

Expression and characterization of human PDE δ and its *Caenorhabditis elegans* ortholog CE δ

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Abstract Cyclic GMP phosphodiesterase (PDE) is rod photoreceptor disk membrane-associated via C-terminal lipid tails. PDE δ , a recently identified subunit, was shown to disrupt PDE/membrane interaction under physiological conditions, without affecting PDE catalytic activity. We found that a PDE δ ortholog from the eyeless nematode *Caenorhabditis elegans* (termed CE δ) solubilizes bovine PDE in vitro with an EC₅₀ very similar to PDE δ . Immobilized PDE δ and CE δ both bind, in addition to bovine PDE, an N-terminal fragment of human retinitis pigmentosa GTPase regulator, but not rhodopsin kinase and Ran binding protein 1. The results suggest that PDE δ and CE δ may regulate membrane binding of a variety of proteins in photoreceptors and other tissues.

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1. Introduction

Rod photoreceptor phosphodiesterase (PDE) holoenzyme consists of three subunits termed PDE α , PDE β and PDE γ [1–3]. PDE α and PDE β contain the catalytic sites of the enzyme, PDE γ acts as an inhibitor [4]. PDE α and PDE β are anchored to the rod disk membrane via farnesyl- and geranylgeranyl side chains, respectively, which are attached to the C-terminal cysteine residue [5–7]. A putative fourth subunit, termed PDE δ , co-purifying with bovine PDE, was identified [8] and subsequently cloned and expressed [9]. The recombinant protein was shown to solubilize PDE very similarly to the native protein [9]. Proteolytic removal of the C-terminal region of PDE catalytic subunits resulted in loss of interaction between PDE δ and PDE suggesting involvement of the lipid side chains. The PDE δ gene, located on human chromosome 2q35–36, expresses a single mRNA species [10,11]. Multiple tissue Northern blots suggested that PDE δ is expressed in tissues other than retina [9,12], while rod PDE is considered retina-specific. Results from a yeast two-hybrid screening suggested that human PDE δ interacts with RPGR [12], a GTPase regulator involved in X-linked retinitis pigmentosa [13], and the small GTP binding protein, rab13 [14], both of which are ubiquitously expressed.

A gene with similar exon/intron arrangement was identified in the eyeless nematode *Caenorhabditis elegans* [15] as part of

the genome sequencing project, with the systematic identification tag C27H5.1. The gene product, in the following termed CE δ , was shown to be very similar in sequence to PDE δ , and in part, at the C-terminal domain, to the retina-specific protein HRG4 [16]. Both CE δ and HRG4 have no known function. HRG4 is a homolog of the *C. elegans* gene unc-119 that is expressed in *C. elegans* neurons [17]. To investigate whether hPDE δ , CE δ , and HRG4 share similar functions, we cloned and expressed these polypeptides in bacteria and analyzed their ability to solubilize PDE, and to interact with other retinal proteins.

2. Materials and methods

2.1. Cloning of CE δ and HRG4

The cloning of human PDE δ was described previously [10]. The coding region of CE δ was amplified from a mixed stage *C. elegans* cDNA library (from Dr. Susan Mango, University of Utah) with N-terminal sense primer 5'-CAC ATA TGG CCA CTA CGC TAC TCG and C-terminal antisense primer 5'-TAT TAG TCG TAA TAA AGA CGG AC. Human RG4 cDNA was amplified from a human retinal cDNA library (obtained from Dr. Jeremy Nathans, Johns Hopkins University) using an N-terminal sense primer 5'-GCA TAT GAA GGT GAA GAA GAA GG and λ gt10 reverse primer. *Nde*I sites (underlined) were introduced at N-termini to facilitate subsequent cloning into expression vectors. The PCR products were cloned into PCR2.1 vector (Invitrogen) and sequenced from both strands with the tagged primers using a Licor L40000 automatic sequencer [18].

2.2. Site-directed mutagenesis of human PDE δ

QuickChange site-directed mutagenesis kit (Stratagene) was used to create C-terminal truncations of hPDE δ . The primers for truncating the last 10 amino acid of PDE δ are 5'-GAC GAC GAT CTT TGA GTA AGC ACA TCC (sense) and 5'-GGA TGT GCT TAC TCA AAG ATC GTC GTC (antisense); the primers for truncating the last 20 amino acids are 5'-GGG AAC GTT ATC TGA GAA ACA AAG TTT (sense) and 5'-AAA CTT TGT TTC TCA GAT AAC GTT CCC (antisense). In-frame stop codons are underlined. The resulting mutants (M1 and M2) were sequenced on both strands.

