8 encodes cGB-PDE.

Expression of cGB-PDE in COS-7 Cells—The ability of cGB-PDE cDNA to encode a functionally intact phosphodiesterase was demonstrated by transfecting COS-7 cells (42) with the cGB-G-8 cDNA clone. Transfection of COS-7 cells with the pCDM8-cGB-PDE construct (see "Experimental Procedures" for explanation) resulted in the expression of approximately 15-fold higher levels of cGMP phosphodiesterase activity than in mock-transfected cells, or in cells transfected with pCDM8 vector alone (Fig. 3A). No increase in cAMP phosphodiesterase activity over mock or vector-only transfected cells was detected in extracts from cells transfected with pCDM8-cGB-PDE (Fig. 3A). Thus, under the assay conditions described in this report (20 μM substrate) the expressed cGB-PDE cDNA protein product hydrolyzed cGMP, whereas no hydrolysis of cAMP was detected. This degree of substrate specificity was also observed with the nonrecombinant cGB-PDE purified from bovine lung, which exhibited no detectable cAMP hydrolysis at concentrations up to 50 μM (10).

The relative potencies of PDE inhibitors for inhibition of cGMP hydrolysis by the expressed cGB-PDE cDNA protein product are shown in Fig. 3B. Zaprinast, a relatively specific inhibitor of cGB-PDE (10), dipyridamole, an effective inhibitor of selected cAMP- and cGMP-specific phosphodiesterases (43), was also a potent inhibitor of the expressed cGB-PDE (IC50 = 3.5 μM). 3-Isobutyl-1-methyl-methoxymethylxanthine (MeOxMeMIX), a relatively selective inhibitor of calcium/calmodulin-stimulated phosphodiesterases (CaM-PDEs) (45, 46), was approximately 10-fold less potent than zaprinast and dipyridamole (IC50 = 30 μM). Rolipram, a potent inhibitor of low Ki, cAMP phosphodiesterases (47, 48), but a poor inhibitor of cGB-PDE (10), was also a poor inhibitor of the expressed cGB-PDE cDNA protein product (IC50 > 500 μM). The relative potencies of this battery of PDE inhibitors for inhibition of cGMP hydrolysis by the expressed cGB-PDE cDNA protein product (Fig. 3B) were identical to those relative potencies reported for cGB-PDE purified from bovine lung (10). These findings provided strong evidence that the cGB-8 cDNA encodes cGB-PDE.

Interestingly, the absolute IC50 values for all inhibitors tested were 2–7-fold higher for the recombinant cGB-PDE expressed in COS-7 cells as compared to cGB-PDE purified from bovine lung. This difference could not be attributed to the effects of any factors present in COS-7 cell extracts on cGMP hydrolytic activity, since purified bovine lung cGB-PDE exhibited kinetics of inhibition identical to those previously reported, when assayed as a pure enzyme, or when assayed in the presence of extract from mock-transfected COS-7 cells (described under "Experimental Procedures") (data not shown). This apparent difference in pharmacological sensitivity could have been due to a subtle difference in the structure of the expressed cGB-PDE cDNA protein product and the cGB-PDE purified from bovine lung, such as a difference in post-translational modification at or near the catalytic site. Alternatively, this difference could have been due to an alteration of the catalytic activity of bovine lung cGB-PDE during the purification.

The cGMP binding activity of the expressed cGB-PDE cDNA protein product was characteristic of the cGB-PDE purified from bovine lung in that it was stimulated by IBMX. In the presence of 0.2 mM IBMX and 1 μM [3H]cGMP, extracts from COS-7 cells transfected with pCDM8-cGB-PDE exhibited 8-fold higher cGMP binding activity than did extracts from mock or vector-only transfected cells (Fig. 3C). This cGMP binding activity was stimulated approximately 1.8-fold by the addition of the IBMX (Fig. 3C). This effect of IBMX, a competitive inhibitor of cGMP hydrolysis, in stimulating cGMP binding 2–5-fold is a distinctive property of the cGMP-binding phosphodiesterases, having been observed for the cGB-PDE from rat (21), and bovine lung (10), as well as for the photoreceptor-PDEs from Rana catesbiana rod (8) and bovine cone (9), and for the bovine heart cGS-PDE (15). IBMX-stimulated binding occurs under conditions in which no cGMP hydrolysis is detected, thereby eliminating the possibility of substrate protection as the mechanism for increased binding (10). Rather, it is thought that IBMX elicits this effect on cGMP binding by generating a conformational change in the PDE upon interacting with the catalytic site (49). The finding that recombinant cGB-PDE, as expressed in COS-7 cells, exhibited IBMX-stimulated binding suggested that its conformation is similar to that of the nonrecombinant enzyme. No IBMX stimulation of the low level of background cGMP binding was observed.

