Evolution of self-organisation in Dictyostelia by adaptation of a non-selective phosphodiesterase and a matrix component for regulated cAMP degradation

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SUMMARY

Dictyostelium discoideum amoebas coordinate aggregation and morphogenesis by secreting cyclic adenosine monophosphate (cAMP) pulses that propagate as waves through fields of cells and multicellular structures. To retrace how this mechanism for self-organisation evolved, we studied the origin of the cAMP phosphodiesterase PdsA and its inhibitor PdiA, which are essential for cAMP wave propagation. D. discoideum and other species that use cAMP to aggregate reside in group 4 of the four major groups of Dictyostelium. We found that groups 1-3 express a non-specific, low affinity orthologue of PdsA, which gained cAMP selectivity and increased 200-fold in affinity in group 4. A low affinity group 3 PdsA only partially restored aggregation of a D. discoideum pdsA-null mutant, but was more effective at restoring fruiting body morphogenesis. Deletion of a group 2 PdsA gene resulted in disruption of fruiting body morphogenesis, but left aggregation unaffected. Together, these results show that groups 1-3 use a low affinity PdsA for morphogenesis that is neither suited nor required for aggregation. PdiA belongs to a family of matrix proteins that are present in all Dictyostelia and consist mainly of cysteine-rich repeats. However, in its current form with several extensively modified repeats, PdiA is only present in group 4. PdiA is essential for initiating spiral cAMP waves, which, by organising large territories, generate the large fruiting structures that characterise group 4. We conclude that efficient cAMP-mediated aggregation in group 4 evolved by recruitment and adaptation of a non-selective phosphodiesterase and a matrix component into a system for regulated cAMP degradation.

KEY WORDS: Morphogenetic signalling, cAMP oscillations, Cyclic nucleotide phosphodiesterase, CTDC domain, Gene co-option, Evolution of development

INTRODUCTION

One of the more surprising outcomes of comparative genome analysis is that the phenotypic complexity of species is only marginally related to the number of protein-coding genes in their genome, as illustrated by the fact that both humans and single-celled protozoa, such as ciliates, have ~25,000 protein-coding genes (Human Genome Sequencing Consortium, 2004; Eisen et al., 2006). This illustrates the fact that unicellular organisms are by no means simple. Their single cells can execute the multitude of functions, such as food uptake, metabolism, reproduction, directional movement, secretion, environmental sensing, prey hunting and predator evasion, for which higher organisms use many specialised cell types, organs and tissues. The genomes of complex multicellular organisms do contain much more non-coding DNA, with both a direct role in cis-regulation of gene expression, and an increasingly recognised enormous potential for post-transcriptional gene regulation (Zhou et al., 2010). This underpins the dependence of both the embryonic development and the adult physiology of multicellular organisms on accurately controlled expression of genes by their specialised cells at the right time and place. A hallmark of the evolution of multicellularity is the extensive use of communication between cells to regulate gene expression.

To understand how multicellular organisms evolved, it is therefore essential to retrace how complex intercellular communication emerged from more simple environmental sensing systems. We study the evolution of cell communication in the social amoebas, a highly tractable model system that combines a unicellular growth stage with an intricately orchestrated social amoebas, a highly tractable model system that combines a unicellular growth stage with an intricately orchestrated social amoebas, a highly tractable model system that combines a unicellular growth stage with an intricately orchestrated social amoebas, a highly tractable model system that combines a unicellular growth stage with an intricately orchestrated.

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cAMP between pulses. PdsA is extensively regulated at both the transcriptional and post-translational level. The PdsA gene is transcribed from three promoters during aggregation, growth and post-aggregative (late) development, respectively, with the late promoter being closest to the coding sequence (Faure et al., 1990). The PdsA protein can be both displayed on the exterior face of the plasma membrane, or cleaved off and released extracellularly. The released form facilitates cAMP wave propagation at low initial cell densities (Palsson, 2009). In the absence of extracellular cAMP an inhibitor protein, PdiA, is released, which increases the affinity (Km) of the secreted form of PdsA from 5 μM to 5 mM. In the presence of cAMP, PdiA expression is inhibited, whereas PdsA expression is strongly enhanced (Yeh et al., 1978; Franke et al., 1987). pdsa-null mutants can no longer aggregate (Sucgang et al., 1997), whereas pdsA-null mutants can no longer produce spiral cAMP waves, and consequently have 50 times smaller aggregation territories than those of wild-type cells (Palsson et al., 1997).

