Chapter 1

The Amoebozoa

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Abstract

The model organism *Dictyostelium discoideum* is a member of the Amoebozoa, one of the six major divisions of eukaryotes. Amoebozoa comprise a wide variety of amoeboid and flagellate organisms with single cells measuring from 5 μm to several meters across. They have adopted many different life styles and sexual behaviors and can live in all but the most extreme environments. This chapter provides an overview of Amoebozoan diversity and compares roads towards multicellularity within the Amoebozoa with inventions of multicellularity in other protist divisions. The chapter closes with a scenario for the evolution of Dictyostelid multicellularity from an Amoebozoan stress response.

Key words Amoebozoa, Protista, Aggregative multicellularity, Encystation, Sporulation, Morphogenesis, Cyclic AMP signaling, Phylogeny

1 Introduction

The Dictyostelids have fascinated biologists for over 150 years with their ability to assemble up to a million amoebas into a tactile migrating organism, which, after seeking out a site for spore dispersal, transforms into a well-balanced fruiting structure. The development of a range of molecular genetic and cell biological procedures for the species *Dictyostelium discoideum* over the past 30 years has established this species as an important model organism for the study of fundamental cell biological and developmental processes (1). More recently, the evolution of social behavior and the study of genes associated with human diseases and bacterial infections have been added to the repertoire of research questions that can be addressed in Dictyostelia (2).

With putative applications of research in mind, findings obtained in *D. discoideum* are usually extrapolated to and compared with research in higher vertebrates. However, we should not lose sight of the fact that *D. discoideum* is evolutionary very distant from Metazoa, including vertebrates, and represents an independent invention of

multicellularity in an entirely different eukaryotic lineage (3). To understand and recognize the core components of any process under study and to separate these components from species- or clade-specific adaptations, comparisons with more related organisms are much more informative. Until recently, such comparative studies were hampered by the fact that beyond morphological descriptions very little was known about any or just a few of the closer cousins of *D. discoideum*. A number of advances are changing this state of affairs. DNA- or protein sequence-based phylogenetic analyses have clarified relationships between the major divisions of eukaryotes and groups within these divisions. Dictyostelia are now robustly placed within Amoebozoa, a deeply rooted diverse group of mostly unicellular organisms. Genome sequencing projects, particularly the recent advent of high throughput genome sequencing, have revealed the protein coding potential of protists that are representative of major groups and divisions. Development of gene manipulation strategies has made more protists amenable for studies into the molecular mechanisms that control their physiology and life cycle transitions.

In this chapter, we firstly discuss the classification of Amoebozoa, their position in the tree of life and the morphologies and life styles that define the major groups. We next discuss roads to multicellularity in all eukaryote divisions and finally zoom in on the Dictyostelia and summarize recent insights into the evolution of multicellularity in this group.

