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INTEGRATION OF SIGNALING NETWORKS THAT REGULATE *DICTYOSTELIUM* DIFFERENTIATION

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■ **Abstract** In *Dictyostelium* amoebae, cell-type differentiation, spatial patterning, and morphogenesis are controlled by a combination of cell-autonomous mechanisms and intercellular signaling. A chemotactic aggregation of $\sim 10^5$ cells leads to the formation of a multicellular organism. Cell-type differentiation and cell sorting result in a small number of defined cell types organized along an anteroposterior axis. Finally, a mature fruiting body is created by the terminal differentiation of stalk and spore cells. Analysis of the regulatory program demonstrates a role for several molecules, including GSK-3, signal transducers and activators of transcription (STAT) factors, and cAMP-dependent protein kinase (PKA), that control spatial patterning in metazoans. Unexpectedly, two component systems containing histidine kinases and response regulators also play essential roles in controlling *Dictyostelium* development. This review focuses on the role of cAMP, which functions intracellularly to mediate the activity of PKA, an essential component in aggregation, cell-type specification, and terminal differentiation. Cytoplasmic cAMP levels are controlled through both the regulated activation of adenylyl cyclases and the degradation by a phosphodiesterase containing a two-component system response regulator. Extracellular cAMP regulates G-protein–dependent and –independent pathways to control aggregation as well as the activity of GSK-3 and the transcription factors GBF and STATa during multicellular development. The integration of these pathways with others regulated by the morphogen DIF-1 to control cell fate decisions are discussed.

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INTRODUCTION

Dictyostelium multicellular development is dependent on the availability of food. When nutrients are available, cells grow vegetatively as single-celled amoebae. The developmental program is initiated by starvation and leads to the formation of a multicellular organism with a defined spatial pattern of cell types organized along an anterior/posterior (AP) axis (Firtel 1995, 1996; Loomis & Cann 1982; Parent & Devreotes 1996). Analysis of the pathways that regulate *Dictyostelium* differentiation has been greatly accelerated by the development of genetic tools that include transformation with extrachromosomal and integrating vectors, homologous recombination permitting gene knockouts and gene replacement, and restriction enzyme-mediated integration (REMI), a method of insertional mutagenesis (Kuspa & Loomis 1992, Mann et al 1994) that has allowed the identification and analysis of new genes and pathways. These techniques, combined with a relatively simple developmental program, make *Dictyostelium* ideal for dissection of signaling pathways involved in controlling multicellular development. Studies on *Dictyostelium* have yielded new insights into the strategies used by multicellular systems to control spatial patterning and cell fate decisions. This review examines the contributions of recent findings to our understanding of signaling networks, including the parallel pathways controlling cell fate in metazoans, which orchestrate the *Dictyostelium* differentiation program. We further consider how this information may help unravel the regulatory pathways controlling cell fate decisions in more complex organisms.

AGGREGATION AND FORMATION OF THE MULTICELLULAR ORGANISM

In *Dictyostelium* multicellularity is achieved by the aggregation of individual cells in response to nanomolar pulses of chemoattractant extracellular cAMP (Firtel 1995, Williams 1995). Cells stimulated by the chemoattractant respond by chemotactic movement toward the source of the cAMP and amplification and relay of the signal as they synthesize and release cAMP (Chen et al 1996, Devreotes 1989, 1994, Firtel 1995, Parent & Devreotes 1996). When cells form an aggregate, cAMP concentration is thought to rise to the micromolar range (Abe & Yanagisawa 1983). A transcriptional cascade is activated, leading to the emergence of different cell-types that self organize within the aggregate (Firtel 1995, 1996; Kimmel & Firtel

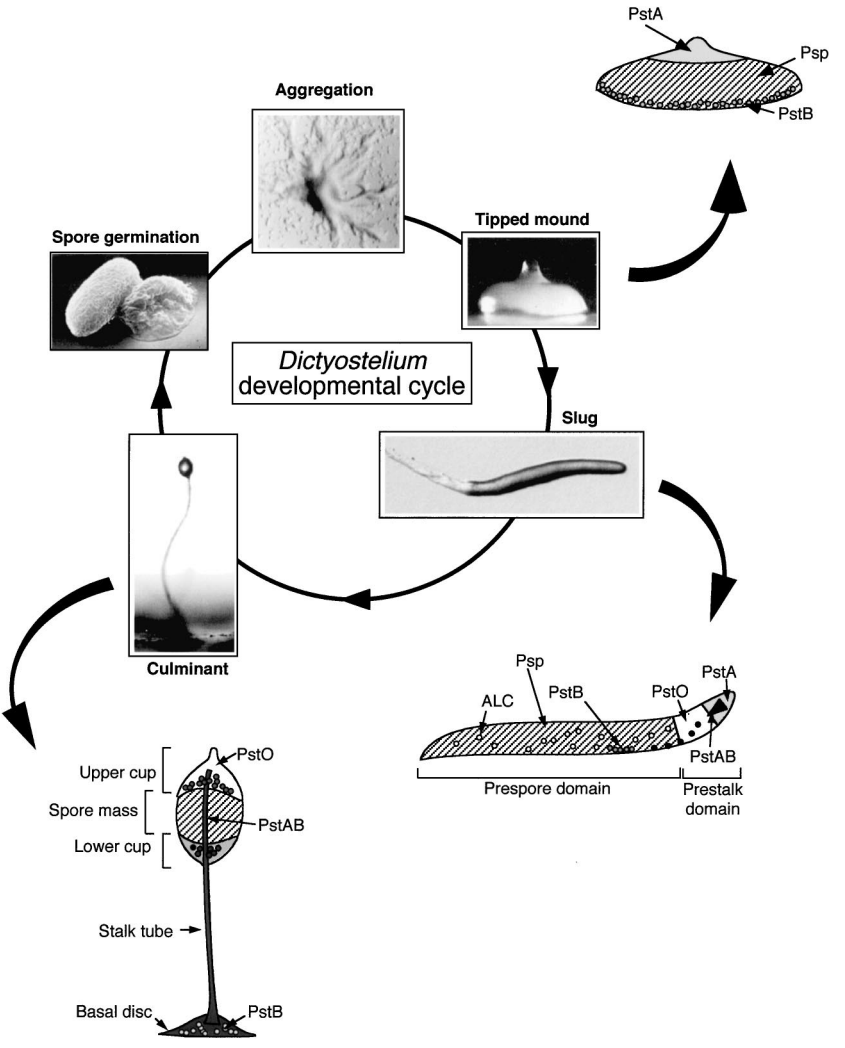
1991; Loomis 1996). Two major precursor cell types that differentiate within the aggregate are the prespore cells, which represent ~70% of the total, and the prestalk cells (Figure 1). A spatially defined pattern of cell types is obtained after sorting of prestalk cells to the top of the mound where a tip is formed. This tip elongates and falls onto the substratum, generating a migrating slug or pseudoplasmodium. In response to intrinsic and extrinsic signals, the pseudoplasmodiun undergoes coordinated morphogenetic movements leading to the formation of a mature, differentiated fruiting body. Prestalk cells terminally differentiate into a cellulose-containing vacuolated stalk that supports a prespore-derived spore mass.

During aggregation, oscillatory waves of cAMP are generated from the center of the aggregating territory and are propagated toward neighboring cells. The intracellular pathways activated by the pulses of cAMP adapt rapidly (Dinauer et al 1980, Kesbeke et al 1985), and adaptation persists until extracellular cAMP is degraded by an extracellular membrane-bound and -secreted cAMP-phosphodiesterase (PDE; Franke & Kessin 1992). Disruption of the gene encoding PDE or overexpression of PDE protein impedes aggregation as a consequence of unregulated cAMP degradation. After cAMP degradation, cells regain sensitivity within a few minutes, resulting in the periodic emission of cAMP pulses every 6 min. PDE activity is regulated by a secreted glycoprotein phosphodiesterase inhibitor (PDI) of the PDE. PDE and PDI expression are reciprocally regulated by extracellular cAMP levels [high cAMP concentrations lead to increased PDE expression, whereas reduced cAMP concentrations enhance PDI expression (Franke et al 1991)], thus providing a mechanism to control the level of extracellular cAMP and PDE activity. The succession of transiently refractory and responsive states is essential for the outward propagation of the cAMP waves and gradient sensing and allows a tight control of the directionality of the migration process toward the center of the aggregate (Tomchik & Devreotes 1981, Van Haastert et al 1987). Propagation of the cAMP waves can be monitored by dark-field or phase contrast video microscopy, in the form of optical density waves, because cells that are alternately responsive and moving or adapted display a different pattern of light scattering (Gross et al 1976, Siegert & Weijer 1991, 1992). Oscillatory cAMP signaling similar to that observed during aggregation is maintained in the multicellular organism during the later stages of development (Steinbock et al 1993). The tip of the mound controls development and acts similarly to a classical embryological organizer, functioning as an autonomous cAMP oscillator. A differential responsiveness of the different cell-type populations to cAMP is involved in the coordination of cell sorting and the establishment of cellular pattern and morphogenetic shape changes. The signaling pathways controlling aggregation are presented in Figure 2.

Molecular Pathways Regulating Aggregation

Two cAMP Receptors, cAR1 and cAR3, Mediate the Effect of cAMP During Aggregation Secreted cAMP is a key regulator of aggregation and later development as a chemoattractant and morphogen. Extracellular cAMP is detected by

four distinct cell-surface cAMP receptors (designated cAR1–4) belonging to the serpentine/G-protein–coupled receptor family; these receptors regulate developmental functions that include cell movement, cell fate determination, and cellular patterning (Johnson et al 1993, Klein et al 1988, Louis et al 1994, Saxe et al 1991, 1993, Sun & Devreotes 1991). The four subtypes of receptors differ by their timing, spatial (cell-type) pattern of expression during development, and affinity for cAMP (Ginsburg et al 1995); cAR1 and cAR3 are high-affinity cAMP receptors with molecular sizes of 200 and 700 kDa, respectively, whereas cAR2 and cAR4 have affinities ~2 orders of magnitude lower than that of cAR1 (Johnson et al 1992, Kim et al 1996, Klein et al 1988). The cAR1 receptor is expressed



throughout development. During aggregation, cAR1 expression is controlled by a promoter that is induced by cAMP pulses and directs its expression in all cells. About 5–10 $\times$ 10^4 receptors are found on each cell (Chen et al 1996). This aggregation stage promoter is repressed as extracellular cAMP increases and control of cAR1 expression is taken over by a late promoter that responds to a high, continuous level of the chemoattractant (Louis et al 1993). Expression is maintained at a much lower level during the multicellular stages. A similar regulatory pathway mediated by cAMP pulses controls the expression of many, but not all, genes that function during aggregation, including the Gα protein subunit Gα2, which couples to cAR1, and contact sites A (csA, gp80) involved in mediating cell-cell contacts (Kumagai et al 1991; Mann & Firtel 1989, 1987; Noegel et al 1985, 1986; Okaichi et al 1992; Wang et al 1986). Transcription factors thought to play a role in cAMP pulse regulation of cAR1 expression and possibly the expression of other aggregation-stage genes have been identified (Mu et al 1998, Otsuka & Van Haastert 1998). The cAR3 receptor is induced next as the mound forms (Johnson et al 1993). Initially present in all cells, cAR3 protein is later confined to prespore cells as the organism reaches the slug stage (Gollop & Kimmel 1997, Yu & Saxe 1996). Induction of cAR2 and cAR4 expression parallels the increase in extracellular cAMP concentration expected as the mound becomes more compact, and it occurs slightly after cAR3 induction. Their expression is more prestalk-specific cells (Louis et al 1994, Saxe et al 1996).

Gene disruption of *cAR1* has demonstrated that cAR1 functions during aggregation as the main cAMP receptor mediating this event as well as aspects of multicellular development (Sun & Devreotes 1991). Cells lacking cAR1 fail to aggregate because of their inability to activate adenylyl cyclases (ACs) and

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Figure 1 *Dictyostelium* development cycle and position of cell types within the multicellular differentiating organism. Upon starvation, individual amoebae aggregate via a chemotactic process to form a mound. The tip of the mound elongates and falls on the substratum, forming a migrating slug. The precursor cell types include prespore cells (Psp) and prestalk cells (Pst), the latter ones being subdivided into PstA, PstB, PstO, and PstAB cells, plus anteriorlike cells (ALCs). These cell-types differentiate at the mound-stage and sort within the multicellular aggregate to generate a defined spatial pattern. At slug stage, the cell types are organized along a well-defined anteroposterior axis. The slug is surrounded by a sheath composed of secreted extracellular matrix proteins and cellulose. The sheath plays a role in the migration of the slug and is probably important in regulating the architecture of the multicellular organism. Under appropriate conditions of light, humidity, and external ammonia concentration, culmination is initiated, leading to a well-proportioned fruiting body composed of a vacuolated stalk supporting a mass of spores. During culmination, morphogenesis results in a complete reorganization of the various cell types, leading to formation of the mature fruiting body. The positions of the different cell types are shown at tipped mound, slug, and culmination stages (see text for details).