D. discoideum is a member of group 4 of the Dictyostelid social amoebas (Schaap et al., 2006). Most, if not all, members in this group use cAMP to aggregate, but none of the species in groups 1-3 (Schaap et al., 2006) (A. Skiba and P.S., unpublished). Glorin, a modified dipeptide of glutamate and ornithine is used by Polysphondylium violaceum and a range of non-group 4 species (Shimomura et al., 1982; Asghar et al., 2012), whereas a folate derivative is used by D. minutum in group 3 (De Wit and Konijn, 1983). Remarkably, even species that do not use cAMP for aggregation, do use cAMP oscillations to coordinate slug and fruiting body morphogenesis (Schaap et al., 1984; Fukushima and Maeda, 1991). Consistent with their post-aggregative use of cAMP, non-group 4 species express cAR genes from a single promoter after aggregation (Alvarez-Curto et al., 2005). Group 4 species have an additional distal promoter that activates cAR expression just before aggregation (Luis et al., 1993; Alvarez-Curto et al., 2005). Proximal post-aggregative and more distal aggregative promoters are also hallmarks of the Pdsa and ACA genes in D. discoideum (Faure et al., 1990; Galardi-Castilla et al., 2010), suggesting that the novel role of cAMP signalling during aggregation of group 4 species was achieved by distal insertion of new cis-regulatory regions in existing cAMP signalling genes. Such a scenario is in good agreement with a growing body of evidence showing that the evolution of new morphologies in animals and plants is largely dependent on elaboration of the cis-regulatory regions of genes, rather than their coding regions (Wray et al., 2003; Carroll, 2005).

To assess to what extent changes in gene regulation and protein function contributed to the evolution of cAMP signalling in the Dictyostelia, we studied conservation and change in both the regulation, function and biochemical properties of the PdsA genes throughout the Dictyostelid phylogeny. Our results show that elaboration of protein function also played a major role in enabling Dictyostelid amoebas to use cAMP oscillations to mediate efficient aggregation and formation of large fruiting structures.

MATERIALS AND METHODS

Cell culture

D. rosarium N45 (Droc), D. mucoroides S28 (Dmuc), P. violaceum P6 (Pvio), D. minutum 71-2 (Dmin), D. rhizopodium AusKV4 (Drhi), P. pallidum PN500 (Ppal), A. stoloniferum YAs6 (Das) and D. fasciculatum SH3 (Dfas) spores were grown in association with Escherichia coli 281 on 0.1% lactose/peptone (LP) agar plates (Raper, 1984). To obtain large quantities of cells, 1.5 × 10⁹ amoebas were inoculated in 300 ml 10 mM sodium/phosphate buffer, pH 6.5 (PB), supplemented with a resuspended E. coli 281 pellet from a 300 ml stationary culture in LB. The suspension was shaken at 200 rpm until the amoeba had reached a density of ~3 × 10⁹ to ~5 × 10⁹ cells/ml. Ddis was grown in HL5 medium. For developmental time courses, growing cells were washed free from bacteria with PB, and incubated at 22°C on non-nutrient (NN) agar (1.5% agar in PB) at 5 × 10⁶ cells/cm². Activated charcoal was placed in the lids to improve synchronous development.

Gene identification and analysis

Genomic DNA (gDNA) of species that represent all Dictyostelid groups was isolated as described earlier (Kawabe et al., 1999; Schaap et al., 2006). Degenerate oligonucleotide primers were designed to match amino acid sequences that are conserved between the PDE-II domains of Ddis PdsA and Saccharomyces cerevisiae PDE-II (Genbank id: EDV10448). Primers PDEF1 and PDER1 (supplementary material Table S1) yielded 633 nt products from Dmuc, Dros and Ddis gDNAs, which were validated by nested PCR with primer pair PDEF1 and PDERN1. The PCR products were sub-cloned in the pGEM-T Easy vector (Promega) and their sequence was determined from three independent clones. The Dmin PCR product was used to retrieve a full length Dmin PdsA coding sequence from a gDNA library (see below). Conserved regions between the Dmin- and Ddis PdsAs were used subsequently to design primers for nested PCR for amplification of PdsAs from more distant species. The first PCR was run using combinations of forward primers PdsA-51A and PdsA51B with reverse primers PdsA-3E and PdsA-3N, and the nested PCR used combinations of forward primers PdsA-51A and PdsA51B with reverse primer PdsA-32. The PCR program consisted of 35 cycles, with 30 seconds at 95°C, 50°C and 68°C each, and yielded products of about 300 bp from Pvio, Drhi, Aana, Daus and Dfas gDNAs. Full length D. purpureum (Dpur), Dfas, Ppal, Acytostelium subglobosum (Asub) and D. lacteum (Dlac) PdsA genes are recently available from ongoing genome sequencing projects (Heidel et al., 2011; Sucgang et al., 2011) (http://acetylodb.tsi.ius.kyoto.ac.jp/cgi-bin/index.cgi?org=as) (P.S. and G. Gloeckner, unpublished results).

To determine the nucleotide sequence of the Ppal PdsA mRNAs, polyA+ RNA was isolated from Ppal cells developed for 12 hours. Full length cDNAs were subsequently synthesised by RNA-ligation mediated rapid amplification of 5’ and 3’ cDNA ends (RLM-RACE) and RT-PCR using the GeneRacer kit (Invitrogen) and primers PdsA-R54 to PdsA-R37 (supplementary material Table S1) according to the manufacturer’s instructions.