2   Classification of Amoebozoa

2.1   Position in the Tree of Life and General Morphology

The morphology-based five kingdom classification of all living organisms (bacteria, protists, animals, plants, and fungi) has in the past 20 years been thoroughly uprooted by molecular sequence data. Instead, now three domains of life are recognized—eubacteria, archaea, and eukaryotes—(4) and eukaryotes are now partitioned into six kingdoms or divisions—Excavata, Rhizaria, Chromalveolata, Plantae, Opisthokonta, and Amoebozoa (3, 5, 6). There is furthermore reasonable molecular and morphological support for a basic dichotomy of the eukaryotes into two superclades, unikonts and bikonts. Unikonts comprise the Amoebozoa and *Opisthokonta*, a clade that contains the Metazoa, Fungi, and associated unicellular relatives, while bikonts comprise the remaining divisions (Fig. 1a). Unikonts usually have only one cilium or flagellum with an associated centriole, whereas bikonts ancestrally harbor two centrioles and cilia. Bikonts undergo ciliary transformation by converting a younger anterior cilium into a modified older posterior cilium. Unikonts may have two or more cilia or flagella, but in such cases the anterior one never transforms into a posterior one. Several groups within the bikonts have acquired photosynthetic endosymbionts or chloroplasts, but this is not the case for unikonts (7–9).
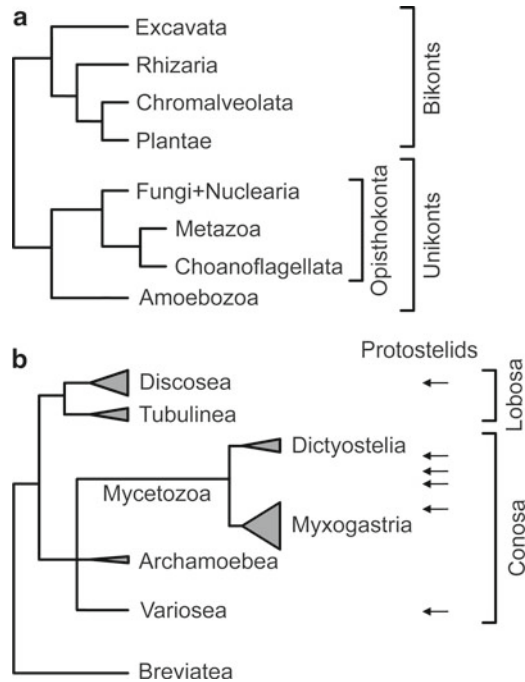
Fig. 1 Phylogenetic relationships between major eukaryote divisions and Amoebozoa. (**a**) Schematic representation of the eukaryote tree of life. The eukaryotes are currently subdivided into the six major divisions of Excavata, Rhizaria, Chromalveolata, Plantae, Opisthokonta, and Amoebozoa, with the latter two considered to form a larger unikont clade, while the remaining divisions group together as bikonts (3, 5, 6). (**b**) Relationships between major groups of Amoebozoa. The current consensus phylogeny of Amoebozoa is based mainly on SSU rRNA sequences and morphological features (18, 28, 31). The positions of the polyphyletic protostelids are indicated by *arrows*. *Triangles* indicate relative species richness of groups, but are not exactly to scale

However, the novel classifications which are mostly based on sequences of just one gene (small subunit ribosomal RNA) are still in a state of flux, and phylogenies based on more molecular markers are badly needed.

Amoebozoa characteristically have no defined shape and are constantly changing form by extending protrusions known as pseudopodia. However, this property is not unique to Amoebozoa or even to unikonts; other amoeboid groups, such as the Heterolobosea and Filosea, are actually members of the bikont divisions *Excavata* and *Rhizaria* (10, 11). Many Amoebozoa alternate a unicellular trophozoite stage with one or several different life cycle transitions. The most common transition is the formation of a dormant cyst in response to environmental stress. The protostelid amoebas can additionally form a stalked spore. Sexual fusion of myxogastrid amoebas followed by nuclear division results in a large syncytial cell

that continues to feed and eventually forms spore-bearing structures in response to environmental cues (12). Sexual fusion of Dictyostelid amoebas followed by cannibalistic engulfment of other amoebas leads to production of a dormant macrocyst (13), while colonial assembly of amoebas to form spore-bearing structures is common to both Dictyostelid and Copromyxid amoebas (14). Amoebas can range in size from a tiny 3–5 μm for the aptly named *Parvamoeba* (15) up to 5 mm for *Pelomyxa palustris* (16). The stalks of the Dictyostelid *D. giganteum* can reach over 7 cm (17), and the plasmodia of Myxogastrids can cover areas of up to several square meters, making them the largest unicellular organisms (12).

