Adenylate cyclase A amplification and functional diversification during Polyspondylium pallidum development

Yoshinori Kawabe
University of Dundee

Pauline Schaap (✉ p.schaap@dundee.ac.uk)
University of Dundee

Research Article

Keywords: excitable systems, cAMP oscillations, adenylate cyclase A, coordinated cell migration, cell aggregation, morphogenetic movement, Dictyostelia,

Posted Date: July 25th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1888328/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Background: In Dictyostelium discoideum (Ddis) adenylate cyclase A (ACA) critically generates the cAMP oscillations that coordinate aggregation and morphogenesis. Unlike group 4 species like Ddis, other groups do not use cAMP to aggregate. However, deletion of cAMP receptors (cARs) or extracellular phosphodiesterase (PdsA) in Polyspondylium pallidum (Ppal, group 2) blocks fruiting body formation, suggesting that cAMP oscillations ancestrally control post-aggregative morphogenesis. In group 2, the acaA gene underwent several duplications. We deleted the three Ppal aca genes to identify roles for either gene and tested whether Ppal shows transient cAMP-induced cAMP accumulation, which underpins oscillatory cAMP signalling.

Results: Unlike Ddis, pre-aggregative Ppal cells did not produce a pulse of cAMP upon stimulation with the cAR agonist 2'H-cAMP but acquired this ability after aggregation. Deletion of Ppal aca1, aca2 and aca3 yielded different phenotypes. aca1 cells showed relatively thin stalks, aca2 showed delayed secondary sorogen formation and aca3 formed less aggregation centers. The aca1aca2 and aca1aca3 mutants combined individual defects, while aca2aca3 and aca1aca3aca2 additionally showed >24 h delay in aggregation, with only few aggregates with fragmenting streams being formed. The fragments developed into small fruiting bodies with stalk and spore cells. Aggregation was restored in aca2aca3 and aca1aca3aca2 by 2.5 mM 8Br-cAMP, a membrane-permeant activator of cAMP-dependent protein kinase (PKA). Like Ddis, Ppal sorogens also express the adenylate cyclases ACR and ACG. We found that prior to aggregation, Ddis aca/ACG cells produced a pulse of cAMP upon stimulation with 2'H-cAMP, indicating that cAMP oscillations may not be dependent on ACA alone.

Conclusions: The three Ppal replicates of acaA perform different roles in stalk morphogenesis, secondary branch formation and aggregation in Ppal, but act together to enable development by activating PKA. While even an aca1aca3aca2 mutant still forms (some) fruiting bodies, suggesting little need for ACA-induced cAMP oscillations in this process, we found that ACG also mediated transient cAMP-induced cAMP accumulation. It therefore remains likely that post-aggregative Ppal morphogenesis is organized by cAMP oscillations, favouring a previously proposed model where cAR-regulated cAMP hydrolysis rather its synthesis dominates oscillatory behaviour.

Background

Developing organisms need to coordinate cell differentiation with the generation of form. While differentiation largely results from regulation of gene expression, form can be generated by coordinated cell movement, cell division or changes in cell shape, either of which can act alone or in combination with others [1]. D. discoideum (Ddis) amoebas survive starvation by aggregating to form a migrating sorogen or slug, which turns into a fruiting body, consisting of spores and stalk cells. The chemotactic cell movements that cause aggregation as well as slug and fruiting body morphogenesis are organized by pulses of cAMP that are initially secreted by the most food-deprived cells and propagate as waves.
through the population by cAMP-induced cAMP synthesis [2–4]. The oscillating centre becomes the organizing tip of aggregates, slugs and fruiting bodies

Dictyostelia can be subdivided into four major groups, with *Ddis* residing in group 4. While the other group 4 species that all use cAMP as chemoattractant [5], are likely to use the same mechanism to coordinate morphogenesis, this is not clear for species in the other three groups, which use the dipeptide glorin and other compounds as chemoattractant for aggregation [5–9]. Nevertheless, deletion of either cARs or PdsA from the group 2 species *P. pallidum (Ppal)* disorganized post-aggregative morphogenesis [10–12], while *D. minutum* in group 3 showed cAMP-induced cAMP synthesis and oscillatory cell movement only after aggregation [13, 14]. This suggested that non-group 4 species use cAMP oscillations to coordinate morphogenesis in the slug and fruiting body stage, while using other chemoattractants for aggregation.

To test this hypothesis we deleted the three acaA homologs from *Ppal* individually and in combination. While single knock-outs in *aca1*, *aca2* or *aca3* showed subtle defects in primary stalk, side-branch formation and aggregation, respectively, triple *aca1aca3aca2* were very delayed in aggregation, but still formed some small fruiting bodies after a long delay. We explored whether other dictyostelid adenylate cyclases could also participate in pulsatile cAMP signalling.

