Identification and characterization of DdPDE3, a cGMP-selective phosphodiesterase from Dictyostelium

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In Dictyostelium cAMP and cGMP have important functions as first and second messengers in chemotaxis and development. Two cyclic-nucleotide phosphodiesterases (DdPDE 1 and 2) have been identified previously, an extracellular dual-specificity enzyme and an intracellular cAMP-specific enzyme (encoded by the psdA and regA genes respectively). Biochemical data suggest the presence of at least one cGMP-specific phosphodiesterase (PDE) that is activated by cGMP. Using bioinformatics we identified a partial sequence in the Dictyostelium expressed sequence tag database that shows a high degree of amino acid sequence identity with mammalian PDE catalytic domains (DdPDE3). The deduced amino acid sequence of a full-length DdPDE3 cDNA isolated in this study predicts a 60 kDa protein with a 300-residue C-terminal PDE catalytic domain, which is preceded by approx. 200 residues rich in asparagine and glutamine residues. Expression of the DdPDE3 catalytic domain in Escherichia coli shows that the enzyme has Michaelis–Menten kinetics and a higher affinity for cGMP (K_m = 0.22 μM) than for cAMP (K_m = 145 μM); cGMP does not stimulate enzyme activity. The enzyme requires bivalent cations for activity; Mn^{2+} is preferred to Mg^{2+}, whereas Ca^{2+} yields no activity. DdPDE3 is inhibited by 3-isobutyl-1-methylxanthine with an IC_50 of approx. 60 μM. Overexpression of the DdPDE3 catalytic domain in Dictyostelium confirms these kinetic properties without indications of its activation by cGMP. The properties of DdPDE3 resemble those of mammalian PDE9, which also shows the highest sequence similarity within the catalytic domains. DdPDE3 is the first cGMP-selective PDE identified in lower eukaryotes.

Key words: chemotaxis, cloning, guanylate cyclase, mutant stmF.

INTRODUCTION

Eukaryotes are endowed with a large family of cyclic-nucleotide phosphodiesterase (PDE) genes [1–3]. The deduced amino acid sequences share a common element of approx. 300 amino acid residues that forms the catalytic domain. Some enzymes specifically hydrolyse cAMP (PDE4, PDE7 and PDE8) or cGMP (PDE5, PDE6 and PDE9), whereas other enzymes show dual specificity. Besides the putative catalytic domain, many enzymes possess additional domains that provide regulation by calmodulin (PDE1) or cGMP, which can be stimulatory (PDE2 or PDE5) or inhibitory (PDE3). The enzymic activities of PDEs might simply serve to degrade cAMP and cGMP. However, the complexity of the PDE superfamily provides the potential for additional regulatory circuits. For instance, the activation of guanylate cyclase and the accompanying increase in cGMP levels might indirectly alter the levels of cAMP, which will decrease when cAMP is hydrolysed by a cGMP-stimulated cAMP-PDE and will increase when cAMP is hydrolysed by a dual-specificity enzyme that is subject to competitive inhibition by the increased cGMP levels.

In Dictyostelium, cAMP and cGMP have pronounced roles as first and second messengers [4,5]. Dictyostelium grows as single cells in the soil, feeding on bacteria. During this stage of the life cycle, cAMP and cGMP are probably not essential for growth. However, cyclic nucleotides are important when cells are starved. Depletion of the food source induces a developmental programme, leading to a multicellular stage. An extracellular cAMP signalling system is induced that is composed of the adenylate cyclase ACA, the G-protein-coupled cAMP surface receptor cAR1, and specific G-protein subunits. This signalling system produces extracellular cAMP that actually accumulates and disappears in an oscillatory manner. Degradation of cAMP by a PDE that is present in the extracellular medium and on the cell surface is essential to the generation of such cAMP pulses [6,7].

Extracellular cAMP induces chemotaxis: starving cells move towards the source of cAMP, thereby forming a slug-shaped multicellular structure. In this slug, cell differentiation towards pre-stalk and pre-spore cells takes place, finally leading to a fruiting body composed of a stalk tube supporting a droplet of spores. Intracellular cAMP is essential for pre-spore differentiation. It is synthesized by the G-protein-coupled adenylate cyclase ACA [8], and by a G-protein-independent enzyme ACB [9] encoded by the acrA gene [10]. Intracellular cAMP is degraded predominantly by a cAMP-specific enzyme encoded by the regA gene [11,12].

During cell aggregation, extracellular cAMP induces a rapid transient increase in cGMP levels [5]. This nucleotide is produced by a guanylate cyclase that is presumably directly activated by G-proteins. In cell lysates, cGMP is degraded predominantly by a cGMP-specific enzyme that is activated by cGMP [13–15]. The cGMP-stimulated cGMP-PDE activity from Dictyostelium is absent from stmF mutant NP368, which is characterized by prolonged chemotactic movement [14,16,17]. In this mutant, the cAMP-stimulated cGMP accumulation proceeds for a longer period, reaches higher concentrations and recovery of basal levels is retarded approx. 6-fold. Quantitative analysis of these data indicates that the cGMP-PDE that is absent from stmF accounts for approx. 80% of the cGMP hydrolysis in wild-type cells and another unidentified enzyme for approx. 20% [18].

