DdPDE4, A NOVEL cAMP-SPECIFIC PHOSPHODIESTERASE AT THE SURFACE OF DICTYOSTELIUM CELLS

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Running title: DdPDE4, a Dictyostelium cell surface cAMP phosphodiesterase

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Dictyostelium discoideum cells possess multiple cyclic nucleotide phosphodiesterases that belong either to class I enzymes that are present in all eukaryotes, or to the rare beta-lactamase class II. We describe here the identification and characterization of DdPDE4, the third class I enzyme of Dictyostelium. The deduced amino acid sequence predicts that DdPDE4 has a leader sequence, two transmembrane segments and an extracellular catalytic domain that exhibits a high degree of homology with human cAMP-specific PDE8. Expression of the catalytic domain of DdPDE4 shows that the enzyme is a cAMP-specific phosphodiesterase activity with a $K_M$ of 10 µM; cGMP is hydrolyzed at least 100-fold more slowly. The full length protein is shown to be membrane-bound with catalytic activity exposed to the extracellular medium. Northern blots and activity measurements reveal that expression of DdPDE4 is low during single cell stages, and increases at nine hours of starvation, corresponding with mound stage. A function during multicellular development is confirmed by the phenotype of ddpde4− knock-out strains, showing normal aggregation but impaired development from the mound stage on. These results demonstrate that DdPDE4 is a unique membrane-bound phosphodiesterase with an extracellular catalytic domain regulating inter-cellular cAMP during multicellular development.

During different developmental stages of Dictyostelium discoideum the cyclic nucleotides cAMP and cGMP play a central role in diverse signal transduction processes. cAMP and cGMP mediate chemotaxis during cell aggregation, and controls gene expression during development. cGMP regulates cytoskeletal organization affecting shape, stability and motility of single cells. The intracellular concentration of the second messengers cAMP and cGMP is determined by the combined action of production and removal. Production directly depends on the enzymatic activity of the adenylyl cyclases and guanylyl cyclases to form cAMP and cGMP, respectively. Removal of intracellular cAMP or cGMP depends on the activity of phosphodiesterases (PDE) that hydrolyze cAMP to form 5'-AMP and cGMP to form 5'-GMP, and on the ability of Dictyostelium discoideum cells to secrete cAMP. This extrusion mechanism is pivotal in the formation of extracellular cAMP waves that mediate chemotaxis during aggregation, mound formation and slug movement.

In Dictyostelium discoideum three different adenylyl cyclases and two guanylyl cyclases have been identified (see (1) and (2)). In addition, five different phosphodiesterases have been reported and characterized in Dictyostelium discoideum. These PDEs belong to two classes that exhibit distinct differences in the amino acid sequence of the putative catalytic domains, namely class I, which is ubiquitous in eukaryotes, and class II that predominantly occurs in lower eukaryotes.

PdsA (or DdPDE1) is a class II dual specificity PDE that degrades cAMP as well as cGMP and is exposed on the cell surface or secreted in the medium. It is the main PDE that degrades extracellular cAMP and thereby essential for shaping cAMP waves (3-13). RegA
(or DdPDE2) encodes a cAMP-specific class I PDE localized in the cytosol of the cell where it degrades intracellular cAMP. The knock-out cell strain \textit{regA} develops very small aggregates and shows defects in spore formation. Additionally \textit{RegA} has been implicated in suppression of pseudopodium formation (14-22). DdPDE3 is a cGMP-specific class I PDE that consists of a catalytic domain without regulatory domains, and is constitutively active. The \textit{pde3} null cell strain shows a moderate phenotype with increased basal levels of cGMP, revealing that the enzyme accounts for about 20% of total cGMP-PDE activity in unstimulated cells (23). DdPDE5 (also named GbpA or PDED) is characterized as a cGMP-stimulated cGMP-specific class II PDE. The \textit{pde5} null cell strain exhibits a phenotype with strongly elevated levels of cGMP implying that it is the main intracellular cGMP-PDE accounting for about 75% of the cGMP-PDE activity. The biochemical phenotype of this \textit{pde5} null cell is similar to what is observed in the mutant NP368, a Streamer F cell strain (24-26). Finally, DdPDE6 (GbpB or PDEE) is a dual specificity class II PDE that degrades extracellular cAMP and cGMP, and accounts for only 5% of the total cGMP-PDE activity (24,26-28).

In searches of the then ongoing \textit{Dictyostelium} genome sequencing project we identified a putative PDE that was named DdPDE4 for its order of initial recognition. DdPDE4 appears to be a class I phosphodiesterase with unusual properties. DdPDE4 is a transmembrane phosphodiesterase with its catalytic domain exposed to the extracellular medium. It degrades extracellular cAMP together with the class II enzymes DdPDE1. Cells lacking DdPDE4 are defective in development at the mound stage, which is also the stage of maximal expression of \textit{ddpde4}.

