Adenylate cyclase A amplification and functional diversification during Polyspondylium pallidum development

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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 extracellular 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: In contrast to 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. aca1ˉ cells showed relatively thin stalks, aca2ˉ showed delayed secondary sorogen formation and aca3ˉ formed less aggregation centers. The aca1ˉaca2ˉ and aca1ˉaca3ˉ mutants combined individual defects, while aca2ˉaca3ˉ and aca1ˉaca3ˉaca2ˉ 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 aca2ˉaca3ˉ and aca1ˉaca3ˉaca2ˉ 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 acaˉ/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, but act together to enable development by activating PKA. While even an aca1ˉaca3ˉaca2ˉ 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 than its synthesis dominates oscillatory behaviour.

Keywords: Excitable systems, CAMP oscillations, Adenylate cyclase A, Coordinated cell migration, Cell aggregation, Morphogenetic movement, Dictyostelia

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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. The other group 4 species also use cAMP as chemoattractant [5] and are likely to use cAMP pulses to coordinate morphogenesis as well. However, 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 aca1 aca3 aca2 cells 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 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: Fig. S1). 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 (Additional File 1: Fig. S1).

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 streaming 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 acaAs 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 file 1: Fig. S2). 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 *aca1 aca2* and *aca1 aca3* double knock-outs, but not for the *aca2* or *aca3* knock-outs. The *aca1 aca3* phenotype combined features of *aca1* and *aca3* knock-out mutants. Similar to *aca3*, *aca1 aca3* formed few but

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**Fig. 2** Development of single *aca*, *aca1 aca2* and *aca1 aca3* mutants. Wild type (WT) *Ppal* and *aca1*, *aca2*, *aca3*, *aca1 aca2* and *aca1 aca3* knock-outs were incubated at 22 °C on NN agar at 10⁶ cells/cm². **A** Aggregation: the images show aggregates after 6 h or 8 h (*aca3* and *aca1 aca3*) of starvation. Bars: 1 mm. **B** Tip formation: aggregation streams of *aca3* and *aca1 aca3* forming tips at 20 h. Bars: 1.0 mm. **C** Whorl mass separation: *aca1*, *aca2* and WT at 20 h and *aca1 aca2* 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 *aca1 aca3* took 32 h and *aca1 aca2* 48 h to complete their fruiting bodies. Bars: 0.5 mm.
large streaming aggregates (Fig. 2A, B), while the fruiting bodies showed thinner and longer stalks, like theaca1-aca2-aca3- mutants (Fig. 2D). The aca1 'aca2' 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, aca1 'aca2' 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 aca1 'aca3' and to generate aca3 'aca2' and aca1 'aca3 'aca2' knock-outs.

Compared to wild-type, aca1 'aca2', aca1 'aca3' and single aca knock-outs, which all initiated aggregation within 8 h of starvation, the aca3 'aca2' 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 aca1 'aca3 'aca2' phenotype combined features of the aca1 'aca2' and the aca3 'aca2' mutant. Similar to aca3 'aca2', 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 aca1 'aca2' (Fig. 3B). Staining of the aca1 'aca3 'aca2' stalk and spore cells with the cellulose dye Calcofluor 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 had already formed fruiting bodies (Fig. 2C). As a result, aca1 'aca2' made very tall fruiting bodies with side branches only at the upper stalk (Fig. 2D).