Results

**Spatio-temporal expression patterns of P. pallidum acaA homologs**

In *D. discoideum (Ddis)*, *acaA* shows complex expression from different promoters. The promoter proximal to the coding sequence directs expression high expression at the slug tip, the central promoter directs low expression in the prespore region, while the most distal promoter directs high expression during aggregation [15]. *P. pallidum (Ppal)* has three *acaA* genes, *aca1*, *aca2* and *aca3* (Additional file 1, Figure A1). Comparative transcriptomics shows that these and other *acaA* genes across taxon groups are upregulated after starvation, with group 4 *acaA* genes showing peak expression during aggregation. *Aca* genes are most highly expressed in stalk cells in groups 1–3, but in group 4 expression is highest in cup cells, which are unique to group 4 (Fig. A1).

To investigate the spatial expression pattern of *Ppal aca1*, *aca2* and *aca3*, their promoter regions were fused to the LacZ reporter gene and transformed into *Ppal* cells. Developing structures were fixed and incubated with X-gal to visualize β-galactosidase activity. *Aca1* was not expressed during aggregation and started to be expressed weakly at the utmost tip region of the primary sorogen, and later sometimes in the tip of secondary sorogens (Fig. 1A). *Aca2* and *aca3* were already expressed in aggregates and more strongly during post-aggregative development (Fig. 1B,C). In primary sorogens, *aca2* was expressed throughout the structure but most strongly at the tip region. *Aca3* expression was more specific to the tips of primary and secondary sorogens. Overall, the post-aggregative expression pattern of the three *Ppal acas* resembles that of *Ddis acaA* with strongest expression at the sorogen tips [15, 16].

**Deletion of aca genes in P. pallidum**
To assess the biological roles of the three *Ppal aca* genes, we replaced essential regions in each gene with the LoxP-NeoR cassette, in which *NeoR*, the single selectable marker of *Ppal* is flanked by *loxP* sites (Additional file1, Figure A2). The *aca1* clones aggregated normally and formed fruiting bodies with somewhat thinner and longer stalks than those of wild type *Ppal* (Fig. 2A, D). The *aca2* mutant aggregated and formed the primary sorogen normally but showed delayed formation of the first whorls of secondary sorogens (Fig. 2C). Such whorls arise at regular intervals when a posterior segment of the primary sorogen pinches off, while forming several regularly spaced tips, which each give rise to a small side branch. As a result, the branch-less lower stalk of *aca2* fruiting bodies was longer than in wild-type (Fig. 2D). The *aca3* mutant formed few aggregation centres with long streams (Fig. 2A), that partitioned into many tip-forming small aggregates that each gave rise to a small fruiting body. The central, large *aca3*-aggregate produced a normal fruiting body (Fig. 2B,D). Overall, the phenotypes of single *aca* knock-out mutants were subtle. We tried to generate double and triple *aca* knock-outs by recycling the LoxP-NeoR cassette using the cre-recombinase expression vector pA15NLS.Cre [17]. This succeeded for the *aca1* mutant, allowing us to generate *aca1aca2* and *aca1aca3* double knock-outs, but not for the *aca2* or *aca3* knock-outs. The *aca1aca3* phenotype combined features of *aca1* and *aca3* knock-out mutants. Similar to *aca3*, *aca1aca3* formed few but large streaming aggregates (Fig. 2AB), while the fruiting bodies showed thinner and longer stalks, like the *aca1* mutants (Fig. 2D). The *aca1aca2* cells aggregated and formed primary sorogens normally. However, the separation of the first whorl only occurred after 28 h of starvation, when WT, *aca1*, and *aca2* had already formed fruiting bodies (Fig. 2C). As a result, *aca1aca2* made very tall fruiting bodies with side branches only at the upper stalk (Fig. 2D).

The failure to recycle LoxP-NeoR cassette of *aca2* or *aca3* mutants was probably due to limited selectability of cells transformed with pA15NLS.Cre with its G418 selection cassette. We found that *Ppal* growth is also inhibited by the antibiotic Nourseothricin. This allowed us to use a Cre-recombinase expression vector pDM1483 [18] with a Nourseothricin selection cassette to eliminate LoxP-NeoR from *aca3* and *aca1aca3* and to generate *aca3aca2* and *aca1aca3aca2* knock-outs.