**EXPERIMENTAL PROCEDURES**

**Identification and reconstruction of the ddpde4 gene**

The \textit{Dictyostelium} genome database (genome.imb-jena.de/dictyostelium) was screened for sequences potentially encoding type I phosphodiesterases. At the initial phase of the sequencing project we identified clone JAX4b25f06. The encoding DNA fragment was obtained by PCR with primer 99B245 CATGGGATCCGGAATTAATGAGTATTG and primer 99B301 CATGGGATCCGGAATTAATGAGTATTG on genomic DNA. With this fragment as a probe a cDNA library (kindly provided by Dr. R. H. Gomer) was screened. Clone pdek9 had the largest insert of 2150 base pairs, but lacked the start of the open-reading frame. The \textit{Dictyostelium} databases were searched with the pdek9 insert revealing that 400 base pairs of coding sequence were missing. The first 100 base pairs were obtained by PCR with primers 00B369 (ATCATGGGATCCAAAAATGATTATATATTATTITTTTAAT) and 00B368 (CTAGGTCTGTGATACCCAC) and the subsequent 300 base pairs with primers 00B345 (CTAGGTCTGTGATACCCAC) and 00B345 (CTAGGTCTGTGATACCCAC). These two fragments were combined using primers 00B369 and 00B345 to create the 400 base pair fragment. This way a BglII site was removed without destroying the amino acid sequence, this action was convenient for further cloning. Together with the pdek9 insert these 400 base pairs comprise the complete \textit{ddpde4} gene. The sequence has been deposited in GenBank (AY211984) and is confirmed by the now completed \textit{Dictyostelium} genome (DDB083560).

**Cluster analysis**

Multiple sequence alignments were constructed using the CLUSTAL W program (29), followed by manual optimization. Distance matrices were constructed from the alignments with the PROTDIST program of the PHYLIP package, which uses the Dayhoff PAM 001 matrix for the calculation of evolutionary distances (Phylip 3.5, J.Felsenstein, 1993 (30)). Phylogenetic trees were generated by using the FITCH program of the PHYLIP package, with 1000 bootstrap replications to assess the reliability of the nodes.

**Construction of the overexpressor**

A genomic DNA fragment of 1450 base pairs comprising the catalytic domain of DdPDE4 was amplified by PCR using the primers PDE4catF: ATCATGGGATCCAAAAATGAGTATTG and PDE4catR: CATCTAGAAACCACTATTATATTATATTATTTTGTTT. Subsequently the BamHI-Xbal fragment
was cloned into the BglII-SpeI site of the vector MB74-GFP (31), resulting in the plasmid MB74-PDE4cat-GFP. Finally AX2 Dictyostelium cells were transfected with 5 µg plasmid yielding many cells overexpressing amino acids 264 – 771 of DdPDE4 comprising the catalytic domain. We will refer to this overexpressor as DdPDE4catOE.

To express the full length protein, a DNA fragment was amplified by RT-PCR using the primers PDE4F: GAGGATCCATGATTATATATTATTTTTAT and PDE4catR. The BamHI-XbaI fragment was cloned in the BglII-SpeI site of the vector MB74-GFP (31), resulting in the plasmid MB74-PDE4-GFP. DH1 Dictyostelium cells were transfected with 5 µg plasmid yielding cells overexpressing the full length PDE4 protein. We will refer to this overexpressor as DdPDE4OE.

**Culture and incubation conditions**

AX2, AX3, knock-out and overexpressor Dictyostelium strains were grown on HG5 medium (14.3 gr/l Oxoid Peptone, 7.15 gr/l Yeast extract, 1.36 gr/l Na2HPO4.12H2O, 0.49 gr/l KH2PO4, 10.0 gr/l glucose) either on 9 cm dish or in shaking culture at 22 °C. After transformation the knock-out and overexpressor cell lines were selected and grown on HG5 medium supplemented with Blasticidin (10 µg/ml).