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 glucor [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
Figure 5A shows that cells at all stages contain a basal level of 3–6 pmol 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 pmol/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 pmol above basal levels and then decreased to 5 pmol. 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 aca1^-aca3^-aca2^- cells, because only few aggregates are formed at different times,
Fig. 4  Effect of 8Br-cAMP on aggregation of aca1ˉaca3ˉaca2ˉ cells. Ppal aca1ˉaca3ˉaca2ˉ and wild-type cells were incubated for 24 h on NN agar with and without 2.5 mM 8Br-cAMP and imaged in situ at the indicated timepoints. Bar: 1 mm

Fig. 5  cAMP relay in Ppal wild-type and D.discoideum. 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 10⁸ cells/ml and stimulated at t = 0 min with 10 μM 2H-cAMP and 5 mM DTT. Reactions were terminated with 1.75% perchloric acid at the indicated timepoints and cAMP was assayed by isotope dilution assay. B. D. discoideum Vegetative WT AX3 and aca7/ACG cells, and cells starved on NN agar for 4 h were resuspended in PB to 10⁶ cells/ml and stimulated with 5 μM 2H-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 Ppal sorogens and two experiments in triplicate for other stages and cell lines. The experiments in panel B were performed twice more in triplicate with aca7/ACG cells in the absence of IPA, with similar results.
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 *P. pallidum* sorogenes [28]. The experiment in Fig. 5A does not identify the adenylate cyclase responsible for the cAMP increase. While currently not feasible in *P. pallidum*, 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 accumulation provides some resolution for the contrasting effects on wild-type and Aca1/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 pmol/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 cAR1 stimulation. The apparent ability of other adenylate cyclases than ACA to participate in transient cAR1 mediated cAMP accumulation provides some resolution for the contrasting effects on *P. pallidum* 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 *P. pallidum* and three times in *A. subglobosum* (Additional File 1, Fig. S1). 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 *P. pallidum* 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*, *P. pallidum* multicellular morphogenesis is organized by cAMP pulses. The *P. pallidum* attractant for aggregation is likely the dipeptide glorin, since starving *P. pallidum* cells chemotactically respond to glorin [9] and their aggregation is disrupted by including glorin in the supporting agar [5].

We here show that *P. pallidum* aca1 was poorly expressed and only visible in the tips of primary and secondary sorogens, while aca2 and aca3 expression was already visible in aggregates. Both genes were preferentially expressed in tip and stalk cells, but aca2 was also expressed in prespore cells. The latter expression likely explains why double loss of *acrA* and *acgA*, which in *Ddis* leads to complete loss of prespore and spore differentiation [31], only mildly affects *P. pallidum* sporulation [20]. The post-aggregative expression pattern of either *P. pallidum* aca resembles that of *Ddis* acaA, which is also preferentially expressed in the organizing tip, from which the organizing cAMP waves emanate [4, 15, 16].

**P. pallidum** aca3 is required for PKA activation and early development

Deletion of individual *P. pallidum* aca genes caused subtle developmental defects (summarized in Table 1), with

| Mutant | Phenotype | Aggregation | Morphogenesis |
|--------|-----------|-------------|---------------|
| aca1⁻  | Normal    | Normal      | Somewhat thinner and longer stalks |
| aca2⁻  | Normal    | Delayed, few centers, long streams | Delayed whorl separation |
| aca3⁻  | Normal    | Delayed, few centers, long streams | Normal |
| aca1⁻ acrA²⁻ | Normal | Delayed, few centers, long streams | Long whorl-free lower stalk, thin tall fruiting bodies |
| aca1⁻ acrA²⁻ | Delayed, few centers, long streams | Normal |
| aca3⁻ acrA²⁻ | Very delayed, few centres, long streams | Long whorl-free lower stalk |
| aca³⁻ acrA²⁻ | Very delayed, very few centres, long streams | Thin tall fruiting bodies, whorls only near top |
acr1⋅ displaying longer, thinner stalks, acr2⋅ delayed separation of the first whorl from the main sorogen and acr3⋅ showing delayed and reduced formation of aggregation centres, giving rise to extensive streaming (Fig. 2). Double acr1acr2⋅ and acr1acr3⋅ knock-outs combined the phenotypes of the individual knock-outs, but acr3acr2⋅ was very delayed in aggregation and like acr1acr3acr2⋅ 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.