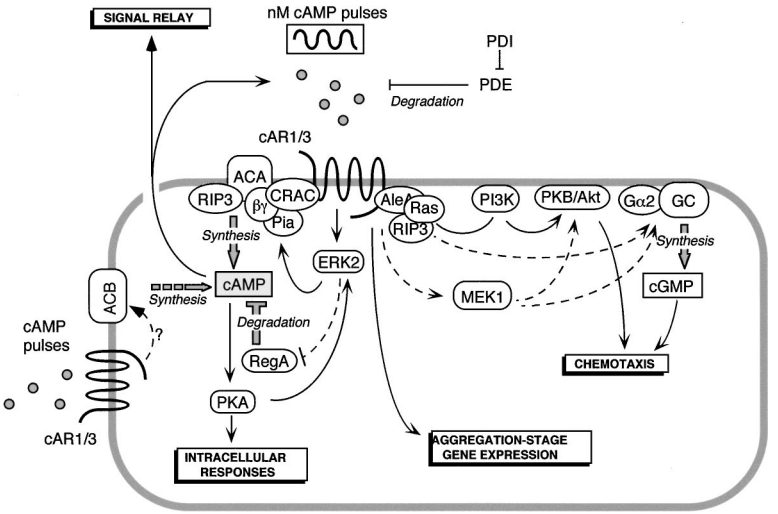

Figure 2 Signaling network functioning during aggregation. During aggregation, *Dictyostelium* cells respond to nanomolar pulses of cAMP detected by the G-protein–coupled cAMP receptors cAR1 and cAR3. The cAMP binding to the receptors leads to the activation of adenylyl cyclase (ACA) involved in cAMP synthesis and signal relay, activation of guanylyl cyclase (GC) required for chemotaxis, activation of Akt/PKB (PKB/Akt) required for proper chemotaxis, and induction of the expression of a number of aggregation-stage genes. Activation of ACA and the production of cAMP involve several proteins in addition to ACA and the G$\beta\gamma$ subunit, including the MAP kinase ERK2, components of the Ras pathway, and cytosolic regulators such as CRAC and Pianissimo (Pia). Stimulation of GC requires the function of the MAP kinase pathway containing the MAP kinase kinase MEK1, the Ras exchange factor Aimless, and the Ras-interacting protein RIP3. Rises in intracellular cAMP lead to the activation of the protein kinase PKA. Whereas extracellular cAMP is degraded to 5′-AMP by a secreted/membrane-bound phosphodiesterase (PDE), breakdown of intracellular cAMP is performed by the two-component system response regulator RegA containing a cAMP-specific PDE. Recently, a novel ACA (ACB) has been identified in *Dictyostelium* that may play an important role in regulating the level of cAMP production during aggregation and/or multicellular development. How ACB is regulated is not known (see text for references).

guanylyl cyclases (GCs), essential elements of the aggregation response. However, the *car1*-null strain can proceed further and form fruiting bodies when subjected exogenously to a higher concentration of cAMP (Insall et al 1994b). The *car3* disruptants do not exhibit aggregation defects, and they form well-proportioned fruiting bodies (Johnson et al 1993). The cAR3 receptors can mediate most cAR1-dependent signaling pathways when cells are examined in suspension culture, although they do so less efficiently than do wild-type cells, presumably because

of a low level of expression of this receptor subtype. Overexpression of cAR3 can partially compensate for the loss of cAR1 during early development. The double mutant *car1/car3*-null, on the other hand, is completely insensitive to cAMP stimulation, consistent with the fact that cAR3 is partially redundant in function to cAR1 for cAMP signaling, at least during early development (Insall et al 1994b, Soede et al 1994).

As expected from their temporal expression pattern, cAR2 and cAR4 do not have any necessary functions during aggregation; cells lacking either of these two receptors aggregate normally. However, *car2*- and *car4*-null mutants arrest development as they reach the multicellular stages (Louis et al 1994, Saxe et al 1993). The functions of cAR2 and cAR4 receptors during later development are described later in the review.

Intracellular Responses Elicited by Extracellular cAMP During aggregation, cAR1 receptors signal through adapting and nonadapting pathways via well-characterized G-protein–dependent and –independent cascades (Milne & Devreotes 1993, Parent & Devreotes 1996). Binding of extracellular cAMP onto cAR1 receptors elicits three rapid responses: (*a*) the activation of the aggregation stage AC (ACA) responsible for cAMP synthesis and thus signal propagation (secretion of newly synthesized cAMP allows recruitment of adjacent cells to the aggregation process), (*b*) the activation of the GC required for the chemotactic process, and (*c*) the activation of the phosphatidylinositol lipid kinase-regulated serine/threonine protein kinase Akt/PKB (Meili et al 1999). The cAR1-coupled, G-protein–dependent activation of ACA, GC, and Akt/PKB is transient because all three effectors adapt quickly after each pulse of cAMP (Van Haastert & Van der Heijden 1983, Van Haastert et al 1992). The *aca*-null mutants are unable to aggregate, although they can move via chemotaxis toward exogenous cAMP, thus restricting ACA function to the synthesis of cAMP (Pitt et al 1992). The activation pathway leading from cAR1 to ACA has been particularly well studied and involves proteins other than the heterotrimeric G protein containing the Gα2 subunit whose deletion also compromises aggregation, including the following: the MAP kinase ERK2; the cytosolic regulators CRAC, Pianissimo, and Vagabond; and components of the Ras pathway (the Ras GTP exchange factor Aimless and RIP3, a Ras-interacting protein) (Chen et al 1997, Insall et al 1994a, 1996, Lilly & Devreotes 1994, Maeda et al 1996, Segall et al 1995; S Lee & R Firtel, submitted for publication). The MAP kinase ERK2 is essential for cAMP production (Segall et al 1995). It is transiently activated by cAMP pulses via a cAR1-dependent but G-protein–independent pathway (Knetsch et al 1996, Maeda et al 1996). ERK2 is thought to function, at least in part, as a negative regulator of RegA, an intracellular, cAMP-specific PDE that is a two-component system response regulator system (Shaulsky et al 1996, 1998, Thomason et al 1998; see below). The cAMP-induced activation of GC results in the accumulation of the second messenger cGMP involved in the rearrangement of the actin and myosin cytoskeletons and the chemotactic response (Liu et al 1993, Liu & Newell 1994, Van Haastert 1997).

This includes the activation of the myosin II heavy-chain kinase MHCK (Abu-Elneel et al 1996, Dembinsky et al 1996, Kolman et al 1996). Recent work has shown that cell polarization during chemotactic movement relies on the activity of Akt/PKB (Meili et al 1999).

The components of the cAMP-signaling response (cAR1, ACA, Gα2, and PDE) are expressed at low levels during the first hours of starvation as two cell density-sensing proteins, PSF (prestarvation factor) and CMF (conditioned medium factor), reach a threshold concentration in the extracellular medium (Clarke & Gomer 1995, Gomer et al 1991, Mehdy & Firtel 1985, Van Haastert et al 1996). However, stimulation with cAMP pulses quickly results in a substantial induction of these aggregation stage-specific genes and the subsequent amplification of the cAMP-signaling response via a positive-feedback loop (Kimmel & Firtel 1991, Mann & Firtel 1987).

Intracellular cAMP Activates the cAMP-Dependent Protein Kinase Although most of the cAMP synthesized by the AC during aggregation is secreted in the extracellular medium and recruits adjacent cells to the aggregative process, the remaining intracellular cAMP regulates pathways dependent upon cAMP-dependent protein kinase (PKA) (Firtel 1996, Loomis 1998, Reymond et al 1995). In *Dictyostelium*, PKA is a heterodimer containing the single catalytic subunit PKA-C (PKAcat) associated with a single regulatory subunit PKA-R, not two of each subunit as in most other organisms (Burki et al 1991, Mann et al 1992, Mutzel et al 1987, Simon et al 1992). PKA, as in other systems, is activated by cAMP, which binds to the regulatory subunit and dissociates the dimer, releasing the catalytically active protein PKA-C. Mutations in PKA-R (PKA-Rm) that abrogate cAMP binding result in a dominant negative form of the protein that binds to but is unable to dissociate from the catalytic subunit (Harwood et al 1992a). PKA is not essential for vegetative growth, as determined from the analysis of *pkacat*-null cells, and the expression of both PKA-R and PKA-C subunits is induced as development proceeds (Anjard et al 1993, Mann & Firtel 1993). Constitutive expression of PKA from the Actin 15 promoter or from disruption of PKA-C prevents aggregation, partly because of an inability of cells to induce ACA expression (Firtel & Chapman 1990, Harwood et al 1992a, Mann et al 1997, Schulkes & Schaap 1995, Simon et al 1989). However, constitutive expression of ACA is not sufficient to restore a normal aggregation of the *pkacat*-null strain (Mann et al 1997). It is clear that PKA regulates multiple aspects of aggregation. In particular, PKA is part of the signaling pathway controlling cAMP production, as a regulator of ERK2 activity (Aubry et al 1997).

Intracellular cAMP concentration and, therefore, tuning of PKA activity depend on the balance between cAMP synthesis and breakdown. Until recently, only two AC genes had been cloned in *Dictyostelium*. ACA is highly expressed and rapidly activated in response to cAMP pulses during aggregation (Pitt et al 1992). Its level of expression decreases as development proceeds. A second AC, ACG, is expressed only in the mature spores and seems to function as an osmosensor

to control spore germination (Pitt et al 1992, Van Es et al 1996). Surprisingly, the *aca*-null mutants exhibit some PKA-dependent gene expression under certain conditions and, therefore, some PKA activity, suggesting the presence of another AC. Recently, Schaap and collaborators identified a novel AC (ACB), which is active during aggregation and multicellular development (HJ Kim et al 1998). Whereas ACA is the major source of cAMP, it is very possible that ACB also regulates the activity of endogenous PKA. Although degradation of extracellular cAMP is carried out by a classical cAMP PDE, an element of a two-component signal transduction system (RegA) participates in intracellular cAMP breakdown. RegA is a composite protein with a cAMP-specific PDE activity and an N-terminal region homologous to response regulators of two-component systems (Shaulsky et al 1996, 1998, Thomason et al 1998). RegA accumulates during aggregation, and its expression is maintained throughout development. It is thus probable that this protein regulates the level of PKA activity via the regulated degradation of cAMP at each stage in which PKA functions (Loomis et al 1998). Details about this pathway are provided below, as the role of RegA during terminal differentiation has been more thoroughly analyzed.

Molecular Pathways Regulating Cell-Type Differentiation

Developmental Switch from Aggregation to Multicellular Development: Role of the Transcription Factor GBF As the aggregate forms, a developmental switch occurs in response to the expected rise of extracellular cAMP concentration within the aggregate, leading to the adaptation/downregulation of the aggregation stage signaling pathways and the activation of postaggregative gene expression (Abe & Yanagisawa 1983, Firtel 1995, Kimmel & Firtel 1991, Mann & Firtel 1989, Mehdy et al 1983b, Schnitzler et al 1994, Williams 1991). This is followed by the induction of cell-type differentiation. The postaggregative genes induced by high, continuous levels of cAMP include key regulatory genes required for further development such as the transcription factor GBF (G-box–binding factor), the cell surface-signaling molecule LagC, and the small-G protein RasD (Dynes et al 1994, Reymond et al 1986, Schnitzler et al 1994).

