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The cyclic AMP phosphodiesterase RegA critically regulates encystation in social and pathogenic amoebas☆

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ABSTRACT

Amoebas survive environmental stress by differentiating into encapsulated cysts. As cysts, pathogenic amoebas resist antibiotics, which particularly counteracts treatment of vision-destroying *Acanthamoeba* keratitis. Limited genetic tractability of amoeba pathogens has left their encystation mechanisms unexplored. The social amoeba *Dictyostelium discoideum* forms spores in multicellular fruiting bodies to survive starvation, while other dictyostelids, such as *Polysphondylium pallidum* can additionally encyst as single cells. Sporulation is induced by cAMP acting on PKA, with the cAMP phosphodiesterase RegA critically regulating cAMP levels. We show here that RegA is deeply conserved in social and pathogenic amoebas and that deletion of the *RegA* gene in *P. pallidum* causes precocious encystation and prevents cyst germination. We heterologously expressed and characterized *Acanthamoeba RegA* and performed a compound screen to identify RegA inhibitors. Two effective inhibitors increased cAMP levels and triggered *Acanthamoeba* encystation. Our results show that RegA critically regulates Amoebozoan encystation and that components of the cAMP signalling pathway could be effective targets for therapeutic intervention with encystation.

1. Introduction

Differentiation into dormant encapsulated cysts, or encystation, is the main differentiation process of amoebas and most other unicellular eukaryotes. Encystation is triggered by starvation and other environmental challenges [1], and as cysts the organisms can withstand these challenges for months up to years [2]. Encystation is of considerable medical importance, because cysts of pathogenic amoebas are impervious to immune attack and treatment with antibiotics or antiseptics [3–7]. This is a particular problem in the treatment of eye infections caused by opportunistic pathogens, such as *Acanthamoeba castellani.* This common inhabitant of soil and surface waters also colonizes other habitats, such as drinking water and air-conditioning ducts [8]. The eye infections are most prevalent in careless contact lens wearers, with outbreaks being caused by substandard lens cleaning fluids [9,10]. The infections require months of painful treatment with a cocktail of antibiotics and antiseptics. They are often recurrent because the therapeutic challenge causes the amoebas to encyst, and frequently leads to the loss of the cornea or eye [7,11,12]. Amoebozoan cysts are also exploited by bacterial pathogens, such as *Legionella*, MRSA and *Vibrio cholerae,* as vectors for long time survival and air-borne dispersal [13–15]. Lack of gene disruption procedures applicable to free-living Amoebozoa, has left the mechanisms that control encystation largely unexplored.

The social amoeba *Dictyostelium discoideum* (*Ddis*), also a member of Amoebozoa, is a popular genetic model system for investigating problems in cell- and developmental biology. It has adopted a novel survival strategy in response to nutrient stress: the starving amoebas aggregate to form multicellular fruiting bodies, in which a proportion of cells differentiates into dormant walled spores, while the remainder differentiate into a stalk that supports the spore mass. Spore differentiation is triggered by extracellular cAMP acting on G-protein coupled receptors [16,17] and intracellular cAMP acting on cAMP-dependent protein kinase (PKA) [18,19]. *Ddis* does not form cysts, but in other Dictyostelia, such as *Polysphondylium pallidum* (*Ppal*), amoebas still encyst individually under wet and dark conditions that are unfavorable for aggregation and fruiting body formation. Spores have a much thicker wall than cysts and are more dehydrated [20], which probably makes them even more environmentally resilient than cysts.

The adenylate cyclases ACG and AcrA have overlapping roles in synthesizing cAMP for activation of PKA in sporulation [21,22]. Particularly ACG then also acts in the spore to prevent germination under conditions that do not favor the proliferation of amoebas [23]. However,

Abbreviations: PDE, phosphodiesterase; cAMP, 3′5′-adenosine monophosphate; PKA, cAMP dependent protein kinase; ACG, adenylate cyclase G; AcrA, adenylate cyclase R; MRSA, methicillin resistant *Streptococcus aureus*; *Acas*, *Acanthamoeba castellani*; *Ddis*, *Dictyostelium discoideum*; *Ppal*, *Polysphondylium pallidum*; KO, knock-out; RI, random integrant.