**Phosphodiesterase assays**

*Dictyostelium* cells were washed with PDE lysis buffer (40 mM HEPES/NaOH, pH 7.0, 1 mM EGTA) and resuspended at a density of 10⁸ cells/ml in PDE lysis buffer supplemented with 0.25 M sucrose. The cells were lysed at 4°C by passage through a 0.45 µm Nuclepore filter. The lysate was centrifuged for 2 minutes at 14000 g and the supernatant was used; the pellet was washed once with lysis buffer, resuspended in the original volume of lysis buffer and used in the assays. PDE activity at the surface of intact cells was assayed using freshly washed cells resuspended in lysis buffer. The PDE assay mixture (final concentrations) contained PDE assay buffer (40mM HEPES/NaOH, pH 7.0, 1mM EGTA, 0.25 M sucrose, 5 mM MgCl₂ and 5 mM MnCl₂), 10 nM [³H]cAMP or 10 nM [³H]cGMP as substrate, unlabelled cAMP or cGMP as indicated, and 30 µl of lysate in a total volume of 100 µl. The assay mixture was incubated at room temperature for 15 minutes and the reaction was terminated by boiling for 1 minute. The product was dephosphorylated by calf intestine phosphatase (1 unit of enzyme in 50 µl of CIP buffer incubated for 1 hour at 37 °C). Finally, 150 µl of DOWEX AG1X2 was added to remove remaining substrate. After 10 minutes of incubation under regular mixing, samples were centrifuged for 2 minutes at 14000 g, and the radioactivity in 200 µl of the supernatant was determined.

**Construction of Knock-outs**

Pdek9 was cloned into pBluescript SK+ with BamHI and XhoI. The Bsr gene (32) was cloned into the HindIII site. The knock-out fragment was amplified by PCR using primer 00B254 ATCATGGATCCAAAATGCCAGAGATAACGATCAGG and primer 00B260 CACTTGTGACTCTATTGACC. The PCR product was purified with the QIAquick PCR Purification kit by Qiagen (Westburg), and 20 µg was electroporated into Dictyostelium cells. Two knock-out cell strains were obtained that showed the altered fragments of the ddpde4 gene with expected size on Southern blots.

**Aggregation tests**

Dictyostelium cells were grown on a 9 cm dish with HG-5 medium to an amount of 2x10⁷ cells. Cells were collected, washed two times in PB (10 mM KH₂PO₄/Na₂HPO₄, pH 6.5) and suspended in 100 µl PB. Subsequently cells were placed on non nutrient agar plates (15.0g/l agar in PB). After 0, 5, 10, 15, 20, and 27 hours of starvation pictures were taken with an Olympus DP10 camera.

**RESULTS**

**DNA Sequence and protein topology**

The *Dictyostelium* DNA database (genome.imb-jena.de/dictyostelium) was searched for sequences similar to two stretches of amino acids that are conserved in the catalytic domains of mammalian phosphodiesterases (23). These sequences are HDyDhpGTtNqFIvmtKSeLaILYndESVMEnHH and DLSnpTKplplyRrwAELImeEFFxQGDkEKeMG.
Capitals represent amino acids that are identical in (almost) all phosphodiesterases and lower case letters represent amino acids that are conserved in the major part of the mammalian phosphodiesterases. With this method we identified a sequenced clone JAX4b25f06. The 665 base pair insert coded for part of a putative phosphodiesterase catalytic domain. This sequence was used to screen a cDNA library and to search further in the Dictyostelium genomic database. Eventually we identified and assembled by RT-PCR the entire open reading frame of 2.6 kb (for detailed information see Materials and Methods). The start ATG is preceded by a long AT stretch. The ddpde4 gene has one intron of 100 base pairs at base pair position 603. The deduced amino acid sequence is 771 amino acids long and predicts a protein of 85 kDa (Fig. 1). As will be shown below, DdPDE4 encodes for a cAMP-specific phosphodiesterase.

According to the SMART topology program (smart.embl-heidelberg.de) and TMPred (www.ch.embnet.org/cgi-bin), DdPDE4 is predicted to contain a signal sequence (amino acids 1-29), two transmembrane regions (amino acids 42-62 and 88-108), and the phosphodiesterase catalytic domain (309-688; Fig. 1A). Topology prediction programs at www.expasy.org/tools/ strongly suggest that the signal sequence is cleaved, and that the new N-terminus is located at the outside of the cell. The short extracellular segment is followed by the two membrane-spanning segments with a short intracellular loop. Consequently, the catalytic domain of DdPDE4 is strongly predicted to be localized in the extracellular space (Fig. 1B), as will be demonstrated experimentally below. In addition to these topology-determining segments, DdPDE4 has a coiled coil region (117-145), and a region in front of the catalytic domain (146-255) that is homologous to a region found in mammalian HsPDE8.