The transcription factor GBF was purified by virtue of its binding to a regulatory element called G-box, identified in the promoter region of several postaggregative and cell-type–specific genes (Schnitzler et al 1994). Promoter analysis, including point mutations and deletions, has demonstrated that such G-boxes are essential for expression of these genes (Ceccarelli et al 1992, Datta & Firtel 1988, Fosnaugh & Loomis 1993, Haberstroh & Firtel 1990, Haberstroh et al 1991, Hjorth et al 1989, 1990, Pears & Williams 1988, Powell-Coffman et al 1994). Consistent with these data, analysis of the *gbf*-null cell line provided additional evidence that GBF is a key component of the development switch between aggregation and multicellular differentiation as a general inducer of postaggregative and cell-type–specific gene expression (Schnitzler et al 1994, 1995). The *gbf*-null cells aggregate and express pulse-induced genes normally, but as cells reach the loose mound stage,

the postaggregative signaling machinery is not induced, and subsequent morphogenesis is not initiated. The mounds disaggregate and reaggregate several times in a cyclic fashion before the cells eventually die, being unable to proceed further in the developmental program (Schnitzler et al 1994). GBF function is rapidly activated by a high cAMP concentration via a G-protein–independent pathway involving the same high-affinity cAMP receptor cAR1 that regulates aggregation (Schnitzler et al 1995). This analysis has been confirmed through the analysis of a strain expressing a Gβ subunit temperature-sensitive mutation (Jin et al 1998). It is interesting that, via the same receptor cAR1, cells perceive two types of cAMP signals (oscillatory and continuous) and respond by triggering the activation of distinct signaling cascades. The cAR1 activation of GBF-mediated pathways requires micromolar continuous levels of cAMP, conditions in which the receptor is fully saturated and in an adapted state for aggregation stage pathways; during aggregation, the same receptor detects nanomolar cAMP pulses to activate the aggregation stage pathways (Figure 3). The cAR1 response variability might be caused by a conformational change, dependent on the type of signal perceived, that triggers association with different downstream effectors, including heterotrimeric G proteins.

Concomitant with the activation of GBF by high cAMP is the tyrosine phosphorylation of the signal transducer and activator of transcription (STAT) factor STATa (Araki et al 1998). STATa was identified biochemically, as the TTGA-binding factor that binds in vitro to the *ecmA* activator and *ecmB* repressor elements (Kawata et al 1996, 1997), and genetically in a REMI screen for mutants that are blocked in fruiting-body formation (Mohanty et al 1999). *Dictyostelium* STATa shares the same functional structure as metazoan STATs, with a DNA-binding domain, an SH2 domain, and a C-terminal regulatory tyrosine phosphorylation site. In mammalian cells, STAT proteins are major components of the cytokine and growth factor-induced signaling pathways (Darnell 1997, Hoey & Schindler 1998, Liu et al 1998). STATs are rapidly activated by tyrosine phosphorylation. For cytokine signaling, this activation has been shown to be regulated, by the Janus kinases, which are activated by transphosphorylation induced by ligand-induced oligomerization of cell surface receptors. Phosphorylation of STATs triggers their dimerization by reciprocal interaction of each SH2 domain with the phosphorylated tyrosine near the C terminus of each STAT monomer. STAT dimers are translocated to the nucleus, where they bind to specific target gene promoters.

As with GBF, extracellular cAMP is the inducer of STATa tyrosine phosphorylation and its nuclear translocation (Araki et al 1998). At the mound stage, most cells exhibit nuclear localization of STATa. Whereas STATa protein remains in all cells, its nuclear localization is lost in most cells except in the prestalk A (pstA) domain as cells reach the slug stage. This change in the nuclear localization of STATa occurs during cell-type differentiation and may require the function of STATa for this process. As with the activation of GBF function, cAMP-mediated STATa activation functions through cAR1 and occurs independently of heterotrimeric G proteins. Thus, two major developmental switches that occur at the mound stage—the activation of GBF function and STATa tyrosine phosphorylation—occur via the same receptor that regulates aggregation via distinct pathways. *STATa*-null cells

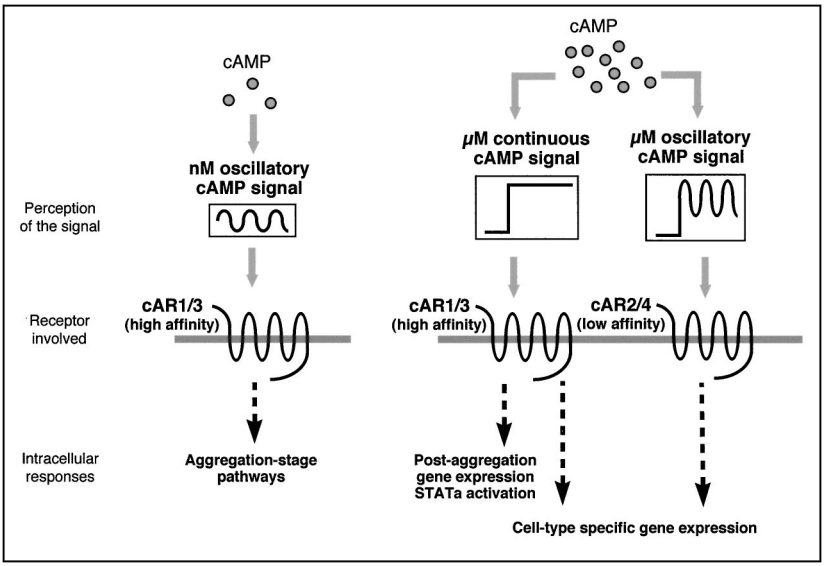

Figure 3 Role of cAMP receptors during *Dictyostelium* multicellular development. Four distinct cAMP receptors are expressed during the different stages of development and differentiation. The high-affinity cARs, cAR1 and cAR3, regulate aggregation by activation of downstream signaling pathways in response to nanomolar pulses of cAMP. These same receptors detect and respond to the high extracellular concentration of cAMP proposed to accumulate as cells form a tight aggregate. Under conditions in which cAMP is in the micromolar range, cAR1 and cAR3 are expected to be in a fully adapted state. These conditions lead to the induction of postaggregative and cell-type–specific genes required for further development and the tyrosine phosphorylation and nuclear localization of the STAT transcription factor STATa. At this stage and later in development, cAMP is released as an oscillatory signal detected by the low-affinity receptors cAR2 and cAR4, which mediate the activation of parallel and convergent pathways, allowing a tight control of morphogenesis and cell-type differentiation (see text for references).

show defects in aggregation; however, the major effect on *Dictyostelium* development is in spatial patterning and expression of *ecmB*, the stalk marker (Mohanty et al 1999). The developmental function of STATa in controlling cell-fate decisions, spatial patterning, and stalk cell differentiation are examined in the last section of the review.

Two Morphogens Are Required to Establish the Prespore and Prestalk Populations: cAMP and DIF The different cell populations that arise at the mound stage are distinguishable in the slug, because they are organized along a well-established anteroposterior axis. The position of each cell type within the multicellular organism has been mapped at different times of development by using *lacZ*

and β-glucuronidase reporter constructs driven by various promoters (Dingermann et al 1989, Early et al 1993, Esch et al 1992, Gomer et al 1986, Haberstroh & Firtel 1990, Jermyn & Williams 1991). The prespore cells, which terminally differentiate into spores, compose 70% of the cells and are localized in the posterior part of the slug (Figure 1). The anterior region consists of prestalk cells that can be divided into several prestalk subcell types, based on their expression of specific marker genes and their fate in the mature fruiting body (Early et al 1993, Jermyn et al 1989, Jermyn & Williams 1991, Williams et al 1993). The pstA cells express high levels of the extracellular matrix protein ecmA and later participate in stalk formation (Jermyn et al 1989). At the slug stage, pstA cells localize to the front half of the prestalk region. The prestalk O (pstO) cell population, which expresses a lower level of *ecmA*, is positioned immediately behind the pstA compartment at the boundary with the prespore compartment and forms part of the upper cup that lies on top of the spore mass in the mature fruiting body. Depending on the cell type, *ecmA* expression is directed by distinct regulatory sequences within the promoter. In pstA cells, sequences proximal to the transcriptional start site direct ecmA expression in pstA cells, whereas pstO cells control *ecmA* expression through the more distal sequences (Early et al 1993). Through the identification of these promoter elements, Early et al have defined cell-type–specific prestalk reporters. The *ecmAO/lacZ* construct, which contains the entire cloned ecmA promoter, has an expression pattern similar to that of the endogenous *ecmA* gene. The *ecmA/lacZ* and *ecmO/lacZ* reporters are expressed in pstA and pstO cells, respectively. A third prestalk cell type, the pstAB cells, localize to a cone-shaped area in the anterior part of the slug surrounded by pstA cells (the area where the stalk formation will initiate during culmination) (Ceccarelli et al 1991, Jermyn et al 1989, Jermyn & Williams 1991, Sternfeld 1992). At the slug stage, some of these cells prematurely enter the stalk differentiation process and express both ecmA and another extracellular matrix protein, ecmB, which is a marker for stalk-cell differentiation (hence the name prestalk AB or pstAB cells) (Sternfeld 1992). A last population of prestalk cells, designated anteriorlike cells (ALCs), is heterogeneous and expresses *ecmA* and/or *ecmB* (Devine & Loomis 1985, Gaskell et al 1992, Sternfeld & David 1981, 1982). The heterogeneity probably depends on a cell's origin. The ALCs have been implicated in the maintenance of cell-type proportioning through a transdifferentiation process (see below) via their conversion into either prespore or prestalk cells, in agreement with the following scheme: prespore $\leftrightarrow$ ALCs $\leftrightarrow$ pstO $\leftrightarrow$ pstA (Abe et al 1994, Sternfeld & David 1982). ALCs are characterized by the expression of proteins involved in signal transduction, such as the MAP kinase ERK1, the protein tyrosine phosphatases PTP1 and PTP3, and the G-protein subunit Gα4 (Gamper et al 1996, Gaskins et al 1994, Hadwiger et al 1994, Howard et al 1994). These cells are found scattered throughout the prespore domain, and they later contribute to the formation of the basal disc, which anchors the stalk to the substratum and the lower cup. ALCs are enriched at the very posterior end of the slug, the rearguard region, and are lost as the slug migrates. Another population of cells that express *ecmB* but not *ecmA* is found in the basal disc and

the lower cup, the pstB cells (Dormann et al 1996, Jermyn et al 1996). The cell-autonomous and -nonautonomous pathways controlling cell-type differentiation are outlined in Figure 4.

Two essential morphogens regulate cell-type differentiation: cAMP and differentiation-inducing factor (DIF) (Williams 1989). External cAMP is needed for the prespore and prestalk differentiation pathways, partly to activate the GBF-mediated pathways and induce expression of genes that do not require GBF, such as Spalten (Spn). In contrast, extracellular cAMP inhibits the terminal differentiation of prestalk cells into stalk cells (Berks & Kay 1988). During multicellular development, cAMP functions in combination with another diffusible signal molecule, DIF-1, to specify cell fate (Berks & Kay 1990, Berks et al 1991). DIF-1 is a dichlorinated hexaphenone whose synthesis is induced at the mound stage by high cAMP concentrations (Brookman et al 1982, Morris et al 1987). DIF-1 was initially identified as a lipophilic factor that caused cells in monolayers to differentiate into vacuolated stalk cells (Kay et al 1983, Town & Stanford 1979, Town et al 1976). DIF-1 is now considered a general inducer of prestalk cell differentiation. Cells that are unable to express DIF-1 in sufficient amounts (strain HM44) arrest development at the mound stage and do not induce the prestalk pathway unless DIF-1 is provided exogenously (Kopachik et al 1983). cAMP acts in synergy with DIF-1 to induce the prestalk gene *ecmA* while it antagonizes the DIF-1–inducing effect on *ecmB* expression (Berks & Kay 1988, 1990, Williams et al 1987). Alternatively, transcription of prespore genes is induced by cAMP and repressed by DIF-1 (Early & Williams 1988). It is surprising that the concentration of DIF is higher in the prespore region than in the prestalk region because of the DIF-induced expression by the prestalk cells of a DIF-dechlorinase, an enzyme responsible for the degradation of the morphogen (Insall et al 1992). This negative-feedback loop regulates DIF breakdown and is proposed to contribute to the establishment of an anteroposterior DIF gradient within the multicellular structure (Kay et al 1993). The exact mode of function of DIF-1 is unknown.