The binding activity of the protein product encoded by the cGB-PDE cDNA was highly specific for cGMP as compared to cAMP. Less than 10-fold higher concentrations of unlabeled cGMP were required to lower [3H]cGMP binding by 50%, whereas approximately 100-fold higher concentrations of cAMP were required for the same effect (Fig. 3D). 100-fold excess concentrations of unlabeled cAMP were also required to produce 50% inhibition of the cGMP binding activity of the nonrecombinant cGB-PDE purified from bovine lung (10).

AA Sequence Analysis and Domain Structure Assignment—A search of the SWISS-PROT and GenEmbl data banks revealed that no protein or nucleic acid sequences other than those of PDEs displayed significant homology to the deduced cGB-PDE sequence. Like all mammalian phosphodiesterases sequenced to date, cGB-PDE contained a conserved segment of sequence, approximately 250 residues in length, located in the carboxyl-terminal half of the protein. Several pieces of evidence supported the proposal that this region of conserved sequence comprises the catalytic site of PDEs (for review, see Ref. 1). This segment included residues 578–812 (Fig. 4) of cGB-PDE (see "Experimental Procedures" for explanation of
FIG. 1. mRNA sequence and predicted amino acid sequence of the cGβ-PDE. The position of the last nucleotide in each line is indicated by the *top numbers* to the right. The sequence of the PCR clone used as a probe for cDNA library screening is enclosed in brackets. The consensus sequence for the translation initiation site is underlined. The in-frame stop codon directly upstream of the initiator methionine is indicated. The translation termination signal is indicated by (**). The positions of the amino acid residues predicted from the large opening reading frame of the cGβ-PDE cDNA (cGβ-8) nucleotide sequence indicate are indicated by the *bottom numbers* to the right which are shown in bold type. Peptide sequences enclosed in boxes correspond to sequences observed in cGβ-PDE purified from bovine lung.
strategy used to determine boundaries of conserved regions of sequence). Fig. 5 shows an alignment of this conserved region of cGB-PDE with the corresponding region of eight other PDEs. Comparison of this segment of cGB-PDE with the corresponding segments of the other PDEs in Fig. 5 yielded Dayhoff scores ranging from 16 to 31, with 28-48% sequence identity (see “Experimental Procedures” for explanation of alignment scores).

The catalytic region of cGB-PDE more closely resembled the catalytic region of the photoreceptor-PDEs than it did the corresponding region of other PDEs (45-48% sequence identity). This finding was not surprising since previous studies demonstrated that the hydrolytic activities of cGB-PDE and the photoreceptor-PDEs share several characteristics, including a strong specificity for cGMP relative to cAMP, and sensitivity to the PDE inhibitors zaprinast and dipyriramole (10, 50). Several segments of sequence contained residues that were invariant among the photoreceptor-PDEs and cGB-PDE, but that were not present in other PDEs (Fig. 5). These regions of high homology among cGB-PDE and the photoreceptor-PDEs may serve important roles in conferring the specificity for cGMP hydrolysis as compared to cAMP hydrolysis, or in conferring sensitivity to specific pharmacological agents.