The delay in aggregation caused by loss of acr3 was somewhat enigmatic, since no such delay occurred in Ppal carAcarB⋅ or pdsA− mutants, which cannot detect or hydrolyse extracellular cAMP, respectively. Exposure of the acr1acr3acr2⋅ 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 acr3⋅ is also restored by wild-type cells, this likely means that PKA induces genes required for glorn synthesis. In early D.dis development PKA also acts to induce expression of aggregation genes [19].

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

Despite its long delay in forming aggregates Ppal acr1acr3acr2⋅ 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 acr− background, D.dis ACG displays a fairly high level of constitutive activity in the growth stage that is activated by high osmolarity [32]. In wild-type D.dis, 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 sporation is less pronounced [20], which may be due to the additional activity of Acrl, 2 and 3 in sorogens.

ACG mediates cAMP stimulated transient cAMP accumulation

Ppal cells at the sorogen stage show transient 2′HCAMP-induced cAMP synthesis (Fig. 5A), consolidating evidence from Ppal car− and pdsA− mutants that cAMP pulses coordinate postaggregative morphogenesis [11, 12], but the Ppal acr1acr3acr2⋅ mutant still makes fruiting bodies, suggesting that this is not the case. To resolve this conundrum, we explored whether other adenylate cyclases might mediate pulsatile signaling. We found that when aggregation competent D.dis acr⋅/ACG cells are stimulated with 2′HCAMP, ACG mediates a transient accumulation of cAMP (Fig. 5B). 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.

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 PI3K, 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 it is mostly cAMP degradation that is under

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**Fig. 6** Proposed models for ACA mediated oscillatory cAMP signalling See main text for explanation. Abbreviations: PdsA phosphodiesterase A, car1 cAMP receptor 1, G2 heterotrimeric G-protein 2, RasC small GTPase C, PI3K phosphoinositide 3-kinase, PI3K phosphatidylinositol-(3,4,5)-triphosphate, CRAC cytosolic regulator of adenylate cyclase, ACA adenylate cyclase A, ERK2 extracellular signal-regulated kinase 2, RegA cAMP phosphodiesterase with response regulator, PKA cAMP dependent protein kinase. Schematics are based on Fig. 6 in [33] and Fig. 2 in [36].
positive and negative feedback regulation, making it conceivable that a constitutively active adenylate cyclase-like ACG or ACR could still display apparent transient activation.

Conclusions
In *D. discoideum*, 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 *P. pallidum*, which likely uses glorin for aggregation. *P. pallidum* has 3 *aca* genes, which similar to *D. discoideum* *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 being formed that attracted 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 *P. pallidum* ACAs are needed to activate PKA.

While the formation of fruiting bodies by the *P. pallidum* *aca1*/*aca3*/*aca2* 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 *P. pallidum* cells produce a cAMP pulse when stimulated with 2′H-cAMP, a cAMP receptor agonist. 3. In *D. discoideum* other adenylate cyclases, such as ACG also mediate transient 2′H-cAMP-induced cAMP accumulation, at a stage when cARs and PdsA are also present.

Overlapping roles of ACG and ACR were detected in *D. discoideum* sporulation [31] and *P. pallidum* encystation, while the *P. pallidum* 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 (*Pp*) was routinely grown in association with *Escherichia coli* on LP agar or 1/5th SM agar (Formedium, UK). For multicellular development, cells were harvested in 20 mM K-phosphate, pH 6.5 (KK2), washed free from bacteria and incubated at 10⁶ cells/cm² and 22 °C on NN agar (1.5% agar in 8.8 mM KH₂PO₄, 2.7 mM Na₂HPO₄) until the desired developmental stages had been reached. *D. discoideum* (*Ddis*) AX3 and *aca1*/ACG cells [29] were grown in HL5 axenic medium, which was supplemented with 20 µg/ml G418 for *aca1*/*ACG*.

DNA constructs and transformation

**Pp* *aca* promoter-lacZ constructs and analysis**
To construct a gene fusion of the *Pp* *aca1* promoter and lacZ, an *aca1* (PPL_01657) fragment 3511 nt upstream and 93 nt downstream of the start ATG was amplified from *Pp* 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 [37], 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 [37], 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.

