Recombinant Expression of a Type IV, cAMP-Specific Phosphodiesterase: Characterization and Structure−Function Studies of Deletion Mutants†

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Received June 6, 1996; Revised Manuscript Received November 13, 1996⊗

ABSTRACT: A potential role for cAMP in regulating the differentiation of myoblasts has led us to examine the components of the cAMP signaling system, including the type IV, cAMP-specific phosphodiesterases. The full coding sequence of the phosphodiesterase PDE4D1 was inserted in the bacterial expression vector pGEX-KG. N- and C-terminal truncations were also placed in the same vector, allowing the expression and purification of glutathione *S*-transferase (GST)−PDE fusion proteins using glutathione-Sepharose. The purified PDE was active [V_{max} = 318 ± 18 nmol min^{-1} (mg of protein)$^{-1}$] and inhibited by RO 20-1724, rolipram, and MIX (IC_{50} values of 2, 0.4, and 40 μM, respectively). The requirement of PDE4D1 for a divalent cation was also examined. It was able to use Mg^{2+}, Co^{2+}, and Mn^{2+}, but not Zn^{2+}, suggesting that it is not a zinc hydrolase as has been proposed for other PDE types. Deletion of both C- and N-terminal regions affected the apparent native size of the enzyme. The C-terminal region was involved in dimer formation, whereas an N-terminal region was responsible for larger aggregates. Removal of the last 35 amino acids of an N-terminal 80-residue highly conserved region (UCR2) resulted in a 6-fold increase in PDE activity, providing evidence that this part of the molecule acts as an intramolecular inhibitor. The availability of a highly purified, enzymatically active protein in substantial quantities has allowed us to directly examine PDE4D1 for the first time.

The cAMP[1] second messenger system is involved in the regulation of a vast array of biological processes from metabolism to cellular differentiation, and the control of intracellular cAMP levels has therefore received a correspondingly large amount of attention. These levels are regulated by the relative rates of formation from ATP by adenylate cyclase [reviewed in Taussig and Gilman (1995)] and of degradation by cyclic nucleotide phosphodiesterases [reviewed in Beavo et al., (1994), Bolger (1994), Conti et al. (1995b), and Manganiello et al. (1995)], and multiple types of both enzymes have been identified. The cyclic nucleotide phosphodiesterases (PDEs) have generally been classified on the basis of substrate specificity (cAMP and/or cGMP), regulation (Ca^{2+}/CaM, cGMP, etc.), inhibitor susceptibility, and kinetics. Due to the explosion of information derived from the cloning of both cDNAs and genes for PDEs, the field has recently undergone a reform in classification and nomenclature (Beavo et al., 1994). Seven gene families are now recognized in both humans and rats, each containing from one to four known genes. Further complexity is derived from multiple mRNA transcripts via alternative splicing. The type IV or low-K_m, cAMP-specific PDEs (PDE4 gene family) are among the largest of these families; both rats and humans have four separate genes, some of which produce five mRNA splice variants. Because the type IV PDEs are present in only trace amounts and are very susceptible to proteolysis, their examination at the biochemical level has not maintained pace with our expanded understanding at the genetic level.

Sequencing of the cDNAs from multiple PDEs has revealed several regions that are conserved to a greater or lesser degree. A strongly conserved catalytic domain of about 270 residues is generally located near the C-terminal end of the enzymes (Charbonneau et al., 1986). In the type IV cAMP-specific PDEs and the type I 61 kDa CaM-PDE, there are extended regions of homology on both ends of this catalytic domain. Additional conserved regions located on the N-terminal side of the catalytic domain have also been identified; these are conserved within the different classes of PDEs but not between classes. The type IV PDEs have two such additional conserved domains, called upstream conserved regions (UCRs) 1 and 2 (Bolger et al., 1993). Alternative splicing of mRNA transcripts often completely removes UCR 1 and in some cases portions of UCR 2 [reviewed in Bolger (1994)]. For example, the rat RNPDE4D gene produces three mRNA transcripts referred to as PDE4D1, PDE4D2, and PDE4D3 (previously ratPDE 3.1, 3.2, and 3.3, respectively) (Swinnen et al., 1989a,b; Monaco et al., 1994), but only one (PDE4D3) contains both UCR regions. PDE4D1 lacks UCR 1, while part of UCR 2 is also deleted in rat PDE4D2. The presence of similar sets of alternative N-terminal regions in the other type IV PDEs suggests an important functional role for these regions.

The significance of these secondary homologies is not clear in the type IV PDEs, but some evidence implicates involvement in regulation of PDE activity, as seen in other classes of PDEs [reviewed in Conti et al. (1995b)]. A deletion in PDE4D1 of the region that includes the entire UCR 2 results in an activation of PDE activity (Jin et al., 1992). Recently,

† This work was supported by a grant from the Medical Research Council of Canada.

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⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1997.

[1] Abbreviations: βME, β-mercaptoethanol; DMSO, dimethyl sulfoxide; MIX, methylisobutylxanthine; TBS, Tris-buffered saline [20 mM Tris (pH 7.4) and 146 mM NaCl]; PDE, 3′,5′-cyclic nucleotide phosphodiesterase; cAMP, 3′,5′-cyclic adenosine monophosphate; SDS, sodium dodecyl sulfate; UCR, upstream conserved region.

S0006-2960(96)01348-7 CCC: $14.00

it has been demonstrated that phosphorylation of the UCR 1 domain of PDE4D3 by protein kinase A increased PDE activity (Sette et al., 1994a,b). Otherwise, the functional significance of these domains in the type IV PDEs is unknown.

Our interest in the process of myogenesis, and the possible role of cAMP in differentiation (Wahrmann et al., 1973; Hu & Olson, 1988; Salminen et al., 1991; Li et al., 1992; Winter et al., 1993; Baek et al., 1994), has led us to examine PDEs in myoblasts. Most cells contain multiple types of cyclic nucleotide phosphodiesterases, but column chromatography of L6 myoblast lysates has shown that, while several different molecular-sized forms of PDE are present, myoblasts are unusual in having primarily a single type, the type IV or low-K_m, cAMP-specific PDE (Ball et al., 1979, 1980; Narindrasorasak et al., 1982). This makes myoblasts a particularly good system in which to study the control of this type of PDE activity. Regulation of PDE activity in response to elevated cAMP was found to occur via two distinct mechanisms: a rapid short term activation that probably involves phosphorylation and a slower long term activation (Ball et al., 1979, 1980). The long term rise in PDE activity was inhibited by cycloheximide and actinomycin D, indicating that transcriptional effects were involved. Recently, we have identified a type IV isozyme, PDE4D1, in L6 myoblasts that is induced by cAMP treatment of the cells (Kovala et al., 1994). A more detailed study of this enzyme required purification and kinetic characterization. To avoid the problems posed by the low abundance of the protein, we have used expression in *Escherichia coli*. In this report, we describe the bacterial expression, purification, and characterization of PDE4D1, and some deletion mutants.