2.3. Bacterial expression vectors and purification of hPDE δ -thioredoxin and glutathione S-transferase (GST) fusion proteins

Human PDE δ cDNA lacking the 5' untranslated region was amplified with N-terminal primer 5'-GCC ATA TGT CAG CCA AGG ACG and T7 and cloned into PCR2.1 vector. The thioredoxin expression vector was constructed by cloning the *Eco*RI-*Pst*I digested fragment of human PDE δ into ThioHisA vector. The plasmids thio-H δ , thio-M1 and thio-M2 were transformed into Top10 cells (Invitrogen). The *Nde*I-*Eco*RI fragments of PCR products were cloned into *Nde*I-*Eco*RI sites of pGEX-2TKcs [19]. The recombinant plasmids GK-hPDE δ , GK-M1, GK-M2, GK-CE δ and GK-HRG4 were transformed into BL21 (DE3) plyS strain (Novagen).

The thioredoxin fusion proteins (thio-hPDE δ , thio-M1 and thio-M2) were isolated by a procedure slightly modified from the manufacturer's protocol (Invitrogen). Expressed proteins were denatured with 8 M urea and dialyzed against lysis buffer before they were purified on Probond columns. GST fusion proteins (GK-H δ , GK-CE δ and GK-HRG4) were isolated as described previously [7]. Fusion

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protein was cleaved by treating with biotinylated human thrombin (thrombin cleavage capture kit, Novagen) at room temperature for 2 h. The recombinant protein was dialyzed against 50% glycerol, 100 mM NaCl, 50 mM Tris, pH 8.0, 1 mM EDTA and stored at -20°C .

2.4. Preparation of bovine (ROS) and extract of ROS proteins

Bovine ROS membranes were prepared from fresh bovine retinas [20]. 0.5 ml ROS (bleached, 3 mg rhodopsin/ml) were washed twice in 2 ml hypotonic buffer (20 mM Tris, 0.1 mM DTT, 0.1 mM EDTA) with 1% Triton X-100, 2 mM GTP and 1 mM PMSF. The homogenate was centrifuged at 15000 rpm for 30 min and the supernatant containing solubilized proteins was collected.

2.5. Protein determinations, SDS-PAGE and immunoblotting

The concentrations of proteins [21], SDS-PAGE [22] and Western blot were carried out as previously described [1,7].

2.6. PDE activity assay

Purified recombinant proteins were incubated with ROS membrane diluted in ROS buffer and incubated for the time and temperature as indicated. The samples were centrifuged at 15000 rpm for 30 min at 4°C to separate supernatants and the membranes [9]. PDE activity was measured by recording release of protons with a pH microelectrode (MI-410, Microelectrodes, Inc.) in a buffer containing 20 mM MOPS pH 8.0, 0.15 M KCl, 2 mM MgCl_2 . The final concentration of cGMP was 2 mM [23].

2.7. In vitro transcription/translation

For construction of vectors expressing human RPGR and Ran binding protein 1 (Ran BP1) in vitro, the N-terminus (residues 1–372) of human RPGR (GenBank accession number Q92834) and the coding region of human Ran BP1 (GenBank accession number D38076) were amplified and cloned into *Nde*I and *Xba*I sites of the pAGA vector [24] (constructs T7-RPGRN and T7-BP1). For construction of vectors expressing and mouse rod PDE α and PDE β , the respective coding regions were cloned into pAGA (constructs T7-PDE α and T7-PDE β , respectively). The constructs were linearized and expressed under the control of T7 promoter in TNT coupled reticulocyte lysate system (Promega) for 90 min at 30°C , with incorporation of [^{35}S]methionine. 5 μl of the final translation mixture was

added to 20 μl of SDS sample buffer, and 5 μl was analyzed by SDS-PAGE.

2.8. In vitro binding assay

The binding of retina proteins (or in vitro translated proteins) with immobilized GK-hPDE δ and GK-CE δ was performed as described [25]. 20 μl GST fusion proteins immobilized on glutathione beads were incubated at room temperature for 30 min, with 50 μl of ROS extract or 10 μl of in vitro translation products in 500 μl binding buffer (20 mM Tris pH 7.5, 1 mM MgCl_2 , 0.1 mM EDTA, 100 mM NaCl, 1 mM DTT, 1 mM PMSF, 2.5 mg/ml BSA). Protein complexes were washed three times with 1 ml binding buffers and twice with 1 ml binding buffer without BSA. The bound proteins were released after adding 20 μl 2 \times SDS sample buffer. 5–10 μl were analyzed on SDS-PAGE.