DNA constructs and transformation

Cloning and expression of Dmin PdsA

The Dmin PdsA PCR product was used to screen a custom-made Dmin λZAPII gDNA library (Alvarez-Curto et al., 2005), which yielded six positive plaques. Their pBluescript phagemids were isolated by in vitro excision and the inserts were sequenced. The sequences could be assembled into a 9 kb contig, which contained full length Dmin PdsA as well as one complete and two partial flanking genes (supplementary material Fig. S3).

To generate an expression construct for gene complementation, a 1.3 kb fragment consisting of the complete coding region of Dmin PdsA without its single intron, was amplified from λZAPII-derived plasmid P6 with primers Dmin-PdsA-Ex3’5’ , which spans the intron that is positioned at 8 nt from the start codon, and Dmin-PdsA-Ex3’, which includes the stop codon. The primers contained a BamHI and XhoI restriction site, respectively (supplementary material Table S1). After BamHI/XhoI digestion, the PCR product was cloned into the similarly digested vector pDV-CYFP (Meima et al., 2007), which deletes the YFP sequence from the vector and places the product was cloned into the similarly digested vector pDV-CYFP (Meima et al., 2007), which deletes the YFP sequence from the vector and places the

Development
Both PCR products were cloned into vector pLoxP-NeoIII, yielding pPal PdsA-KO. To construct pLoxP-NeoIII, the Xhol site in pLoxP-Neo (Kawabe et al., 2009) was destroyed by digestion with Xhol, fill-in with Klenow and self-ligation with T4-ligase. The A6Neo cassette with flanking LoxP sites was next excised with BamHI/HindIII and cloned into pBluescript SK–, creating pLoxP-NeoIII.

The Ppal PdsA-KO vector was digested with SacI and Xhol and co-electroporated into Ppal cells with 100 μM of primers PdsA-53Xh and PdsA-32S to enhance the efficiency of homologous recombination (Kuwayama et al., 2008). Transformants were selected on lawns of autoclaved bacteria, supported by agar containing 300 μg/ml G418 (Kawabe et al., 1999). Out of 60 clones, obtained from two separate experiments, 55 showed the same aberrant phenotype. Southern blots were performed on gDNAs from one normal and four aberrant clones, and from untransformed cells. All four aberrant clones showed disruption of PdsA, but two clones harboured an additional random vector integration. The normal clone did not harbour a PdsA disruption (supplementary material Fig. S4).

PdsA promoter-lacZ constructs and analysis
To construct gene fusions of the PdsA415 and PdsA802 promoters and lacZ, the 2.2 kb PdsA415 promoter was amplified from Ppal gDNA using primers PdsA-P51X and PdsA-P31B, whereas for the 3.6 kb PdsA802 promoter primers PdsA-P52X and PdsA-P32B were used. Both primer sets contain XhoI and BamHI restriction sites (supplementary material Table S1). After digestion with XhoI/BamHI, the 2.2 kb PCR product was ligated into the BamHI/XhoI digested pDDGal17 vector (Harwood and Drury, 1990), yielding vector P415-gal, and the 3.6 kb PCR product was ligated into the BamHI/XhoI digested pDDGal16 vector (Harwood and Drury, 1990), yielding P802-Gal. Ppal cells were transformed with either of the vectors. β-Galactosidase activity was visualised with X-gal in developing Ppal structures as described previously (Dingermann et al., 1989; Kawabe et al., 2009).

PDE assays
To measure PDE-II activity, intact cells were serially diluted between 10^7 and 3x10^3 cells/ml in PB to yield activities that hydrolysed <60% of the substrate. Alternatively, cells were lysed through nucleopore filters (pore size 3 μm) at 10^7 cells/ml. Lysate (1 ml) was centrifuged for 10 minutes at 14,000 g to separate the cytosol and particulate fraction, with the latter being resuspended in 1 ml PB, and both diluted to the equivalent of 10^7 cells/ml. Cells or cell fractions were incubated for 30 minutes at 22°C with 10 nM or 100 nM [2,8-3H]cAMP ([3H]cAMP, Amersham, UK) in a total volume of 20 μl in the presence of 0.2 mM of the PDE-I inhibitor 3-isobutyl-1-methylxanthine (Sigma). Unlabelled cAMP or cGMP was added as indicated in the figure legends. Reactions were terminated by boiling and [2,8-3H] 5’AMP was hydrolysed further with 10 μg of Naja messambica snake venom (SA Venom Suppliers, Louis Trichardt, SA, USA) to [2,8-3H]adenosine, which was separated from [3H]cAMP by adsorption of the latter to Dowex anion exchange resin.