2.2 Phylogenetic Relationships and Specializations

Amoebozoa can be further divided into the phyla *Conosa*, which either have cilia or a flagellum or have secondarily lost them; *Lobosa*, which never have cilia or flagella; and the free-living, anaerobic, flagellated *Breviatea* (Fig. 1b) (18, 19). *Breviata anathema* is a marine amoeboflagellate with irregular, pointed, and sometimes branched pseudopodia. Cells are sometimes multinucleate and *Breviata* can form cysts. Although it lacks mitochondria, *Breviata* contains nuclear mitochondrial genes, indicating that mitochondria were lost during its adaptation to an anaerobic habitat (19, 20).

The phylum *Lobosa* can be divided into the well-supported *Tubulinea* and the less well-defined *Discosea*. *Tubulinea* have a more or less cylindrical shape and show typical amoeboid movement through pseudopod extension and cortical contraction. *Tubulinea* comprise the naked amoeba genera *Amoeba*, *Chaos*, *Copromyxa*, *Hartmanella*, *Leptomyxa*, *Gephyramoeba*, and *Echinamoeba* and the testate amoebas or *Arcellinida* (5, 18). Testate amoebas possess an outer shell with a single opening, which consists either entirely of secreted proteins as in *Arcella*, a mixture of secreted and captured organic material as in *Difflugia*, or secreted anorganic material as in *Quadrulella*. All testate amoebas are free living in soil and freshwater and can encyst inside the shell under unfavorable conditions. Within *Discosea*, amoebas with variable, often flattened shapes are combined, and they do not necessarily form a natural group (18, 21). Typical examples of Discosea are *Acanthamoeba*, *Vanella*, *Dermamoeba*, and *Thecamoeba*. Cyst formation occurs in *Acanthamoeba* and some other *Discosea*, but the life cycles of most *Discosea* are unknown.

The phylum *Conosa* comprises the *Variosea*, *Archamoebae*, and *Mycetozoa* (18, 22). Flagellated species of *Conosa* are characterized by a cone of microtubules that connects the mostly single basal body to the nucleus, forming a so-called karyomastigont. *Variosea* contain dissimilar species, such as the flagellate *Phalansterium* (22) and the multiciliated amoeba *Multicilia marina* (23), in which, respectively, the flagellum or cilia each have an apposed basal body. *Phalansterium solitarium* is a solitary species that can form cysts, while *Phalansterium digitatum* forms colonies in which cells are

embedded in a globular organic matrix (22, 24). The Varipodida are also grouped with *Variosea* and contain species with thin and sometimes branched pseudopods like *Acramoeba*, *Grellamoeba*, *Filamoeba*, and *Flamella* (22).

The *Archamoebae*, which have secondarily lost their mitochondria (25), contain the anaerobic Mastigamoebida, comprising *Mastigamoeba*, *Endamoeba*, *and Endolimax*, and the Pelobiontida, comprising *Pelomyxa*, *Entamoeba*, and *Mastigina*. In *Mastigamoeba* the nucleus is physically attached to the basal body of a forward facing flagellum and can be protracted. It can both encyst and form multinucleate cells. *Endamoeba* and *Endolimax* are found in the guts of animals and spread as cysts. *Pelomyxa palustris* is a multinucleate amoeba, containing up to several thousand nuclei (26). *Pelomyxa* lives in anaerobic freshwater sediments, where it indiscriminately takes up material and digests usable constituents. Besides inclusions like sand and diatom shells, the cytoplasm harbors several bacterial endosymbionts, some of which are methanogen. Its life cycle is complex with binucleate cells being derived by plasmotomy from larger cells or hatched from cysts and cysts with four nuclei. Most *Entamoeba* species are harmless commensals residing in the large intestine of animals. Lacking mitochondria, they can only survive outside the body as dormant cysts. *E. histolytica* is an important human pathogen in developing countries, which causes amoebic dysentery and often lethal liver abscess (27).