Along with GBF, Other Genes Are Required for the Initial Steps Leading to Cell-Type Differentiation Currently, the mechanisms responsible for the initial cell-type divergence within the mound are not fully elucidated. At the loose-mound stage, only a few scattered cells express the prestalk marker *ecmA* or the prespore marker *SP60* (Datta et al 1986, Haberstroh & Firtel 1990, Williams et al 1989). Those cells seem to be randomly organized within the aggregate, which suggests that the initial cell fate decision is cell autonomous and does not result from positional information. However, as differentiation extends to more cells, both positional information and cell-autonomous mechanisms cooperate to establish proportioning and patterning via cell sorting (Early et al 1995). The position of the cells in the cell cycle at the time of starvation influences this initial cell fate decision (Araki et al 1994, Gomer & Firtel 1987, Maeda 1993, Weijer et al 1984a). Cells arrested early in the cell cycle [S phase and early G2; there is no G1 phase in the *Dictyostelium* cell cycle (Maeda 1986, Weijer et al 1984b)] when

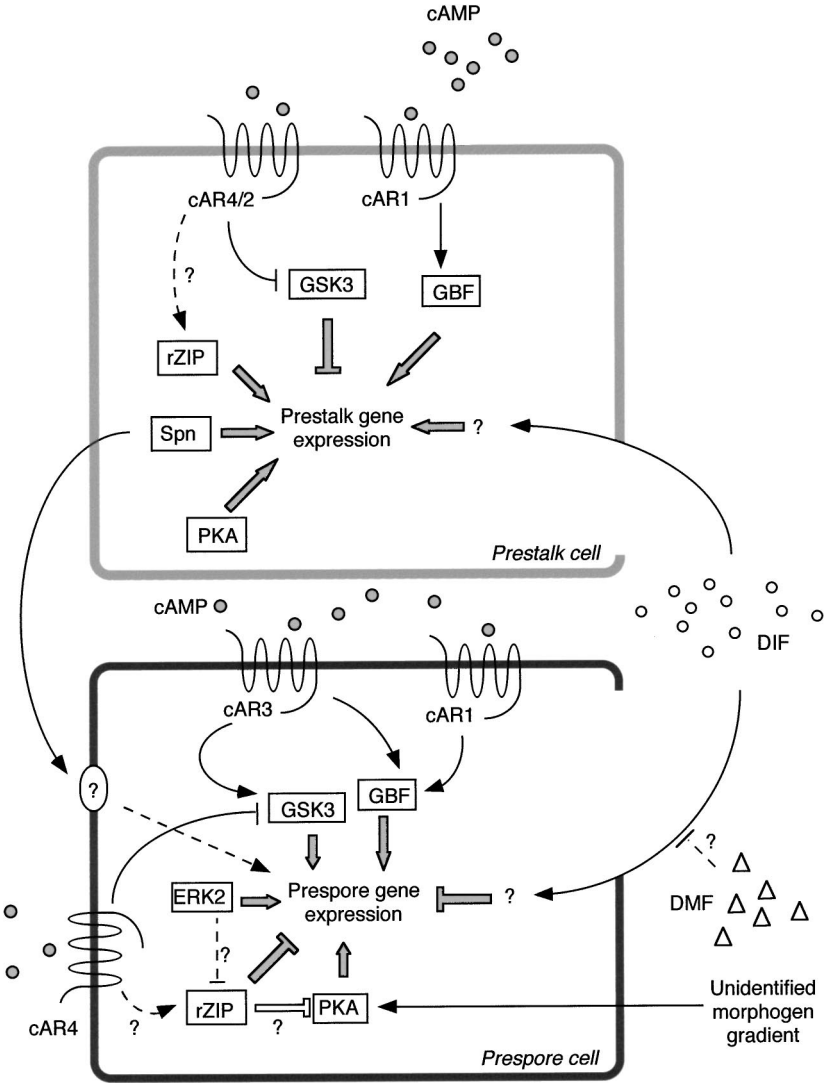

Figure 4 Signaling pathways regulating cell-type differentiation. In addition to the G-box–binding (GBF) transcription factor, a number of gene productions are required for initial cell-type divergence and maintenance of cell fate. These proteins function either via cell-autonomous pathways to control prespore and prestalk differentiation or in a cell-nonautonomous fashion via secreted signaling molecules (chemoattractants and morphogens; see text for details).

nutrient removal happens tend to differentiate into prestalk cells, whereas cells arrested later have a propensity to differentiate into prespore cells. However, several experiments strongly indicate that position in the cell cycle does not definitely predetermine cell fate. Synchronized cells form aggregates that initially have aberrant cell-type proportioning; however, with slug migration, slugs having a normal prespore/prestalk ratio are formed, suggesting that correction mechanisms function later (Wang et al 1988, Weijer et al 1984a). Additionally, slugs deprived of their prestalk-anterior or prespore-posterior compartments by microsurgery are able to regenerate the missing part (via transdifferentiation of the cell types) and form a fairly well-proportioned fruiting body (Raper 1940, Sakai 1973). Such plasticity of differentiation demonstrates the involvement of additional signaling mechanisms. It is, however, possible that cell cycle position at the time of starvation influences a cell's responsiveness to later cell fate determinants (Araki et al 1997, Early et al 1995). Specification of cell fate appears to be the result of the complex interplay of two types of factors: extrinsic signals (morphogens and chemoattractants) that regulate nonautonomous pathways and intrinsic factors that function through cell-autonomous mechanisms.

Analysis of GBF-overexpressing cells provided an understanding of the cascade of events preceding cell-type differentiation and mediating the switch from unspecialized cells to specific cell types. The *gbf*-null cells are unable to express postaggregative genes (Schnitzler et al 1994, 1995). This block can be overcome by overexpression of GBF under the actin 15 promoter, which expresses the transcription factor constitutively from the vegetative state to multicellular development. However, GBF overexpression does not lead to a premature expression of postaggregative genes, an indication that other conditions are required. It is possible to reconstitute the succession of stage-specific gene inductions observed during multicellular development in suspension cultures in which cells are submitted to exogenously controlled extracellular conditions (cAMP pulses/high cAMP concentrations, fast- or slow-shaking conditions to control cell-cell interactions) (Mehdy & Firtel 1985, Mehdy et al 1983a). Such experiments demonstrated that high cAMP concentrations are required in addition to GBF to complement the *gbf*-null phenotype. Cells starved for a few hours can induce postaggregative genes within a few minutes after high cAMP treatment. Cell-type–specific gene expression is restored in these conditions, but only if cells are allowed to establish cell-cell contacts. This induction takes place several hours after cAMP treatment, indicating the requirement of the function of the initial wave of postaggregative genes induced by cAMP (Schnitzler et al 1994). Currently, we know only a limited number of the essential components of the pathway that regulates cell-type differentiation and the specification of cell-type populations. The expression of some of these genes, such as *lagC*, is under direct control of GBF. The *lagC*-null cells exhibit developmental phenotype very similar to that of *gbf*-null cells, and it is very likely that some of the phenotypic defects of the *gbf*-null strain are a consequence of the absence of LagC protein expression (Dynes et al 1994, Sukumaran et al 1998). Analysis of the null mutant and LagC-overexpressing strains revealed that LagC is necessary

for the formation of tight mounds and the expression of cell-type–specific genes. Cell-type–specific genes can be induced when *lagC*-null cells are developed in chimeras with wild-type cells, only if a majority of the cells in the chimera are wild type. LagC is predicted to be a transmembrane protein with a long extracellular domain. This structure, together with the cell-mixing experiments, is consistent with LagC functioning cell nonautonomously as a signaling molecule on the cell surface and not through the release of a diffusible molecule. LagC may mediate the cell-cell interactions required for further multicellular development.

The developmental phenotype of cells carrying a disruption in the gene Spalten is reminiscent of that of *gbf*- and *lagC*-null cells (Aubry & Firtel 1998). Spalten (*Spn*) is expressed throughout development, with a peak of expression at the tight mound stage. This peak of *Spn* expression is cAMP dependent but GBF independent. Cells aggregate normally but fail to proceed further. The mounds disaggregate and split into smaller structures that become tighter in an attempt to reinitiate development. Aggregation and postaggregation gene expression kinetics mirror the phenotype perfectly, being sequentially repressed and reinduced as aggregates oscillate between loose- and tighter-mound stages. The *spn*-null cells are unable to induce cell-type differentiation, as indicated by the absence of *ecmA* and SP60 marker expression. Prestalk cell differentiation is not rescued if *spn*-null cells are developed in chimeras with wild-type cells. Thus, Spalten functions in a cell-autonomous manner to control prestalk cell differentiation. However, some prespore- and spore-specific gene expression can be obtained in a coaggregate with wild-type cells, as well as with the *pslA*-null mutant that is able to differentiate only prestalk cells. *Spn* expression is highest in ALCs at the onset of multicellular development, and then it is mainly expressed in the prestalk O region in the slug. These results support a model in which *Spn* functions within the prestalk cell population to regulate prestalk differentiation. Moreover, these data strongly point to a direct role of prestalk cells, via a cell-nonautonomous pathway, in the induction of prespore cells. Such a model requires the postulation of a not-yet-identified prespore differentiation factor released by the prestalk cells that would be required for prespore cell differentiation. In chimeras with wild-type cells, prespore-specific expression in the *spn*-null cells, although partly restored, is delayed, indicative of a complex regulatory mechanism. *Spn* is a bimodular protein with a PP2C-like phosphatase activity and a C-terminal domain homologous to Gα subunits of heterotrimeric G proteins. Deletion and point mutations within the *Spn* gene have shown that the phosphatase domain is the effector domain of the protein, whereas the Gα-like domain regulates *Spn* function through a GTP/GDP-binding–dependent mechanism and *Spn* subcellular localization. The intracellular function of *Spn* would thus consist of the control of the phosphorylation level of a substrate whose dephosphorylation state is required for development to proceed. Recently, a putative Ser/Thr kinase (homologous to members of the Raf kinase family) has been isolated in a second-site suppressor screen. Disruption of the gene encoding this kinase partially rescues the *spn*-null mutant phenotype. Analysis of this novel protein and how it integrates in the signaling cascade downstream of *Spn* should

further elucidate the role of the *Spn*-mediated pathway in the establishment of the prestalk and prespore populations.

After the first dichotomous decision that governs induction of unspecialized cells into either prestalk-type or prespore cells, a second cell fate choice occurs to specify the distinct prestalk subtypes that differentiate within the prestalk population. The pstA and pstO cell types were initially defined from the spatially restricted expression patterns of subdomains of the *ecmAO* promoter (the cloned promoter of the *ecmA* gene) that were preferentially expressed in the pstA and pstO regions of the slug (Early et al 1993). The results of using these promoters as markers for the earliest stages of prestalk cell differentiation support a model in which the pstA and pstO cells arise independently in spatially localized subdomains of the mound (Abe et al 1994, Early et al 1995). Differential cell movement of the pstA and pstO cells in response to cAMP (pstA cells move more rapidly) results in a sorting pattern with the pstA cells at the apical tip and the pstO cells lying below them (Early et al 1995). However, one cannot know, using these reporters, whether there is an earlier, common cell type that expresses a distinct prestalk cell marker.

Analysis of the *tagB*-null mutant has provided clues about the mechanisms regulating prestalk cell differentiation (Shaulsky et al 1995). The TagB gene and its homolog TagC encode closely related bimodular proteins that may function as a heterodimer. TagB/C contains an extracellular domain with a serine protease activity coupled to a transmembrane domain homologous to the ABC family of ATP-driven transporters. The *tagB*-null cells arrest development at the mound stage before tip formation. In contrast to the mound-arrested phenotypes of *gbf*-, *lagC*-, and *spn*-null mutants, *tagB*-null cells express prespore-specific genes and the prestalk-specific reporter *ecmA/lacZ* to a low level. These *ecmAO*-expressing cells sort to the apical tip and thus behave somewhat like pstA cells. This strain, however, does not express the pstO-specific marker *ecmO/lacZ*. It is not clear whether this is owing to an inability to induce pstO cells or an inability to express this part of the *ecmA* promoter to a sufficiently high level to be detected in the assays, because the stronger *ecmAO/lacZ* reporter is expressed only at a low level. The inability to express *ecmO/lacZ* can be rescued when *tagB*-null cells are codeveloped with wild-type cells. Because TagB expression is restricted to prestalk cells, a simple model is that TagB functions cell autonomously to control prestalk cell differentiation and, as they differentiate, these cells produce a signal to enhance the expression of prestalk cell markers. Thus, TagB would function in an autoregulatory loop required for maximal prestalk cell differentiation. Because TagB is a membrane protease, it is likely that TagB participates in the processing of such a signal, which is then released in the extracellular medium. TagB function is not restricted to prestalk-fate decisions: the *tagB*-null cells are unable to generate spores from prespore cells, but sporulation is restored in *tagB*-null/wild-type chimeras, which suggests a similar role for TagB in peptide processing for TagB-dependent spore encapsulation. This role is detailed in the last section of the review. TagB is thought to play a role in producing factors that function through membrane two-component histidine kinases controlling the activity of the response regulator cAMP-specific

PDE RegA and PKA during terminal differentiation (Anjard et al 1998a,b). It is possible that TagB serves a similar function at the mound stage. PKA activity is required for the full induction of prestalk-specific gene expression (Mann et al 1997, Zhukovskaya et al 1996). The endogenous *ecmA* gene is expressed at only low levels in *pka-C*–null cells in response to high cAMP, which suggests that PKA is required for this process. This is consistent with an inhibition of prestalk cell differentiation by *ecmAO*/PKA-Rm and with overexpression of PKA-C or deletion of RegA bypassing the *tagB*-null phenotypes (Anjard et al 1998b, Loomis 1998, Shaulsky et al 1996). The results support a model in which TagB/C is required to produce an extracellular factor that inhibits RegA PDE activity in prestalk cells, allowing cAMP and thus PKA-C activity to rise and maximally induce prestalk cell differentiation.