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it is actually the cAMP phosphodiesterase RegA that plays the most pivotal role in controlling intracellular cAMP levels. The phosphodiesterase (PDE) activity of RegA is controlled by phosphorylation of its intrinsic response regulator domain by sensor histidine kinases/phosphatases. There are 15 sensor histidine kinases/phosphatases in the *Ddis* genome and at least four of these are receptors for signals that control the timely formation and germination of spores in an intricate network of communication between the maturing spore and stalk cells [24–32].

In this work we used the genetically tractable encysting Dictyostelid *Ppal* to investigate whether RegA critically regulates encystation. We show that this is the case and then identified and expressed a *RegA* gene from *Acanthamoeba castellani (Acas)*. By using a pharmacological approach, we also established an essential role for RegA in encystation of this pathogen.

2. Materials and methods

2.1. Gene disruption, cloning and expression

2.1.1. P.pallidum RegA gene disruption

To disrupt *P.pallidum* (*Ppal*) *RegA1*, two *RegA1* fragments comprising base pairs 139–1333 (A) and 1896–2833 (B), respectively, were amplified from *Ppal* PN500 genomic DNA, using primer pairs PpRegAI5′/PpRegAI3′ and PpRegAII5′/PpRegAII3′ (Table S1). The primers generated KpnI/BamHI and HindIII/HindIII restriction sites, flanking the two fragments. After HindIII digestion, fragment B was inserted into HindIII site vector pLox-NeoI, which, after selection of a construct with the appropiate orientation of fragment B, was further complemented after KpnI/BamHI digestion with KpnI/BamHI digested fragment A, yielding pRegA1KO (Supplementary Fig. S2A).

Ppal PN500 cells were transformed by electroporation with the linearized vector pRegA1KO according to established procedures [33]. Genomic DNA was isolated from G418 resistant clones and screened by two PCR reactions and Southern blot to diagnose *RegA1* gene disruption by homologous recombination (Fig. S2B,C). Four knock-out (KO) clones and four random integrants (RIs) were identified from two independent transformations.

2.1.2. Cloning and expression of Acas RegA

The partially assembled *Acas* genome http://blast.hgsc.bcm.tmc.edu/blast.hgsc?organism=AcastellaniNeff was queried by tBlastn with *Ddis* RegA, yielding hits on 3 contigs, which after assembly yielded about 3.3 kb of coding sequence homologous to the query sequence, but containing many introns. To identify intron positions, we amplified a cDNA from *Acas* mRNA by reverse transcripion PCR. Total *Acas* RNA was isolated using the Qiagen RNeasy Mini Kit and reverse transcribed with SuperScript III First-Strand Synthesis System (Invitrogen, Paisley, UK), using primers AcRegAF and AcRegAR, that contained NheI and EcoRI sites respectively, followed by cDNA amplification with Phusion High-Fidelity DNA Polymerase (NEB, Ipswich, MA). The cDNA was cloned after NheI/EcoRI digestion into similarly digested pET28a (Novagen, Leuven, Belgium), yielding plasmid pET-AcRegA, in which *Acas* RegA is fused at the N-terminus to a hexahis-tag. The DNA sequence was determined from three clones and showed an open reading frame of 1863 bp.

To obtain *Acas* RegA protein, plasmid pET-AcRegA was transformed into *E.coli* BL21DE3. Bacteria were grown overnight at 37 °C in LB containing 30 μg/ml kanamycin. The culture was then diluted 1:40 in LB, incubated for 2 h at 30 °C and supplemented with 1 mM IPTG. After 4 h, cells were lysed using BugBuster® Protein Extraction Reagent (Novagen), the *Acas* RegA his-tag fusion protein was purified using Ni-NTA His.Bind® Resin (Novagen) and stored at −80 °C.