Amino acid sequence and cluster analysis of the catalytic domain

Eleven subfamilies of mammalian phosphodiesterases have been identified. Each subfamily has a variety of phosphodiesterase genes (A,B,C etc.) that often display different splice variants. The sequence identity of the catalytic domains within members of a subfamily is approximately 90%, whereas the sequence identity between subfamilies is 45%. We have used one member of each subfamily in the sequence analysis of DdPDE4. Figure 2 shows an alignment of the catalytic domain of DdPDE4 with Dictyostelium RegA (or DdPDE2) (19,33), DdPDE3 (23) and the eleven mammalian phosphodiesterases (34-46). DdPDE4 shows high sequence identity with the other phosphodiesterases. Of the 40 amino acids that are identical or conserved in all the other depicted phosphodiesterases, DdPDE4 has 38 amino acids that are identical or conserved. The exceptions are substitutions of a Tyrosine for a Histidine at position 412 of the DdPDE4 amino acid sequence (in figure 2). The two metal binding sites depicted in figure 2 are also conserved in DdPDE4 (* and + in figure 2). We identified previously two amino acids, an aspartic acid and an arginine, that are conserved in all cAMP-specific PDEs, but not in cGMP-specific enzymes or enzymes with dual-specificity (23). DdPDE4 contains this aspartic acid and arginine (symbol ] in figure 2), suggesting that DdPDE4 belongs to the group of cAMP-specific enzymes, which is confirmed below in biochemical experiments.

The DdPDE4 amino acid sequence directly N-terminal to the catalytic domain (named the precatalytic region; amino acids 146 to 255, figure 1A) shows homology to an amino acid sequence preceding the catalytic domain of PDE8 isoforms found in vertebrates (figure 3). Members of this PDE subfamily with a precatalytic region were also found in the insects Drosophila melanogaster and Apis mellifera and the worm Caenorhabditis elegans (figure 3). In vertebrate and insect PDE8 this precatalytic region is preceded by a PAS domain (43) that is not present in worms and Dictyostelium DdPDE4.

To further explore the relationship of DdPDE4 with other class I enzymes, we performed a cluster analysis of the catalytic domains as defined in figure 2 of DdPDE4, one member of each of the eleven subfamilies of mammalian enzymes, and the three PDE8 isoforms of insects and worms. Two groups of phosphodiesterases can be distinguished based on the bootstrap values (figure 4), similar to what has been reported previously (47). One group
harnors PDEs that are cGMP-specific or have a cAMP/cGMP dual specificity (HsPDE2A, HsPDE5A, BtPDE6A and HsPDE10A and HsPDE11). The second group consists of cAMP-specific and dual specificity enzymes (HsPDE1A, HsPDE3A, HsPDE4A, HsPDE7A, HsPDE8A, HsPDE11A). DdPDE4 belongs to this second group. The catalytic domains of PDEs from insects and worms are clearly PDE8 isoforms. *Dictyostelium* DdPDE4 is placed relatively close to this PDE8 subfamily. However, bootstrap values show that the node of DdPDE4 is not very reliable.

The other *Dictyostelium* phosphodiesterases (DdPDE2 and DdPDE3) and HsPDE9A do not fall into one of these groups.

**Catalytic activity and localization of DdPDE4**

The DdPDE4 catalytic domain (amino acids 264-771) was expressed in vegetative AX2 cells. We measured PDE activity in the cytosolic fraction of a cell lysate of these DdPDE4catOE cells and used PDE activity in lysates from the wild type AX2 cell strain as a control. The results in figure 5A show that the overexpressor DdPDE4catOE provides a threefold increase of cAMP phosphodiesterase activity compared to the control. There was no difference in cGMP PDE activity between DdPDE4catOE and the control, suggesting that DdPDE4 may be cAMP-specific. The increased cAMP hydrolysis of DdPDE4catOE cells compared to AX2 cells was observed only in the soluble fraction and not in the particulate fraction of a cell lysate (data not shown). Subsequently, the hydrolysis of [3H]cAMP in the presence of increasing concentrations unlabelled cAMP or cGMP was measured for both cell lines. We calculated the hydrolysis of [3H]cAMP in lysates form DdPDE4catOE cells after subtracting [3H]cAMP hydrolysis in lysates from AX2 cells, providing direct characterization of DdPDE4. The result in figure 5B show half-maximal inhibition of [3H]cAMP hydrolysis at 310 µM cAMP, whereas no inhibition was observed at 100 µM cGMP, indicating that DdPDE4 is at least 100-fold more specific for cAMP than for cGMP. The kinetic data for DdPDE4 were obtained from an Eadie-Hofstee plot (figure 5C); linear regression analysis yields a slope of $K_M = 9.6 \mu M$ cAMP and an intercept at $V_{max} = 4200$ pmol/min/mg. The absence of detectable cGMP hydrolysis above control levels suggest that the $K_M$ for cGMP is above 1 mM and the $V_{max}$ below 100 pmol/min/mg. DdPDE4 activity can be inhibited by the general class I inhibitor IBMX, but rather high concentrations are requires (half-maximal inhibition at 300 µM IBMX, data not shown).