Abbreviations used: 8Br-cGMP, 8-bromoguanosine 3’5’-monophosphate; CIP, calf intestine alkaline phosphatase; EST, expressed sequence tag; IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase.

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To characterize further the role of cGMP in *Dictyostelium* chemotaxis, we have started to clone the genes that encode the enzymes synthesizing or degrading intracellular cGMP. The *Dictyostelium* sequencing project in Japan [19] provided a partial cDNA sequence encoding an amino acid sequence that exhibited a significant degree of identity with the catalytic domain of mammalian PDEs. We obtained a full-length sequence for this DdPDE3, and expressed the protein in *Escherichia coli* and *Dictyostelium*. DdPDE3 seems to degrade cGMP specifically but the enzyme is not activated by cGMP and is still present in mutant *stim*F. This suggests that DdPDE3 represents the residual cGMP-hydrolysing activity in mutant *stim*F.

**MATERIALS AND METHODS**

**Materials**

[2.8-^3^H]cAMP (1.85 Bq/mmoll) and [8-^3^H]cGMP (0.55 Bq/mmoll) were obtained from Amersham; cAMP, cGMP, 8-bromoguanosine 3′,5′-monophosphate (8Br-cGMP), calf intestine alkaline phosphatase (CIP) and IPTG (isopropyl-β-D-thiogalactoside) were purchased from Boehringer Mannheim. 3-Isobutyl-1-methylxanthine (IBMX) was obtained from Calbiochem; pRSET came from Invitrogen.

**Strain and culture conditions**

*Dictyostelium* AX3 (‘wild-type’), PDE3 cells overexpressing DdPDE3, and A^3^ΔG/GCA/PDE3 cells overexpressing both DdGCA and DdPDE3, were grown in HG5 medium (HL5 medium with 10 g/litre glucose). When growing cells under selection, HG5 medium was supplemented with 10 μg/ml blasticidic S or 10 μg/ml geneticin (neomycin), depending on the selection marker present (blasticidin deaminase and neomycin phosphotransferase genes respectively). *Stim*F mutant NP368 and its isogenic parental strain XP55 were grown in association with *Klebsiella aerogenes*.

Cells were starved for up to 5 h by being shaken in 10 mM phosphate buffer, pH 6.5, at a density of 10^6^ cells/ml for the indicated durations. For longer starvation periods, cells were transferred to non-nutrient agar plates [1.5%, (w/v) agar in 10 mM phosphate buffer, pH 6.5] and incubated at 22°C. For DNA transfection by electroporation, cells were grown to exponential phase at a density of (1–3)×10^6^ cells/ml. Approximately 10^5^ cells were mixed with 1–10 μg of DNA. After electroporation [20] and 5 h of recovery in a 10 cm dish, selection medium was added. Medium was changed after 24 h for the first time and thereafter approximately every 3 days.

**Cloning of DdPDE3**

The expressed sequence tag (EST) clone SSB116 was identified as a potential PDE-encoding sequence in the Japanese cDNA project by using bioinformatics. The clone was kindly provided by Dr T. Morio [19] and sequenced completely. The insert was used as a probe to screen a λZAP cDNA library kindly provided by Dr R. A. Firtel. In a screen of 10^6^ clones, 21 positive clones were identified. Inserts from 14 clones were rescued as Bluescript plasmids, with insert sizes from 1.2 to 1.9 kb. LambdaZAP clone 116-6 had the largest insert and was sequenced completely.

**Northern and Southern analysis**

RNA was isolated from 3×10^7^ cells at the indicated developmental stage with the Qiagen Rneasy minikit by using the supplier’s protocol for animal cells. After separation of the RNA samples on a formaldehyde/1% (w/v) agarose gel, the samples were transferred to a Nytran filter (Schleicher & Schuell).

Genomic DNA was purified with a minipreparation as described in [21]. Approximately 0.25 μg of genomic DNA was digested with the indicated restriction enzymes, separated on a 0.7% agarose gel and transferred to a Nytran filter. Northern and Southern blots were preincubated for 2 h at 65°C in hybridization solution [0.5 M sodium phosphate buffer (pH 7.0)/7%, (w/v) SDS/0.2 mg/ml BSA]. Hybridization was performed in the same prehybridization solution with the addition of a radioactive probe. Probes were labelled with ^32^P by the random primer method (High Prime; Boehringer Mannheim). Blots were incubated for 16 h, washed at high stringency with wash solution 1 [0.5M phosphate buffer/1% (w/v) SDS (pH 7.0) at 65°C] and wash solution 2 [0.1 M phosphate buffer/1% (w/v) SDS (pH 7.0) at 65°C] and exposed with a PhosphorImager.

**Expression of DdPDE3 catalytic domain in E. coli**

A 0.9 kb region of cDNA 116-6 starting at bp 888 was amplified by PCR with the universal sequencing primer as the 3′ primer and the gene-specific 5′ primer 5′-ATCATGGATCCAAAA-TGCCGGTTATAGTAAATAGT-3′; a BamHI restriction site is underlined; the DdPDE3 sequence is indicated in bold. The PCR product was digested with BamHI and KpnI and subcloned in pUC21, yielding pUC21-PDE3. After sequencing, the BamHI/KpnI insert was ligated into the BamHI and KpnI sites of the bacterial expression plasmid pRSET-A, yielding pRSET-PDE3. The encoded protein lacks the first 201 residues of DdPDE3 consisting of the glutamine and asparagine repeats. Translation starts with the amino acid sequence MASMTGG-QQMGRRGSKM (single-letter codes) derived from pRSET and the 5′ region of the PCR primer. It continues with the DdPDE3-derived amino acid sequence starting with R^386^GYNDDN, which is approx. 35 amino acids before the start of the conserved catalytic domain.