Compared to wild-type, *aca1aca2*, *aca1aca3* and single *aca* knock-outs, which all initiated aggregation within 8 h of starvation, the *aca3aca2* mutant only started to aggregate at 24 h or later (Fig. 3A). Only few aggregation foci were formed, which attracted very long aggregation streams. Starting from the initial (small) focus, mounds appeared at intervals within the streams, which each attracted downstream cells. Each of these mounds gave rise to a small, branched fruiting body, which, similar to *aca2*, showed a longer whorl-free lower stalk (Fig. 3B). The *aca1aca3aca2* phenotype combined features of the *aca1aca2* and the *aca3aca2* mutant. Similar to *aca3aca2*, aggregation was much delayed with long streams appearing only after 24–48 h of starvation (Fig. 3A), which eventually broke up and gave rise to small fruiting bodies. These fruiting bodies showed delayed side-branch formation, like *aca1aca2* (Fig. 3B). Staining of the *aca1aca3aca2* stalk and spore cells with the cellulose dye Calcoflour showed that it formed a normal primary and secondary stalk and elliptical spores encapsulated in cellulose walls (Fig. 3C), and this was also the case for all other *aca* mutants (not shown).
To investigate whether the aggregation phenotypes of the \( aca3\aca2 \) or \( aca1\aca3\aca2 \) mutants were cell-autonomous, the mutants were developed as chimeras with wild-type cells. Introduction of 10% wild-type cells was sufficient to restore delayed aggregation of both mutants (Fig. 3A). The mixtures aggregated within 8 h of starvation like wild-type cells, but still formed larger aggregation streams. Also, the formation of secondary sorogens in \( aca1\aca3\aca2 \) chimeras with wild-type was not as delayed as in \( aca1\aca3\aca2 \) alone, resulting in formation of more normal fruiting bodies (Fig. 3B). These experiments show that the defects in aggregation and whorl separation of the \( aca \) mutants are non-cell autonomous.

We also tested microcyst formation in \( aca \) knock-out mutants. Incubation with 0.2 M sorbitol for 24 h induced cyst formation in both wild type and \( aca1\aca3\aca2 \) to the same degree (Additional File1, Figure A3), indicating that the \( aca \) genes are not required for encystation.

**Restoration of \( aca1\aca3\aca2 \) aggregation by 8Br-cAMP.**

The strongly reduced initiation of aggregation centres and extensive delay in aggregation of both the \( aca3\aca2 \) and \( aca1\aca3\aca2 \) was unexpected, since \( Ppal \) does not use cAMP as attractant for aggregation, but most likely glorin [5, 9]. However, both \( Ddis \) and \( Ppal \) require PKA activity and therefore likely intracellular cAMP to develop competence for aggregation [19, 20]. To investigate whether lack of PKA activation due to the absence of intracellular cAMP cause the aggregation abnormalities in \( aca3\aca2 \) and \( aca1\aca3\aca2 \), \( aca1\aca3\aca2 \) cells were developed on agar containing 2.5 mM 8Br-cAMP, a membrane-permeant PKA agonist. While without 8Br-cAMP cells had not yet started to aggregate after 24 h of starvation, the 8Br-cAMP treated cells initiated many aggregation centres and almost completed aggregation within 6 h (Fig. 4). The aggregates remained however blocked in the mound stage and did not form fruiting bodies. This was however also the case for most \( Ppal \) WT aggregates developed on 8Br-cAMP agar. These results show that the \( aca1\aca3\aca2 \) aggregation defect was likely caused by insufficient intracellular cAMP for PKA activation.

**cAMP relay in \( Ppal \) and in \( Ddis \) aca-/ACG cells**

Despite the loss of all ACA activity the \( aca1\aca3\aca2 \) cells still made relatively normal fruiting bodies after a long delay. cAMP-induced excitation and adaptation of ACA underpins pulsatile cAMP signalling and wave propagation in \( Ddis \) [21, 22], with cAMP receptors (cARs) and extracellular cAMP phosphodiesterase (PdsA) as essential components to respectively detect secreted cAMP and to hydrolyse it between pulses [23–25]. From earlier findings that cAR or PdsA null mutants in \( Ppal \) were specifically defective in fruiting body morphogenesis [11, 12], we concluded that cAMP waves mediated this process as they do in \( Ddis \) [4]. The present data imply that this is either not the case, or that the \( aca1\aca3\aca2 \) cells have a means to compensate for loss of ACA activity.

To investigate whether \( Ppal \) also shows transient cAMP induced cAR mediated accumulation of cAMP, we stimulated wild-type \( Ppal \) cells at different stages of development with the cAR agonist 2'H-cAMP in the presence of the PdsA inhibitor DTT [26]. Figure 5A shows that cells at all stages show a basal level of
3–6 pmoles cAMP/mg protein. Starving cells or cells from streaming aggregates showed none or marginal responses to 2'H-cAMP, while cells from tipped mounds showed a 5 pmoles/mg protein increase in cAMP, which then levelled off. However, cells from dissociated sorogens showed transient increase that peaked after 3 min after stimulation at 11 pmoles above basal levels and then decreased to 5 pmoles. These data indicate that Ppal can relay a pulse of cAMP, but only after tips and sorogens have formed. In Ddis, which unlike Ppal also uses cAMP to aggregate, cAMP relay is highest at the aggregation stage [27].