A simple mode of regulation can be proposed to explain the pstO/pstA fate decision: In the apex of the mound, randomly distributed cells initially start their differentiation into pstA and pstO cells. As cells differentiate, they secrete factors that control the fate of the neighboring cells, further inducing the prestalk pathway. Later in development, pstA and pstO differentiation is tightly orchestrated by cell-autonomous and -nonautonomous pathways and reciprocal-signaling pathways mediated by extracellular molecules, allowing cell sorting, establishment of cellular patterning, and maintenance of cell-type ratio. A similar model can be envisioned to explain the initial prestalk/prespore dichotomous choice.

cAMP Functions Through cARS to Regulate Cell-Type Differentiation The pathways mediating cAMP effects are likely to be more complex during later development when compared with aggregation, because all four cAMP receptor subtypes can signal to downstream effectors in response to cAMP stimulation (Kim et al 1998b, Verkerke van Wijk et al 1998). Once past mound stage, all four cARs are expressed and can thus mediate distinct responses. The cARs exhibit distinct spatial expression patterns and different affinities for cAMP (Johnson et al 1993, Louis et al 1993, 1994, Saxe et al 1991, 1993, 1996). Postaggregative and cell-type differentiation pathways are activated in response to high, continuous concentrations of cAMP, which suggests distinct roles for each receptor (Fosnaugh & Loomis 1991, Jin et al 1998, Kimmel & Firtel 1991, Mehdy & Firtel 1985, Mehdy et al 1983b, Verkerke van Wijk et al 1998). On the other hand, cAMP waves are still observed originating from the apical tip of the multicellular structure at the tipped mound stage and later (Siegert & Weijer 1991, 1995). During these stages, overexpression of PDE under a prestalk-specific promoter disrupts cAMP signaling and prevents harmonious morphogenesis (Hall et al 1993, Traynor et al 1992). Given the different affinity of the two classes of receptors [low (cAR2 and cAR4) and high (cAR1 and cAR3)], the oscillatory signal should be perceived by the cAR2/4 receptors in the mound, organizing morphogenesis via pathways that parallel those that control aggregation [activation of AC (signal relay) and GC (chemotaxis)] and cell-type differentiation. Conversely, the same oscillatory signal is likely to be perceived as a continuous signal by cAR1/3 receptors, assuming

that the external cAMP concentration is above the threshold that keeps the receptors saturated. Once activated, these receptors could mediate separate intracellular pathways. Analysis of *car*-null and -overexpressing strains has contributed to understanding the functions of each receptor, antagonistic responses of the cAMP receptors, and pathways in which downstream effectors are activated when cARs are stimulated with the morphogen.

Loss of *car2* expression strongly affects morphogenesis and postaggregative development (Saxe et al 1993). Cells aggregate with normal, wild-type kinetics; however, they are unable to generate a tip and develop past the tight-mound formation. Cell-type–specific gene expression is also affected. Loss of *cAR2* expression results in an ~10-fold enhancement in prespore-specific gene expression and a significant reduction in the expression of the prestalk marker *ecmB*, without obvious changes in the level of expression of *ecmA*. Because the *ecmB*-expressing cell population represents only a small percentage of the whole organism and prespore cells represent ~70% of the cells of the organism, these changes cannot be caused by an interconversion of the pstB cells into prespore cells. In addition, cAR2 expression is particularly enriched in pstA cells (>10-fold) localized at the anteriormost region of the slug (Ginsburg et al 1995, Saxe et al 1996). This prestalk-specific expression pattern supports the idea that cAR2 receptors control prespore differentiation through a cell-nonautonomous mechanism. Because Spalten also regulates prespore gene expression through its control of prestalk cell differentiation, it is possible cAR2 and Spn may be components of a common pathway.

The low-affinity receptor cAR4 is preferentially expressed in prestalk cells, although it is present in prespore cells at a lower level (sixfold less abundant) (Ginsburg et al 1995, Louis et al 1994). Disruption of *cAR4*, like *cAR2*, results in an increase in prespore- and a decrease in prestalk-specific gene expression. Morphological consequences of *cAR4* loss of function are less severe than those exhibited by *car2* null strains. However, cellular patterning is strongly disrupted, with prespore cells present in the anterior prestalk compartment (Ginsburg & Kimmel 1997). The sharp boundary normally present between the prespore and prestalk domains of wild-type slugs is lacking in the mutant. These patterning and gene expression defects do not result from reduced levels of cAMP or DIF. The prestalk differentiation defect of *car4*-null cells is not rescued in chimeras with wild-type cells. This suggests that cAR4 functions in a cell-autonomous manner to control prestalk-cell differentiation. Prespore differentiation, on the other hand, is regulated by cAR4 through both cell-autonomous and -nonautonomous mechanisms: (*a*) cAR4 inhibits prespore-specific gene expression cell autonomously, probably by antagonizing the function of another cAR, because cAMP is an inducer of prespore differentiation; (*b*) cAR4 regulates prespore cell differentiation via a postulated extracellular prespore stimulatory factor, DMF (DIF modulation factor), which modulates the sensitivity of prespore cells to the inhibitory effect of DIF. DMF has no effect on prestalk cells (Ginsburg & Kimmel 1997).

Before the slug stage, the expression of the high-affinity receptors cAR3 and cAR1 is observed in all cells. Later in development, cAR1 messenger RNA

(mRNA) becomes enriched in prestalk cells, whereas cAR3 becomes restricted to prespore cells (Gollop & Kimmel 1997, Yu & Saxe 1996). The cAR1 and cAR3 postaggregative expression is dependent on the transcription factor GBF, which is required for transcriptional control and cell-type specificity of expression (Gollop & Kimmel 1997). Despite the absence of morphological defects of *car3*-null mutants (Johnson et al 1993), recent work indicates that cAR3 regulates the activity of GSK3, an essential regulator of cell fate decisions (Harwood et al 1995, Plyte et al 1999). These observations have positioned the serine-threonine kinase GSK3 at the convergence point of the different cAR-mediated pathways leading to cell fate specification. GSK3 plays major roles in morphogenesis of more complex organisms, such as *Xenopus* and *Drosophila* (Diaz-Benjumea & Cohen 1994, Dierick & Bejsovec 1998, He et al 1995, Itoh et al 1998, Ruel et al 1993a,b, Siegfried et al 1992, Simpson et al 1992). In *Drosophila*, GSK3 is encoded by the *Zeste-white3/shaggy* gene and is part of the wingless (Wg) signaling pathway. Loss of *shaggy* function leads to segment and imaginal disc polarity defects as well as misspecification and abnormal patterning of cell types in neurectoderm structures. Similarly, in *Xenopus* embryos, ectopic expression of an active GSK3 in dorsal structures induces their ventralization. In 1995, work from Harwood et al established that GSK3 is a central component of the signaling network leading to cellular patterning in *Dictyostelium* (Harwood et al 1995). GSK3 is required at the mound stage to control prespore and pstB cell differentiation. The *gsk3* disruptants have a dramatically impaired prespore/prestalk ratio with a particularly reduced prespore induction and an expansion of the pstB cell population. This aberrant distribution results in an abnormal morphology of the multicellular organism; the terminal structures exhibit a short stalk with an enlarged basal disc supporting (occasionally) a tiny spore head. As mentioned earlier, extracellular cAMP induces prespore cell formation while it represses pstB cell differentiation. Both effects of cAMP are lost in a mutant that lacks *gsk3*, suggesting that GSK3 could mediate the cAMP regulatory effect on prespore and pstB cell populations. GSK3 kinase activity increases just before mound formation, consistent with the requirement for GSK3 in the initial stages of multicellularity for correct proportioning of prespore and pstB cells. Moreover, the upregulation of GSK3 activity is not observed in *car3*-null mutants, although the level of GSK3 protein remains constant (Plyte et al 1999). These results identified the cAMP receptor cAR3 as the upstream activator of the GSK3 pathway. In that case, how can one explain the absence of a strong developmental phenotype in the *car3*-null strain (Johnson et al 1993)? A closer analysis of the *car3*-null strain, using monolayer differentiation assays and several reporter constructs to monitor cell-type emergence and sorting in differentiated organisms, revealed similarities with the *gsk3*-null phenotype: significant reduction of the prespore/pstB cell ratio at the mound stage, reduced efficiency of prespore cells to differentiate into spores, and absence of cAMP repression on stalk differentiation in monolayer assays (Plyte et al 1999). Nevertheless, *car3* disruption has less severe consequences than the *gsk3*-null mutation. Even though the initial proportioning defect is accompanied by a prolonged arrest

at the mound stage, the *car3*-null mutant ultimately forms wild-type–looking culminants, implying a partial functional replacement of cAR3 by another receptor expressed at that time of development, possibly cAR1. Reestablishment of a wild-type–looking cellular pattern in the later stages of *car3*-null–cell development has been proposed to result from the combination of a massive loss of extranumerous prestalk cells at the rearguard of the slug and an active transdifferentiation of the pstB cells into other cell types.

Recent data indicate a connection between GSK3 and the cAMP receptor cAR4 through a pathway that directly antagonizes the cAR3 activation pathway at the level of GSK3. The *car4*-null cells exhibit a phenotype opposite that of *gsk3*-null cells for *ecmB* and prespore gene expression, and these phenotypes can be rescued by treatment with 5–10 mM LiCl, an inhibitor of GSK3 activity (Ginsburg & Kimmel 1997, Klein & Melton 1996). Consistent with this, treatment of wild-type cells with LiCl results in a phenocopy of the *gsk3* null strain (Sakai 1973, Van Lookeren Campagne et al 1988). These results strongly suggest participation of cAR4 in cell-type specification and positioning via a negative modulation of GSK3 kinase activity. GSK3 would thus be submitted to both positive and negative controls in response to extracellular cAMP binding to distinct cAMP receptors.

The cAR4-mediated inhibition of GSK3 activity that is proposed for *Dictyostelium* to control cell fate determination is reminiscent of observations in metazoans. In a similar regulatory scheme, GSK3 is inhibited in vertebrates and *Drosophila* in response to the Wnt-1/Wg morphogen binding to the seven-transmembrane domain receptor Frizzled, which is involved in the correct establishment of the dorsoventral axis and segment polarity (He et al 1996, Torres et al 1996). So far, no ligand-induced stimulation of GSK3 activity has been described in metazoans. In early embryogenesis of *Xenopus*, the morphogen Wnt-5 has been found to antagonize the dorsalizing action of Wnt-1. If the multireceptor-mediated regulation of GSK3 activity established in *Dictyostelium* is a generally conserved mode of control of body plan determination, one might expect a convergence of the Wnt-5A and Wnt-1 pathways at the level of GSK3.

Regulation of Cell-Type Differentiation by PKA The absolute requirement for PKA in several steps of development is clearly established. Strains overexpressing the dominant negative regulatory subunit PKA-Rm under various cell-type–specific promoters exhibit developmental defects. Expression of Rm under the prestalk *ecmA* promoter prevents *ecmA* and *ecmB* expression as soon as the intracellular level of Rm protein increases (Harwood et al 1992b, Hopper et al 1993a, Zhukovskaya et al 1996). Similarly, expression of Rm under the prespore *pspA* promoter blocks the expression of other prespore-specific genes such as *SP60/CotC* and *SP70/CotB* (Hopper et al 1993b, 1995). *PspA* expression is not affected and thus is not dependent on PKA, which suggests that there are at least two classes of prespore genes (Hopper et al 1993b, 1995, Mann et al 1997, Mann & Firtel 1993). PspA expression, unlike the expression of *SP60/cotC* and *SP70/cotB*, is induced in aggregation streams and thus may be induced earlier in development via a different

regulatory pathway, although both classes of prespore genes require GBF function (Hopper et al 1995, Schnitzler et al 1994). These data provide evidence for a major role of PKA in the regulation of prespore- and prestalk-specific gene expression.