The *Mycetozoa*, characterized by spore-bearing fruiting bodies, are the most diverse group within the *Amoebozoa* and comprise some protostelids, the Myxogastria and the Dictyostelia. The protostelids are however a larger polyphyletic assemblage and fall into different lineages within the *Amoebozoa* (Fig. 1b) (28). Most protostelids form a single spore on top of a thin hollow stalk that is secreted by the same cell, but species like *Protosporangium* (29) can have up to four spores. Many species also form cysts and in some species, amoeba fuse to form small multinucleate plasmodia. The Myxogastria group into two clades containing either the dark-spored *Physarida* and *Stemonitida* or the bright-spored *Liceida* and *Trichiida* (30, 31). Myxogastrids are amoeboflagellates and hatch from spores either as amoebas or biflagellated cells. These forms can also interconvert, with wet conditions favoring the flagellate form. Upon nutrient depletion either cell type encysts to form a dormant microcyst. In addition, both amoebas and flagellates can fuse to form a zygote when compatible mating types are present. The zygote then goes through multiple synchronous nuclear divisions without cytokinesis and continues to feed, thus causing large to enormous single-celled plasmodia to form. Under dry and/or cold conditions, the plasmodium converts into irregular hardened masses of dormant macrocysts, called sclerotia. Other environmental stimuli, such as light, induce cleavage of the plasmodium into segments with single nuclei that mature into haploid spores, after going

through one round of meiosis. The remainder of the protoplasm forms often quite intricate structures to elevate the spore mass above the substratum (12, 32).

The Dictyostelia form multicellular fruiting bodies by aggregation of amoebas and are commonly found in forest soils. They have been isolated from the Arctic to the tropics (33), but there are no marine species and as yet only one description of a pathogenic *D. polycephalum* isolate (34). The amoebas phagocytose bacteria and small yeasts, although one species, *D. caveatum*, can also eat other amoebas by nibbling (35, 36). Upon starvation, amoebas secrete a chemoattractant, which can be cAMP, glorin, folate, a pterin, or an as yet unidentified compound, and form an aggregate consisting from around ten to a million cells (1, 37). Some species form an intermediate pseudoplasmodium or "slug" that moves towards warmth and light to find a suitable spot for fruiting body formation. Once aggregated, the amoebas initiate differentiation into condensed encapsulated spores and highly vacuolated stalk cells. Stalk cells are encased by a cellulose wall and are collectively shaped into a rigid column by a cellulose stalk tube, which carries the spore mass above the substratum (38). Ancillary structures, called upper and lower cups, which support the spore mass, and a basal disk to anchor the stalk to the substratum can also be present (39). The spores are hydrophilic and are most likely dispersed by rain and melting snow, but small soil invertebrates and even birds and bats may also aid in spore dispersal (40, 41).

Many Dictyostelid species can also encapsulate individually as microcysts or engage in sexual fusion and form macrocysts, a process in which the zygote attracts and ingests other amoebas before surrounding itself with a thick wall. After a long period of dormancy, the macrocyst undergoes meiosis and multiple mitoses and eventually hatches to yield several haploid amoebas (13, 42). Population genetics of wild isolates indicates that mating occurs frequently in nature (43). Specific environmental conditions trigger entry into the alternative pathways of fructification, microcyst-, or macrocyst formation. Fructification requires an air-water interface and is stimulated by light. Microcysts are formed under dark, humid, or submerged conditions with high solute or ammonia levels as additional stimuli (44). Macrocyst formation usually requires the presence of a compatible mating type and is stimulated by ethylene, darkness, and submersion (45).

Traditionally, the Dictyostelia have been divided into the genera *Acytostelium*, with a secreted acellular stalk, *Dictyostelium* with unbranched or irregularly branched sorocarps, and *Polysphondylium* with regular whorls of side branches (44). However, molecular phylogenetic analysis revealed that Dictyostelia can be subdivided into four major groups, called groups 1–4, with Dictyostelids being present in each group and multiple independent origins for Polysphondylid-like species (46). Extension of taxon sampling

indicated that a few group-intermediate species, such as *P. violaceum*, *D. polycarpum*, and *D. polycephalum*, may represent additional minor clades (47).