EXPERIMENTAL PROCEDURES

RT-PCR Cloning and Sequencing. Polyadenylated mRNA was purified from L6 myoblasts using Fast Track mRNA isolation kits (Invitrogen Corp., San Diego, CA) according to the manufacturer's instructions. The day 3 myoblasts had first been treated for 16 h with dibutyryl cAMP and MIX. cDNA was synthesized using Moloney Murine Leukemia Virus reverse transcriptase and 400 pmol of random hexanucleotide primers in PCR buffer [10 mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% gelatin, and 1% Triton X-100] with each dNTP (1mM) in a final volume of 20 μL. For PCRs, the first-strand cDNA reaction mixtures were diluted to 100 μL with PCR buffer containing the appropriate primers. Three primers were designed as consensus sequences on the basis of conserved regions within known PDEs. The sequences of these homology-based primers were 5′-CAAAGCTTGCATGCCTGCAGGACTCTAGAAGACCACTACCA (H1), 5′-GGCGAATTCGAGCTCGGTACCATGTAGTCGATGAAGCCCACCTG (H2), and 5′-TCGCCCGGGTCGACCATGCTGACCCGGGAGCTCACACACCT (H3). The first two consensus primers are derived from highly conserved regions within the cyclic nucleotide PDE catalytic domain (Chen et al., 1986; Swinnen et al., 1989a), and the third is derived from a conserved region found only in type IV PDEs. The PCR product produced with primers H1 and H2 (clone E) which has been used previously as a probe in Northern blots (Kovala et al., 1994) was used here to raise anti-PDE antibodies (see below). Two additional primers that specifically matched the 5′ and 3′ ends of rat PDE4D1 and included the start and the stop codons were also used [5′-TCGCCCGGGTCGACCATGAAGGAGCAGCCCTCATGT (P5) and 5′- TGCGCATGCAAGCTTACACTGTTACGTGTCAGGAC (P3) respectively]. All the primers contained restriction sites added to the 5′ ends. The PCR cycle consisted of denaturation for 30 s at 95 °C, annealing for 30 s at 45 °C, and extension for 1 min at 72 °C, and was repeated for 45 cycles. The amplified sequences were subcloned into pGEM3Zf+ (Promega, Madison, WI). Single-stranded DNA generated with R408 helper phage was sequenced with Sequenase 2.0 (United States Biochemical, Cleveland, OH).

Initial RT-PCR cloning used primers H1 and H2. Both primers matched at a minimum of 17 out of 21 bases the known rat PDEs. A cDNA clone (clone E) of approximately 700 bp was produced and found to have a sequence nearly identical to PDE4D1 [rat PDE 3.1 (Swinnen et al., 1989a)]. Each of the primers (H1 and H2) introduces single-base pair changes which had no effect on the amino acid sequence. Clone E also contained a single substitution due to error introduced during PCR, G1341A, which again had no effect on the amino acid residue present at this position. The third consensus-based primer (H3), which was used in the production of clone D, introduces four sequence changes into the clone: T414G, A416C, T417C, and C429A. The combination of changes at positions 416 and 417 produced an amino acid substitution (N98T), while the other substitutions were silent.

Both combinations of consensus sequence primers (H1 and H2, and H3 and H2) led to the amplification of parts of PDE4D1 mRNA. Therefore, two additional primers, P5 and P3, were designed which exactly matched, respectively, the 5′ and the 3′ ends of the PDE4D1 open reading frame. Primers P5 and H2 were used to produce the 1.4 kb clone A, and primers H3 and P3 were used to create the 1.5 kb clone D (Figure 1). Sequencing demonstrated that both were nearly identical to the published sequence of PDE4D1. In clone A, two changes in the base composition were found, a silent C to G substitution at position 435 (C435G) and a T1403A substitution which results in a F418T change in the amino acid. In clone D, there were also two substitutions, T1277C and A1756T, producing L376P and T536S mutations, respectively. The full length rPDE4D1 clone was constructed by ligating together the 5′ end of clone A and the 3′ end of clone D using a *Bam*HI site at position 1301. This left only the conservative T536S substitution. In the equivalent position of other type IV PDEs, both threonine and serine are found, along with alanine and asparagine, suggesting that the threonine is not vital.

Production of Antibodies to PDE4D1. Clone E (Kovala et al., 1994) was inserted into the *Hin*d III site of the bacterial expression vector pATH-10 (generously provide by P. Greer) to create a TrpE−PDE fusion protein (Koerner et al., 1991). Inclusion bodies were isolated, and the protein was solubilized in Laemmli sample buffer (Laemmli, 1970). After SDS−PAGE, the protein was visualized with 4 M sodium acetate, excised, and eluted from the gel (Harlow & Lane, 1988). The fusion protein was then mixed with Hunter's Titer Max (CytRx Corp., Atlanta) and injected into rabbits.

Construction of GST-rPDE4D1 Fusion Clones. The bacterial expression vector pGEX-KG (Guan & Dixon, 1991) was used for the creation of GST−PDE fusion clones. Clones A and D were first ligated into pGEX-KG separately. Clone D encodes amino acids 87−584 of PDE4D1. In both

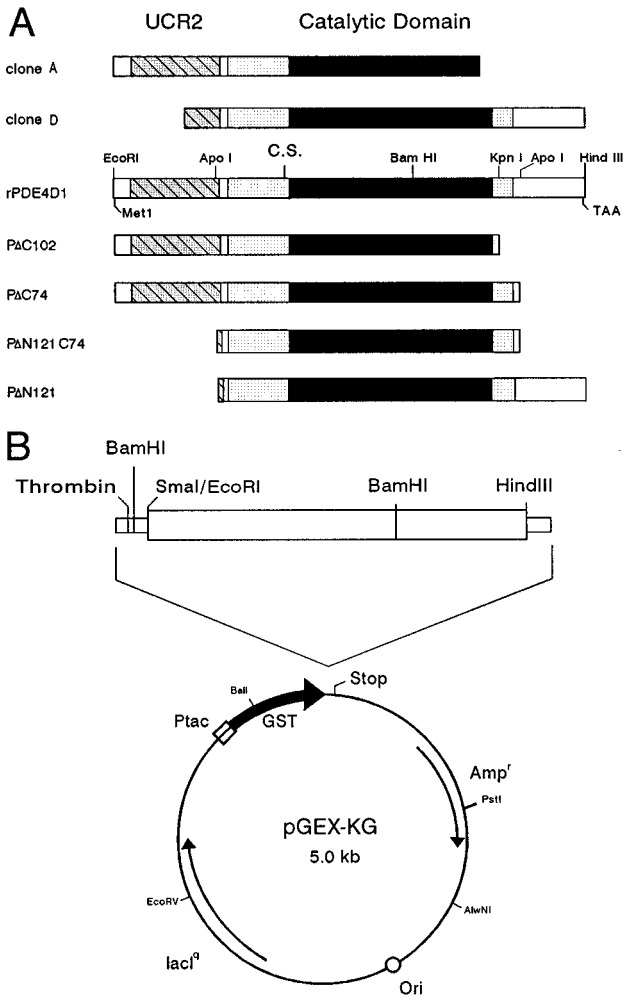

FIGURE 1: Creation of full length and deletion clones of rPDE4D1. The top two diagrams (clones A and D) represent the PCR products from which a full length cDNA clone (rPDE4D1) was constructed using the *Bam*HI site. The locations of restriction sites used and of the proteolytic cleavage site (C.S.) are indicated on the full length clone. The first methionine (Met1) and the termination codon (TAA) are marked. Below rPDE4D1 are the deletion clones produced with the appropriate restriction sites. The location of UCR2 is indicated by the striped area. The catalytic domain is shown in black, with the conserved type IV PDE extensions on either end indicated by the stippled area. Below is shown the pGEX-KG expression vector with the rPDE4D1 insert.