3. Results and discussion

3.1. Recombinant hPDE δ and CE δ solubilize rod PDE from ROS membrane

The PDE δ amino acid sequence is nearly identically conserved among species [10]. Bovine PDE δ was previously expressed in insect cells and shown to be biologically active [9]. To facilitate the purification of the native and mutant proteins, we expressed hPDE δ , CE δ , HRG4, M1 and M2 as GST fusion proteins. The GST polypeptides GK-hPDE δ , GK-CE δ and GK-HRG4 were mostly soluble, and after cleavage with thrombin, the resulting K-hPDE δ , K-CE δ and K-HRG4 (K denotes the kinase domain which is retained after cleavage with thrombin) were used for activity assays. SDS-PAGE analysis showed that the purified recombinant proteins are homogeneous and exhibit the correct mobility (Fig. 1A). To test for activity of the recombinant proteins, excess amounts were incubated with purified ROS membrane in isotonic solution (Fig. 1B), conditions that favor membrane attachment of PDE. The membrane portion and soluble portion of PDE

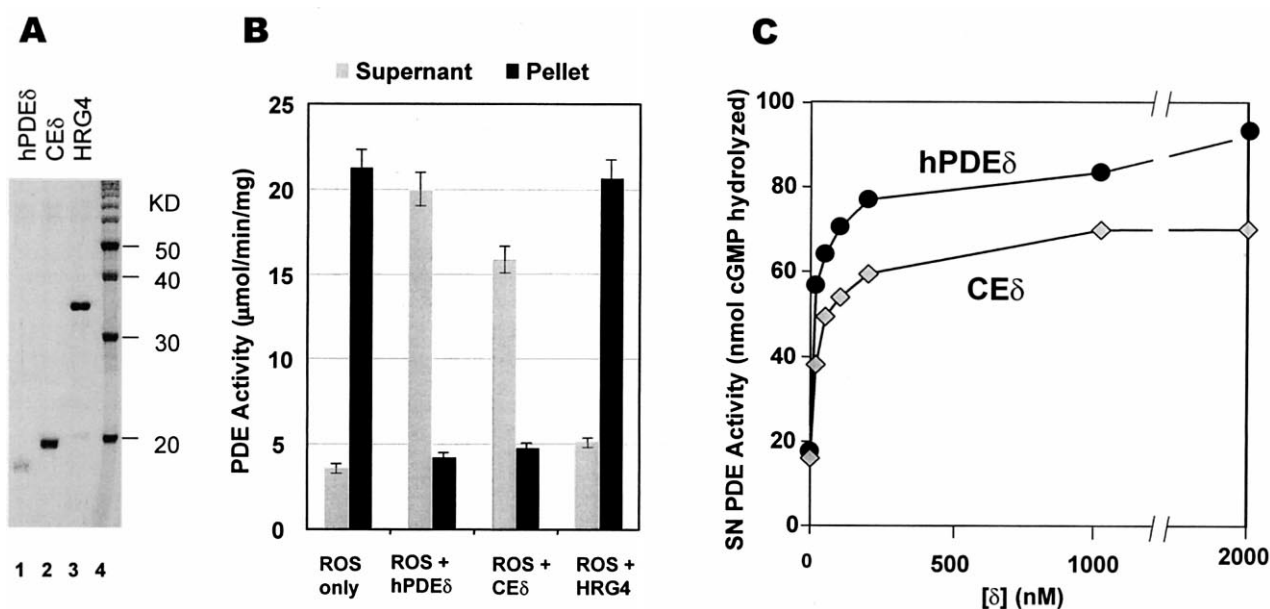


Fig. 1. Expression of GST fusion proteins of hPDE δ , CE δ , and HRG4. A: The fusion proteins were cleaved with thrombin, retaining the kinase domain (K). Lane 1, purified K-hPDE δ . Lane 2, K-CE δ . Lane 3, K-HRG4. Lane 4, 10 kDa protein ladder (Gibco BRL). B: Activity of the purified proteins. ROS membranes (final concentration: 1.5 mg/ml) were incubated with purified recombinant protein at 30°C for 60 min, pelleted, and the supernatant and pellet fractions assayed for PDE activity after trypsin activation, as described in Section 2. The results are the average of two assays. C: Dose-dependent solubilization of ROS-PDE activity by recombinant hPDE δ and CE δ . Purified K-hPDE δ or K-CE δ were incubated with ROS membranes and PDE activities were measured with the pH assay.