RESULTS
Identification of PdsA genes in group representative taxa
Most Dictyostelia are members of four major taxon groups that combine to form two branches, containing groups 1 and 2 and groups 3 and 4, respectively (Schaap et al., 2006) (A. Skiba and P.S., unpublished). Only group 4 taxa use cAMP to aggregate (Schaap et al., 2006) (A. Skiba and P.S., unpublished) and require PdsA to generate steep chemotactic gradients (Darmon et al., 1978). To investigate whether PdsA is conserved in the Dictyostelids, we used a PCR approach with degenerate oligonucleotide primers to amplify homologues of the D. discoideum (Ddis) PdsA gene from group-representative taxa. Figure 1A shows a schematic representation of the SSU rRNA phylogeny of the Dictyostelia and illustrates the position of the test species. The first set of primers was designed to match regions conserved between the PDE-II domain of Ddis PdsA and non-dictyostelid PDE-II type enzymes. This set only yielded products from two other group 4 taxa, D. mucoroides (Dmuc) and D. rosarium (Dros), and from a group 3 taxon D. minutum (Dmin). The Dmin PdsA product was subsequently used to screen a genomic library. This yielded a full-length PdsA gene (see below), the sequence of which was used to design more specific primers for dictyostelid PdsAs.

The new primers yielded a single fragment from another group 3 taxon D. rhizopodium (Drhi), the group-intermediate taxon P. violaceum (Pvio), the group 1 taxa D. aureo-stipes (Daus) and D. fasciculatum (Dfas), and two sequences from the group 2 taxon A. anastomosans (Aana). Complete coding sequences for D. purpureum (Dpur), D. lacteum (Dlac), A. subglobosum (Asub), P. pallidum (Ppal) and D. fasciculatum (Dfas) PdsAs and for the Ddis PdsA homologue, Pde7 (Bader et al., 2007), were recently obtained from ongoing (Asub, Dlac) and completed genome sequencing projects (Eichinger et al., 2005; Heidel et al., 2011; Sugang et al., 2011). All derived partial and complete PdsA amino acid sequences were aligned with the closest PdsA homologues in non-dictyostelid organisms, which are three PDE-II sequences from Naegleria gruberi, Legionella pneumophila and Flavobacterium johnsoniae (supplementary material Fig. S1).

The sequence alignment was used to determine phylogenetic relationships between the PdsA sequences (Fig. 1B). The analysis shows that the group 4 PdsAs form two sister clades, whereas the group 3 PdsAs cluster together. The single Ppal PdsA clusters with one of the Aana and two Asub PdsAs, whereas the other Asub- and Aana PdsAs cluster with the group 1 PdsAs from Dfas and Daus. The second Dfas PdsA is more related to the cluster containing Ppal PdsA. These data suggest that two PdsA genes were present in the last common ancestor (LCA) to groups 1 and 2; one gene was lost in Ppal, whereas the other was duplicated in Asub. The LCA to groups 3 and 4 contained a single PdsA, which was duplicated twice in group 4.

Ddis PdsA is inhibited by a secreted inhibitor protein, PdiA, which, apart from a hydrophobic leader peptide, also contains five 24-amino acid (aa) cysteine-rich repeats that are found in large numbers in extracellular matrix proteins, such as EcmA and EcmB (Williams et al., 1987; Wu and Franke, 1990). These repeats were recently classified as Dicty-CTDC domains (PFAM accession number PF00526). BLAST queries with PdiA identified two and four PdiA-like genes in the genomes of the group 4 taxa Ddis and Dpur, respectively (Fig. 1C). However, in group 1, 2 and 3 genomes only large EcmA-like proteins were hit. The two Dpur proteins are the closest homologues of Ddis PdiA, but seem to have fewer CTDC domains. However, the aligned Ddis and Dpur sequences show only small variations over the regions that encompass the missing repeats (supplementary material Fig. S2) and high (62%) sequence identity over the entire length of the three proteins. In short, it appears that Dpur probably has one or two functional PdiAs, but that PdiA is not present in group 1, 2 or 3 taxa.