clones, a *Sma*I site introduced by the 5′ PCR primers was ligated into a blunted *Eco*RI site. Directional cloning was provided by restriction sites incorporated into the 3′ PCR primers, a *Sac*I site in clone A and a *Hin*dIII site in clone D. In order to create a full length clone (named rPDE4D1), the 5′ *Bam*HI fragment of pKG-D was replaced by the 5′ *Bam*HI fragment of pKG-A. Clones were screened by restriction mapping and by monitoring expression of fusion proteins on SDS−PAGE. After cleavage by thrombin, this construction results in the full length protein with a 15-amino acid N-terminal extension derived from the vector (sequence GSPGISGGGGGIGST).

Deletion clones were constructed from full length rPDE4D1 (see Figure 1); clone names are based on the amino acid residues deleted and their location (N- or C-terminal end). To make PΔC102, clone rPDE4D1 was first digested with *Kpn*I and *Hin*dIII and treated with Klenow, and the large fragment was religated. In this plasmid, codon 482 is followed by a termination signal. The PΔN121-C74 clone was produced by inserting the central *Apo*I fragment (which contains the conserved catalytic domain) into the *Eco*RI site of pGEX-KG, resulting in a truncated protein (amino acids 122−510, codon 511 is a terminator). PΔC74 was produced by an incomplete digestion of rPDE4D1 with *Apo*I such that the *Eco*RI/*Sma*I site used originally to insert the clone A and the second internal *Apo*I site were cut. This left the first internal *Apo*I site, which had been used in PΔN121-C74, untouched. The PΔN121 clone was made by replacing the *Bam*HI fragment from PΔN121-C74 with the *Bam*HI fragment from rPDE4D1. Both clones were cut at the internal *Bam*HI site and a site located in the vector, allowing the addition of the 3′ end from the rPDE4D1 clone to PΔN121-C74. Clones truncated at N121 gave an 11-amino acid N-terminal extension (GSPGISGGGGG) derived from the vector; others had the 15-amino acid extension found in the full length rPDE4D1.

Purification of the GST−rPDE4D1 Fusion Protein. Small (10 mL) overnight cultures of the *E. coli* strain RR1 containing the expression vector were diluted 10-fold in 2X YT medium and incubated for 1 h at 37 °C. Following a shift to room temperature, IPTG was added to a final concentration of 1 mM and the incubation was continued for 3 h. Cells were harvested with a 10 min centrifugation at 3500*g* and resuspended in 1 mL of TE (pH 8), to which was added 10 mM β-mercaptoethanol, 0.2 mM TSF, 20 μM TLCK, 0.1 mM TPCK, 8 μg/mL leupeptin, and 8 μg/mL pepstatin A. Proteins were isolated as described (Ball et al., 1995). Briefly, cells were lysed by sonication (2 × 30 s at setting 5) and diluted 4-fold with TE containing 10 mM βME before centrifugation for 20 min at 12500*g*. NaCl was added to the supernatant to a final concentration of 0.15 M and Triton X-100 to 1%, after which it was incubated for 30 min at room temperature with 10 mL of glutathione-Sepharose beads (Pharmacia) in a 50 mL tube. The beads were washed four times with 40 mL of TE containing 10 mM βME and 0.1% Triton X-100 and then split into two 15 mL tubes and washed twice with 8 mL of thrombin cleavage buffer (TBS containing 2.5 mM $CaCl_2$, 10 mM βME, 0.05% Triton X-100, and 10% glycerol). The beads were then resuspended in 3 mL of cleavage buffer containing 4 μg/mL thrombin and incubated at room temperature for 30 min. Supernatant was recovered after a brief centrifugation; the beads were washed with another 3 mL of buffer, and the supernatant was pooled with the initial elutions. Aliquots were stored at −70 °C. For the recovery of complete, uncleaved fusion protein, an elution buffer [50 mM Tris (pH 7.5), 0.2 M NaCl, 0.1 mM EDTA, 0.05% Triton X-100, 10 mM βME, and 10% glycerol) containing 5 mM glutathione was used.

Protein Determination, SDS−PAGE, and Western Blotting. Protein was quantified using the Peterson modification (Peterson, 1983) of the Lowry assay (Lowry et al., 1951). Samples of protein were run on 8.5% SDS−polyacrylamide gels as described by Laemmli (1970). The molecular mass markers used were fibronectin (240 and/or 220 kDa), myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (94 kDa), bovine serum albumin (68 kDa), catalase (60 kDa), ovalbumin (43 kDa), lactate dehydrogenase (34 kDa), and carbonic anhydrase (30 kDa). Proteins were blotted onto Immobilon-P membranes (Millipore) using a Bio-Rad Trans-blot apparatus. Blots were stained with 0.0025% amido black in a 50% methanol and 10% acetic acid solution,

blocked for 1 h with 4% casein in TBS, and then washed three times with wash buffer (TBS, 0.5% triton X-100, 0.1% SDS, and 0.5 mM EDTA). The anti-rPDE antibody was diluted 1/1000 in incubation buffer (1% casein in TBS) and incubated with the blot for 1 h. Following three washes as above, the secondary antibody was added for 1 h. Peroxidase-conjugated goat anti-rabbit antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD) were used as the second antibody. After washing as above, the blot was incubated with chemiluminescent substrates according to the manufacturer's instructions. Kodak XAR-5 film was used to visualize the blots.

To remove pre-existing rabbit antibodies to bacterial proteins, a 200 mL overnight culture of *E. coli* strain RR1 containing the pGEX-KG vector was centrifuged and resuspended in 5 mL of TE at pH 8. Following sonication (2 × 45 s at setting 5), SDS was added to a final concentration of 0.1% and the sample boiled for 2 min. For preabsorption, 10 μL of rabbit anti-PDE antibody was incubated with 1 mL of the bacterial lysate in a total volume of 10 mL of antibody incubation buffer overnight at 4 °C. The insoluble fraction was removed by centrifugation and the supernatant used directly for probing western blots.