Plasmid pRSET-PDE3 was used to transform *E. coli* BL21DE3. Bacteria were grown overnight at 37°C in Luria–Bertani medium containing 25 μg/ml ampicillin and 50 μg/ml chloramphenicol. The culture was then diluted 1:40 in the same medium. After growth for 2 h at 30°C, 25 μg/ml IPTG was added and growth was continued for 3 h at 30°C. A 10 ml aliquot of the culture was centrifuged for 5 min at 3000 g, the pellet was resuspended in 15 ml of ice-cold PDE buffer [20 mM Hepes/NaOH/1 mM MgCl_2 (pH 7.0)] and cells were lysed by sonication with four pulses of 3 s each. PDE activity was measured in the crude lysate; the activity was stable for several months when the lysate was stored at −80°C.

**Expression of DdPDE3 catalytic domain in Dictyostelium**

The *Bam*HI/XbaI insert of pUC21-PDE3 was ligated into the *Bgl*II/SpeI sites of the *Dictyostelium* expression plasmid HK12N, yielding HK12N-PDE3. The plasmid HK12N is a derivative of the extrachromosomal plasmid MB12N [22], with a small multiple cloning site in the original *Bgl*II site that allows the directional ligation of genes to be expressed. Translation from HK12N-PDE3 starts with the first ATG codon after the *Bam*HI site of the PCR primer; after translation the start methionine is followed by the DdPDE3-specific sequence beginning with the motif R^386^GYNDDN.

HK12N-PDE3 was used to transform *Dictyostelium* wild-type strain AX3 and strain A^3^ΔG/GCA-neo, which overexpresses a guanylate cyclase under G418 selection (J. Roelofs, H. Snipe, R. G. Kleineidam and P. J. M. Van Haastert, unpublished work). Transformants were selected in HG5 medium in the presence of 10 μg/ml blasticidic. Cells were grown at 22°C to a density of 3×10^6^ cells/ml, washed in 10 mM phosphate buffer, pH 6.5, and.
cGMP-phosphodiesterase from Dictyostelium

Figure 1 Scheme and composite sequence of DdPDE3

Top panel: scheme of DdPDE3. The coding region is shown as a box; the 5' and 3' untranslated regions are depicted as lines. The putative catalytic domain is shown hatched and the stretches of polyglutamine and polyasparagine are indicated as Q and N respectively. The upper numbers refer to amino acids; the lower numbers indicate nucleotides. Southern blot analysis with the indicated restriction sites ScaI and BglII revealed the predicted 1.7 kb fragment. Lower panel: starved for 1 h in 10 mM phosphate buffer, pH 6.5, at a density of 10^6 cells/ml. Cells were washed and resuspended in ice-cold lysis buffer [20 mM Hepes/NaOH/1 mM EGTA (pH 7.0)] to a density of 10^6 cells/ml, then lysed by forced filtration through a Nucleopore filter (pore size 0.45 µm). The lysate was centrifuged for 5 min at 14000 g; the supernatant was used in the PDE assays.

Assays for PDE, cGMP response and chemotaxis

PDE activity was assayed in mixtures of 100 µl containing PDE buffer [20 mM Hepes/NaOH/1 mM MgCl₂ (pH 7.0)], 10 nM [³²P]cAMP or [³²P]cGMP, unlabelled cAMP or cGMP as indicated, and 30 µl of enzyme (diluted lysates from E. coli or Dictyostelium to achieve between 10 and 30% hydrolysis). The reactions were conducted at 22 °C for 15 min, terminated by boiling for 1 min, and followed by dephosphorylation of the hydrolytic product with phosphatase (1 h incubation at 37 °C with 100 µl of CIP buffer containing 1 unit of CIP). Finally, 500 µl of a 50% (w/v) slurry of DOWEX AG1-X2 was added, samples were incubated for 15 min at 22 °C, centrifuged for 2 min at 14000 g, and the radioactivity in 250 µl of the supernatant was determined.

For measurement of the cGMP response, cells were starved for 5 h, washed and then resuspended in 10 mM phosphate buffer, pH 6.5, to a density of 10^6 cells/ml. Cells were stimulated with 0.1 mM cAMP; at the times indicated, samples of the cell suspension were added to an equal volume of 3.5% (w/v) HClO₄. The cGMP content was measured with a radioimmunoassay in these lysates after neutralization with KHCO₃ [23]. Chemotaxis towards cAMP and folic acid was measured by the small-population assay [24].