In summary, the Amoebozoa are a division of amoeboid or amoeboflagellate organisms that most commonly alternate a trophozoite feeding stage with a dormant cyst stage. Sexual fusion is also common, leading either to formation of dormant zygotic cysts or to multinucleate cells of varying sizes. Several subdivisions have evolved forms that elevate one or a few spores above the substratum, but only *Copromyxa* and Dictyostelia construct fruiting structures from more than ten cells.

2.3 Many Roads to Multicellularity

Multicellularity arose several times independently during evolution but is commonly perceived to be only present in plants, animals, and fungi. However, most eukaryotic divisions and phyla show independent inventions of multicellularity. In the bikonts, multicellular photosynthetic organisms evolved independently from unicellular green algae (all land and many marine plants), brown algae (kelps and stramenopiles), and red algae (many seaweeds). Not only Dictyostelia and *Copromyxa* in *Amoebozoa* but also unrelated amoebas like *Acrasis* and *Pocheina* in *Excavata* (48) and *Fonticula alba* in *Opisthokonta* (49) form fruiting bodies from hundreds to up to a million cells. The *Opisthokonta* on the unikont side is particularly prone to multicellularity with colonial forms in the choanoflagellates (50), both unicellular and multicellular species in *Fungi* and unconditional multicellularity in *Metazoa*. Both *Metazoa* and vascular green plants generate the multicellular form from a zygote through cell division and cell differentiation. In *Fungi*, mycelia of interconnected cells can develop by cell division from either a zygote, a spore, or asexual propagates. On the other hand, aggregative multicellularity does not necessarily depend on cell divisions and almost always results in formation of a spore- or cyst-bearing structure. In the following paragraphs, we describe organisms with aggregative multicellularity in more detail.