DdPDE4 has a putative signal sequence and two transmembrane segments, predicted it to be a plasma membrane protein with the catalytic domain exposed to the outside of the cell. The topology of DdPDE4 was investigated by over-expressing the full length protein in wild-type cells, yielding strain DdPDE4OE. To determine the location of the catalytic domain of DdPDE4 at the outside or inside of the plasma membrane, we measured [3H]cAMP hydrolysis using intact cells and the particulate fraction of a cell lysate (figure 6). Wild-type cells contain on their surface the very active DdPDE1 (3,000 fmol/min/10^7 cells) whose activity can be inhibited strongly with dithiothreitol (DTT) (48). Wild type cells possess a small residual activity in the presence of 5 mM DTT (29 fmol/min/10^7 cells). DdPDE4OE cells contain approximately the same amount of total PDE activity compared to control cells (2,900 fmol/min/10^7 cells), but about 4-fold higher DTT-resistant [3H]cAMP hydrolysis activity than control cells. When the activity was measured with [3H]cGMP as substrate, very little activity was observed in control cells with DTT (18.1 +/- 1.8 fmol/min/10^7 cells) which was not elevated in DdPDE4OE cells (17.7 +/- 1.1 fmol/min/10^7 cells). This result confirms the cAMP-specificity of DdPDE4, and also indicates that cells are not leaky for the very active cytosolic cGMP-specific enzymes. Membranes isolated from wild type cells contain slightly less [3H]cAMP hydrolysis activity than intact cells, suggesting that little or no phosphodiesterase activity is exposed to the cytoplasmic face of the plasma membrane. Similarly, PDE activity in membranes isolated from DdPDE4OE cells is slightly less than the PDE activity of intact DdPDE4OE cells. The results strongly suggest that the catalytic domain of DdPDE4 is exposed to the extracellular medium, confirming the topology programs predicting a signal sequence, two transmembrane regions and extracellular N- and C-termini. Programs predict the cleavage of the signal
sequence, for which we have no experimental evidence.

Expression of mRNA
To investigate the expression level of *ddpde4* at different time intervals during development, we isolated mRNA at 0 to 21 hours after starvation on non-nutrient agar. A Northern blot was made with the isolated mRNAs and it was probed with part of the catalytic domain of *ddpde4* (see M&M). Figure 7A shows a mRNA with an approximate size of 3 kb that is present in the wild type *Dictyostelium* strain AX3 predominantly at 9 hours of starvation, during the mound stage. Expression is very low during growth and cell aggregation (up to 6 hours), maximal during the mound stage, but *ddpde4* remains being expressed at significant levels during the other stages of multicellular development.

To assay for DdPDE4 activity at different stages of development, we made use of the localization of DdPDE4 at the cell surface, and its resistance to DTT and inhibition by IBMX. We define DdPDE4 activity as the IBMX-mediated decrease of phosphodiesterase activity measured with intact cells in the presence of 10 mM DTT. The results of figure 7B demonstrate very low DdPDE4 activity in single cells starved for 1 and 5 hours. The activity starts to increase at 7 hours of development when cells are in loose aggregates. A substantial increase of activity is observed between 7 and 9 hours of development when tight aggregates are formed. DdPDE4 activity increases slightly in slugs at 12 hours of development. The biochemical assays confirm the Northern blot, showing that DdPDE4 is active predominantly in the multicellular stage. It should be mentioned that DdPDE4 is active at low levels in the cell cycle regardless of the developmental stage (note the differences in axes for total PDE and DdPDE).

Knock-out
Two independent knock-out strains with a deletion of the *ddpde4* gene were created. Phosphodiesterase assays show a reduction of PDE activity on the cell surface from 29 fmol/min/10^7 cells in control AX2 cells to 9 fmol/min/10^7 cells in *ddpde4* null cells (figure 6). Assays showed a slower development of the knock-out strains in comparison to AX3. Aggregation speed was similar, but from the mound stage until fruiting body formation the knock out strains were about five to ten hours slower than wild type AX3 (figure 8). Cell aggregation was nearly completed at 10 hours after the onset of starvation in both AX3 and *ddpde4* null cells. These aggregates developed into slugs at 15 hours in AX3 cells, but largely remained as mounds in *ddpde4* null cells. At 20 hours after the onset of starvation, the knockouts are still in early mound stage, while the wild type strain AX3 starts to culminate. At 27 hours of starvation fruiting body formation was completed in AX3 whereas many mounds were still present in *ddpde4* null cell (figure 8). Thus, especially slug formation and culmination appear to be affected in *ddpde4* null cells; these processes were not only slower but also less slugs and fruiting bodies were formed.

The expression of full length DdPDE4OE in *ddpde4* null cell restored the phenotypic defects of the null cell line, and no deteriorating effects on development were observed (figure 8).