PDE Assays, Inhibitor Studies, and Metal Ion Dependence. Enzyme assays were performed as previously described (Ball et al., 1979). Samples of either bacterial lysates or the purified PDEs were diluted to ensure that the kinetics remained in the linear range and that no more than 20% of the substrate was consumed. To 100 μL sample aliquots was added 100 μL of 2X reaction mix [0.05 μM [^{3}H]-cAMP, 2 μM cAMP, 20 mM $MgCl_2$, 0.3 mg/ml bovine serum albumin, and 100 mM Tris-HCl, (pH 8.0)]. The reaction mixture was incubated at 30 °C for appropriate time periods and the reaction stopped by boiling for 1 min. Ten microliters of 2.5 mg/mL snake venom (as a source of 5′-nucleotidase) was added, followed by a further incubation of 10 min at 30 °C. One milliliter of BioRad AG-1X2 resin (as a 1/3 slurry with H_2O) containing 0.1 mM adenosine as a carrier was added to each sample. Samples were vortexed and centrifuged briefly in a microcentrifuge. Supernatant (0.5 mL) was removed and added to 10 mL of scintillation fluid for scintillation counting. Blank values were obtained using buffer alone. For inhibition studies, the assays were performed as above except that the concentration of cAMP used in the assay was varied. The inhibitors RO 20-1724 and rolipram were prepared as 50-fold concentrated stocks in DMSO, and DMSO was added to a final concentration of 2% (v/v) in all samples assayed.

Sephacryl S-300 Column Chromatography. Two milliliters of purified protein was loaded on a 1.6 × 48 cm column of Sephacryl S-300. The buffer that was used consisted of 20 mM Tris-HCl (pH 8), 1 mM β-mercaptoethanol, 50 mM NaCl, and 10% glycerol. Fractions of 1.8 mL were collected and assayed for PDE activity. The molecular mass markers used were blue dextran (2 × 10^6 Da), ferritin (440 kDa), catalase (232 kDa), alcohol dehydrogenase (150 kDa), and cytochrome *c* (12.4 kDa).

RESULTS

Purification of rPDE4D1. PDE activity induced by IPTG in plasmid-carrying bacteria was first examined in the soluble fraction of bacterial lysates. Expression of clone A gave no detectable PDE activity beyond that of *E. coli* (0.54 ± 0.06 vs 0.47 ± 0.04 pmol min^{-1} μg^{-1}). In contrast, both clone D and rPDE4D1 fusion protein exhibited up to 5−7-fold increases in PDE activity (2.33 ± 0.24 and 3.66 ± 0.18 pmol min^{-1} μg^{-1}, respectively). The induction of full length GST−rPDE4D1 at 37 °C gave mainly insoluble protein, but at 22 °C, the majority of the fusion protein was soluble (data not shown).

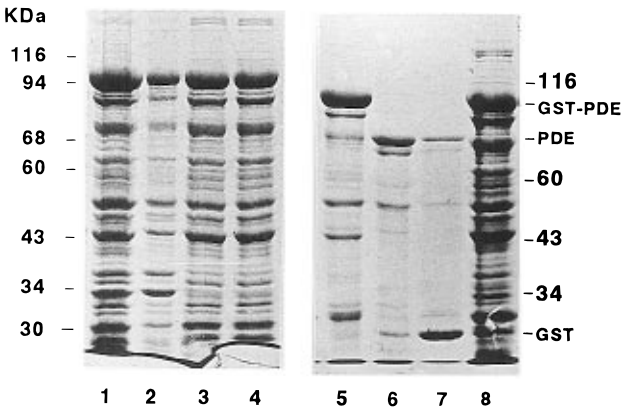

FIGURE 2: Purification of bacterially expressed rPDE4D1. SDS−PAGE analysis of the various fractions from the purification of a GST−rPDE4D1 fusion protein from *E. coli* using glutathione-sepharose beads. The lanes are (1) total cell lysate, (2) insoluble pellet, (3) soluble fraction, (4) supernatant following fusion protein absorption, (5) proteins bound to beads, (6) thrombin-eluted rPDE4D1, (7) beads after elution, and (8) total lysate.

Table 1: Purification of rPDE4D1

fraction	total protein (mg)	specific activity[a] [pmol min^{-1} (μg of protein)$^{-1}$]	yield (%)
lysate	100.5	0.91	100.0
soluble	74.4	0.57	46.1
pellet	33.6	0.22	8.1
thrombin elution	0.15	78.4	12.8

[a] Specific activity was determined with 5 μM cAMP.

The full length rPDE4D1 was purified using glutathione affinity chromatography (shown in Figure 2). A substantial portion of the fusion protein (M_r = 98 000 Da) remains in the insoluble pellet (lane 2) even when the cells are grown at 22 °C. However, this represents less than 10% of the total PDE activity (Table 1), suggesting that most of this insoluble PDE is inactive, probably due to its precipitation in inclusion bodies. Only a small proportion of the GST−PDE was bound to the glutathione-Sepharose (compare lanes 3 and 4 in Figure 2), but this was not due to the inactivity of the rPDE4D1 left in the supernatant (0.63 pmol min^{-1} μg^{-1}) which is virtually identical to that of the initial soluble fraction. Thrombin cleavage and elution of the full length rPDE4D1 from glutathione-Sepharose beads gives one major band of 73 000 Da and several minor bands on SDS−PAGE (lane 6 of Figure 2). Among the minor bands, one migrated with the cleaved GST. A 50 000 Da M_r band was present in variable amounts when the GST−rPDE4D1 fusion protein was cleaved with thrombin or when eluted using 5 mM glutathione (data not shown). This indicated that the 50 kDa band was not simply due to thrombin cleavage of rPDE4D1. Both the 50 and 73 kDa bands were recognized on Western blots by the anti-PDE antibody (see below). To identify these bands, N-terminal amino acid sequencing of the blotted proteins was undertaken. The major 73 kDa band was found

to be intact rPDE4D1, whereas the N-terminal sequence of the 50 kDa band matched nine internal residues (XLKT-FKIPVD) in rPDE4D1, starting at L210, indicating that this band is due to proteolytic cleavage. The conserved catalytic domain common to all PDEs begins at K214, raising the possibility that the fragment was catalytically active. However, comparison of various preparations in which the proportion of 50 kDa band differed showed that the specific activity decreased as the fraction of the total protein contributed by this band increased (data not shown). Yields of rPDE4D1 from 200 mL cultures of *E. coli* harvested and run on 5 mL of glutathione-*S*-sepharose columns were in the 0.15−0.5 mg range. The rPDE4D1 band generally represented 60−80% of the protein in the preparations, determined by densitometric scanning of stained gels. Elution with glutathione gave a higher yield (but of the fusion protein), perhaps due to the tendency of the PDE to aggregate without the GST portion (see below). A comparison of the GST fusion protein with the thrombin-released rPDE4D1 revealed no kinetic differences, nor did thrombin treatment affect the activity of the fusion rPDE4D1. The thrombin-released nonfusion protein was used for further studies.

Characterization of the Full Length rPDE4D1. The Type IV PDEs are by definition susceptible to inhibition by rolipram and RO 20-1724. We therefore used these type IV-specific inhibitors and the nonspecific PDE inhibitor MIX to characterize the purified rPDE4D1. The concentration dependence for these inhibitors is shown in panel A of Figure 3. IC_{50} values derived from these studies were 0.4 μM for rolipram, 2 μM for RO 20-1724, and 40 μM for MIX. In panel B of Figure 3, the Lineweaver−Burke plot for RO 20-1724 is indicative of competitive inhibition. A K_i value of 1.8 ± 0.4 μM for RO 20-1724 was derived from Dixon plots (panel C).