The *Chromalveolate* ciliate species *Sorogena stoianovitchae* feeds on the smaller ciliate *Colpoda*. When starved at high cell density, *Sorogena* ciliates aggregate by cell adhesion to form a mound encased in a mucous sheath (Fig. 2a). This sheath then contracts and elongates to form an acellular stalk that lifts the cell mass above the water surface, followed by encystation of the ciliate cells (51, 52). Some heterolobose amoeba genera in the *Excavates*, such as *Acrasis spp.* and the related *Pocheina rosea*, either encyst individually or amoebas aggregate to form a mound that is lifted above the substratum by virtue of cells encysting at the base of the structure. *Acrasis* fruiting bodies are mostly tree-shaped with the mature spores forming branched chains (Fig. 2b), while *Pocheina* forms a stalk with a globose spore mass. Both stalk and spore cells are viable and only marginally differ from each other. They are also very similar
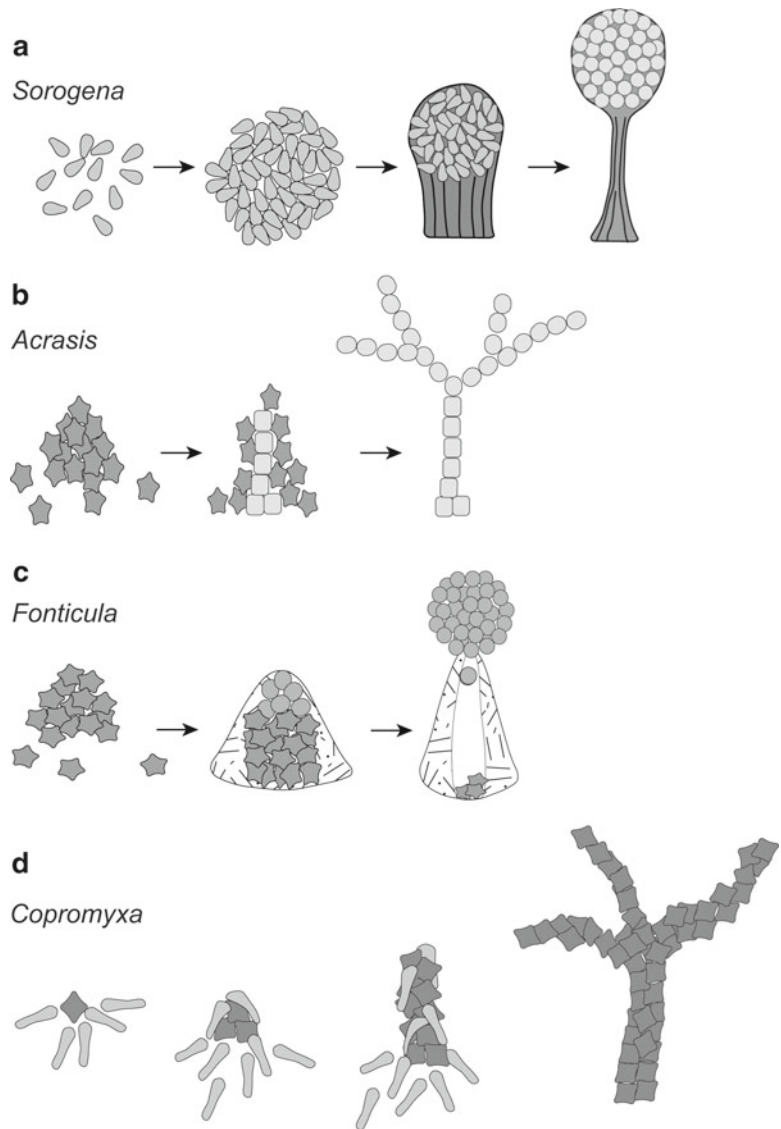
Fig. 2 Fruiting body formation in various organisms with aggregative multicellularity. (**a**) The ciliate *S. stoianovitchiae* aggregates by adhesion and forms a sheath that contracts to form a stalk, while the cells encyst. (**b**) *Acrasis* amoebas aggregate and form a stalk by encysting at the base of the structure, while more apical cells rearrange themselves into chains and then encyst. (**c**) *F. alba* amoebas aggregate and deposit a cone-shaped matrix around the cell mass. Amoebas differentiate into spores and are expulsed through the apex. (**d**) *Copromyxa* amoebas are attracted to a few encysted founder cells. Once aggregated, cells crawl on top of existing cysts and then encyst themselves

to cysts but additionally have plinth-like connecting structures, called hila (5, 48, 53, 54).

Amoebas of the amoeboid *Opisthokont Fonticula alba* collect into aggregates and secrete an extracellular matrix that forms a volcano-shaped enclosure around the cells (Fig. 2c). When the amoebas mature into spores, the apex opens eruptively and deposits the spores as a droplet on top of the structure, leaving some undifferentiated amoebas behind at the base (49, 55). The Amoebozoan *Copromyxa protea* feeds on bacteria in dung and, similar to Dictyostelia, can enter upon three alternative survival strategies when starved. Amoebas can differentiate into round or "puzzle-piece"-shaped (micro)cysts or fusion of two amoebas results in the formation of dormant double-walled spherocysts. Alternatively, some amoebas encyst first and then become founder cells, which attract other amoebas to form an aggregate (Fig. 2d). The amoeba in the aggregate form a branched fruiting structure by crawling on top of each other and in turn forming so-called sorocysts that are morphologically identical to microcysts (14, 56)

Evidently, the formation of fruiting bodies by aggregation has evolved several times independently. Despite occurring in very diverse genetic lineages, these forms of multicellularity all resemble Dictyostelia in the fact that they are a response to starvation and generate a structure that elevates dormant spores or cysts above an air/water interface. Most aggregating amoebas or ciliates use fructification as an alternative strategy to encystation of individual cells in situ. However, Dictyostelia are unlike all aggregating amoebas by differentiating into at least two morphologically distinct cell types and by the sophistication of their aggregation process and morphogenetic program.