Discussion
The completed genome sequence of *Dictyostelium discoideum* (49,50) uncovers several PDEs, of which five enzymes have been characterized in detail. In this paper we described the sixth member, DdPDE4, with unusual properties. The predicted topology of this PDE strongly suggests that DdPDE4 is a membrane bound phosphodiesterase with an extracellular catalytic domain. Biochemical experiments of the expressed protein confirm this topology. Since in wild type cells DdPDE4 is expressed during the onset of multicellular development and has cAMP phosphodiesterase activity we suggest that it is involved in regulating inter-cellular cAMP levels in the mound and slug stage. This is also indicated by the retarded multicellular development of the knock out cell line of *ddpde4*.

*Dictyostelium* cells contain three pools of cyclic nucleotides with different functions: 1) extracellular cAMP as signal molecule for chemotaxis and morphogenesis, 2) intracellular cAMP as source for cAMP secretion and inducing development, and 3) intracellular cGMP as mediator of chemotaxis (Table I). The
characterized PDEs of *Dictyostelium* belong to two different classes (I and II) with three members in each class. It is striking that each cyclic nucleotide pool is regulated by a combination of a class I and a class II phosphodiesterase. Intracellular cGMP is degraded by cGMP-specific class I DdPDE3 (23) and class II DdPDE5 (and to a lesser extent by the dual specificity DdPDE6) (22,24,26-28). Intracellular cAMP is degraded by the cAMP-specific class I DdPDE2 (RegA) and by the dual specificity class II DdPDE6 (14-22). Extracellular cAMP is degraded by the class II DdPDE1 (PdsA) (3,4,6-11), and class I DdPDE4. It is of further interest that each cyclic nucleotide pool is degraded by at least two enzymes with different kinetic properties: one enzyme has a high affinity for the substrate and a relatively low capacity, while the other enzyme has a lower affinity and higher capacity. These properties allow the degradation of the substrate over a wide range of concentrations to occur with approximately first order kinetics. At low substrate concentrations degradation mainly takes place by the high affinity enzyme. At intermediate substrate concentration the high affinity enzyme becomes saturated, and the low affinity enzyme takes over. Due to the high capacity of this low affinity enzyme, substantial degradation of substrate is possible even at very high concentrations.

Northern blot indicate that DdPDE4 is expressed mainly in the multicellular stage, while expression in the single cell stages is undetectable, confirming biochemical experiments. The phenotype of *ddpde4*-null cells also suggests that DdPDE4 does not significantly contributes to the degradation of extracellular cAMP during the single cell stage, because cell aggregation is not affected. The *ddpde4*-null mutant exhibits defective development from the mound stage till culmination, which are the developmental stages in which wild type cells shows high expression of DdPDE4. In *ddpde4*-null cells the normal shaped mounds develop more slowly to slugs and fruiting bodies, which are much smaller than in control cells. The apparent *K_m* of 0.8 µM cAMP for DdPDE1 (8) matches the dynamic range of the cAMP concentration during aggregation where cAMP waves reach concentrations up to approximately 1 µM (51). During mound and slug stage intercellular cAMP is expected to reach significantly higher levels due to the small intercellular space (1). At such high cAMP levels DdPDE1 will be saturated with cAMP. DdPDE4 with a *K_m* 10 µM may be in a better position to match the dynamic range of cAMP in the multicellular stage.

**ACKNOWLEDGMENTS**

We are indebted to all of the teams involved in the *Dictyostelium* sequencing projects.

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LEGENDS TO THE FIGURES

Figure 1. Predicted topology of DdPDE4.
Panel A, predicted domain composition of DdPDE4 and HsPDE8. The DdPDE4 sequence from amino acids 1 to 29 depicts a signal sequence. Amino acids 42 to 62 and amino acids 88 to 108 are transmembrane regions. Amino acids 117 to 145 are predicted by SMART to be a coiled coil region. Amino acids 146 to 255, indicated as precatalytic region, form a potential regulatory domain also found in human PDE8. The catalytic domain lies in between amino acids 256 and 688 (with a stretch of repetitive sequence from amino acid 471 to 580). The topology of HsPDE8A is depicted below that of DdPDE4. It has a PAS domain, a precatalytic region and a catalytic domain. Panel B, proposed topology of DdPDE4. The signal sequence leads the DdPDE4 protein through the membrane, and is supposed to be cleaved off. From this it follows that the two transmembrane regions enter the membrane in the depicted fashion, and consequently the remaining part of the protein resides at the extracellular side of the membrane, as demonstrated in figure 6.