The structural similarity between cGB-PDE and the other cGMP-binding PDEs (cGS-PDE and the photoreceptor-PDEs) was not limited to the conserved catalytic region, but also included an additional region of sequence homology located in the amino-terminal half of the protein. Optimization of the alignment (see “Experimental Procedures”) between cGB-PDE, cGS-PDE, and the photoreceptor-PDEs indicated that this conserved amino-terminal segment included residues 142–526 of cGB-PDE (Fig. 4). Earlier comparison of the deduced AA sequences of the cGS-PDE and the photoreceptor-PDEs led to the proposal that this segment of conserved sequence comprised a noncatalytic cGMP binding region (5). Alignment of the proposed cGMP binding region of cGB-PDE with the corresponding region of the photoreceptor-PDEs and cGS-PDE produced Dayhoff alignment scores of 14–18 standard deviations (see “Experimental Procedures” for explanation of alignment scores) with 26–28% sequence identity. A multiple sequence alignment of the proposed cGMP binding regions of several PDEs is shown in Fig. 6. Within this region containing approximately 400 residues, 38 positions were invariant among all cGMP-binding PDEs listed. Neither the catalytic nor the noncatalytic cGMP binding regions of cGB-PDE showed statistically significant homology to the cyclic nucleotide-binding domains of the E. coli catabolite gene activator protein (CAP) family of proteins (which includes the CAP protein, cAK, cGK, and the cyclic nucleotide-gated channels) (for review, see Ref. 51). Additionally, neither region contained the guanine nucleotide-binding elements of G-proteins (14).

The proposal that the cGMP binding region is located within the conserved segment of cGB-PDE sequence near the amino terminus is supported by previous biochemical studies of the purified lung cGB-PDE. Partial proteolysis of purified cGB-PDE with chymotrypsin produced two fragments (35 and 45 kDa) which exhibited \[^{32}P\]cGMP binding activity and specific photoaffinity labeling with \[^{32}P\]cGMP (10). The fragments were identical in their amino-terminal sequences, TSPRFNDLEG, suggesting that the 35-kDa fragment was derived from a second chymotryptic cleavage of the 45-kDa fragment. If the molecular weights determined by SDS-PAGE were accurate, both fragments would span most of the more amino-terminal conserved segment. These data provided direct evidence that this segment of conserved sequence serves as a cGMP binding region of cGB-PDE. It is of interest to note that earlier studies of purified cGB-PDE suggested that the dimerization domain is located within or adjacent to this cGMP binding region (10).

Like the photoreceptor-PDEs and cGS-PDE, the proposed cGMP-binding domain of cGB-PDE contained two internally homologous repeats. Optimization of the alignment of these two segments of cGB-PDE sequence (see “Experimental Procedures”) suggested that the repeats span at least residues 228–311 (labeled repeat a in Fig. 4) and 410–500 (repeat b in Fig. 4). Alignment of cGB-PDE repeat a with cGB-PDE repeat b gave a Dayhoff alignment score of 16 with 43% sequence identity (Table I). Since boundary assignments were not rigorously defined (see “Experimental Procedures”), some degree of internal homology may extend beyond the core of conserved sequence defined by regions a and b. Alignment of the repeated segments of cGB-PDE with the corresponding regions of cGS-PDE and the photoreceptor-PDEs showed that both segments a and b of cGB-PDE were homologous to each segment a and b from all the cGMP-binding PDEs (Table I). Fig. 7 shows a multiple sequence alignment of the regions corresponding to repeats a and b from several cGMP-binding PDEs.

The internally homologous repeats present in the cGMP binding region of cGB-PDE, as well as of the other cGMP-binding PDEs, may represent two similar, but distinct, cGMP-binding sites. A tandem repeat pattern is also present in the sequences of cAK and cGK and, in these instances, represent two distinct cyclic nucleotide-binding sites on both kinases (51). Studies of the association and dissociation patterns of cGMP binding to cGB-PDE are suggestive of the existence of more than one type of cGMP-binding site (10, 49). Additionally, Scatchard analysis of cGMP binding indicated the existence of two classes of non-catalytic sites for cGMP binding to cGS-PDE (15), and ROS-PDE (8, 52). If the tandem repeats of sequence in the cGMP-binding PDEs represent two distinct cGMP-binding sites, amino acids essential for cGMP binding should be conserved in both regions.
Fig. 3. Expression of cGB-PDE in COS-7 cells. COS-7 cells were subjected to mock transfection, or were transfected with either vector DNA (pCDM8) or cGB-PDE cDNA ligated into pCDM8 (pCDM8-cGB-PDE). After 48 h, cells were harvested and resulting extracts were assayed for phosphodiesterase activity and cGMP binding activity as described under "Experimental Procedures." Panel A, cell extracts were assayed for phosphodiesterase activity using either 20 μM cGMP or 20 μM cAMP as the substrate. Results are shown as pmol of cyclic nucleotide hydrolyzed/((min)(mg of total protein in cell extracts)). All assays were performed in duplicate. Error bars represent standard deviation. Panel B, extracts from cells transfected with pCDM8-cGB-PDE were assayed for cGMP phosphodiesterase activity (20 μM cGMP) in the presence of increasing concentrations of phosphodiesterase inhibitors. PDE activity in the absence of inhibitor is taken as 100%. Each point represents the average of two separate determinations. All assays were performed in duplicate. Error bars represent standard deviation. Panel C, cell extracts were assayed for [3H]cGMP binding activity (concentration of [3H]cGMP = 1 μM) in the absence or presence of 0.2 mM IBMX. Results are shown as pmol of cGMP bound/mg total protein in cell extract. The results shown are averages of three separate transfections. All assays were performed in duplicate. Error bars represent standard deviation. Panel D, extracts from cells transfected with pCDM8-cGB-PDE were assayed for [3H]cGMP binding activity (concentration of [3H]cGMP = 2.5 μM) in the presence of excess unlabeled cAMP or cGMP at the concentrations indicated. cGMP binding in the absence of unlabeled competitor was taken as 100%. Each point represents the average of three separate determinations. All assays were performed in duplicate. Error bars represent standard deviation.