Functional analysis of a group 3 PdsA
The partial PdsA PCR product from the group 3 taxon Dmin was used to screen a Dmin AZAP genomic library. Six positive clones were identified, which could be assembled into a 9.02 kb contig (supplementary material Fig. S3). This contig contains Dmin PdsA and three flanking genes, which are most similar to the Ddis genes rhm8A, DDB G10286003 and AbcF1, which occupy the same positions relative to Ddis PdsA on chromosome 4, indicating that Dmin PdsA is a true orthologue of Ddis PdsA.
To investigate whether the PdsAs from taxa that do not use cAMP as attractant are functional cAMP phosphodiesterases, we expressed the Dmin PdsA gene from the constitutive A15 promoter in the Ddis pdsA-null mutant Uk7. This mutant is defective in aggregation and fruiting body formation, and is fully restored by expression of Ddis PdsA (Sucgang et al., 1997). At a high cell density, the Uk7 cells formed a few small aggregates and misshapen fruiting bodies (Fig. 2A). Aggregation of Uk7/A15::Dmin PdsA cells, though still incomplete, was improved and more normal fruiting bodies were formed. At three- to tenfold lower cell densities, A15::Dmin PdsA became progressively less efficient in restoring Uk7 aggregation (data not shown). To assess whether Dmin PdsA is fully functional, we measured the activity of the expressed enzyme. Figure 2B shows that intact Uk7/A15::Dmin PdsA cells hydrolyse 100 nM 3HcAMP almost as rapidly as wild-type Ddis cells, whereas Uk7 cells show only negligible activity. To determine whether Dmin PdsA has a similar affinity and selectivity for cAMP as Ddis PdsA, we measured hydrolysis of 10 nM 3HcAMP in the presence of increasing concentrations of unlabelled cAMP or cGMP. Figure 2C shows that about 200-fold higher cAMP concentrations are required to compete for 3HcAMP hydrolysis by Dmin PdsA than for Ddis PdsA, and Dmin PdsA has also lost most of its selectivity for cAMP over cGMP. Km values for Ddis and Dmin PdsA are 2 μM and 460 μM, respectively, as derived from a Hanes plot (Fig. 2D) (Biswanger, 2002). The poor ability of Dmin PdsA to restore Uk7 aggregation is very likely to be a result of its low affinity for cAMP.

Developmental regulation of PdsA expression

Ddis PdsA is transcribed from three different promoters during growth, aggregation and post-aggregative development that yield mRNAs of 1.9, 2.4 and 2.2 kb, respectively (Faure, et al., 1990). For at least one synchronously developing species in each of the taxon groups, we investigated whether its PdsA gene was under similar complex regulation (Fig. 3). The Ddis 2.4 and 2.2 kb mRNA bands were not clearly resolved, but at least two differentially regulated PdsA mRNA species are present in...
another group 4 taxon Dros. Group 3 Dmin PdsA mRNA appears shortly after aggregation. Ppal in group 2 transcribes a >2.2 kb mRNA throughout development and a <1.9 kb mRNA after aggregation. In group 1, Daus transcribes two mRNA species during growth and early development, with one transcript re-appearing during aggregation, whereas Dfas transcribes two or three mRNAs during growth and retains the largest during post-aggregative development. It should be noted that northern blots are indicative, but not reliably diagnostic for mRNA heterogeneity; mRNAs of similar size might not be resolved, and band displacement artefacts can occur when mRNAs have the same size as the highly abundant ribosomal RNAs. This seems to be the case for the two identically regulated Dmin mRNA bands. Nevertheless, there appears to be considerable heterogeneity in the complexity of PdsA transcription that does not show any obvious group-specific trend.

P. pallidum PdsA gene structure and expression pattern of isoforms

Ppal is thus far the only non-group 4 species that is amenable to genetic manipulation and we therefore focussed research into the role and regulation of PdsA on this species. To define the promoters and determine the sequences of the two PdsA mRNAs that are transcribed in Ppal (Fig. 3), we performed RT-PCR and oligo-capping RACE on mRNA isolated at 12 hours of development. Four different PdsA mRNA sequences were obtained, encoding PdsA proteins with different N-termini (Fig. 4, supplementary material Fig. S5). The shortest 1.35 kb mRNA encodes a 415 aa protein (PdsA415) that is somewhat smaller than Ddis PdsA (452 aa). Ppal PdsA415 has no obvious N-terminal signal peptide (supplementary material Fig. S1), which is essential for cell surface and extracellular localisation of Ddis PdsA (Franke et al., 1991). Of the others, the 2.55 and 2.81 kb mRNAs encode the same 802 aa protein (PdsA802), owing to extension of the N-terminal region, which now also includes a signal peptide. The position of the first intron downstream of the start codon is the same in the longer mRNAs, but the upstream intron boundaries vary. The longest 2.82 kb mRNA contains an ATG in the first exon, but this open reading frame terminates immediately at the second exon, suggesting that this mRNA is not functional.

We next examined the spatial expression patterns of the PdsA415 and PdsA802 isoforms. For the PdsA415 isoform, we amplified 2.18 kb of 5'UTR, upstream from its putative start codon (Fig. 4A, 415 promoter) and for the PdsA802 isoform we amplified 3.62 kb of 5'UTR upstream of the putative start codon in the 2.55 and 2.81 kb mRNAs (Fig. 4A, 802 promoter). These sequences were fused to the lacZ reporter gene, yielding P415-gal and P802-gal, and transformed into Ppal cells. Intact developing structures were stained with X-Gal to visualise the lacZ expression pattern (Fig. 4B,C). P415-gal was first expressed weakly in streaming aggregates (Fig. 4Ba). In the newly formed sorogen, expression was strongest at the tip region (Fig. 4Cb), disappearing almost completely from cells below the tip (Fig. 4Bc,d). The secondary sorogons that are formed from cell masses at the rear of the main sorogen usually showed enriched 415-gal expression at their tips (Fig. 4Be,f). P802-gal was already expressed in aggregation streams (Fig. 4Ca,b) and remained expressed in the whole structure throughout fruiting body formation, with somewhat higher expression in the tip region (Fig. 4Dc-f). It should be noted that P802-gal could contain the promoters for both the 2.55 and 2.81 kb mRNAs.
Evolution of PdsA and PdiA