Figure 2. Alignment of the catalytic domain of the class I PDEs.
Multiple sequence alignments were made by using the CLUSTAL W program with improvements made by hand. All sequences are from published reports (Genbank® accession numbers AY162269, U60170, U40370, U67733, M91667, U68532, AF043731, M27541, U67932, O60658, AF048837, AB020593, and AB036704). To improve the alignment, insertions of 44 and 47 residues in HsPDE3A, 60 amino acids in HsPDE1A and 95 residues in DdPDE4 relative to other PDEs have been deleted and are indicated by [44], [47], [60] and [95] respectively. * and + indicate two metal binding sites; [ refers to two amino acids that are conserved in all cAMP-specific enzymes including DdPDE4, but different in cGMP-specific or dual-specificity enzymes. Black boxes represent positions where all 14 sequences are identical or conserved. Positions where 10-13 amino acids are identical or conserved are represented by grey boxes. Conserved amino acids are EQDN, KRH, FYW, MVLI and GASTP (single letter codes).
**Figure 3. Alignment of the pre-catalytic region of Dictyostelium DdPDE4 and members of the PDE8 subfamily.**

Multiple sequence alignments were made by using the CLUSTAL W program with improvements made by hand. All sequences are from GenBank® with accession numbers: HsPDE8A, O60658; HsPDE8B, NP003710; DmPDEA, AAR96128; AmPDE, XP392234; CePDE6, AAF60898. Species abbreviations are Hs, Homo sapiens, Dm, Drosophila melanogaster; Am, Apis mellifera; Ce, Caenorhabditis elegans. Black boxes represent positions were all 6 sequences are identical or conserved. Positions where 4 or 5 amino acids are identical or conserved are represented by grey boxes. Conserved amino acids are EQDN, KRH, FYW, MVLI and GASTP (single letter codes).

**Figure 4. Cluster analysis of Dictyostelium discoideum and mammalian PDE catalytic domains.**

Cluster analysis was based on the alignments of figure 2 (with additional sequences for the PDE8 subfamily). The analysis was generated by using the FITCH program of the PHYLIP package, with 1000 bootstrap replications to assess the reliability of the nodes. The bootstrap values are indicated in percentages. The catalytic domains of the phosphodiesterases from figure 2 fall into 2 groups. DdPDE4 groups with cAMP and dual specificity phosphodiesterases. The other group consists of cGMP specific and dual specificity mammalian phosphodiesterases. The other class I Dictyostelium phosphodiesterases (DdPDE2 or RegA and DdPDE 3) and HsPDE9A do not fall into either group.

**Figure 5. Biochemical analysis of the DdPDE4 overexpressor.**

All phosphodiesterase assays contain 5 mM DTT, which inhibits by more than 99% the abundant DdPDE1. Panel A, cAMP and cGMP phosphodiesterase activity measured in lysates from control cells (AX2) and from cells overexpressing the catalytic domain of DdPDE4 (DdPDE4catOE) using 10 nM [3H]cAMP or 10 nM [3H]cGMP. Panel B, inhibition of PDE activity using 10 nM [3H]cAMP by varying amounts of unlabelled cAMP and cGMP. PDE activity in this competition assay was measured in lysates from DdPDE4catOE and AX2 cells; the PDE activity measured in AX2 cells was subtracted from the activity measured in DdPDE4cat-GFPOE. Half-maximal inhibition of [3H]cAMP hydrolysis occurs at a concentration of 3-10 µM cAMP, while 100 µM cGMP has no effect. Panel C, Eadie-Hofstee plot of cAMP hydrolysis by DdPDE4 in the overexpressor cell line. Linear regression yields a slope of $K_M = 9.6$ µM and an intercept at $V_{max} = 4200$ pmol/min/mg.

**Figure 6. DdPDE4 is localized at the cell surface.**

cAMP phosphodiesterase activity of intact cells (c) or the membrane fraction of a lysate (m) from control cells (AX2), cells overexpressing the full length DdPDE4 (PDE4), and cells with a deletion of the the ddpde4 gene (pde4 null). Activity was measured using 10 nM [3H]cAMP in the presence of 10 mM DTT.

**Figure 7. Expression of DdPDE4 mRNA and activity during development.**

Panel A, Northern blot showing mRNA expression of ddpde4 in Dictyostelium strain AX3. RNA was isolated from wild-type AX3 cells at the developmental times indicated, size-fractionated and transferred to a Nytran filter. The blot was probed with part of the catalytic
domain of DdPDE4, detecting a mRNA with an estimated size of approximately 3 kb. The mRNA bands are interrupted by the 3 kb ribosomal RNA band. DdPDE4 is being expressed in AX3 cells at very low levels during early development, maximally at 9 hours of starvation during mound stage, and at somewhat lower levels during further development. The lower portion of the blot depicts rRNA stained with ethydium bromide to show loading. Panel B, DdPDE activity was measured using intact cells with $[^{3}H]cAMP$ in the presence of 10 mM DTT, with or without 0.8 mM IBMX. DTT inhibits DdPDE1, while IBMX inhibits DdPDE4; therefore, DdPDE4 activity is defined as the reduction of activity by IBMX. DdPDE4 activity is very low during the first 5 hours of development, increases strongly between 7 hour (loose aggregates) and 9 hours (tight aggregates), and is maximal at 12 hours in slugs.