The multidomain adapter protein rZIP is also involved in the regulatory network controlling cell fate specification. The rZIP protein contains a RING-type zinc-binding domain, a leucine zipper, and an SH3-binding motif (Balint-Kurti et al 1997). The RING/leucine zipper region is required for in vitro homodimerization of rZIP. The rZIP protein is ~40% identical in the RING finger domain with the mouse protooncogene c-*Cbl* and its related human protein Cbl-b, proteins that exhibit the C3HC4 zinc finger, a leucine zipper, and SH3-binding motifs (Keane et al 1995, Langdon et al 1989). Cbl family members have been proposed to be negative regulators of receptor tyrosine kinase-mediated signaling pathways (Galisteo et al 1995, Yoon et al 1995). SLI-1, a *Caenorhabditis elegans* homolog of c-Cbl, negatively regulates the EGF tyrosine kinase receptor homolog LET23 during vulval development (Yoon et al 1995). The rZIP protein is expressed in all cell types throughout development, with a low level of expression in vegetative cells that peaks around the mound stage (Balint-Kurti et al 1997). Overexpression of rZIP seriously compromises the anteroposterior prespore/prestalk cellular patterning as a consequence of a high induction of prestalk-specific gene expression, with a precocious stimulation of *ecmB* expression and repression of prespore-specific genes. Conversely, disruption of rZIP leads to the opposite phenotype, which promotes prespore-specific gene expression and inhibits prestalk-specific gene expression. The *rzpA*-null cells have defects in the transdifferentiation of prespore cells into prestalk cells. Analysis of chimeric organisms carrying various cell-type–specific promoter-reporter constructs indicated a dual function for rZIP within the prespore domain: (*a*) rZIP inhibits, in a cell-nonautonomous manner, the accumulation of an anteroposterior graded signal needed for prespore differentiation, and (*b*) rZIP functions with PKA to promote prespore gene transcription (Balint-Kurti et al 1998). Cell transplantation experiments and dissection of the promoter region of the prespore-specific gene *SP60/cotC* by deletions and point mutations revealed a spatial control of prespore-specific gene expression, which suggests the possibility that morphogen gradients might regulate prespore fate within the prespore domain (Buhl et al 1993, Haberstroh & Firtel 1990, Haberstroh et al 1991). Both wild-type and *rzpA*-null cells exhibit a homogeneous expression of the *SP60/cotC* gene in the posterior region of the slug (used to define the prespore compartment) (Balint-Kurti et al 1997). However, in chimeric organisms of 90% wild-type cells and 10% null cells carrying the reporter constructs SP60:*lacZ*, β-galactosidase staining is restricted to the most anterior part of the prespore domain despite a homogeneous distribution of *rzpA*-null cells throughout the prespore compartment (Balint-Kurti et al 1998). These results established the existence of a gradient of a nonautonomous prespore-activating signal responsible for prespore cell differentiation. Only prespore-specific genes requiring PKA for their transcriptional activation are subject to this regulation, implying that PKA is a component of the intracellular pathway activated in response to the proposed extracellular gradient.

Within its proline-rich stretch, rZIP contains an MAP kinase consensus phosphorylation site (Balint-Kurti et al 1997). The MAP kinase ERK2 plays a major role during aggregation as part of the signaling cascade that activates AC and is crucial during prespore differentiation for sustained expression of prespore-specific genes (Gaskins et al 1996, Maeda et al 1996), although it is not known whether ERK2 functions by negatively regulating RegA or through another pathway. In vitro, rZIP interacts with the SH3 domain of the mammalian adapter Nck, which suggests that this multidomain protein may function in a multiprotein complex including a Nck-like adapter (Balint-Kurti et al 1997). A proposed model is that ERK2 could modulate the interactions within the complex by phosphorylating the SH3-binding domain of rZIP.

In *Dictyostelium*, *rzpA*- and *car4*-null mutants exhibit phenotypic similarities, which suggests that rZIP may lie in a pathway originating from the receptor cAR4 to control cell-type specification. During aggregation, ERK2 is activated in response to cAMP via a cAR1-dependent G-protein–independent pathway (Aubry et al 1997, Knetsch et al 1996, Maeda et al 1996). Through the analysis of an ERK2 temperature-sensitive mutant, it has been established that ERK2 is required for prespore cell differentiation (Gaskins et al 1996). However, it is not known whether another cAR substitutes for cAR1 in the later developmental stages to regulate ERK2 activity. As described above, cAR4 participates in the regulation of GSK3 activity. Further analysis of the rZIP pathway and dissection of its possible interactions with the serine-threonine kinases GSK3 and ERK2 and the cAMP receptors cAR4/cAR2 and cAR3 should delineate the general network in which these components integrate to regulate cell fate decisions.

MAINTENANCE OF CELL-TYPE PROPORTIONING

A simple spatial pattern is established at the mound stage as prestalk cells sort to the apex of the mound and organize along an anteroposterior axis as the tip elongates to form a slug (Firtel 1995, Jermyn & Williams 1991, Raper 1940, Williams 1995, Williams et al 1989). Although significant variability is observed in the size of the organism (from <100 to 10^5 cells), cell-type patterning and proportions remain surprisingly constant (Loomis & Cann 1982). Understanding how cellular patterns are generated and maintained is a major challenge in developmental biology and embryology. In *Dictyostelium*, experimental results suggest the existence of cell-cell communication networks between the cell types that probably function by induction and inhibition processes controlling cell-type proportioning within the differentiating organism. Removal of the posterior-prespore or the anterior-prestalk region of a migrating slug is followed by the regeneration of the missing part and reestablishment of the original pattern within $\sim$8 h in the absence of cell division (Raper 1940, Sakai 1973). This indicates that some of the remaining cell type must "transdifferentiate" into the cell type that is lacking to produce a smaller, properly proportioned organism. Additional supporting data derive from

experiments showing that in situ poisoning of prespore cells by expression of toxic proteins such as ricin from prespore-specific promoters leads to the killing of the multicellular organism (Shaulsky & Loomis 1993). As the prespore cells differentiate and die because of expression of ricin, they are replaced by some prestalk cells that differentiate into prespore cells and die. In contrast to the data obtained from the slug microdissection experiment, prespore cells fail to convert into and replace prestalk cells poisoned by the expression of ricin under the *ecmAO* promoter. In normal situations, some transdifferentiation of cell types into others has been observed (Abe et al 1994, Detterbeck et al 1994, Kakutani & Takeuchi 1986). As the slug migrates, prestalk cells belonging to the pstAB subtype are lost from the rearguard region (Sternfeld 1992). For the 4:1 prespore-to-prestalk ratio to be maintained, considering that no cell division occurs during *Dictyostelium* development to maintain the prestalk cell number (Shaulsky & Loomis 1995), transdifferentiation of some prespore cells into prestalk cells must occur. Such a process is shared by *Dictyostelium* and some metazoan embryos. Cell-type–specific surface labeling of developing cells has allowed a more in-depth analysis of the transdifferentiation process that occurs within the migrating slug, by tracking the morphogenetic movement of marked cells and monitoring the expression of the cell-type–specific reporter constructs (Abe et al 1994). The ALCs have been implicated in the maintenance of the prestalk-to-prespore ratio as intermediary cells able to convert to either cell type, in agreement with the following scheme: prespore ↔ ALC ↔ pstO ↔ pstA (Blaschke et al 1986, Early et al 1995, Kakutani & Takeuchi 1986). ALCs are recruited to replace the prestalk cells in a slug deprived of its anterior region (Sternfeld & David 1982). The ALCs may be involved in the signaling pathway that controls interconversion of the cell types. In the ricin poisoning experiment, it is possible that the inability of prespore cells to convert into prestalk cells results from the killing of the ALCs present in the prespore region, leading to the loss of signaling by the ALCs.

In metazoans, homeobox-containing transcription factors regulate anteroposterior body plan establishment. In *Dictyostelium*, two homeobox-containing genes have been cloned that are preferentially expressed at mound stage: *DdHbx1* (named Wariai) and *DdHbx2* (Han & Firtel 1998). The *wariai*-null cells show a twofold increase of the pstO compartment, with a concomitant reduction of the prespore domain. The pstA and pstAB domains are not affected. The second homeobox-containing gene *DdHbx2* does not lead to an overt developmental phenotype when disrupted, but it seems to potentiate Wariai function. Whereas homeobox-containing genes classically function cell autonomously, Wariai is expressed only in pstA cells, which suggests that it functions in a cell-nonautonomous manner to regulate the posterior boundary of the pstO compartment. The network into which Wariai integrates is not known. Wariai is expected to encode a transcription factor and thus may regulate the expression of genes required for cell-type proportioning. The differentiation of pstA and pstO requires the morphogen DIF, toward which they exhibit a differential sensitivity (Early et al 1995). On the other hand, prespore-specific gene expression is repressed by DIF (Berks & Kay 1990). DIF

is present in the slug along an anteroposterior gradient caused by the expression of the DIF-dechlorinase enzyme (responsible for DIF breakdown) at the tip of the slug (Insall et al 1992, Kay et al 1993). It is possible that Wariai affects the sensitivity of the cells to the morphogen DIF via an unknown extracellular signaling molecule and consequently modulates the prespore/pstO cell ratio. Wariai could regulate prespore and pstO fate, and thus the position of the boundary between those two compartments, by altering the DIF gradient via the control of the level of dechlorinase expression. Analysis of the *gsk3-*, *car4-*, and *rzip*-null strains indicates the participation of additional extracellular signaling molecules in cell fate decisions, which need to be integrated in a more general network before a clear explanation of Wariai function can be provided.

A similar defect in cell-type patterning is obtained by disruption of the putative MAP kinase kinase kinase MEKKα (Chung et al 1998). Deletion of MEKKα and overexpression of a putative dominant negative mutant MEKKα^{K199A} (mutation of the Lys in the ATP-binding site to Ala) result in an expansion of the pstO compartment and a decrease of the prespore domain, with an apparent disruption of the boundary between both domains. Analysis of mosaic organisms of wild-type cells mixed with various mutants of MEKKα (null strains and overexpressors) indicates that the level of expression of MEKKα influences the propensity of cells to differentiate toward the prespore or prestalk pathway and may thus be partly responsible for the maintenance of cell-type ratios, at least by maintaining the prespore state of differentiation. MEKKα contains WD40 repeats, which seem to be responsible for the targeting of the protein to the cortical region of the cell, and an F box, which controls (together with the WD40 repeats) MEKKα degradation via a ubiquitin-mediated proteolysis pathway. Ubiquitination involves a series of reactions catalyzed by specific enzymes, including Ub-activating enzymes (E1), which activate ubiquitin (Ub-76aa) in an ATP-dependent step; Ub-conjugating enzymes (UBCs or E2); and Ub-protein ligases (E3), which perform the covalent binding of Ub onto the protein targeted for degradation (Hershko & Ciechanover 1998, Kalderon 1996, Weissman 1997). A well-described function of polyubiquitination is to direct the modified proteins to the 26S proteasome degradation complex. Ubiquitination is a reversible process. Deubiquitination is catalyzed by specific ubiquitin proteases (UBPs) that cleave the polyubiquitin chain just before protein degradation in the proteasome but that also can remove the ubiquitin molecules from the modified protein and thus rescue proteins from proteolysis. In mammalian cells, ubiquitin-dependent degradation plays key roles in intracellular processes such as signal transduction, gene transcription, and cell cycle progression (Maniatis 1999). The rapid turnover of the tumor suppressor p53 is mediated by ubiquitination. After appropriate stimulation, Iκ-B, a negative regulator of the Rel-family transactivation factor NFκB that sequesters the transcription factor in the cytoplasm, is phosphorylated and subjected to ubiquitination and proteosomal degradation. Limited proteolysis by the proteasome allows the processing of the ubiquitinated p105 precursor form of the NFκB complex component p50 and formation of the active NFκB dimer p50-p65. Cell-cycle progression is

tightly regulated by the sequential activation and inactivation/ubiquitin-mediated degradation of specific cyclin-dependent kinases. Several genes belonging to the ubiquitination cascade have been cloned in *Dictyostelium* : UBCB, UBPA, UBPB, and NosA, a homolog of the yeast protein UFD2 involved in the ubiquitin fusion degradation pathway (Chung et al 1998, Clark et al 1997, Pukatzki et al 1998). Null mutations of these genes result in developmental defects, providing evidence that ubiquitination and subsequent proteasome-dependent degradation are important regulatory process in the control of *Dictyostelium* development. Two of these, UBCB and UBPB, interact with the MEKKα F-box/WD40 repeats in a yeast two-hybrid system and regulate the MEKKα stability in a developmental-stage– and cell-type–specific fashion in vivo (Chung et al 1998). UBPB leads to the F-box/WD40 repeat-dependent degradation of MEKKα in prespore but not prestalk cells, whereas UBPB stabilizes MEKKα. Moreover, *ubpB*-null cells have phenotypes similar to those of *mekkα*-null cells, consistent with UBPB regulating the stability of MEKKα. Western blot analysis of *ubcB*-null cells by using an anti-ubiquitin antibody showed that the ubiquitination pattern of several proteins is affected by the *ubcB* deletion. This result, together with the fact that *ubcB*-null cells arrest before slug formation, indicates that, in addition to MEKKα, other proteins regulated by ubiquitination/deubiquitination pathways are required for coordinated multicellular development. So far, none of the members of the MAP kinase cascade downstream of MEKKα have been identified. However, it is possible that this MAP kinase cascade interferes with the pathways controlled by rZIP and/or cAR4, because strains lacking either gene display a size variation opposite to that of the prespore and prestalk domains (increase of the prespore domain) when compared with *mekkα*-null cells and a disturbed boundary between these two compartments.