Effects of Divalent Cations on PDE Activity. PDEs require divalent cations to catalyze the hydrolysis of the cAMP or cGMP. In fact, the type V cGMP-specific PDE has been proposed to be a zinc hydrolase due to its ability to bind Zn^{2+} and because Zn^{2+} can support PDE activity (Francis et al., 1994). We have therefore examined the effect of zinc and several other common divalent cations on the PDE activity of rPDE4D1 (Figure 4).

The standard conditions for the assay of PDE include 10 mM $MgCl_2$. In assays containing various concentrations of Mg^{2+}, it was found that 200 μM was sufficient to produce an 11-fold increase in activity over the levels seen in the absence of any cations (Figure 4A). Both Mn^{2+} and Co^{2+} support the catalytic activity of the type III (cGMP-inhibited) PDE (Pillai et al., 1994) and the type V (cGMP-specific) PDE (Francis et al., 1994); therefore, the effect of these cations on rPDE4D1 activity was tested. With Mn^{2+}, it was found that 15 μM produced a 20-fold increase in activity. In the case of Co^{2+}, 20 μM produced a 15-fold increase in activity (Figure 4B). Both Mn^{2+} and Co^{2+} proved to support PDE activity in rPDE4D1 better than the Mg^{2+} used in standard assays, producing similar or greater activations at concentrations that are 1 order of magnitude lower. No effect on PDE activity was detected with Zn^{2+} concentrations ranging from 0.01 to 30 μM (Figure 4C).

Deletion Analysis of rPDE4D1. The constructed deletion mutants were expressed, purified, and thrombin-released in the same fashion as the full length protein (Figure 5A). They produced proteins with similar yields except for the PΔC102

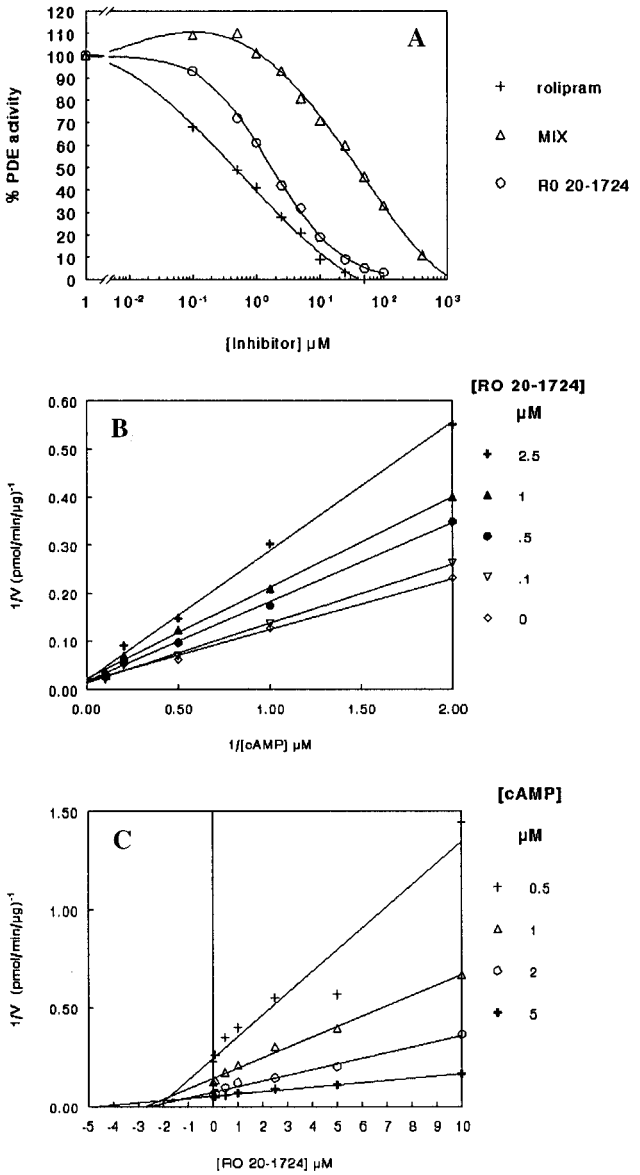

FIGURE 3: Characterization of the purified rPDE4D1. Panel A shows the inhibition curves for rPDE4D1. IC_{50} values at a concentration of 1 μM cAMP were determined from the inhibition curves for the specific type IV PDE inhibitors rolipram (IC_{50} = 0.4 μM) and RO 20-1724 (IC_{50} = 2 μM) and the nonspecific inhibitor MIX (IC_{50} = 40 μM). Panel B shows the Lineweaver−Burke plot for rPDE4D1 with various concentrations of RO 20-1724. Panel C shows a Dixon plot for RO 20-1724 from which a K_i value of 1.8 ± 0.4 μM was derived.

clone (lane 7) which gave a largely insoluble protein. The apparent M_r's of the expressed proteins reveal an interesting pattern. Proteins that include the C-terminal region migrated more slowly than predicted, giving apparent relative molecular masses 6−9000 kDa higher (e.g. PΔN121, predicted 53 000 Da, actual 62 000 Da; full length, predicted 67 000 Da, actual 73 000 Da). C-terminal deletions, on the other hand, migrate virtually as predicted. This may be explained by the concentration of acidic amino acid residues in the C-terminal area (31 of the last 102 residues are Glu or Asp); that often leads to slower migration in SDS−PAGE (Takano et al., 1988; Bryan, 1989; Hayashi et al., 1989).

Some contaminating bands are present. In particular, a 50 kDa band is evident in lanes 3−5. This band was also recognized by anti-PDE antibody (Figure 5B) and corresponds to the C-terminal proteolytic cleavage product orig-