**Figure 8. Phenotype of ddpde4 null cells.** Development of AX3 (left panel), the ddpde4 null cell strain (middle panel), and ddpde4 null cell strain expressing DdPDE4$^{OE}$ (right panel) on non-nutrient agar after 10 to 27 hours of starvation. Cell aggregation at 10 hours is not influenced by deletion of ddpde4 (upper panels), but multicellular development after 15 hours is severely retarded in ddpde4 null cells. At 20 hours of starvation, AX3 cells are forming fruiting bodies, whereas culmination has not yet started in the knock-out cells. At 27 hours of starvation AX3 have formed full grown fruiting bodies, whereas the knock-outs are in the process of forming fruiting bodies, with many mounds still present. Expression of full length DdPDE4$^{OE}$ in ddpde4 null cell completely restored the phenotypic defects of the null cell line. The bars indicate 1 mm.
| Cyclic nucleotide pool | PDE  | Class | Localization     | cAMP/cGMP specificity | Kinetic properties |
|-----------------------|------|-------|------------------|-----------------------|-------------------|
|                       |      |       |                  |                       | K_m (µM)          | V_max (pmol/min/mg) |
| Extracellular cAMP     | PDE1 | II    | Cell surface     | 3.2                   | 1                 | 100               |
|                       | PDE4 | I     | Extracellular    | >10                   | 10                | 13                |
| Intracellular cAMP     | PDE2 | I     | Cytosolic        | >100                  | 5                 | 50                |
|                       | PDE6 | II    | Cytosolic        | 8.7                   | 200               | 5200              |
| Intracellular cGMP     | PDE3 | I     | Cytosolic        | 0.04                  | 0.2               | 2                 |
|                       | PDE5 | II    | Cytosolic        | 0.008                 | 5-20              | 400               |

The cAMP/cGMP specificity is the ratio of hydrolysis measured at 10 nM cAMP and cGMP. The kinetic properties are for the substrate of the indicated pool, and are for 5h starved cells. The PDE activity of intact slug cells at 12h (figure 7B) suggest that the V_max for PDE1 is approximately the same as at 5h, while the V_max for PDE4 increases to about 300 pmol/min/mg protein.
Bader et al, Figure 1

A

DdPDE4

- Transmembrane: 211 - 324
- Signal sequence: 146 - 255
- Coiled coil: 256 - 688
- Precatalytic: 385 - 483
- Catalytic domain: 484 - 821

HsPDE8A

- Precatalytic: 385 - 483
- Catalytic domain: 484 - 821

B

Plasma membrane

- DdPDE4 (in - out)
Bader et al, Figure 3

pre-catalytic region <-> catalytic domain
Bader et al, Figure 4
Bader et al, Figure 5

A

PDE activity (pmol/min/mg protein)

[ ] (µM)

v (pmol/min/mg protein)

cAMP AX2  cAMP PDE4  cGMP AX2  cGMP PDE4

B

PDE activity (%)

[S] (M)

[ ] (M)

cAMP  cGMP

C

v (pmol/min/mg protein)

v[S] (pmol/min/mg protein/µM)

0  100  200  300  400  500

0  1  2  3  4  5  6  7

0  20  40  60  80  100  120

-8  10  -7  10  -6  10  -5  10  -4  10
Bader et al, Figure 6

PDE activity (fmol/min/cell equivalent)

|       | control | PDE4   | pde4-null |
|-------|---------|--------|-----------|
| c     |         |        |           |
| m     |         |        |           |
| c     |         |        |           |
| m     |         |        |           |
| c     |         |        |           |
| m     |         |        |           |
A

3 kb

rRNA

B

DdPDE4

total

Development (hours)

0 2 4 6 8 10 12 14

Development (hours)

0 2 4 6 8 10 12 14

Total PDE activity (fmol/min/10^7 cells)

0 200 400 600 800

PDE4 activity (fmol/min/10^7 cells)

0 200 400 600 800
DdPDE4, a novel cAMP-specific phosphodiesterase at the surface of dictyostelium cells
Sonya Bader, Arjan Kortholt, Helena Snippe and Peter J. M. Van Haastert

J. Biol. Chem. published online April 27, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M600040200

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