We could not meaningfully measure 2'H-cAMP induced cAMP accumulation in the aca1aca3aca2 cells, because only few aggregates are formed at different times, which then fragment and fairly rapidly mature into fruiting bodies. This means that at any time only a very small fraction of cells is in the sorogen stage.

Apart from Aca1, Aca2 and Aca3, two other adenylate cyclases, AcgA and AcrA are expressed in Ppal sorogens [28]. The experiment in Fig. 5A does not identify the adenylate cyclase responsible for the cAMP increase. While currently not feasible in Ppal, a Ddis acaA knock-out is available that expresses AcgA (ACG) from the constitutive actin 15 promoter [29]. During growth, this mutant synthesizes cAMP at a constant rate [30], but it is unknown whether cAMP synthesis comes under cAR regulation at a later stage. We compared 2'H-cAMP induced cAMP accumulation between Ddis wild-type and aca/ACG cells in vegetative and 4 h starved cells, which are just starting to aggregate. To validate that the observed responses are mediated by Ddis cAR1, we included the cAR1 antagonist 2'3'-O-isopropylidene adenosine (IPA) in control assays. Figure 5B shows that wild-type Ddis shows no 2'H-cAMP induced cAMP accumulation in the vegetative stage and a 70 pmoles/mg protein increase in 4 h starved cells that peaks at 5 min. This response is almost completely inhibited by IPA. Vegetative aca/ACG cells show a steady increase in cAMP levels after addition of 2'H-cAMP/DTT that is only slightly reduced in the presence of IPA. However, 4 h starved aca/ACG cells show a faster transient increase of cAMP that peaks at 3 min after 2'H-cAMP/DTT stimulation. This response is also strongly reduced by IPA. These data indicate that in early aggregating Ddis cells, ACG is also controlled by cAR stimulation. The apparent ability of other adenylate cyclases than ACA to participate in transient cAR mediated cAMP accumulation provides some resolution for the contrasting effects on Ppal fruiting body morphogenesis of aca deletion on one hand and cAR or pdsA deletion on the other.

**Discussion**

**Gene amplification of aca genes and their expression in P. pallidum**

Representative species of the Dictyostelium taxon groups 1, 3 and 4 have a single gene each of the adenylate cyclases acaA, acrA and acgA, but in taxon group 2, the ancestral acaA gene was amplified twice in Ppal and three times in A. subglobosum (Additional File 1, Figure A1). In Ddis, a signaling network that critically incorporates ACA, cAR1 and PdsA generates the cAMP pulses that coordinate aggregation and cell movement in the multicellular stage [24, 29]. Deletion of the two Ppal car genes, carA and carB, or its single pdsA gene had no effect on aggregation but disorganized the subsequent formation of sorogens and fruiting bodies [11, 12]. This suggested that similar to Ddis, Ppal multicellular
morphogenesis is organized by cAMP pulses. The \textit{Ppal} attractant for aggregation is likely the dipeptide glorin, since starving \textit{Ppal} cells chemotactically respond to glorin \cite{9} and their aggregation is disrupted by including glorin in the supporting agar \cite{5}.

We here show that \textit{Ppal aca1} was poorly expressed and only visible in the tips of primary and secondary sorogens, while \textit{aca2} and \textit{aca3} expression was already visible in aggregates. Both genes were preferentially expressed in tip and stalk cells, but \textit{aca2} was also expressed in prespore cells. The latter expression likely explains why double loss of \textit{acrA} and \textit{acgA}, which in \textit{Ddis} leads to complete loss of prespore and spore differentiation \cite{31}, only mildly affects \textit{Ppal} sporulation \cite{20}.

The post-aggregative expression pattern of either \textit{Ppal aca} resembles that of \textit{Ddis acaA}, which is also preferentially expressed in the organizing tip, from which the organizing cAMP waves emanate \cite{4, 15, 16}.