Fig. 3. Developmental regulation of PdsA transcription in group-representative taxa. Ddis, Dros, Dmin, Ppal, Dfas and Daus cells were freed from bacteria and incubated at 22°C on NN agar until fruiting bodies had formed. Total RNA was extracted at 2-hour intervals and northern transfers were probed at high stringency with [32P]dATP-labelled PCR products amplified from the PdsA genes in each of the species. The same blots were re-probed at medium stringency with the constitutively expressed Ddis IG7 gene. All transfers showed equal sample loading (data not shown), except Ppal, for which the first four slots received less total RNA. The progression of aggregation and fruiting body formation are outlined underneath the time courses by grey and black triangles, respectively, and an intervening migrating slug stage is indicated by a white box. Arrows indicate the position of the 1.9 kb 17S rRNA band.

Developmental role of Ppal PdsA
To understand the role of PdsA in species that do not use cAMP for aggregation, we disrupted the single PdsA gene of Ppal by homologous recombination (supplementary material Fig. S4). The pdsA- cells showed normal aggregation with inflowing streams of amoebas (compare Fig. 5A and 5B). However, thereafter, the formation of slugs and fruiting bodies was slow and aberrant. Only small club-like structures were formed (Fig. 5C), without an obvious stalk or spore head, or the whorls of side branches that are characteristic for this species (Fig. 5D). The aberrant pdsA- fruiting structures were stained with Calcofluor, which stains the cellulose-rich wall of mature spores and stalk cells, to assess whether terminal cell differentiation had occurred. Wild-type fruiting bodies form stalks that are one cell thick, and elliptical spores (Fig. 5E). In pdsA- fruiting bodies, the stalk cells are more disorganised, and the sori or the cell masses that are clinging to the stalk contain encapsulated round cells (Fig. 5F). Ppal amoebas can differentiate directly to spherical cysts without aggregating. However, electron-microscopic examination of the pdsA- spores revealed that they display the condensed cytoplasm and thick three-layered cell wall that characterises wild-type spores (compare Fig. 5G and 5I). Cysts are less dense and are surrounded by a thinner two-layered cell wall (Fig. 5H) (Hohl et al., 1970; Kawabe et al., 2009).

We examined next whether the phenotype of the pdsA- mutant is cell autonomous by mixing pdsA- and wild-type cells at different ratios and allowing the mixtures to form fruiting bodies (Fig. 6). Chimeric fruiting bodies with 50% wild-type cells showed wild-type morphology (compare Fig. 6A and 6B). With only 10% wild-type cells, the chimeric fruiting bodies showed normal spore heads and main stalks. However, the number of side branches on fruiting bodies was reduced (Fig. 6C). Calcofluor staining revealed that elliptical spore morphology and regular stalk formation were fully restored by mixing pdsA- with 10% wild-type cells (Fig. 6D). We also measured the ratio of wild-type to pdsA- spores in the chimeric fruiting bodies. Figure 6E shows that chimeric fruiting bodies formed from mixtures with 10% or 50% wild-type cells contained 9% or 47% wild-type spores, respectively. This means that the tendency of wild-type and pdsA- cells to differentiate into either spore or stalk cells in chimeric structures is almost the same. Combined, these data show that PdsA is required for post-aggregative morphogenesis and spore elongation in Ppal in a non-cell autonomous manner.

Activity and cellular localisation of the P. pallidum PdsA
The non-cell autonomous phenotype of the Ppal pdsA-null mutant suggests that PdsA is only essential for extracellular cAMP signalling, despite the fact that the PdsA415 isoform does not have a signal peptide. To confirm that the Ppal pdsA-null mutant is defective in extracellular cAMP hydrolysis, we measured 3HcAMP hydrolysis in intact and fractionated cells from early culminants. Figure 7A shows that wild-type culminant cells hydrolyse 3HcAMP about six times faster than pdsA- cells, all PdsA specific 3HcAMP hydrolysis is localised in the particulate fraction, indicating that all measured PdsA activity is membrane associated. Growth stage cells show no PdsA specific 3HcAMP hydrolysis. We also determined the Km and substrate affinity of PdsA for cAMP is very low (Km: 200 μM) and the enzyme is not selective for cAMP over cGMP.