RESULTS

Cloning of DdPDE3

Two stretches of amino acids that are conserved in the putative catalytic domain of mammalian PDEs were used to search for similar sequences in the Dictyostelium cDNA database, HDyDHp GTtNqFlVntKSeLAlLYndESVMEnHH and DLSnPpTKplplyRrwAELImeEFFxQGDkEKeMG. In these peptide sequences, capital letters show residues that were kept constant, while at the positions of lower case letters conservative substitutions or other amino acids found frequently in PDEs were accepted. With this method we identified a 0.7 kb cDNA, SSB116, that had been partly sequenced. Its deduced amino acid sequence showed a moderate degree of sequence identity with a small part of mammalian PDE enzymes. We determined the complete nucleotide sequence of this 0.7 kb cDNA, which seemed to encode a complete putative PDE catalytic domain. Because the cDNA hybridizes to an mRNA of approx. 1.9 kb (see below), the missing 1.2 kb sequence might encode regulatory domains. Therefore a complete cDNA (clone 116-6) was isolated from a kZAP cDNA library.

The kZAP cDNA 116-6 is 1882 bp in length. At its 3' end the complete nucleotide and deduced amino acid sequence. The basis of the sequence, including the 5' and 3' untranslated regions are depicted as lines. The putative catalytic domain is shown hatched and the stretches of polyglutamine and polyasparagine are indicated as Q and N respectively. The upper numbers refer to amino acids; the lower numbers indicate nucleotides. Southern blot analysis with the indicated restriction sites ScaI and BglII revealed the predicted 1.7 kb fragment. Lower panel: starved for 1 h in 10 mM phosphate buffer, pH 6.5, at a density of 10^6 cells/ml. Cells were washed and resuspended in ice-cold lysis buffer [20 mM Hepes/NaOH/1 mM EGTA (pH 7.0)] to a density of 10^6 cells/ml, then lysed by forced filtration through a Nucleopore filter (pore size 0.45 µm). The lysate was centrifuged for 5 min at 14000 g; the supernatant was used in the PDE assays.

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Figure 2 Alignment of the catalytic domain of DdPDE3 with DdPDE2 (RegA) and ten mammalian PDEs

All sequences are from published reports (GenBank accession nos U60170, U40370, U67733, M19667, U68632, AF043731, M27541, L12052, AF048837 and AB020593). Alignment was made with the program ClustalW version 1.8, followed by improvements by hand. Positions where all 12 sequences are identical or conserved are depicted within a black box; positions with 9–11 identical or conserved amino acids are indicated by grey shading. Conserved amino acids are ILVM; STA; DE; NQ; YFW; KRH (single-letter codes). To improve the alignment, insertions of 44 and 47 residues in PDE3A and 60 amino acids in PDE1 relative to other PDEs have been deleted and are indicated by ‘[44]’, ‘[47]’ and ‘[60]’ respectively. The symbol $i$ indicates amino acids that are different in DdPDE3 relative to all other PDEs; $*$ and $\ast$ indicate two metal-binding sites. Open squares indicate amino acids that are conserved within the group of mammalian cAMP-specific or cGMP-specific enzymes, but are different between the groups; the filled squares indicate two amino acids that are conserved in all cAMP-specific enzymes, including DdPDE2. The $y$ symbols indicate amino acids that have been mutated in PDE5 [43] to investigate the specificity of substrate binding. Numbers indicate the amino acid positions in the complete proteins.
and HsPDE10. In the third group DdPDE3 is placed together with the cAMP-specific DdPDE2 and the cGMP-specific HsPDE9. The position and composition of the first two groups are well defined by different methods (see the legend to Figure 3), but the position of the third group relative to the other two groups is less well defined; the Dayhoff method places DdPDE2 somewhat closer to HsPDE7 than does Kimura’s method. The analysis indicates that there is no strict separation between cAMP-specific, cGMP-specific and dual-specificity enzymes at the amino acid level.

Expression of DdPDE3 catalytic domain in E. coli

The N-terminal 200 residues of DdPDE3 are composed nearly exclusively of repeated Asn/Gln sequences that might cause difficulties in expressing the protein in E. coli. Expression was therefore started at residue 202, approx. 36 amino acids before the putative catalytic domain of the enzyme. Lysates from vector-control and DdPDE3-transformed E. coli cells were assayed for PDE activity with [3H]cAMP or [3H]cGMP as a substrate. DdPDE3 expression resulted in a moderate increase in cAMP-hydrolytic activity (means ± S.D., n = 3) from 0.94 ± 0.06 to 3.0 ± 0.4 pmol/min per mg of protein (Figure 4A). In contrast, the hydrolysis of cGMP increased from a barely detectable 0.024 ± 0.04 pmol/min per mg in control lysates to as much as 50 ± 4 pmol/min per mg in lysates containing recombinant DdPDE3. Thus the catalytic domain of DdPDE3 hydrolyses cGMP approx. 25-fold faster than it hydrolyses cAMP, both at 10 nM substrate concentration. The specificity of DdPDE3 was investigated further with competition experiments, showing that increasing concentrations of unlabelled cGMP or cAMP inhibit the hydrolysis of [3H]cGMP or [3H]cAMP (Figure 4B). Half-maximal inhibition of the hydrolysis of 10 nM [3H]cGMP was observed at 0.22 μM cGMP or 156 μM cAMP. Inhibition of the hydrolysis of the less preferred substrate [3H]cAMP yielded similar values, with half-maximal inhibition at 145 μM cAMP or 0.33 μM cGMP. Thus the catalytic domain of DdPDE3 binds cGMP with approx. 650-fold higher affinity than cAMP. An Eadie–Hofstee plot for the data on cGMP hydrolysis yielded a linear curve (r² = 0.99), indicating that the enzyme showed normal Michaelis–Menten kinetics with a K_m of 0.22 μM (Figure 4C).