\textbf{Ppal aca3 is required for PKA activation and early development}

Deletion of individual \textit{Ppal aca} genes caused subtle developmental defects (summarized in Table 1), with \textit{aca1} displaying longer, thinner stalks, \textit{aca2} delayed separation of the first whorl from the main sorogen and \textit{aca3} showing delayed and reduced formation of aggregation centres, giving rise to extensive streaming (Fig. 2). Double \textit{aca1aca2} and \textit{aca1aca3} knock-outs combined the phenotypes of the individual knock-outs, but \textit{aca3aca2} was very delayed in aggregation and like \textit{aca1aca3aca2} only formed a few aggregates on an entire plate of cells. The latter mutant did however form small fruiting bodies with some whorls of side branches near the top after a very long delay (Fig. 3. Both the delayed aggregation and delayed secondary sorogen formation are non-cell autonomous defects as they are restored by chimeric development of the mutants with 10% wild-type cells.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aggregation</td>
</tr>
<tr>
<td>\textit{aca1}</td>
<td>normal</td>
</tr>
<tr>
<td>\textit{aca2}</td>
<td>normal</td>
</tr>
<tr>
<td>\textit{aca3}</td>
<td>delayed, few centers, long streams</td>
</tr>
<tr>
<td>\textit{aca1aca2}</td>
<td>normal</td>
</tr>
<tr>
<td>\textit{aca1aca3}</td>
<td>delayed, few centers, long streams</td>
</tr>
<tr>
<td>\textit{aca3aca2}</td>
<td>very delayed, few centres, long streams</td>
</tr>
<tr>
<td>\textit{aca1aca3aca2}</td>
<td>very delayed, very few centres, long streams</td>
</tr>
</tbody>
</table>

Table 1
Phenotypes of \textit{Ppal aca} knock-outs
The delay in aggregation caused by loss of aca3 was somewhat enigmatic, since no such delay occurred in Ppal carAcarB or pdsA mutants, which cannot detect or hydrolyse extracellular cAMP. Exposure of the aca1aca3aca2 mutant to the membrane-permeant PKA agonist 8Br-cAMP restored normal aggregation (Fig. 4) indicating that Aca3 provides cAMP for activation of PKA in early development. Because defective aggregation of aca3 is also restored by wild-type cells, this likely means that PKA induces genes required for glorin synthesis. In early Ddis development PKA also acts to induce expression of aggregation genes [19].

The Ppal aca genes are not essential for post-aggregative morphogenesis

Despite its long delay in forming aggregates Ppal aca1aca3aca2 cells were still able to form relatively normal fruiting bodies with stalk and spore cells. In view of observations that Ppal carAcarB or pdsA cells are highly defective in fruiting body morphogenesis, this suggests that perhaps a static gradient of cAMP produced by ACR or ACG is sufficient to organize morphogenesis or that either or both of these adenylate cyclases can also participate in an oscillatory network. When expressed from the constitutive A15 promoter in an aca background, Ddis ACG displays a fairly high level of constitutive activity in the growth stage that is activated by high osmolarity [32]. In wild-type Ddis, ACG has an overlapping role with ACR in induction of spore formation and inhibition of spore germination ([31, 33]. In Ppal ACG and ACR critically regulate encystation, but their role in sporulation is less pronounced [20], which may be due to the additional activity of ACA1, 2 and 3 in sorogens.

ACG mediates cAMP stimulated transient cAMP accumulation

We found here that when aggregation competent Ddis aca/ACG cells are stimulated with 2'HcAMP, ACG mediates a transient accumulation of cAMP. Like ACA mediated transient cAMP synthesis, the response is inhibited by the cAR antagonist IPA, indicating that it is mediated by cARs. The experiment shows that at least one of the other dictyostelid adenylate cyclases could also give rise to oscillatory cAMP signalling, which provides some resolution to the conundrum of cARs and PdsA, but not Aca1, 2 and 3 being required for Ppal morphogenesis.

How the apparent transient ACG activation occurs is unresolved. Oscillatory cAMP signalling depends on both positive and negative feedback loops acting on cAMP production (Fig. 6). ACA activation involves positive feedback loop where cAMP synthesized by ACA process binds to cAR1 and initiates a multistep process that causes activation of ACA (see [34]. Negative feedback on ACA may involve PIP3, one of the activating intermediates, also causing inhibition of ACA after a delay [35]. In an alternative model (Fig. 6B), the positive feedback loop involves both cAMP activation of ACA, as above, and of the protein kinase ERK2, which next inactivates the intracellular cAMP phosphodiesterase RegA, enabling cAMP to increase. The negative loop involves PKA activation by cAMP synthesized by ACA, inactivation of ERK2 and thereby activation of RegA [36]. In this model the negative feedback loop acts on intracellular cAMP, which contrast with early findings that inhibition of ACA by cAR1 activation also occurs when no
intracellular cAMP is synthesized [37]. It is plausible that cells utilize more than one inhibitory feedback loop to improve the robustness of oscillatory signalling. In the second model it is mostly cAMP degradation that is under positive and negative feedback regulation. It is therefore conceivable that a constitutively active adenylate cyclase like ACG or ACR could still display apparent transient activation. Alternatively, ACG may have come under cAR1 control itself, requiring further study of involvement of any other ACA activating proteins.