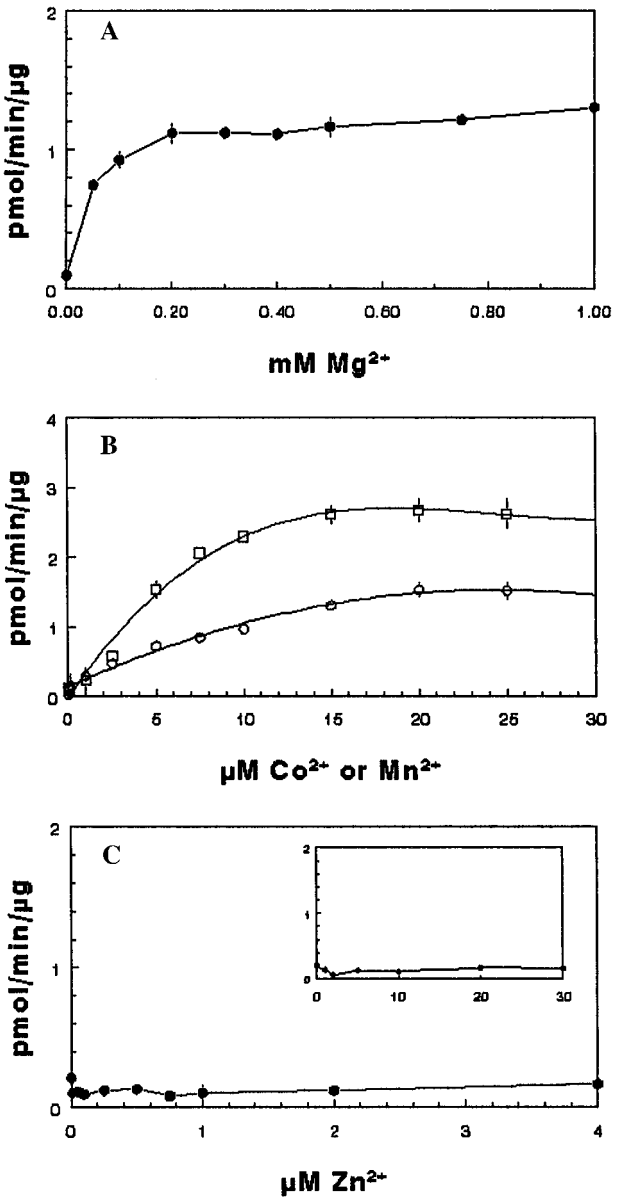

FIGURE 4: Effect of metal ions on PDE activity. Panel A shows the concentration dependence of Mg^{2+} effects on PDE activity. In panel B, the effects of Co^{2+} (○) and Mn^{2+} (□) are compared. The lack of effect on PDE activity by various concentrations of Zn^{2+} is shown in panel C. The inset in panel C has the same axis units as the main figure.

inally identified in the purification of the full length rPDE4D1. As expected, this band is absent in those clones producing PDE fragments in which the final 74 amino acids (residues 511−584) of the C-terminal region of the protein have been deleted, namely PΔN121-ΔC74, PΔC74, and PΔC102 (lanes 2, 6, and 7, respectively). Since the 50 kDa band lacks N-terminal residues 1−209, the copurification of this PDE fragment with the full length rPDE4D1 is likely due to binding in either the catalytic domain or the C-terminal region. The absence of a second band equivalent to the 50 kDa band in those PDEs which lack the C-terminal 75 residues suggests that a sequence in this C-terminal region is responsible for the association.

In order to further examine the effect of the deletions on quaternary structure, the complete GST−rPDE4D1, the thrombin-cleaved rPDE4D1, and thrombin-treated deletion proteins were examined by Sephacryl S-300 column chro-

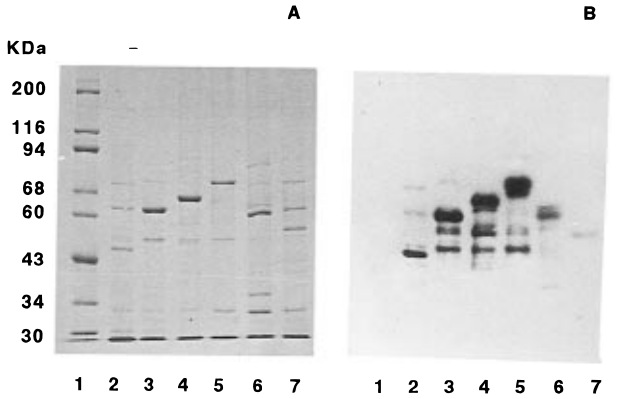

FIGURE 5: SDS−PAGE and Western blot analysis of full length and deletion clones. Panel A shows a Coomassie blue-stained SDS−PAGE gel, while panel B shows a similar gel that has been blotted and probed with an anti-PDE antibody. The lanes in both panels and predicted M_r's are (1) molecular mass markers, (2) PΔN121-C74 (44 000), (3) PΔN121 (53 000), (4) clone D (58 000), (5) rPDE4D1 (67 000), (6) PΔC74 (59 000), and (7) PΔC102 (56 000). Approximately 1.5 µg of protein per lane was loaded.

matography (Figure 6). The fusion protein GST−PDE eluted at an apparent size of 360 kDa compared to the standards, while the thrombin-cleaved rPDE4D1 had an apparent M_r in excess of 2×10^6 Da. Aggregation of PDE has been noted before in the examination of PDE in extracts from both skeletal muscle myoblasts and adult muscle (Narindrasorasak et al., 1982). The GST portion of the fusion protein appears to be able to inhibit this aggregation. The expressed protein from clone D had an apparent M_r of 240 kDa, indicating a multimeric structure. Removal of the N-terminal region in PΔN121 gave an M_r of 120 kDa, suggesting a dimer. Hence, the region between residues 87 and 120 that is deleted from clone D to make PΔN121 is likely responsible, at least in part, for some homophilic interactions. The proteins expressed from clones with C-terminal deletions (PΔC74, PΔN121-ΔC74, and PΔC102) all migrated in the 60−80 000 Da range, consistent with a monomeric size, supporting the presence of a C-terminal site that also participates in quaternary structure.

Effects of Deletions on PDE Activity. The effects of the deletions on enzyme activity were also examined (Figure 7). The full length rPDE4D1 protein had a specific activity of 48.1 (±1.0) pmol min^{-1} (µg of protein)$^{-1}$. Two PDE fragments lacking the C-terminal region (PΔC102 and PΔC74) demonstrated reduced PDE activity (3.7 ± 1.2 and 14.9 ± 0.4 pmol min^{-1} μg^{-1}, respectively). The larger the deletion, the less activity present. Deletions at the N-terminal end had a different effect. Clone D showed moderately decreased activity (28.5 ± 3.4 pmol min^{-1} μg^{-1}); however, it should be noted that this clone also had a mutation in the catalytic domain. A deletion of the N-terminal 121 amino acids gave a dramatic 6-fold increase in specific activity. In PΔN121, the PDE activity was 260 ± 83 pmol min^{-1} μg^{-1}, and in PΔN121-ΔC74, it was 334 ± 102 pmol min^{-1} μg^{-1}. To determine whether this was due to changes in the K_m, $V_{max,}$ or both, the kinetics were examined (Table 2). It is clear that most of the increased activity was due to a 4-fold increase in the V_{max} associated with the N-terminal deletion. PΔN121-C74 and PΔN121 had V_{max} values of 1360 ± 21 and 1325 ± 266 pmol min^{-1} μg^{-1}, respectively, while rPDE4D1 had a V_{max} of 318 ± 18 pmol/min/µg. Both clone D and PΔC102 had V_{max} values that were less than half of