2.2. Cell growth, development and encystation

2.2.1. Growth and development

Ppal, strain PN500, was routinely grown in association with *Klebsiella aerogenes* on 1/5th SM agar and when appropriate in HL5 axenic medium (Table S3). Strain PN500 is naturally axenic and was further trained to grow effectively in HL5 by alternating growth from spores on HL5, and fruiting body formation on non-nutrient (NN) agar (Table S3) for a few months. For multicellular development, *Ppal* cells were harvested in 10 mM Na/K-phosphate, pH 6.5 (PB), washed free from bacteria and incubated at 10^6 cells/cm^2 and 22 °C on NN agar. *Acas*, strain Neff, was grown in AC medium (Table S3) at 21 °C.

2.2.2. Encystation

For quantification of growth and encystation, *Ppal* cells were inoculated in HL5 at 3×10^5 cells/ml and shaken at 150 rpm and 21 °C. Aliquots of 1 ml were sampled at regular intervals, centrifuged at $1000 \times g$ for 1 min, and resuspended in 50 μl PB containing 0.001% calcofluor (which reacts to cellulose in the cyst wall). Total amoeba and cyst densities were determined by counting cells in a haemocytometer under phase contrast and UV illumination, respectively. 100–500 cells were counted for each time point.

Acas encystation was induced by incubating amoebas at 5×10^5 cells/ml in starvation buffer (SB) (Table S3) [34]. Amoeba and cyst cell densities were determined at regular intervals, as described above.

2.3. Enzyme and cAMP assays

2.3.1. Phosphodiesterase

To measure cAMP PDE activity, 0.02 μg of purified RegA was incubated for 30 min at 22 °C with 10 nM [2,8-^{3}H]-cAMP (Perkin Elmer, Waltham, MA) and 1 mM $MgCl_2$ in PB, with unlabelled cAMP, cGMP or PDE inhibitors as indicated. Reactions were terminated by boiling and [2,8-^{3}H] 5′AMP was hydrolysed further with the 5′nucleotidase contained in 10 μg of *Naja messambica* snake venom (SA venom suppliers, Louis Trichardt, South Africa) to [2,8-^{3}H]adenosine, which was separated from [2,8-^{3}H]-cAMP by adsorption of the latter to Dowex anion exchange resin [35], and measured by scintillation counting.

2.3.2. Cellular cAMP

To measure cellular cAMP levels, pellets of 10^7 *Acas* cells were resuspended in 50 μl PB and lysed with 50 μl 3.5% perchloric acid. Samples were neutralized by adding 25 μl 50% saturated $KHCO_3$ and 40 μl cAMP assay buffer (4 mM EDTA in 150 mM K-phosphate, pH 7.5) and centrifuged for 5 min at 13,200 × *g*. cAMP was assayed in 40 μl supernatant. The pellet was resuspended in 500 μl 0.1 M NaOH and assayed for protein.

3. Results

3.1. Deep conservation of RegA

All Genbank eukaryotic genes and amoebozoan genomes that were close to completion were screened for the presence of *Ddis* RegA homologs, which contained both the response regulator domain [36] and the HDc-type phosphodiesterase (PDE) domain [37] that characterize RegA [24,25,38]. One or two copies of RegA were detected in the genomes of *Dictyostelium purpureum* [39], *Dictyostelium lacteum* (Schaap, P. and Gloeckner, G. unpublished), *Ppal* and *Dictyostelium fasciculatum* [40], which, with *Ddis* [41], represent the four major groups of Dictyostelia [42]. A single *RegA* gene was found in the *Acas* genome [43] and in the genome of *Naegleria gruberi* [44]. The *RegA* homologs have a similar domain architecture across species (Fig. 1) with the response regulator domain followed by the PDE domain. The second *Ppal RegA* gene (*RegA2*) is highly derived (Fig. 1) and lacks several essential residues in both the response regulator and catalytic domain (Supplementary Fig. S1).