Three regions of cGB-PDE, beyond the boundaries of the proposed cGMP binding region and proposed catalytic region, showed no significant sequence similarity to other PDEs (Fig. 4). The site (Ser') of phosphorylation of cGB-PDE by cGK (22) was located within the unique amino-terminal region (Fig. 4). The location of the phosphorylated serine near the amino terminus of the deduced AA sequence of cGB-PDE is in good agreement with previous studies of purified cGB-PDE. The peptide sequence of the site of phosphorylation of cGB-PDE, KISASEFDRPLR, (the phosphorylated serine is underlined) was obtained from a tryptic [32P]phosphopeptide derived from purified cGB-PDE, which had been phosphorylated by cGK and [γ-32P]ATP (22). Limited proteolysis of cGB-PDE, phosphorylated by either cGK or catalytic subunit of cAK and [γ-32P]ATP, with chymotrypsin or S. Aureus protease produced 80–85-kDa cGB-PDE fragments which lacked radiolabel (56). The radioactivity remained associated with small fragments of cGB-PDE (10–15 kDa), providing biochemical evidence that the phosphorylated serine was located within 10–15 kDa of one extremity of the cGB-PDE monomer.

Since the amino-terminal segment containing the phosphorylation site of cGB-PDE showed no homology to other phosphodiesterase sequences, and the effect of phosphorylation...
tion by cGK on cGBP-PDE function is unknown, the fact that several phosphodiesterases have been reported to be regulated by phosphorylation (57, 58) could be coincidental. A recent report showed that incubation of partially purified cGBP-specific PDE from guinea pig lung with the catalytic subunit of cAK resulted in a 10-fold increase in the V_max for cGMP hydrolysis (59). It is of interest to note that binding of cGMP to the allosteric site on cGBP-PDE and photoreceptor-PDEs are identical in all PDEs listed are enclosed in open boxes. Shaded boxes designate residues which are identical in the cGBP-PDE and photoreceptor-PDEs only. Gaps introduced for optimal alignment are indicated by periods. Abbreviations for the PDEs listed are as follows: 63 kCaM, bovine 63-kDa calcium/calmodulin-dependent PDE (193-242) (4); 63 kCaM, bovine 63-kDa calcium/calmodulin-dependent PDE (193-242) (60); Rad rundown, rat cAMP-specific PDE (77-316) (61); drosdunce, Drosophila cAMP-specific dunce PDE (1-239) (62); 77 kCaM, bovine 77-kDa calcium/calmodulin-dependent PDE (209-561), 63 kCaM, bovine 63-kDa calcium/calmodulin-dependent PDE (209-561) (63); 63 kCaM, bovine 63-kDa calcium/calmodulin-dependent PDE (209-561) (64); cGS, bovine cGMP-binding, cGMP-specific phosphodiesterase from guinea pig lung (11); cGB-PDE IISFMQVQKC TIFI (578-812). Numbers to the right designate the position of the last residue in that alignment. The alignment was performed using the PILEUP program.