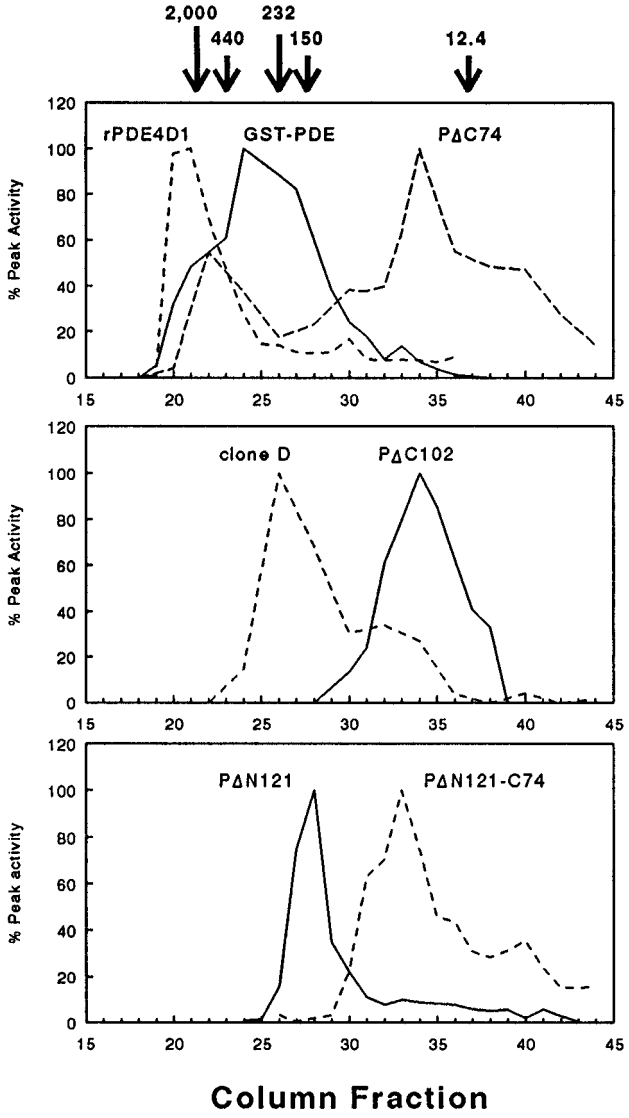

FIGURE 6: S-300 sizing column profiles. The PDE activities of fractions from a Sephacryl S-300 column are shown for the full length and truncated PDEs, all thrombin-treated except for GST−PDE. Activities are expressed as a percentage of the peak activity in each run. Elution of standards and their sizes in kilodaltons are indicated by arrows at the top of the diagram.

that of rPDE4D1 (156 ± 4 and 146 ±18 pmol min^{-1} μg^{-1}, respectively). A slight decrease in K_m with the N-terminal deletions was also evident.

DISCUSSION

In examining the regulation of cAMP levels in L6 rat skeletal myoblasts, we had demonstrated that PDE activity was regulated by two separate mechanisms, one involving a protein kinase A-mediated increase in the transcription rates for a type IV, cAMP-specific PDE known as PDE4D1 (Kovala et al., 1994). Using RT-PCR, we have now cloned and expressed as a GST fusion protein the complete coding sequence for this PDE in bacteria. This is the first report of a recombinant PDE to be purified from bacteria and obtained in relatively large quantities [although PDE4D1 has been expressed before as a nonfusion protein in *E. coli* (Swinnen et al., 1989b; Jin et al., 1992) and many PDE4s have been expressed in eukaryotic cells, e.g. Livi et al. (1990), Torphy et al. (1992), Bates et al. (1993), Bolger et al. (1993), and Monaco et al. (1994)].

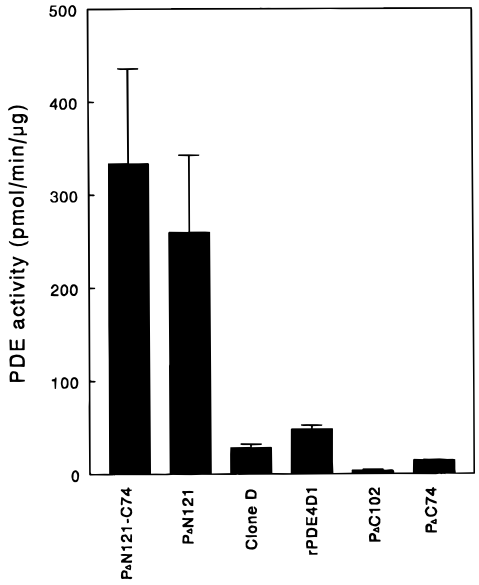

FIGURE 7: PDE activity of the deletion clones. The PDE activity of the purified PDE truncations was determined at 1 μM cAMP in standard assays. Results are the mean of three to five determinations, each in triplicate (±SD), corrected for protein impurities by scanning densitometry of SDS gels.

Table 2: Kinetics of Deletion Clones

clone	V_{max} (pmol min^{-1} μg^{-1})[a]	K_m (μM)
PΔN121-C74	1360 (±20)	1.8 (±0.3)
PΔN121	1325 (±270)	2.8 (±0.5)
clone D	156 (±4)	3.9 (±0.1)
rPDE4D1	318 (±18)	5.2 (±0.1)
PΔC74	146 (±18)	3.2 (±0.5)

[a] Corrected for the proportion of impurities present as measured by scanning of stained SDS gels.

Expression of PDE4D1 and the deletion mutants as GST fusion proteins allowed a rapid and simple method of purification. The actual purity achieved in individual preparations was found to vary, with the major band representing from 30 to 80% of total protein. The major contaminant was identified as a C-terminal 50 kDa fragment presumably produced by proteolytic cleavage in the bacteria. [This band is likely identical to the prominent contaminant seen in the unpurified enzyme by Jin et al., (1992).] Our amino acid sequence data showed it to lack both the UCR 2 and the type IV extension to the catalytic domain. This fragment was strongly associated with the full length rPDE, and attempts to remove it by several chromatographic methods were not successful (data not shown). Although we could not completely remove this fragment, indirect evidence indicated that it was not active and would not interfere with kinetic measurements, as also found by Jin et al. (1992). The yield of purified material (~0.7 mg per liter of culture) is low by some standards but high enough for further characterization and some types of physical studies.

The expressed rPDE4D1 has kinetic characteristics that are typical for members of its class with K_m values in the 2−5 μM range (Colicelli et al., 1989; Swinnen et al., 1989a,b; Henkel-Tigges & Davis, 1990; Jin et al., 1992; Pillai et al., 1993). V_{max} values are more difficult to compare since only a few PDEs have been purified. Partial purification of a rabbit muscle type IV PDE (Narindrasorasak & Sanwal, 1986) gave a V_{max} of 39.1 pmol min^{-1} μg^{-1}, 8-fold lower

than the 318 pmol min^{-1} μg^{-1} observed with rPDE4D1. In the purification of a type IV PDE from postmeiotic male mouse germ cells, a 43 kDa protein with a K_m of 3.3 μM and a very high V_{max} of 10 500 pmol min^{-1} μg^{-1} was isolated (Giorgi et al., 1992). The size of this PDE suggests that it is most likely an active proteolytic fragment, comparable to the 46 kDa protein produced by PΔN121-C74. In the above reports, the actual identity of the PDEs was unknown. A recombinant PDE4A enzyme purified to homogeneity from yeast gave a V_{max} of 800 pmol min^{-1} μg^{-1} (Wilson et al., 1994), comparable to that of the PDE4D1. A type IV PDE purified from Sertoli cells to roughly 20% purity showed a V_{max} of 1000−2000 pmol min^{-1} mg^{-1} (Conti et al., 1995a). The half-maximal inhibition of rPDE4D1 by the type IV-specific inhibitors rolipram (IC_{50} = 0.4 μM) and RO 20-1724 (IC_{50} = 2 μM) was nearly identical to the concentrations reported for PDE4D1 measured in bacterial lysates (Swinnen et al., 1989b; Jin et al., 1992). Higher concentrations are typically required by the nonspecific PDE inhibitor MIX (IC_{50} = 40 μM). Hence, our purified rPDE4D1 matches the known characteristics of type IV PDEs in those aspects that have been tested.