**Pp* gene knock-out constructs**
To disrupt *Pp* *aca1* (PPL_01657), an *aca1* fragment was amplified from *Pp* PN500 genomic DNA using primers Pp-ACA1-P51H and Pp-ACA1-P31B (Additional file 1: Table S1) 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 (Additional file 1: Fig. S2A).

To disrupt *Pp* *aca2* (PPL_12370), an *aca2* fragment was amplified using primers Pp-ACA2-51H and Pp-ACA2-31B (Additional file 1: Table S1) that harbour HindIII and BamHI restriction sites, respectively. The fragment was digested with XbaI and BamHI (using an internal BamHI site) and ligated into the BglII/XbaI digested pDdGal17 [37], yielding vector pPpACA2-LacZ.

**Pp* aca1 KO1 and pACA1-KO2, with loxP-NeoR inserted in *aca1* and EcoRV digested pDdGal16 [37], yielding vector pPpACA1-LacZ. After validation of the plasmids by DNA sequencing, they were transformed into *Pp* wild-type cells. Transformants were selected at 300 µg/ml G418 [38] and pools of 7–10 transformed clones were developed into multicellular structures on dialysis membrane, supported by NN agar. β-galactosidase activity was visualized with X-gal in the structures, as described previously [11, 39].
Pp-ACA2-31S (Additional file 1: Table S1) that harbour KpnI and SacI sites, respectively. The fragment was cloned into KpnI/Sacl digested pBluescript SK+ which was next digested with BamHI/Sall. LoxP-NeoR was excised with BamHI/Sall from pLoxNeoII and ligated into the BamHI/Sall digested aca2 plasmid, yielding vector pACA2-KO with LoxP-NeoR flanked by 1918 bp 5′aca2 sequence and 3086 bp 3′aca2 sequence (Additional file 1: Fig. S2B).

To disrupt Ppal aca3 (PPL_106588), two aca3 sequences, A and B, were amplified using primer pair Pp-ACA3-52K/Pp-ACA3-32X, that harbour KpnI and XbaI sites for A and primer pair Pp-ACA3-52B/Pp-ACA3-32X with BamHI and XbaI sites for B, respectively. Fragment A was digested with KpnI/Sall (using an internal SalI site) and inserted into KpnI/Sall digested pLoxNeoIII, and next BamHI/XbaI digested fragment B was inserted into the BamHI/XbaI sites of the resulting vector, yielding pACA3-KO with 2042 bp 5′aca3 sequence and 2531 bp 3′aca3 and 3′UTR sequence. (Additional file 1: Fig. S2C).

Ppal cells were transformed by electroporation with the linearized vectors according to established procedures [38]. Genomic DNA was isolated from G418 resistant clones and analyzed by PCR and Southern blots to diagnose gene disruption by homologous recombination (Additional file 1: Fig. S2).

To generate double aca1 aca2 or aca1 aca3 knockouts, 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 knockout. To generate an aca3 aca2 double and aca1 aca3 and aca4 triple knockout, aca3 and aca1 aca3 were transformed 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 (Additional file 1: S2).

cAMP relay assays
To measure cAMP-induced cAMP accumulation, Ppal cells were resuspended in PB (10 mM Na/K-phosphate, pH 6.5) at 106 cells/ml, dispersed 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 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 × 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, 40].

Supplementary Information
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Additional file 1: Figure S1. acaA genes across Dictyostelia. Figure S2. Schematics and diagnosis of Ppal aca1, aca2 and aca3 knock-outs. Figure S3. Encystation. Table S1. Oligonucleotide primers used in this work.

Author contributions
YK and PS designed the study, YK performed most experimental work, analyzed the data and wrote the first draft of the manuscript. PS supervised the work, performed the cAMP relay experiments and prepared the final draft. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed 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

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

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

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