**Fig. 5.** Multiple sequence alignment of the cGMP-binding region of several PDEs. Residues which are identical in all PDEs listed are enclosed in open boxes. Shaded boxes designate residues which are identical in the cGBP-PDE and photoreceptor-PDEs only. Gaps introduced for optimal alignment are indicated by periods. Abbreviations for the PDEs listed are as follows: 63 kCaM, bovine 63-kDa calcium/calmodulin-dependent PDE (193-242) (4); 63 kCaM, bovine 63-kDa calcium/calmodulin-dependent PDE (193-242) (60); Rad rundown, rat cAMP-specific PDE (77-316) (61); drosdunce, Drosophila cAMP-specific dunce PDE (1-239) (62); 77 kCaM, bovine 77-kDa calcium/calmodulin-dependent PDE (209-561), 63 kCaM, bovine 63-kDa calcium/calmodulin-dependent PDE (209-561) (63); 63 kCaM, bovine 63-kDa calcium/calmodulin-dependent PDE (209-561) (64); cGS, bovine cGMP-binding, cGMP-specific phosphodiesterase from guinea pig lung (11); cGB-PDE IISFMQVQKC TIFI (578-812). Numbers to the right designate the position of the last residue in that alignment. The alignment was performed using the PILEUP program.

**Fig. 6.** Multiple sequence alignment of the cGMP-binding region of several PDEs. Residues which are identical in all PDEs listed are enclosed in open boxes. Gaps introduced for optimal alignment are indicated by periods. Abbreviations for the PDEs listed are as follows: 63 kCaM, bovine 63-kDa calcium/calmodulin-dependent PDE (193-242) (4); 63 kCaM, bovine 63-kDa calcium/calmodulin-dependent PDE (193-242) (60); Rad rundown, rat cAMP-specific PDE (77-316) (61); drosdunce, Drosophila cAMP-specific dunce PDE (1-239) (62); 77 kCaM, bovine 77-kDa calcium/calmodulin-dependent PDE (209-561), 63 kCaM, bovine 63-kDa calcium/calmodulin-dependent PDE (209-561) (63); 63 kCaM, bovine 63-kDa calcium/calmodulin-dependent PDE (209-561) (64); cGS, bovine cGMP-binding, cGMP-specific phosphodiesterase from guinea pig lung (11); cGB-PDE IISFMQVQKC TIFI (578-812). Numbers to the right designate the position of the last residue in that alignment. The alignment was performed using the PILEUP program.
to an improved understanding of the mechanisms by which the enzymatic activity of cGB-PDE is regulated and of the contributions of cGB-PDE to modulation of cGMP-mediated signal transduction pathways.

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TABLE 1
Homology between internally homologous repeats in cGB-PDE and other cGMP-binding PDEs

| cGB-PDE a | cGS a | ROS-a | ROS-a' | CONE-a | cGB-PDE b | ROS-b | ROS-b' | CONE-b |
|-----------|-------|-------|--------|--------|-----------|-------|--------|--------|
| cGB-PDE a | 7.5   | 9.38  | 10.14  | 8.69   | 16.01     | 15.25 | 8.22   | 8.47   | 7.14   |
| (25)       | (36)  | (40)  | (33)   | (43)   | (46)      | (35)  | (37)   | (34)   | (32)   |
| cGB-PDE b | 16.01 | 5.81  | 7.54   | 7.84   | 7.40      | 10.93 | 10.06  | 9.28   | 7.17   |
| (43)       | (14)  | (27)  | (29)   | (31)   | (38)      | (30)  | (29)   | (32)   |

*% sequence identity in parentheses.

Fig. 7. Multiple sequence alignment of internally homologous repeats a and b from several PDEs. Residues identical in each repeat a and b from all cGMP-binding PDEs are listed in an open box. Arrows represent positions in which all residues are chemically conserved. Residues identical in repeats a and b of cGB-PDE are shown in bold. Abbreviations for PDEs are given in legend to Fig. 5. Residues comprising each repeat are as follows: ROS-a (a = 149-229, b = 355-438); ROS-b (a = 147-227, b = 353-436); CONE-a (a = 146-226, b = 352-450); cGS (a = 284-364, b = 454-536); cGB-PDE (a = 228-311, b = 410-500). Alignment was performed according to the Progressive Alignment Method (39).
cGMP-binding, cGMP-specific Phosphodiesterase

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