SIGNALING PATHWAYS CONTROLLING TERMINAL DIFFERENTIATION

In the wild, environmental factors such as low humidity, overhead light, and reduction of the local NH_4^+ concentration result in an arrest of slug migration and in initiation of culmination, the terminal differentiation of spores and stalk cells, and formation of the mature fruiting body (Newell et al 1969, Raper 1940, Schindler & Sussman 1977, Slifkin & Bonner 1952). The morphogenesis of the mature fruiting body requires a spatial reorganization of the cell populations within the slug. As slug migration arrests, the posterior of the slug moves under the tip, forming a structure that has been referred to as a Mexican hat because of its shape. The rearguard cells, formerly in the posterior of the slug, form the base of the structure, and the prestalk cells previously found at the anterior of the slug form the tip. The prespore cells form the central mass of the structure, which includes the "brim" of the Mexican hat. Fruiting-body formation is initiated when the apically localized prestalk cells invaginate into the prespore mass, differentiate into stalk

cells, and form the stalk tube. The first cells to enter are the pstAB cells, which express prestalk markers. As the pstA cells enter, they induce *ecmB* and commit to stalk cell differentiation. A stalk tube progressively elongates through the prespore mass, raising it off the substratum. Simultaneously, the ALC population migrates to form part of the basal disc and the upper and lower cups (Early et al 1993, Sternfeld & David 1982). As the stalk is formed, prespore cells enter the terminal differentiation step and generate spores.

Several types of experiments indicate that terminal differentiation of stalk and spore cells is triggered by PKA (Harwood et al 1992a, Kay 1989, Loomis 1998, Mann & Firtel 1993, Mann et al 1994, Reymond et al 1995). Overexpression of PKA-C, deletion of PKA-R, deletion of RegA (the intracellular cAMP-specific PDE), or treatment with the membrane-permeable cAMP analog 8-Br-cAMP leads to precocious maturation of both cell types. Conversely, overexpression of the dominant-negative PKA-Rm under the control of cell-type–specific promoters prevents maturation of prestalk and prespore cells. Activation of PKA via a rise in intracellular cAMP is both necessary and sufficient to induce stalk and spore differentiation. Because PKA activity is required for cell-type differentiation, the terminal differentiation of prestalk and prespore cells must be held at bay via a mechanism that regulates the level of PKA activity during multicellular differentiation. Cells are capable, for example, of differentiating into spore cells as soon as prespore cell differentiation is established. Cells carrying an expression cassette in which PKA-C is downstream from the *SP60* prespore promoter enter into spore cell differentiation in <30 min (as determined by expression of the spore marker *SpiA*) after the first detection of endogenous *SP60* prespore-specific gene expression, whereas in wild-type strains, this does not initiate until 9–10 h later (Mann et al 1994). Thus, there must be a signaling pathway that initiates the final step in the development of *Dictyostelium*. This regulation appears to be under the control of a two-component phosphorelay system that includes the cAMP-specific PDE RegA. A diagram depicting the signaling pathways controlling terminal differentiation is presented in Figure 5.

The Two-Component System DhkC/RdeA/RegA Controls Dictyostelium Terminal Differentiation

The prototypical two-component system (H1-D1) is composed of a sensor histidine kinase (H1) and a response regulator (D1) (Alex & Simon 1994, Parkinson & Kofoid 1992). Changes in the extracellular environment are detected by the input domain of the sensor kinase, leading to its auto/transphosphorylation on a histidine residue. The phosphate is then transferred from this donor protein to an aspartate residue on the receiver domain of a response regulator. The phosphorylation state of the response regulator modulates the activity of its output domain, producing a response to the extracellular stimuli. In bacteria, such signaling mechanisms are widely used to regulate cellular processes such as cell division, chemotaxis, and sporulation. In *Saccharomyces cerevisiae*, osmoregulation

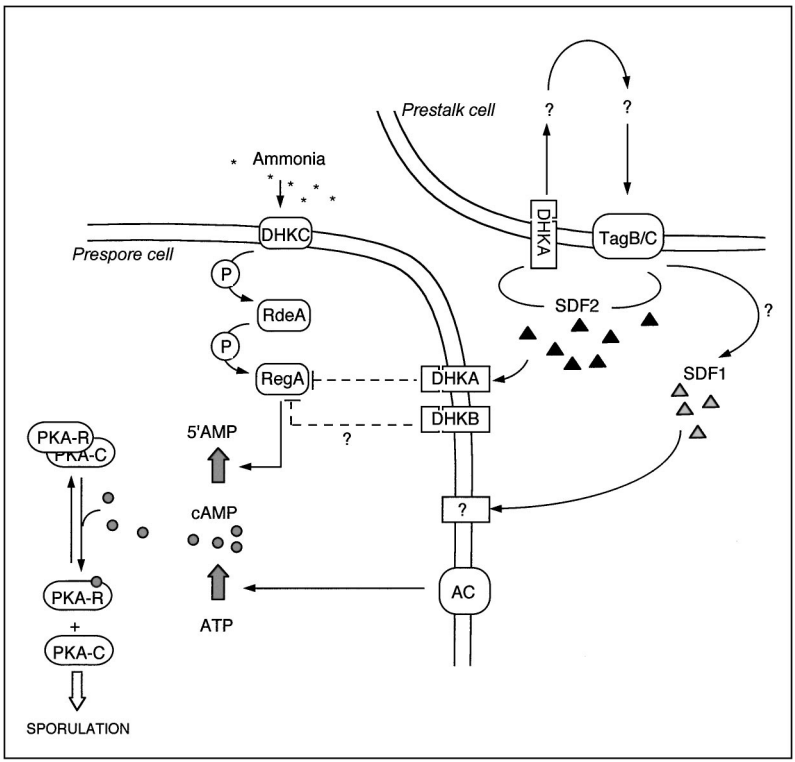

Figure 5 Signaling pathways controlling sporulation. During culmination, protein kinase (PKA) is essential to trigger spore encapsulation. PKA activity is dependent on the level of intracellular cAMP (which controls the dissociation of the PKA-C and PKA-R subunits), which is the result of the balance between synthesis by the adenylyl cyclase (AC) and degradation by the hybrid protein phosphodiesterase/response regulator RegA. The pathway leading to the stimulation of RegA activity is proposed to be regulated by extracellular ammonia accumulation and involves a classical two-component signaling phosphorelay, including the hybrid histidine kinase DHKC and the phosphodonor RdeA. Gene disruption of any of these components results in the interruption of the cascade initiated by DHKC and induces rapid sporulation. In addition to ammonia, two peptides, SDF1 and SDF2 released by the prestalk cells, have been proposed to activate intracellular pathways, the final output of which seems to be a modulation of PKA activity. SDF2 is probably processed by the protease/ABC transporter TagB/C and is probably a ligand for the histidine kinase DHKA. The downstream components of DHKA are so far unknown. DHKA shares partially redundant functions with a third histidine kinase, DHKB, a potential receptor for discadenine, an inhibitor of germination (see text for references).

is governed by a well-characterized two-component phosphorelay system of a higher degree of complexity, because it requires the transfer of the phosphoryl group onto four successive acceptors (H1-D1-H2-D2) (Appleby et al 1996, Maeda et al 1994, Posas & Saito 1997, Posas et al 1996, Wurgler-Murphy & Saito 1997). Sln1p contains an extracellular sensor domain, a catalytic histidine kinase domain (H1), and an attached aspartate relay domain (D1). Under normal osmolarity, Sln1p constitutively initiates a phosphorelay from its H1 and D1 modules to the H2 module, Ypd1, a small cytosolic protein sharing similarities with the histidine phosphorylation domain of some sensor kinases. From Ypd1, the phosphoryl group is passed to Ssk1, the final D2 response regulator, leading to the inhibition of the HOG1 MAP kinase osmoregulatory pathway. Hyperosmolarity interrupts the phosphorelay, allowing activation of the HOG1 pathway and adaptation to osmotic stress. *Dictyostelium* RegA is a composite protein with cAMP PDE activity and an N-terminal region homologous to response regulators of two-component signal transduction systems (Shaulsky et al 1998, Thomason et al 1998). Although RegA is the only identified response regulator in *Dictyostelium*, it is expected that there are others, because multiple two-component-system His kinases have been cloned or potentially identified in the expression sequence tag (EST) database. The analysis of null strains of several of these response regulators indicates distinct phenotypes, which suggests that the different His kinases control different downstream response regulators.

RegA was identified by three independent approaches: (*a*) a second-site suppressor screen, because a mutation can restore the *tagB*-null mutant sporulation defect (Shaulsky et al 1996); (*b*) a screen designed to identify mutations that allow spore and stalk cell maturation in vitro (Thomason et al 1998); and (*c*) a REMI screen for mutations that result in abnormal morphogenesis (L Aubry & RA Firtel, unpublished observations). The *regA*-null phenotype, short stalk, and premature spore encapsulation resemble those aspects of the RdeA and RdeC classes of rapidly developing mutants obtained by chemical mutagenesis (Abe et al 1983, Kessin 1977). A mutation that inactivates the function of the PKA regulatory subunit is *rdeC*, which results in constitutively active PKA (Simon et al 1992). Moreover, overexpression of PKA-Rm, in *regA*-null cells under the prespore-specific *pspA* promoter, causes reduced sporulation efficiency, supporting the model that PKA functions downstream of RegA (Shaulsky et al 1998, Thomason et al 1998). As a cAMP-specific PDE, RegA is likely to directly modulate the level of activity of PKA by controlling the rate of degradation of intracellular cAMP. This leads to a model in which a two-component His kinase pathway controls culmination by regulating intracellular cAMP levels (Thomason et al 1998). RegA PDE enzymatic activity is stimulated by phosphorylation of the expected aspartate phosphoacceptor site. Because RegA activity must decrease, resulting in a rise in intracellular cAMP, for PKA to be activated, signals that initiate culmination must lead to an inhibition in RegA activity through a cessation of the phosphorelay pathway. This is consistent with the *regA*-null phenotype, which results in precocious culmination.

Isolation of mutants belonging to the RdeA complementation group by REMI mutagenesis resulted in the cloning of RdeA and its identification as part of the

multistep phosphorelay system upstream of RegA (Chang et al 1998). RdeA does not show strong similarities to any known proteins except for the presence of a histidine residue (His65) in a short domain related to the yeast phosphoshuttle protein Ypd1. The ability of wild-type yeast Ypd1 and the inability of RdeA His65/Glu to complement the *rdeA* defect in *Dictyostelium* provide strong genetic evidence for a role of RdeA as an H2 module in a two-component system in which RegA is the final response regulator (Chang et al 1998). A direct transfer of phosphate from RdeA to RegA will have to be demonstrated biochemically before unequivocally positioning RdeA as the upstream phosphate donor of RegA. However, the high level of intracellular cAMP detected in vegetative *rdeA*-null cells and the similarity between the *rdeA*- and *regA*-null phenotypes are consistent with a role for RdeA as a phosphoshuttle protein belonging to the RegA-phosphorelay pathway (Abe et al 1983, Coukell & Chan 1980, Shaulsky et al 1998, Thomason et al 1998).

Five members of the *Dictyostelium* two-component histidine kinase family have been cloned and analyzed: DOKA, DHKA, DHKB, DHKC, and DHKD; their characteristics are summarized in Table 1 (Schuster et al 1996, Singleton

TABLE 1 Characteristics of five members of the *Dictyostelium* two-component histidine kinase family

Name	Null phenotype	Function	References
Histidine kinase (H1-D1)			
DokA	Osmosensitivity, few spores	Osmoregulation	Schuster et al (1996)
DhkA	Long thin stalks, reduced number of spores	Receptor for SDF2	N Wang et al (1996, 1999)
DhkB	Premature germination	Possible receptor for discadenine	Zinda & Singleton (1998)
DhkC	Rapid development, no slug migration stage, partial ammonia insensitivity	Ammonia sensor	Singleton et al (1998)
DhkD	Unknown		CK Singleton (personal communication)
Phosphorelay (H2)			
RdeA	High internal cAMP concentration, rapid development	RegA phosphodonor	Chang et al (1998)
Response regulator (D2)			
RegA (phospho-diesterase)	Rapid development	cAMP degradation	Shaulsky et al(1998) Thomasson et al (1998)

et al 1998, N Wang et al 1996, Zinda & Singleton 1998). Sequence analyses suggest that these proteins belong to the hybrid kinase family, because they contain a potential or proven sensor kinase module and an attached receiver module, as identified by their conserved histidine and aspartate phosphorylation boxes. The sensor domain sequences vary from kinase to kinase, as expected for proteins that might bind distinct ligands. The histidine kinase DOKA is involved in osmoregulation; disruption of *dokA* causes a reduced viability of the cells after high osmotic stress (Schuster et al 1996). In addition, the sorus of *dokA*-null strains contains fewer spores, which may be explained by an inability to respond properly to expected changes in osmolarity that are likely to occur during spore formation. Lack of DHKB expression is visibly manifest only during spore germination (Zinda & Singleton 1998). Shortly after their encapsulation, spores swell and germinate within the sorus, an indication that functional DHKB is required to maintain spore dormancy.