Expression of DdPDE3 catalytic domain in Dictyostelium

The catalytic part of the enzyme was also expressed in Dictyostelium by using the extrachromosomal plasmid HK12N. Lysates were assayed in the presence of 5 mM dithiothreitol, which inhibits psdA (DdPDE1) but not the DdPDE3 expressed in E. coli. We also included 50 μM cAMP in the assay; at this concentration the activity of DdPDE3 with [3H]cGMP as a substrate was not inhibited (see Figure 4B), whereas the non-specific DdPDE1 and the cAMP-specific DdPDE2 were inhibited by more than 95% [12]. Under these assay conditions, lysates from control cells exhibited significant hydrolysis of [3H]cGMP (0.4 pmol/min per mg of protein). From previous experiments we know that this activity is derived predominantly from a cGMP-stimulated cGMP-specific PDE. Lysates from cells transformed with DdPDE3 hydrolyse [3H]cGMP at a rate of 3.6 pmol/min per mg of protein, indicating a 9-fold increase in cGMP-PDE activity. Thus the truncated C-terminal part of DdPDE3 is also active in Dictyostelium.

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The putative catalytic domain (residues 202–522) was expressed in *E. coli*. Bacteria were lysed and enzyme activity was measured with 10 nM [3H]cAMP or 10 nM [3H]cGMP. (A) PDE activity of bacteria containing control vector (open bars) and bacteria expressing DdPDE3 (filled bars). (B) Inhibition of PDE activity by cAMP (■) and cGMP (○). Measurements of [3H]cGMP and cAMP reveal that the catalytic domain of DdPDE3 expressed in *E. coli* has a similar specificity to the catalytic domain of DdPDE3 expressed in *D. discoideum*; the reactions were performed with 10 nM [3H]cGMP (○) or 10 nM [3H]cAMP (■). (C) Eadie–Hofstee plot of cGMP hydrolysis by DdPDE3 expressed in *E. coli*. The substrate concentrations used in this experiment were 0.01–3 mM cGMP. Enzyme concentrations and incubation times were optimized to give no more than 25% substrate conversion. (D) Inhibition of [3H]cGMP hydrolysis by DdPDE3 expressed in *D. discoideum* by cAMP (□) and cGMP (○). Error bars indicate S.D. of 3 independent experiments.

**Figures 4-5**

**Table 1**

<table>
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<tr>
<th>Lysate</th>
<th>Without 8Br-cGMP (pmol/min per mg)</th>
<th>With 8Br-cGMP (pmol/min/mg)</th>
<th>(%)</th>
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<tr>
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<td>3.64 ± 0.50</td>
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<td>0.13 ± 0.03</td>
<td>106 ± 30</td>
</tr>
</tbody>
</table>

PDE activity was measured with 10 nM [3H]cGMP in the absence or presence of 1 μM 8Br-cGMP with lysates from *E. coli* or *Dictyostelium* cells. Results are means ± S.D. for 3 independent experiments. Abbreviation: n.d., not determined.

**PDE activity in wild-type and*stmF* mutant Dictyostelium cells**

The hydrolysis of [3H]cGMP in mutant *stmF* was approx. 25% of the activity in the isogenic wild-type strain XP55, measured at 10 nM [3H]cGMP (Table 1). The inhibition of [3H]cGMP hydrolysis by unlabelled cGMP and unlabelled cAMP was used to probe it with a DNA fragment encoding the catalytic domain of DdPDE3 on Northern blots (Figure 5). The results reveal a single band of 1.9 kb present at approximately constant levels throughout development. The size of this band corresponds to the length of the isolated 116-6 cDNA, which is presumed to be full length. The Northern blot also reveals the transcription of the DdPDE3 gene in *stmF* mutant NP368 and its isogenic parental strain XP55. Mutant *stmF* lacks the cGMP-stimulated PDE activity, probably owing to a mutation in the structural gene of this enzyme [38]. DdPDE3 seems to be expressed at a normal level in mutant *stmF*. Furthermore, a Southern blot of restriction-enzyme-digested DNA did not reveal a marked difference between the DdPDE3 gene of wild-type cells and *stmF* mutant cells (results not shown).

Although we cannot exclude point mutations, biochemical results presented below suggest that DdPDE3 is expressed unaltered in mutant *stmF* because the residual cGMP-PDE activity in mutant *stmF* has all the characteristics of DdPDE3. This suggests that DdPDE3 is distinct from the cGMP-stimulated PDE-PDE that is absent from mutant *stmF*.

**DdpDE3 mRNA levels in wild-type Dictyostelium and mutant StmF**

To investigate the expression of DdPDE3, we isolated total mRNA at different times during *Dictyostelium* development and probed it with a DNA fragment encoding the catalytic domain of DdPDE3. The isolation of DdPDE3 mRNA was performed on Northern blots at the developmental times indicated, then size-fractionated and transferred to a nylon filter. The blot was hybridized with the catalytic domain of DdPDE3, detecting an mRNA with an estimated size of approx. 1.9 kb. The hydrolysis of [3H]cGMP or cGMP was determined in lysates from *E. coli* expressing DdPDE3; the reactions were performed with 10 nM [3H]cGMP (Figure 4D). Bacteria were lysed and enzyme activity was measured with 10 nM [3H]cGMP or 10 nM [3H]cGMP. (A) PDE activity of bacteria containing control vector (open bars) and bacteria expressing DdPDE3 (filled bars). (B) Inhibition of PDE activity by cAMP (■) and cGMP (○). Kinetic analysis of the data on cGMP hydrolysis yielded a linear Eadie–Hofstee plot (Figure 4C).