DISCUSSION

PdsA gene regulation only partially reflects its utilisation
PdsA is an essential component of the network that generates cAMP oscillations and enables aggregation and morphogenesis of the model organism D. discoideum (Wu et al., 1995; Sucgang et al., 1997). Its inhibitor protein PdiA is instrumental for long range propagation of spiral cAMP waves in a field of starving cells (Palsson and Cox, 1996; Palsson et al., 1997), but is not expressed during post-aggregative development (Franke et al., 1991). PdsA genes could be identified in all selected test species across the four Dictyostelid taxon groups, indicating that PdsA is likely to be conserved in all Dictyostelia. By contrast, PdiA was only detected in group 4 taxa (Fig. 1).
**Fig. 4. Structure and expression patterns of the Ppal PdsA gene.** (A) Gene structure. Four different PdsA mRNA sequences were obtained by RT-PCR and oligo-capping RACE (Maruyama and Sugano, 1994) performed on Ppal mRNA isolated at 12 hours of development. The exons that make up the four transcripts are outlined in grey boxes and the positions of the putative start and stop codons are indicated. The 1.36 kb mRNA encodes a protein of 415 aa, whereas the 2.55 and 2.81 kb mRNAs yield a 802 aa protein. The largest mRNA has its start and stop codons in the first and second exon, respectively. The putative promoter sequences for the 415 and 802 aa proteins are outlined by black boxes. (B,C) Spatial regulation of promoter activity. The 415 and 802 promoter regions as outlined in panel A were fused to lacZ, yielding P415-gal and P802-gal, respectively, and expressed in Ppal. The P415-gal (B) and P802-gal (C) cells were developed on NN agar and intact developing structures were fixed and stained with X-gal. a, aggregate; b, tip formation; c, primary sorogen formation; d, segregation of whorls; e,f, formation of secondary sorogens. Scale bars: 0.1 mm.

*PdsA* is transcribed as three alternatively spliced mRNA species from three different promoters, which direct expression during growth, aggregation and in the anterior prestalk region of multicellular structures, respectively (Faure et al., 1990; Hall et al., 1993). Group 1 and 2 taxa also transcribed two to three mRNA species, of which at least one was already expressed during growth and aggregation. Only the group 3 taxon *Dmin* did not express *PdsA* during growth and early aggregation (Fig. 3). This evolutionary progression in expression patterns differs strongly from that of the *cAR* genes, which are also essential for oscillatory cAMP signalling. cARs are expressed after aggregation in group 1 and 2 taxa, during both aggregation and late development in group 4 taxa and during growth and late development in *Dmin* (Alvarez-Curto et al., 2005). At least for groups 1 and 2, the cAR expression pattern is consistent with its exclusive role in post-aggregative development (Kawabe et al., 2009). However, both the group 3 *cAR* and group 1 and 2 *PdsAs*, are expressed at stages at which they do not appear to be required.

**PdsA is essential for post-aggregative development in non-group 4 species**

Disruption of the *PdsA* gene in the group 2 taxon *Ppal* did not affect aggregation, but resulted in formation of highly abnormal fruiting bodies with disorganised stalk cells and round spores. This phenotype resembles that of a *Ppal* car-null mutant, but there are also differences. The *car*– spores turned out to be cysts (Kawabe et al., 2009), whereas the *pdsA*– spores showed otherwise normal spore morphology (Fig. 5I). Additionally, in contrast to the *car*– phenotype, the *pdsA*– phenotype is non-cell autonomous, as both fruiting body formation and elliptical spore morphology can be restored by mixing *pdsA*– cells with wild-type cells (Fig. 6).

The *pdsA*– and *car*– phenotypes substantiate conclusions drawn from pharmacological experiments that all non-group 4 taxa use oscillatory cAMP signalling for organisation of post-aggregative morphogenesis, but not for aggregation (Alvarez-Curto et al., 2005; Kawabe et al., 2009) (A. Skiba and P.S., unpublished). The non-cell autonomous nature of the *pdsA*– phenotype indicates that PdsA is involved in mediating cAMP signalling between cells, but not in transduction of the cAMP signal, as is the case for cARs. It is unclear what causes the round spore morphology. The *car*– mutants formed cysts in their fruiting bodies, because they lack cAR-mediated cAMP induction of pre-spore differentiation, but have retained PKA-mediated induction of encystation (Kawabe et al., 2009). The *pdsA*– structures are likely to contain excessively high extracellular cAMP levels, which possibly accelerate spore wall assembly relative to spore elongation.