Conclusions

In *Ddis*, cAMP waves produced by ACA and emanating from aggregation centres and organizing tips control both aggregation and post-aggregative morphogenesis. We here investigated ACA function in *Ppal*, which likely uses glorin for aggregation. *Ppal* has 3 *aca* genes, which similar to *Ddis aca* are most highly expressed in the organizing tip. Deletion of either gene causes different but relatively subtle changes in aggregation and post-aggregative morphogenesis, while deletion of all three *aca* genes causes a long delay in aggregation with only few centres formed that attract long streams of amoebas. The fragmenting streams eventually gave rise to small fruiting bodies. Timely aggregation was restored by including the PKA agonist 8Br-cAMP in the substratum, indicating that in early development the *Ppal* ACAs are needed to activate PKA.

While the formation of fruiting bodies by the *Ppal aca1aca3aca2* mutant argues against a role for ACAs and thereby oscillatory cAMP signalling in fruiting body morphogenesis, several lines of evidence indicate the opposite. 1. Loss of other components that are essential for cAMP oscillations such as cARs or pdsA disorganizes morphogenesis. 2. Post-aggregative *Ppal* cells produce a cAMP pulse when stimulated with 2'H-cAMP, a cAMP receptor agonist. 3. Other adenylate cyclases, such as ACG also mediate transient 2'H-cAMP induced cAMP accumulation, at a stage where cARs and PdsA are also present. The latter finding indicate that apparent oscillatory behaviour is displayed by multiple adenylate cyclases, perhaps following a model where cAMP exerts both positive and negative feedback on its accumulation by regulating the intracellular cAMP phosphodiesterase RegA [36].

Overlapping roles of ACG and ACR were detected in *Ddis* sporulation [31] and *Ppal* encystation, while the *Ppal* ACAs were proposed to overlap with ACG and ACR in induction of sporulation [20]. The current study provides hints that ACG and possibly ACR in turn overlap with ACA in morphogenetic signalling. Altogether, ACA, ACG and ACR may ancestrally have been less specialized and acquired their specific roles in the course of dictyostelid evolution by expression in different cell types and interaction with proteins specific to that cell type.

Methods

Growth and development.
P. pallidum PN500 (Ppal) was routinely grown in association with Escherichia coli on LP agar or 1/5th SM agar (Formedium, UK), respectively. For multicellular development, cells were harvested in 20 mM K-phosphate, pH 6.5 (KK2), washed free from bacteria and incubated at 10^6 cells/cm^2 and 22°C on NN agar (1.5% agar in 8.8 mM KH₂PO₄ and 2.7 mM Na₂HPO₄) until the desired developmental stages had been reached. D. discoideum (Ddis) Ax3 and aca/ACG cells [29] were grown in HL5 axenic medium, which was supplemented with 20 µg/ml G418 for aca/ACG.

**DNA constructs and transformation.**

**Ppal Aca promoter-lacZ constructs and analysis**

To construct a gene fusion of the Ppal aca1 promoter and lacZ, an aca1 (PPL_01657) fragment 3511 nt upstream and 93 nt downstream of the start ATG was amplified from Ppal gDNA using primers Pp-ACA1-P52X with XbaI site and Pp-ACA1-P32. The fragment was digested with XbaI and BamHI (using an internal BamHI site) and ligated into the BglII/XbaI digested pDdGal17 [38], yielding pPpACA1-LacZ. The aca2 (PPL_12370) 3.8 kb 5’intergenic region (-3743 to +86) was amplified using primers Pp-ACA2-P51E and Pp-ACA2-P31B that harbour EcoRI and BamHI respectively. The EcoRI/BamHI digested PCR product was ligated into the EcoRI/BglII digested pDdGal16 [38], yielding vector pPpACA2-LacZ. The aca3 (PPL_10658) 2.6 kb 5’ intergenic region (-2502 to +50) was amplified using primers Pp-ACA3-P52X and Pp-ACA3-P32B that harbour XbaI and BamHI respectively. The XbaI/BamHI digested PCR product was ligated into similarly digested pDdGal17, yielding vector pPpACA3-LacZ. After validation of the plasmids by DNA sequencing, they were transformed into Ppal wild-type cells. Transformants were selected at 300 µg/ml G418 [39] and developed into multicellular structures on dialysis membrane, supported by NN agar. β-galactosidase activity was visualised with X-gal in the structures, as described previously [11, 40].