**Figure 5**

Northern blot of DdPDE3

mRNA was isolated from wild-type AX3 cells and mutant *stmF* strain NP368 at the developmental times indicated, then size-fractionated and transferred to a nylon filter. The blot was hybridized with the catalytic domain of DdPDE3, detecting an mRNA with an estimated size of approx. 1.9 kb.
characterize the enzyme activities in \textit{stmF} and wild-type XP55 (Figure 6). The addition of low concentrations of unlabelled cGMP to the incubations of wild-type lysates resulted in an increase in the hydrolysis of $[^{3}H]$cGMP, whereas high cGMP concentrations led to an inhibition of $[^{3}H]$cGMP hydrolysis. Analysis of these data yields an activation constant, $K_{a}$, of 0.11 mM cGMP, a $K_{m}$ of 5.5 mM cGMP and a $V_{max}$ of 350 pmol/min per mg of protein for the activated enzyme. These kinetic data are similar to those obtained previously for the partly purified cGMP-stimulated cGMP-PDE from \textit{Dictyostelium} [39]. It was this cGMP-stimulated enzyme activity that was shown to be absent from \textit{stmF} mutant NP368 [14]. Indeed, the residual cGMP-hydrolysing activity of \textit{stmF} was not activated by cGMP; low concentrations of unlabelled cGMP inhibited the hydrolysis of $[^{3}H]$cGMP (Figure 6). Kinetic analysis reveals Michaelis–Menten kinetics with a $K_{m}$ of 0.27 mM cGMP and a $V_{max}$ of 2.2 pmol/min per mg of protein. Inhibition of the hydrolysis of $[^{3}H]$cGMP by cAMP occurred only at high concentrations, both for the enzyme activity of XP55 and for that of \textit{stmF} cells, yielding half-maximal inhibition at 550 and 370 mM cAMP respectively.

\section*{Regulation of DdPDE3 by 8Br-cGMP, bivalent cations and IBMX}

Previous experiments have shown that the major cGMP-hydrolysing activity in \textit{Dictyostelium} lysates is activated by cGMP with a cyclic-nucleotide specificity that is different from the specificity of hydrolysis [40]. For instance, 8-Br-cGMP is a potent activator of cGMP hydrolysis ($K_{a} = 0.07 \mu$M) but interacts poorly with the catalytic site ($K_{m} = 50 \mu$M). The experiments presented in Table 1 investigate the effect of 8Br-cGMP on $[^{3}H]$cGMP hydrolysis. In lysates prepared from wild-type cells, 8Br-cGMP stimulates cGMP-PDE activity approx. 3-fold. In contrast, 8Br-cGMP does not alter the cGMP-hydrolysing activity of the DdPDE3 catalytic domain expressed in \textit{E. coli} or \textit{Dictyostelium}. This observation is consistent with the linear Michaelis–Menten kinetics of DdPDE3. 8Br-cGMP also does not affect cGMP hydrolysis by the residual PDE activity of \textit{stmF}.

The amino acid sequence of DdPDE3 exhibits two putative binding sites for bivalent metal ions. DdPDE3 expressed in \textit{E. coli} showed minimal activity in the absence of bivalent cations (Figure 7). Increasing concentrations of Mg$^{2+}$ enhanced the activity, yielding maximal cGMP hydrolysis at 3–10 mM MgCl$_2$. Mn$^{2+}$ was a more potent activator of DdPDE3 than Mg$^{2+}$, whereas Ca$^{2+}$ did not enhance enzyme activity. This cation specificity has been observed in other PDE enzymes, notably in PDE9A [34], and is supported by the proposed two metal-binding sites that have been deduced from the primary structure. The cation specificity of cGMP hydrolysis by \textit{Dictyostelium} lysates is essentially identical with that of the catalytic domain of DdPDE3 expressed in \textit{E. coli}. This was observed for lysates from wild-type cells, from DdPDE-overexpressing cells and from mutant \textit{stmF} cells (results not shown).

The effect of the PDE inhibitor IBMX on enzyme activity was tested for the different enzyme preparations (Figure 8). IBMX...
inhibited cGMP hydrolysis by the catalytic domain of DdPDE3 expressed in E. coli; half maximal inhibition was obtained at approx. 60 μM IBMX. Slightly higher IBMX concentrations were needed to obtain the same inhibition of cGMP hydrolysis by the catalytic domain of DdPDE3 overexpressed in Dictyostelium. In contrast, cGMP hydrolysis in lysates prepared from wild-type cells was poorly inhibited by IBMX, suggesting that the cGMP-stimulated cGMP-PDE is relatively insensitive to IBMX inhibition. Hydrolysis of cGMP by the residual activity of mutant stimF seemed to be inhibited by IBMX with the same sensitivity as exhibited by the activity of DdPDE3 overexpressed in Dictyostelium (Figure 8).