**PdsA is an opportunistic cAMP phosphodiesterase**

In contrast to the *Dmin* *cAR*, which can fully complement the aggregation deficient phenotype of a *Ddis* *car*-null mutant (Kawabe et al., 2009), *Dmin* PdsA can only partially rescue aggregation of a *Ddis* *pdsA*– mutant (Fig. 2). This is most likely due to its 200-fold reduced affinity for cAMP, which it shares with the *Ppal* PdsA from group 2 (Fig. 7B). Both the *Dmin* and *Ppal* PdsAs show no selectivity for cAMP over cGMP. PdsA is a PDE-II type enzyme, which have a catalytic domain that is similar to that of the metallo-beta lactamases, but not to the metazoan PDE-I domain (Conti and Beavo, 2007). In fungi, PDE-II enzymes generally have a lower affinity for cAMP than do PDE-I enzymes (Nikawa et al., 1987; Zhang et al., 2011). PDE-II type genes are present in most major clades of bacteria, but only two genes have been analysed functionally. *Vibrio fischeri*, a symbiotic marine bacterium, exposes a PDE-II type enzyme, CpdP, outward into the periplasm. This enzyme hydrolyses cyclic nucleotides indiscriminately at a very high rate, supposedly to enable *V. fischeri* to utilise cyclic nucleotides, secreted by its hosts, as a carbon source (Callahan et al., 1995). *Myxococcus xanthus* PdeE is
the other characterised PDE-II. Apart from cAMP and cGMP, it also hydrolyses 5’ nucleotides (Kimura et al., 2011). Evidently, the roles of prokaryote PDE-II enzymes are not confined, or perhaps not even related, to cAMP signal transduction.

*Ppal* and *Dmin* PdsA show Kms for cAMP of 0.2 and 0.46 mM respectively, indicating a 100-200 fold lower affinity for their substrate as *PdsA* (Fig. 2D). Combined with their lack of selectivity for cAMP, this strongly suggests that PdsA originally had other substrates, or is a broad-spectrum hydrolase. One can only guess what its actual substrate is or might have been.

Because PdsA is exposed on the cell surface, roles in environmental signalling or detoxification seem likely. Such roles might explain why so many species express PdsA during growth (Fig. 3), without there being an obvious requirement for secreted cAMP at this stage. The low affinity of the non-group 4 PdsAs for cAMP might not be problematic for its role in post-aggregative development, where cAMP released between the tightly packed cells can easily reach the supramicromolar concentrations that are, for example, required for pre-spore gene induction (Schaap and Van Driel, 1985; Oyama and Blumberg, 1986). However, in a field of dispersed starving cells that respond chemotactically to as little as 0.1 nM cAMP (Van Haastert and Konijn, 1982), an enzyme with a Km of 0.4 mM will not be efficacious to generate steep chemotactic gradients. The 200-fold increase in affinity for cAMP of the group 4 PdsAs is therefore likely to have been an essential adaptation to enable cAMP-mediated aggregation in this group.

**Improvement of cAMP signalling dynamics by recruitment of a matrix protein**

The evolutionary history of the PdsA inhibitor PdiA is equally intriguing. PdiA consists of eight 24 aa cysteine-rich repeats, of which five are recognised as Dicty-CTDC domains and the other three are degenerate CTDC domains (supplementary material Fig. S2). Apart from a few prokaryote proteins, CTDC domains are unique for Dictyostelia. They are typically present in long arrays in extracellular matrix proteins, such as ecmA and ecmB (McRobbie et al., 1988), cysteine oxidation of which will form intra- and interprotein crosslinks. The *Ddis* genome contains 20 CTDC domain proteins; most include a signal peptide and ten include a PA14 domain with a proposed role in carbohydrate binding (http://dictybase.org/). The *Ppal* and *Dfas* genomes contain at least 25 and 19 CTDC proteins, respectively (H. Lawal and P.S., unpublished results). Three of the *Ddis* proteins, PsiA, and PsiF(DicA) and cyrA, act as external signals.
controlling gene expression and cell motility (Kolbinger et al., 2005; Yamada et al., 2010; Suarez et al., 2011). Evidently, CTDC domain-containing genes were strongly amplified in early Dictyostelid evolution, and from this pool of matrix components, genes with novel signalling roles emerged. This scenario bears similarity to the evolution of the hedgehog proteins, important signal molecules in animal development, which originated from a cleavable substrate adhesion protein in the unicellular choanoflagellate ancestor (Snell et al., 2006).

PdiA probably emerged late in Dictyostelid evolution, as a putative duplicated orthologue could only be recognised in Dpur, another group 4 species (Fig. 1C). PdiA was theoretically predicted and experimentally confirmed to generate the conditions that allow spiral cAMP waves, which cover large territories, to form in preference to concentric waves, which organise fewer cells into aggregates (Palsson and Cox, 1996; Palsson et al., 1997). Group 4 species stand out by forming relatively large aggregates and fruiting structures (Schaap et al., 2006). It is not unlikely that they owe this feature to recruitment of PdiA from the pool of matrix proteins.

Opportunistic exploitation of existing genes limits gene number expansion

The evolution of the PdsA-PdiA system illustrates in a fascinating manner how a sophisticated intercellular communication system, capable of organising the movement of up to a million cells, emerged by modification of existing genes with fairly mundane or redundant functions. These modifications involved both elaboration of promoter regions as well as mutations in the coding sequence for providing gene sequences. We thank John James and Dr Alan Prescott for expert electron microscopy.

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Competing interests statement

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Supplementary material

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