**Ppal gene knock-out constructs**

To disrupt Ppal aca1 (PPL_01657), an aca1 fragment was amplified from Ppal PN500 genomic DNA using primers Pp-ACA-51H and Pp-ACA-31B (Additional File 1, Table A1) that harbour HindIII and BamHI restriction sites respectively. The fragment was cloned into BamHI/HindIII digested pBluescript SK+, which was next digested with EcoRV. The LoxP-NeoR cassette of pLoxNeoIII [12] was excised with BamHI and HindIII, filled in with Klenow polymerase, and ligated into the EcoRV digested aca1 plasmid, yielding vectors pACA1-KO1 and pACA1-KO2, with loxP-NeoR inserted in aca1 in forward and reverse orientation, respectively, and flanked by 2307 bp 5’UTR and 5’aca1 sequence and 1414 bp 3’aca1 sequence (Fig. A2A). pACA1-KO2 was used for gene disruption.

To disrupt Ppal aca2 (PPL_12370), an aca2 fragment was amplified using primers Pp-ACA2-51K and Pp-ACA2-31S (Table A1) that harbour KpnI and SacI sites, respectively. The fragment was cloned into KpnI/SacI digested pBluescript SK+, which was next digested with BamHI/SalI. LoxPA-NeoR was excised with BamHI/SalI from pLoxNeoIII and ligated into the BamHI/SalI digested aca2 plasmid, yielding vector pACA2-KO with LoxP-NeoR flanked by 1918 bp 5’aca2 sequence and 3086 bp 3’aca2 and 3’UTR sequence (Fig. A2B).
To disrupt *Ppal aca3* (PPL_10658), two *aca3* sequences, A and B, were amplified using primer pair Pp-ACA3-51K/Pp-ACA3-31X, that harbour KpnI and *Xba*I sites for A and primer pair Pp-ACA3-52B/Pp-ACA3-32X with BamHl and *Xba*I sites for B, respectively. Fragment A was digested with *KpnI*/*SalI* (using an internal *SalI*) and inserted into KpnI/SalI digested pLox-NeoIII, and next *BamHl/XbaI* digested fragment B was inserted into the *BamHl/XbaI* sites of the resulting vector, yielding pACA3-KO with 2042 bp 5’ *aca3* sequence and 2531 bp 3’ *aca3* and 3’UTR sequence. (Fig. A2C).

*Ppal* cells were transformed by electroporation with the linearized vectors according to established procedures [39]. Genomic DNA was isolated from G418 resistant clones and analysed by PCR and Southern blots to diagnose gene disruption by homologous recombination (Fig. A2).

To generate double *aca1aca2* or *aca1aca3* knock-outs, the loxP-NeoR cassette was removed from *aca1* by transient transformation with pA15NLS.Cre [17]. Cells that had regained sensitivity to G418 were then transformed with the pACA2-KO or pACA3-KO plasmids. This strategy did not work for the *aca2* and *aca3* knockouts. To generate an *aca3*/*aca2* double and *aca1*/*aca3*/*aca2* triple knockout, we transformed *aca3* and *aca1aca3* with pDM1483 [18], which harbours cassettes for Nourseothricin selection and cre-recombinase expression. Transformants were selected after growth for 3–5 days in the presence of 300 µg/ml Nourseothricin, and, after replica-plating, selected for G418 sensitivity and transformed with the pACA2-KO vector. All gene knock-outs were diagnosed by PCR and/or Southern blot (Fig. A2).

**cAMP relay assays**

To measure cAMP induced cAMP accumulation, *Ppal* cells were resuspended in PB (10 mM Na/K-phosphate, pH 6.5) at 10^8^ cells/ml, dispensed as 25 µl aliquots in microplate wells and, stimulated with 5 µl of 60 µM 2′H-cAMP (2′-deoxyadenosine 3′:5′-cyclic monophosphate, Sigma-Aldrich, in 30 mM DTT (dithiothreitol, Sigma-Aldrich) and gently shaken at 21°C. Reactions were terminated by addition of 30 µl of 3.5% (v/v) perchloric acid. *Ddis* cells additionally received 3 µl of 10 mm IPA (2′,3′-O-isopropylideneadenosine, Sigma-Aldrich) in 10% (v/v) (DMSO) dimethylsulfoxide, Sigma-Aldrich) or 3 µl 10% DMSO (controls) and were stimulated with 3 µl 50 µM 2′H-cAMP in 50 mM DTT. For cAMP assay, samples were neutralized by addition of 15 µl of 50% saturated KHCO₃ and 75 µl of cAMP assay buffer (4 mM EDTA in 150 mM K phosphate, pH 7.5). Protein and perchlorate were precipitated by centrifugation for 5 min at 2000 x g and cAMP was assayed in 50 µl of supernatant by isotope dilution assay using purified PKA regulatory subunit from beef heart as cAMP-binding protein and [2,8-3H]cAMP (Perkin-Elmer) as competitor [26, 41].