Recently, it has been found that the type V cGMP-specific PDE (cG-BPDE) binds tightly to 2 mol of Zn^{2+} per monomer of PDE. At concentrations from 0.05 to 1 μM, it was found that Zn^{2+} could also support catalytic activity, while further concentration increases proved to be inhibitory (Francis et al., 1994). On this basis, and the presence within the catalytic domain of two copies of a possible Zn^{2+} binding motif ($Hx_3Hx_{20-120}E$) also found in thermolysin and other Zn^{2+} metalloproteases [reviewed in Vallee and Auld (1990a,b) Jiang and Bond (1992)], it was proposed that cG-BPDE was a zinc hydrolase (Francis et al., 1994). Because these two Zn^{2+} binding motifs are conserved in all known mammalian PDEs, it was suggested that this could apply to all PDEs. Although we have not directly examined the ability of this motif to bind Zn^{2+} in rPDE4D1, we found that the metal has no effect on activity, suggesting that this enzyme is not a zinc hydrolase. It remains possible that the protein may bind zinc very tightly and retain it during the isolation procedure. If so, a second cation is still required for PDE activity.

Metal ion preferences among the PDE families, while not well-understood, appear to be quite variable. Comparison of the sensitivity of rPDE4D1 with those of other types of PDE shows large differences. For cG-BPDE, the preference is similar (Mn^{2+} > Co^{2+} ≫ Mg^{2+}), but it is about 20-fold more sensitive to Mn^{2+} and 10-fold more sensitive to Mg^{2+} than rPDE4D1 (Francis et al., 1994). The metal requirements for the type III cGI-PDE have been examined by (Pillai et al. (1994), who found that activation by Co^{2+} is poor and that 100 mM Mg^{2+} or 0.3 mM Mn^{2+} is required for maximum activation, much higher than those needed for either rPDE4D1 or cG-BPDE. A pure PDE4A enzyme preferred Mg^{2+} over Mn^{2+} (Wilson et al., 1994). Clearly, the divalent cation requirements of each PDE differ substantially in spite of the similarities in sequence, substrate, and presumably reaction mechanism, suggesting that the cations play an auxiliary role.

Deletions of the cloned PDE4D1 allowed analysis of some possible functions of regions outside the catalytic domain. The strong association of the 50 kDa proteolytic cleavage product with the full length rPDE through purification by glutathione-Sepharose beads was initially noted. As this 50 kDa protein was lacking the N-terminal 213 residues, the binding domain had to reside in the remaining C-terminal portion of the PDE. SDS−PAGE analysis of the series of deletion mutants demonstrated that the 50 kDa protein appeared only in those deletion proteins which retained the C-terminal 75 residues. This strongly suggested that the C-terminal region is involved in the self-association of rPDE4D1. In fact, a dramatic change in the native size of the mutant proteins was evident with the deletion of the C-terminal 75 amino acids. While PΔN121 and clone D eluted with apparent sizes of approximately 120 000 and 240 000 Da, respectively, the products of the three clones with C-terminal deletions (PΔC74, PΔN121-C74, and PΔC102) all appeared in the 60−70000 Da range. These changes in the elution profiles probably reflect a change from a multimeric to a monomeric protein with the deletion of the C-terminal 75 residues. The elution of PΔN121 at 120 000 Da suggests a dimeric form, while clone D elutes at 240 kDa, suggesting a tetrameric structure. The larger native size of clone D may be due either to a second site responsible for quaternary structure in the N-terminal portion of the PDE or a less specific aggregation. In fact, the observation that the presence of the GST at the N-terminal region prevents aggregation (apparent M_r of 360 000 Da vs 2×10^6 Da for rPDE4D1) supports the possibility of a role for the N-terminal region in forming large aggregates. However, aggregation is not seen in those mutant PDEs that retain the N-terminal region but lack the C-terminal region, suggesting a weak interaction between the N- and C-terminal regions to produce very large complexes. Native PDEs have been found to exhibit a range of sizes on gel filtration, some of which have been attributed to artifacts (Ball et al., 1979; Narindrasorasak et al., 1982; DiSanto & Heaslip, 1993), but others may have functional significance.

The effects of the deletions on PDE activity shed some light on how the enzyme is regulated. The C-terminal deletions resulted simply in decreases in PDE activity. In contrast, deletion of the first 121 amino acids either alone (PΔN121) or in the context of a C-terminal deletion (PΔN121-C74) produced a 6-fold increase in PDE activity. An effect of the short N-terminal extensions derived from the vector cannot be completely excluded but seems unlikely. For instance, a similar phenomenon was noted by Jin et al., (1992) in a 97-amino acid internal deletion (residues 45−145) in PDE4D1 that produced an apparent 2-fold increase in activity, although the measurements were carried out with crude enzyme in bacterial lysates. These activating deletions affect the 80-amino acid UCR 2 domain (residues 46−126). Increased specific activity was not evident in clone D (which lacks the first half of UCR 2), limiting the inhibitory region to the second half of the UCR 2, but the presence of a mutation in the catalytic domain of this protein makes the evidence ambiguous. Nonetheless, it is clear that the UCR 2 area or parts of it are responsible for decreasing the specific activity of the enzyme.

Much evidence from other PDEs also points to the presence of regulatory sequences in the N-terminal regions (Conti et al., 1995b; Manganiello et al., 1995; Sonnenburg et al., 1996), and an inhibitory N-terminal region has been described in a PDE4A (Shakur et al., 1995). If the UCR 2 represents an inhibitory domain, then different types of regulation would depend on different means of relieving the

autoinhibition. The multiple splice variations at the 5′ ends of the type IV PDEs would then be responsible for grafting different regulatory peptide sequences onto a basic inhibited unit. This model is supported by the activation of ratPDE 3.3 via phosphorylation that occurs on residue 13 or 54 (Sette et al., 1994b), near the N-terminal region. In the case of PDE4D1, phosphorylation by PKA has not been found and no activator has yet been identified. Activity may be controlled by an associated protein as is the type VI photoreceptor PDE (Tsuboi et al., 1994). Purification of the type IV PDE4D1 and of the derived deletion mutants reported here provides the first opportunity to examine possible modes of control of these regulatory enzymes.

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BI9613483