Phenotype of DdPDE3-transformed Dictyostelium cells

Cells overexpressing the catalytic domain of DdPDE3 showed normal cell aggregation and multicellular development (results not shown). In addition, chemotaxis towards cAMP was not significantly different from that of control cells. To evaluate these observations, we measured basal and cAMP-stimulated cGMP levels. In DdPDE3-overexpressing cells, basal cGMP levels were reduced slightly from 3.44±0.23 to 2.87±0.11 pmol/10^7 cells relative to control-transformed cells; this difference is not statistically significant (Figure 9A). The CAMP-mediated cGMP response is significantly smaller in DdPDE3-overexpressing cells. For a better understanding of the role of cGMP in Dictyostelium we also overexpressed a Dictyostelium guanylate cyclase (DdGCA) (J. Roelofs, H. Snipe, R. G. Kleineidam and P. J. M. Van Haaster, unpublished work), resulting in an approx. 3-fold elevation of basal cGMP levels (Figure 9B). When the catalytic domain of DdPDE3 was overexpressed in these DdGCA-expressing cells, we observed a pronounced decrease in the cGMP concentration to nearly the basal levels of wild-type cells, clearly demonstrating that DdPDE3 is active in vivo. A CAMP-mediated cGMP response was not observed in DdGCA-overexpressing cells, possibly owing to the high constitutive guanylate cyclase activity in these cells. The CAMP-mediated cGMP response remained absent when DdPDE3 was also overexpressed in DdGCA-expressing cells.

Figure 9 Effect of DdPDE3 overexpression on cGMP levels in Dictyostelium cells

After starvation for 5 h, cells were stimulated with 0.1 μM cAMP. At the times indicated, samples were quenched in HClO4 and used to measure cGMP levels. (A) Wild-type HK12N cells (○) and DdPDE3-overexpressing cells (●). (B) Cells overexpressing Dictyostelium guanylate cyclase DdGCA (○) and DdGCA-overexpressing cells that also overexpressed DdPDE3 (●). Error bars indicate S.E.M. of three to five experiments.

DISCUSSION

Spatiotemporal changes in intracellular cGMP levels might have a crucial role in chemotactic signalling of Dictyostelium cells to extracellular cAMP. It is therefore very important to thoroughly characterize guanylate cyclase and cGMP-specific PDE activities at a molecular level. As yet, not all cGMP-PDE activities are accounted for in Dictyostelium. Here we characterize a cGMP-specific PDE, DdPDE3, that is not activated by cGMP. This PDE is the third PDE gene cloned in Dictyostelium, and the first cGMP-specific enzyme cloned in lower eukaryotes.

DdPDE1, encoded by the psdA gene [41,42], is a glycoprotein that is secreted or becomes localized on the cell surface. The deduced amino acid sequence of DdPDE1 shows no sequence similarity with other PDEs, suggesting that it evolved independently from all other PDEs. The enzyme is inhibited by dithiothreitol but not by IBMX or other compounds known to inhibit mammalian PDEs. It hydrolyses cAMP and cGMP at approximately the same rate with slight negative co-operativity and an apparent K_m of approx. 1 μM.

DdPDE2, encoded by the regA gene [11,12], was identified in a genetic screen for suppressors of developmental mutants. The deduced amino acid sequence predicts a protein with a response regulatory domain and a PDE catalytic domain that shows strong sequence similarity to that of mammalian PDEs. Enzyme assays with the expressed protein demonstrate that DdPDE2 is a cAMP-specific PDE with a K_m of approx. 5 μM cAMP. IBMX inhibits DdPDE2 with an IC_{so} of approx. 35 μM. The enzyme is expressed throughout development, although at lower levels during growth.

DdPDE3

DdPDE3 was identified through the Dictyostelium sequencing projects. Cloning of the full-length cDNA and expression studies of the putative catalytic domain characterize DdPDE3 as a cGMP-specific PDE that is not activated by cGMP. The protein consists merely of a C-terminal catalytic domain of approx. 300 amino acids connected to an N-terminal domain of approx. 200 amino acids predominantly harbouring polyglutamine and polyasparagine stretches. No other protein sequence motifs could be discerned. The catalytic domain of DdPDE3 shows a high affinity for cGMP as a substrate, with a K_m of 0.22 μM; it is approx. 600-fold more specific for cGMP than for cAMP. The enzyme requires bivalent cations for enzyme activity, with a preference for Mn^{2+} over Mg^{2+}, and is inhibited by IBMX with an IC_{so} of approx. 60 μM.

Catalytic domain of DdPDE3

The catalytic domains of all PDE enzymes identified so far are positioned at the C-terminus of the protein; regulatory elements, when present, are located at the N-terminus. When comparing the Dictyostelium DdPDE2 and DdPDE3 sequences with ten human PDE sequences with the use of computer programs, we noticed that the putative catalytic domains are well aligned up to residue 653 in HsPDE4 (see Figure 2). Inspection by eye clearly identified an additional conserved region that was not previously recognized. This region ends at a conserved Trp and is located just before the C-terminus of many PDE enzymes. In HsPDE1 and HsPDE3 there is an insertion in this region of 60 and 47 residues respectively.