2.4 Evolution of Morphogenesis in Dictyostelia

Outwith Dictyostelia, aggregating species usually collect into mounds by cell adhesion or by moving towards each other individually. While the latter mode is also observed for some of the smaller species of Dictyostelia, most Dictyostelids aggregate as interconnecting streams of amoebas. Studies in the model organism *D. discoideum* revealed that this mode of aggregation results from relay of chemoattractant waves, in this case cAMP, through the starving population (57). A biochemical network, consisting of the cAMP receptor, cAR1, the adenylate cyclase, ACA, the extracellular phosphodiesterase PdsA, and intracellular proteins, including PKA and RegA, generates pulses of cAMP in a few starving cells (58, 59). In surrounding cells these pulses elicit cAMP-induced cAMP secretion (cAMP relay), which results in propagation of the cAMP pulse throughout the cell population and chemotactic movement of cells towards the cAMP source. Once aggregated, the tips of multicellular structures continue to emit cAMP pulses, which guide and shape the organism during slug migration and fruiting body formation by coordinating the

movement of its component cells (60). Secreted cAMP not only coordinates morphogenesis but also regulates stage- and cell-type-specific gene expression. Nanomolar cAMP pulses accelerate the expression of aggregation genes (61), while micromolar cAMP concentrations induce the expression of prespore genes and inhibit stalk gene expression (62, 63).

In addition to these roles for secreted cAMP, intracellular cAMP acting on PKA also crucially regulates many developmental transitions. Together with secreted cAMP, intracellular cAMP is required for prespore differentiation (64). Furthermore, active PKA crucially triggers spore and stalk maturation and maintenance of spore dormancy in the fruiting body (64–66). For stalk and spore maturation, cAMP is produced by adenylate cyclase R (ACR) and for induction of prespore differentiation and control of spore germination by adenylate cyclase G (ACG) (66–68). ACG harbors an intramolecular osmosensor and is activated by high ambient osmolarity, a condition that keeps spores dormant in the spore head (69, 70). The cAMP phosphodiesterase RegA also plays a crucial role in regulating intracellular cAMP levels (71).

Recent comparative studies into conservation and change in genes involved in synthesis and detection of cAMP throughout the Dictyostelid phylogeny provided insight into the evolutionary origin of cAMP signaling. Osmolyte-activated ACG is functionally conserved throughout the Dictyostelid phylogeny (72). Many early diverging Dictyostelid species have retained the ancestral mechanism of encystation (46). Similar to spore germination, cyst germination is also inhibited by high osmolarity, but unlike spore formation, encystation can be directly induced by high osmolarity. For soil amoebas, high osmolarity is probably a signal of approaching drought, which increases the concentration of soil minerals. Osmolyte-induced encystation is mediated by cAMP production and PKA activation (72), suggesting that the roles of intracellular cAMP and PKA in spore differentiation and germination are evolutionary derived from a similar role in the encystation of solitary amoebas.