**Declarations**

*Ethics approval:* not applicable

*Consent for publication:* not applicable
Availability of data and materials: All data generated or analysed during this study are included in this article and additional file 1. The DNA constructs and knock-out cell lines produced in the study will be deposited in the Dictyostelium Stock Centre http://dictybase.org/StockCenter/StockCenter.html

Competing interests: The authors declare that they have no competing interests.

Funding: This research was funded by BBSRC grant BB/K000799/1 and ERC grant 74228. The BBSRC and ERC grants were used at initial and later phases of the work to fund the salary of YK and the materials to carry out the study.

Authors contributions: YK and PS designed the study, YK performed most experimental work, analysed the data and wrote the first draft of the manuscript. PS supervised the work, performed the cAMP relay experiments and prepared the final draft.

References


Figures

Figure 1

Expression patterns of *P. pallidum* aca genes

*Ppal* wild-type cells were transformed with gene fusions of the *LacZ* reporter and the intergenic regions upstream of the start-codon of the *Ppal* aca1, aca2 and aca3 genes. The cells were developed on NN agar until aggregates (top), primary sorogens (centre) and secondary sorogens (bottom) had formed and intact structures were then fixed with glutaraldehyde and stained with X-gal. Bars: 0.1 mm.

Figure 2

Development of single aca, aca1aca2 and aca1aca3 mutants.

Wild type (WT) *Ppal* and aca1, aca2, aca3, aca1aca2 and aca1aca3 knock-outs were incubated at 22°C on NN agar at 10^6 cells/cm^2^.

A. Aggregation. The images show aggregates after 6 h or 8 h (aca3 and aca1aca3) starvation. Bars: 1 mm.

B. Tip formation. Aggregation streams of aca3 and aca1aca3 forming tips at 20 h. Bars: 1.0 mm.

C. Whorl mass separation. aca1, aca2 and WT at 20 h and aca1aca2 at 28 h of starvation. Bars: 0.5 mm.

D. Fruiting bodies. WT, aca1, and aca2 had formed mature fruiting bodies at 28 h of starvation, while aca3 and aca1aca3 took 32 h and aca1aca2 48 h to complete their fruiting bodies. Bars: 0.5 mm.
Figure 3

Development of *aca3*^-aca2^- and *aca1^-aca3^-aca2^- mutants.

A. Aggregation. *Ppa* *aca3*^-aca2^- and *aca1^-aca3^-aca2^- were starved on NN agar at $10^6$ cells/cm$^2$ on their own or mixed with 10% wild-type *Ppa* for the indicated time periods. Bar: 1 mm.
B. Fruiting bodies. The cells were developed into fruiting bodies, which were imaged in situ. Bars: 0.5 mm.

C. Spore and stalk cells. Ppal WT and aca1aca3aca2 fruiting bodies were transferred to 0.001% Calcofluor and imaged under phase contrast (left) and epifluorescence (right). Bar: 20 µm.

Figure 4

Effect of 8Br-cAMP on aggregation of aca1aca3aca2 cells

Ppal aca1aca3aca2 cells were incubated for 24 h on NN agar with and without 2.5 mM 8Br-cAMP and imaged in situ at the indicated time points. 8Br-cAMP treated wild-type cells are shown for comparison. Bar: 1 mm.

Figure 5

cAMP relay in Ppal wild-type and Ddis aca/ACG

A. P. pallidum. Ppal WT was starved on agar for 4 h or until streaming aggregates, tipped mounds and aerially lifted sorogens had formed. Structures were gently dissociated, resuspended in PB to $10^8$ cells/ml and stimulated at $t=0$ min with 10 µM 2'H-cAMP and 5 mM DTT. Reactions were terminated with 1.75% perchloric acid at the indicated time points and cAMP was assayed by isotope dilution assay.
B. D. discoideum. Vegetative WT Ax3 and aca/ACG cells and cells starved on NN agar for 4 h were resuspended in PB to $10^8$ cells/ml and stimulated with 5 μM 2'H-cAMP and 5 mM DTT in the presence and absence of 1 mM IPA. Reactions were terminated as above, and cAMP was assayed. Data were standardized to the protein content of the cell suspensions and represent means and SE of six experiments performed in triplicate for PpaI sorogens and two experiments in triplicate for other stages and cell lines. The experiments in panel B were performed twice more in triplicate with aca/ACG cells in the absence of IPA with similar results.

Figure 6

Proposed models for ACA mediated oscillatory cAMP signalling.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- KawabeAdditionalFile1.pdf