Multiple sequence alignment of the catalytic domains of DdPDE2, DdPDE3 and HsPDE1–10 reveals two distinct groups (HsPDE1, HsPDE3, HsPDE4, HsPDE7 and HsPDE8, and...
HsPDE2, HsPDE5, HsPDE6 and HsPDE10), and a third group (HsPDE9 and DdPDE2/3) with less well defined branching (Figure 3). The first group contains the cAMP-specific human enzymes and two dual-specificity enzymes, whereas two cGMP-specific enzymes are placed in the second group together with two dual-specificity enzymes. It is possible that the dual-specificity enzymes HsPDE1 and HsPDE3 evolved from or together with the cAMP-specific enzymes and that HsPDE2 and HsPDE10 evolved from cGMP-specific enzymes.

The notion of the close relationship between PDEs that are either cAMP-specific (DdPDE2) or cGMP-specific (HsPDE9 and DdPDE3) might help in the recognition of the amino acids that provide substrate specificity. The sequence alignment of the mammalian enzymes identifies seven amino acids that are different between cAMP- and cGMP-specific PDEs but are conserved within these groups (indicated by open squares in Figure 2). However, on the inclusion of the *Dictyostelium* DdPDE2 and DdPDE3 sequences in this analysis, none of the seven amino acids remain conserved within the groups. For example, the first potentially selective amino acid (at position 426 of HsPDE4) is histidine in mammalian cAMP-PDEs and arginine in mammalian cGMP-PDEs; however, the cAMP-specific DdPDE2 contains an arginine residue. In contrast, two amino acids are conserved in all cAMP-specific enzymes and differ from the cGMP-PDE or cGMP/cAMP-PDE isoenzymes: Asp and Arg/Lys at positions 439 and 607 of HsPDE4 respectively (filled square in Figure 2). However, in cGMP-specific enzymes no conserved amino acids are present at these two positions. Conversely, there is not a single amino acid that is conserved in all cGMP-PDEs while differing from the amino acids of cAMP-PDE at that position. A detailed mutagenesis study on substrate specificity has been performed with the cGMP-specific PDE5 [43]. Four amino acids were selected based on hydropathy calculations (indicated by ▼ in Figure 2). Conversion of these amino acids into the residues present in the cAMP-specific HsPDE4 (from Trp, Gln, Ala and Leu to Tyr, Thr and Arg respectively) changed the specificity approx. 100-fold towards cAMP. Interestingly, none of these amino acids are conserved within the group of cAMP-specific or cGMP-specific PDEs. Apparently, substrate specificity in PDEs is not determined by absolutely conserved amino acids. This situation is different from other nucleotide-binding proteins. For instance, in cAMP-dependent and cGMP-dependent protein kinases, specificity is dominated by a conserved Ala and Thr residue respectively [44].

In adenylate and guanylate cyclases, two amino acids determine substrate specificity, Lys and Asp binding ATP, and Glu and Cys binding GTP [45,46].

### Function of DdPDE3 in *Dictyostelium*

The cGMP-PDE activity in wild-type *Dictyostelium* lysates is characterized by non-Michaelis–Menten kinetics involving an enzyme that is activated by cGMP. This enzyme has been partly purified and is absent from mutant *simF* [14]. The genetic (Northern and Southern blots) and biochemical (Km for cGMP and IC50 for IBMX) properties of the catalytic domain of DdPDE3, suggest that DdPDE3 is not identical with the cGMP-stimulated cGMP-PDE. This would imply that the *Dictyostelium* genome still contains at least one other PDE. The *Dictyostelium* sequencing project does not yet provide convincing evidence for the existence of a fourth PDE gene. At present the chance of finding a gene with 1 kb of coding sequence (300-residue PDE catalytic domain) is approx. 80–90%.

The cGMP-PDE activity in mutant *simF* is approx. 25% of the cGMP-hydrolysing activity of wild-type cells, both measured at low cGMP concentrations. At higher substrate concentrations the contribution of DdPDE3 to cGMP hydrolysis in *Dictyostelium* diminishes owing to its high affinity but relatively low Vmax compared with the cGMP-stimulated PDE. The moderate importance of DdPDE3 in the hydrolysis of cGMP in *Dictyostelium* is also demonstrated on overexpression of the enzyme in *Dictyostelium* cells. When the catalytic domain of DdPDE3 is overexpressed approx. 25-fold compared with the endogenous levels of DdPDE3 in wild-type cells, this results in a non-significant decrease in basal cGMP levels and a 33% decrease in cAMP-mediated cGMP response. Overexpression of the catalytic domain of DdPDE3 in cells that also overexpress guanylate cyclase DdGCA has a more pronounced effect. The approx. 3-fold increase in cGMP levels owing to DdGCA overexpression is decreased by DdPDE3 to nearly the basal cGMP levels of wild-type cells. These combined results suggest that DdPDE3 is involved mainly in regulating basal cGMP levels and has no predominant role in hydrolysing cGMP that is synthesized on receptor stimulation. This conclusion is supported by the lack of an overt phenotype of the DdPDE3-overexpressing cells.

In summary, we have identified a high-affinity cGMP-specific PDE in *Dictyostelium* that shows similarity to mammalian PDE9, in both its biochemical properties and its amino acid sequence. The enzyme contributes at most 30% to the hydrolysis of intracellular cGMP; the remainder is contributed by a cGMP-stimulated cGMP-PDE that is absent from mutant *simF*. Identification and isolation of the gene encoding the latter enzyme will provide the means of full control of cGMP levels in *Dictyostelium* and of establishing the function of cGMP in this organism.

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