were separated by centrifugation and the amount of PDE activity in either portion was determined. The results show that K-hPDE δ was as active as recombinant PDE δ expressed in insect cells. K-CE δ was nearly as active and solubilized the majority of PDE from ROS membrane while K-HRG4 showed little effect. When assayed in a dose-dependent manner (Fig. 1C), the EC₅₀ of CE δ was 25.9 nM, which is very close to the EC₅₀ of PDE δ (14.9 nM) under the same conditions. The results show that a protein that is expressed in an eyeless nematode has nearly the same biological activity as a protein expressed in mammalian photoreceptors. Absence of membrane detachment activity in the recombinant HRG4 suggests a more distant relationship of this protein to CE δ /PDE δ .

3.2. The C-terminus of PDE δ is essential for folding and activity of the protein

The C-termini of PDE δ and CE δ contain Ser-Arg-Val (SRV) and/or Phe-Tyr-Val (FYV) motifs that are thought to be involved in interacting with PDZ domain-containing proteins. The C-terminus of PDE δ is also essential for its correct cellular location when it is expressed in tissue culture [12]. We therefore generated truncation mutants lacking 10 (M1) and 20 (M2) amino acids at the C-terminus. Both GK-M1 and GK-M2, however, are insoluble even after treatment with 8 M urea and dialysis. We therefore expressed hPDE δ and its truncation mutants as thioredoxin fusion protein (thio-hPDE δ , thio-M1, thio-M2) in *Escherichia coli* (Fig. 2). The thioredoxin fusion products were soluble in buffers after 8 M urea treatment, and showed the correct mobility on SDS gels (Fig. 2A). When assayed for PDE membrane detachment, thio-hPDE δ disrupted membrane association in part, but was not as active as PDE δ expressed as GST fusion protein (Fig. 2B). The thio-M1 and thio-M2 mutants, in contrast, were completely inactive. These results suggest that the PDE δ C-terminus is important for the activity and correct folding of the protein.

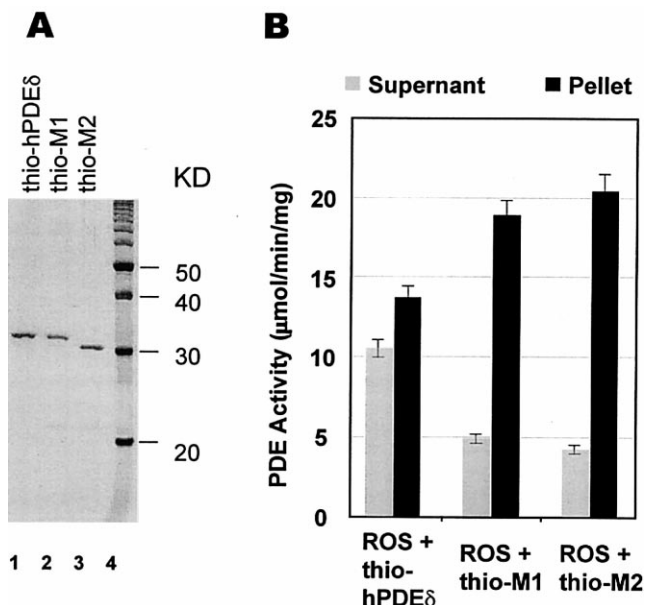


Fig. 2. A: SDS-PAGE analysis (12.5%) of purified recombinant thio-fusion proteins expressed in *E. coli*. Purified thio-hPDE δ (lane 1), thio-M1 (lane 2), and thio-M2 (lane 3). Lane 4 contains 10 kDa protein ladder. B: Activity of thio-hPDE δ , and truncation mutants. Conditions are as in the legend to Fig. 1.