Genes encoding cAR1 and therefore extracellular cAMP signaling are also functionally conserved throughout the *Dictyostelid* phylogeny. In group 4 species, such as *D. discoideum* and *D. rosarium*, cAR1 is expressed from a proximal promoter during postaggregative development and from a distal promoter during aggregation (73, 74). In these species, inhibition of cAR function blocks both aggregation and subsequent development. Remarkably, in groups 1 and 2, cAR1 orthologs are only expressed after aggregation (74), and in either group 1, 2, or 3, abrogation of cAR function disrupts slug and fruiting body formation, but not aggregation (75). The latter effect was not unexpected, since group 1–3 species use other attractants than cAMP to aggregate, with glorin being most prevalent (76). However, the fact that postaggregative morphogenesis is blocked by loss of cAR function suggests that all
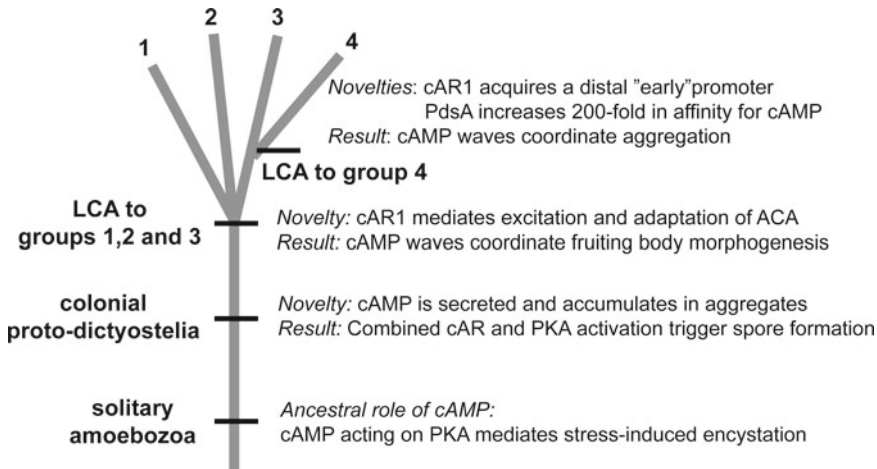
Fig. 3 The evolution of morphogenetic cAMP signaling in Dictyostelia. Putative scenario for the evolution of developmental cAMP signaling in Dictyostelia from a second messenger function in Amoebozoan encystation. *LCA* last common ancestor

Dictyostelia use oscillatory cAMP signaling to coordinate cell movement during slug and fruiting body formation. This hypothesis was further substantiated by recent observations that loss of the *PdsA* gene from a group 2 species also resulted in disruption of postaggregative morphogenesis, while aggregation remained normal. Strikingly, the affinity of PdsA for cAMP in groups 1–3 was low but increased 200-fold in group 4 species. This probably reflects an adaptation from hydrolyzing relatively high extracellular cAMP concentrations within an aggregate to hydrolyzing much lower concentrations in a dispersed field of starving cells (77). Taken together, the data indicate that oscillatory cAMP signaling evolved first to coordinate morphogenesis and that its additional role in mediating aggregation appeared more recently in group 4.

cAR gene disruption in the group 2 species *P. pallidum* yielded stunted fruiting structures that contained cysts instead of spores in the spore head. This was due to the fact that the cAR null mutant no longer expressed prespore genes in response to cAMP stimulation (75). As discussed above, sporulation and encystation both require intracellular cAMP acting on PKA, but sporulation additionally requires extracellular cAMP acting on cARs. With the latter pathway ablated, the cAR null cells reverted to the ancestral strategy of encystation.

Together, these results suggest that cAMP signaling in Dictyostelia evolved from a "classical" second messenger role for cAMP in stress-induced encystation (Fig. 3). Dictyostelia secrete most of the cAMP that they produce but can only accumulate the micromolar concentrations that are required for prespore differentiation, once they have aggregated. In early Dictyostelids, accumulation of secreted cAMP

may therefore have acted as a signal for the aggregated state and have prompted cells to form spores and not cysts. Oscillatory cAMP secretion, which requires cAR-mediated positive and negative feedbacks on cAMP synthesis by ACA evolved next and enabled the cells to form architecturally sophisticated fruiting bodies. cAMP-mediated aggregation was the most recent innovation and only occurred in group 4 (Fig. 3).

While at first sight the multitudinous roles of cAMP in *D. discoideum* in aggregation, morphogenesis, and gene regulation may seem perplexing, evolutionary reconstruction of these roles allows us to separate core ancestral processes from more recent adaptations. In essence, evolutionary reconstruction reveals the underlying logic of convoluted interrelated processes. Comparative analysis and evolutionary reconstruction are therefore powerful tools to unravel complex biological processes.

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