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3.3. Interaction of immobilized GST-hPDE δ and GST-CE δ with other proteins

PDE δ was previously shown to bind PDE catalytic subunits by a gel filtration assay [9]. While rod PDE is considered retina-specific, PDE δ is thought to be expressed also in non-

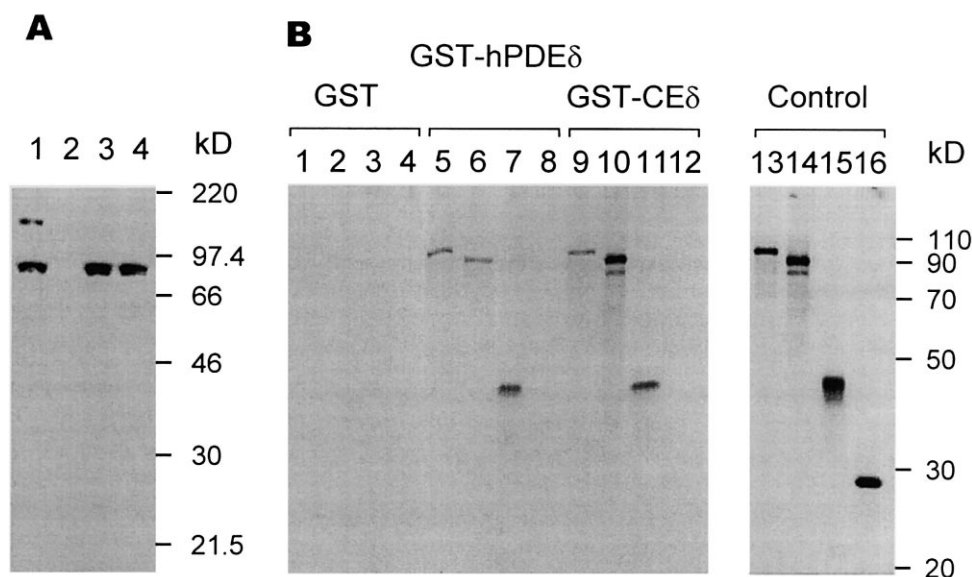


Fig. 3. In vitro interaction of PDE δ or CE δ with PDE, RPGR, and Ran BP1. A: Affinity chromatography and Western blot of hypotonic bovine ROS extracts bound to GST beads (lane 2), GST-hPDE δ (lane 3), and GST-CE δ (lane 4). Lane 1, soluble bovine ROS proteins as a control. The antibody was anti-PDE polyclonal antibody (MOE). B: ³⁵S-labeled in vitro translated mouse PDE α (lanes 1, 5 and 9), mouse PDE β (lanes 2, 6 and 10), human RPGR-N (lanes 3, 7 and 11), and human Ran BP1 (lanes 4, 8 and 12) were incubated with GST alone (lanes 1–4), GST-hPDE δ (lanes 5–8), or GST-CE δ (lanes 9–12). The binding of the proteins was analyzed by SDS-PAGE and autoradiography. Lanes 13–16 are mPDE α , mPDE β , human RPGR-N, and human Ran BP1 translated in vitro.

retina tissues. To identify proteins distinct from PDE that may interact with PDE δ and CE δ , we incubated GST-PDE δ and GST-CE δ immobilized on glutathione beads with bovine ROS components that were solubilized in hypotonic buffer. Proteins binding to GST-hPDE δ or GST-CE δ were identified by Western blotting. The result confirms the binding of hPDE δ to PDE, and show that CE δ binds PDE indistinguishably from hPDE δ (Fig. 3A). When an identical blot was probed with anti-rhodopsin kinase antibody, no binding between PDE δ and rhodopsin kinase was observed (results not shown).

To improve the sensitivity of the binding assay (specific antibodies to other proteins were unavailable), we exposed the immobilized GST proteins to several peptide candidates which were in vitro transcribed/translated using [35 S]-methionine as a tracer (Fig. 3B). The candidates were RPGR, suggested to bind to PDE δ [14], Ran BP1, a ubiquitously expressed protein binding to Ran, a small GTPase involved in nuclear import [26], and luciferase (negative control). As shown in Fig. 3B, both GST-PDE δ and GST-CE δ bind individual PDE subunits PDE α , and PDE β . In addition, a peptide comprising the N-terminal region of RPGR was bound, while Ran BP1 and luciferase (not shown) were not bound. GST alone did not interact with PDE, RPGR, or Ran BP1. The interaction of PDE δ with PDE $\alpha\beta$ or Rab13 implied that the isoprenylated C-termini are necessary for its interaction. However, the N-terminal RPGR peptide is not isoprenylated, and other isoprenylated retinal proteins, such as rhodopsin kinase, do not bind PDE δ . The results suggest that lipid modifications are not essential for the binding.

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