Although DhkA deletion does not cause any clear developmental defects before terminal differentiation, it is expressed earlier in development (N Wang et al 1996). The *dhkA*-null strains produce aberrant fruiting bodies with a long fragile stalk supporting a small spore head. In addition to stalk morphogenesis defects, sporulation is severely reduced. This effect on sporulation is cell autonomous and can be rescued by overexpression of PKA-C or inactivation of RegA or PKA-R (Anjard et al 1998b, N Wang et al 1999). Similarly, treatment of *dhkA*-null strains with 8-Br-cAMP can bypass the DHKA requirement for spore formation, consistent with DHKA functioning upstream of PKA. DHKA was initially proposed as the upstream component of the RdeA-RegA phosphorelay pathway (Shaulsky et al 1998). Such a scheme implied an inhibitory effect of phosphotransfer from DHKA onto RegA on RegA PDE activity. However, because phosphorylation of RegA activates its PDE activity (Thomason et al 1998), it is expected that DHKA is not the initial phosphate donor in the RdeA-RegA cascade but more likely is an antagonist of RegA function regulated by a different ligand.

The most likely candidate for the RegA-RdeA upstream sensor histidine kinase is DHKC (Singleton et al 1998). The *dhkC*-null cells exhibit accelerated morphogenesis, as do *regA*- and *PKC-R*–null strains. Overexpression of a DHKC protein lacking its sensor domain (CHK, which may constitutively transfer phosphates) produces a slowly developing "slugger" phenotype in which the slugs migrate for extended periods and the cells are impaired in the initiation of culmination. This phenotype can be rescued by treatment with 8-Br-cAMP. However, no gain-of-function phenotype was observed when CHK was overexpressed in *regA*-null cells, consistent with RegA being downstream of DHKC signaling. Ammonia, which is produced as a metabolic byproduct of protein degradation during development (Gregg et al 1954), inhibits culmination and prolongs the slug stage of wild-type developing cells for abnormally extended periods, producing a slugger phenotype (Gee et al 1994, Schindler & Sussman 1977). The *dhkC*-null aggregates culminate rapidly, bypassing the slug stage, which cannot be restored by addition of exogenous ammonia (Singleton et al 1998). Many years ago, Sussman

and coworkers demonstrated that a high ammonia concentration causes a drop in the intracellular cAMP concentration and presumably a subsequent inactivation of PKA (Schindler & Sussman 1979). However, the pathways linking ammonia and PKA activity were not elucidated. The results obtained from analysis of the *dhkC*-null and CHK mutants suggest that DHKC could be the initial mediator of the ammonia effect. The N-terminal domain of DHKC resembles a histidine kinase sensor domain, and it is tempting to suggest that DHKC is an ammonia sensor. Ammonia would trigger phosphorelay from the DHKC sensor kinase to RdeA and then to RegA, leading to a reduction in intracellular cAMP and modulation of the PKA activity level.

Spore Maturation Is Controlled by Two Peptides, Spore Differentiation Factor 1 and 2 (SDF1 and SDF2) The spore-specific marker gene *SpiA*, which encodes an inner spore coat protein, is induced in a spatial gradient when the sorus is about halfway up the stalk (Richardson et al 1994). This induction requires spore maturation-inducing factors produced by the differentiating prestalk cells. The initial analysis of *tagB*- and *tagC*-null mutants supported this model (Shaulsky et al 1995). Strains carrying a null mutant in either gene are unable to proceed past the mound stage and cannot form spores and stalk cells, although they can express prespore-specific genes. However, the presence of chimeric organisms containing *tagB*-null cells and wild-type cells suggests that they can induce spore maturation. Because TagB is expressed exclusively in prestalk cells, the results suggest that wild-type prestalk cells induce spore maturation via a secreted signal that could be processed by the protease domains encoded by TagB/TagC. The sporulation defect of the *tagB*-null strain can be rescued by overexpression of a truncated (and thus presumably constitutively active) form of DHKA, which lacks the sensor domain (N Wang et al 1996). This suggests that such factors produced by TagB/C are ligands for the sensor histidine kinase.

Two secreted peptides, SDF1 and SDF2, have been identified as inducers of spore cell terminal differentiation in in vitro low-density monolayer cell culture preparations of sporogenous strains (strains that have a propensity to form spore cells in culture) (Anjard et al 1997, 1998a,b). In addition, comparable in vitro studies indicate that SDF1 and SDF2 regulate stalk cell terminal differentiation. In such assays, SDF1 promotes stalk cell differentiation, whereas SDF2 inhibits stalk cell formation (Anjard et al 1998a). SDF1 is a PKA-phosphorylated/activated peptide released by prestalk cells in a single burst at the onset of culmination. SDF2, on the other hand, accumulates during the early steps of culmination but is released only during late culmination. Response to either peptide is dependent on PKA activity. The SDF1-induced response, however, is slower than the SDF2-induced response and can be inhibited by the protein synthesis inhibitor cycloheximide, suggesting a requirement for de novo protein synthesis for this pathway (Anjard et al 1998a). The intracellular pathway (receptor and downstream effectors) stimulated by SDF1 remains to be unraveled. A proposed function for SDF1 is that

it prepares the cells for culmination, inducing the expression of a battery of genes required for the SDF2-induced terminal differentiation step. One of these genes might be PKA-C, which is expressed from at least three promoters, including one that is induced during culmination.

SDF2 triggers spore encapsulation in responsive cells as soon as they are exposed to the signal. The response of prespore cells to SDF2 is mediated by a DHKA-dependent signaling pathway that can be bypassed by treatment with 8-Br-cAMP (Anjard et al 1998a). Modification of the extracellular domain of DHKA by insertion of a *myc* tag not only prevents the mutated protein from complementing the *dhkA*-null phenotype, but also reduces the sensitivity of the prespore cells to the SDF2 signaling molecule, consistent with SDF2 being the direct ligand of DHKA (N Wang et al 1999). Interestingly, sporulation can be induced in *dhkA*-null cells overexpressing the myc-modified DHKA construct simply by addition of myc antibodies. This result is reminiscent of the activation of lymphocytes by specific antibodies against membrane receptors such as CD40. The ultimate effect of SDF2 on prespore cells would be to activate PKA, presumably by modulation of the intracellular cAMP level. It is possible that DHKA activates a pathway converging at the level of RegA, resulting in the inhibition of the PDE activity of RegA and the subsequent increase in cAMP levels. Mechanisms resulting in RegA downregulation have not been studied. It is possible that RegA catalyzes its own dephosphorylation. However, RegA could be negatively regulated by other pathways. In *Bacillus subtilis*, sporulation is governed by a complex phosphorelay two-component system that integrates extracellular signals via independent histidine kinases. The phosphoryl group is initially transferred from kinases A, B, or C to the response regulator SpoOF, then relayed to the phosphotransferase SpoOB and finally to SpoOA, a response regulator/transcription factor hybrid protein that activates several gene promoters. SpoOF and SpoOA can be deactivated by RAP A/RAP B and SpoOE phosphatases, respectively (Perego & Hoch 1996). Even though no evidence of the existence of such aspartate phosphatases has been found in *Dictyostelium*, it is possible that DHKA-induced phosphorelay leads to the activation of RegA inhibitory phosphatases and modulates the output activity of the response regulator. Recent data indicate that the histidine kinases DHKB and DHKA fulfill partially redundant functions in the sporulation process (N Wang et al 1999). DHKB is expressed in vegetative cells and throughout development, consistent with a role for this protein earlier in development than the germination step (Zinda & Singleton 1998). The *dhkA*-null strain maintains a residual sporulation level that can be slightly increased by overexpression of DHKA carrying point mutations in the conserved histidine (in the catalytic domain) or aspartate (in the response regulatory domain) moieties but fully restored by coexpression of both constructs (Wang et al 1999). The partial complementation, however, is completely compromised by disruption of *dhkB*, which suggests the possibility of cross-talk between the two histidine kinases and a similar output resulting from stimulation of DHKB- and the DHKA-dependent pathways. After spore encapsulation,

DHKB has been proposed to be the receptor of discadenine, a spore germination inhibitor produced during late spore maturation. Discadenine may ensure spore dormancy by maintaining sufficient levels of cAMP to keep PKA activated, possibly by modulation of RegA activity (Abe et al 1976, Zinda & Singleton 1998). During spore maturation, DHKB could compensate for the lack of DHKA activity via a similar pathway, assuming that DHKB receives the appropriate activating signal.

Responses to SDF2 are not limited to prespore cells, because SDF2 production by prestalk cells involves DHKA in addition to PKA and TagB/C through a positive-feedback loop (Anjard et al 1998a). Once terminally differentiated, encapsulated spores and vacuolated stalk cells are static structures unable to undergo further cell sorting or morphogenesis. Regulation of stalk and spore cell terminal-differentiation pathways via prestalk secreted peptides would allow the synchronization of the maturation process and prevent premature final differentiation of the components of the mature fruiting body. SDF1 and SDF2 would act sequentially, producing a highly regulated pathway.

In addition, other less-well-understood pathways regulate terminal differentiation. Overhead light on migrating slugs, which results in the arrest of slug migration and initiation of culmination, produces a rapid activation of phospholipase D (PLD) activity. Whereas the role of PLD is not known, it is regulated by a pathway that involves the heterotrimeric Gα protein subunit Gα1 (Dharmawardhane et al 1994). Because mutations in Gα1 affect culmination, it is possible that Gα1 functions through the regulation of PLD activity.

Transcriptional Regulation of Terminal Differentiation Two transcription factors, Stalky and srfA, are regulators of prespore cell differentiation. Stalky encodes a GATA family member transcription factor (Chang et al 1996). A mutation initially identifying Stalky was discovered 18 years ago (Morrissey & Loomis 1981). The mutation showed prespore cell differentiation, but the prespore cells differentiated into stalk cells rather than spores during culmination. Stalky is expressed in prespore cells and thus is thought to be required for maintenance of this developmental state. The phenotype of *stalky*-null mutants suggests that it is a homeotic mutation, except that expression of Stalky in prestalk cells does not lead to their differentiation into spores during culmination. Thus, Stalky may not cause the induction of spore cells, but it prevents cells from differentiating into stalk cells.

SrfA, a *Dictyostelium* member of the serum response factor (SRF) family of transcription factors, is required for spore terminal differentiation (Escalante & Sastre 1998). The SRF-like family belongs to the group of MADS-box transcription factors, which includes the human, *Xenopus*, and *Drosophila* SRF homologs and the yeast proteins MCM1 and ARG80 (Shore & Sharrocks 1995). In *Drosophila*, SRF is involved in intervein tissue differentiation during wing development and trachea formation. In *S. cerevisiae*, MCM1 controls the transcriptional activation of cell-type–specific genes and is crucial in the pheromone response. *SrfA* is

specifically expressed in prespore and spore cells and highly induced during culmination (Escalante & Sastre 1998). Disruption of *srfA* leads to abnormal spore morphology and loss of spore viability. Temporal and spatial patterns of expression of the prestalk markers *ecmA* and *ecmB* and the prespore marker *SP60/cotC* are not affected in the mutant, which suggests that basic cell-type differentiation and cell fate decisions are not regulated by *SrfA*. However, the expression of the spore marker *SpiA* is strongly reduced in *srfA*-null cells. It is expected that SrfA directly regulates multiple spore-specific genes, because the *srfA*-null mutant displays a stronger spore-defective phenotype than the *spiA*-null mutant. Activation of PKA by 8-Br-cAMP induces *SpiA* expression in *srfA*-null cells but is unable to fully complement the morphological defect of the mutant spores. SrfA could be part of the activating pathway upstream of PKA that controls its expression/activation during spore maturation. Another possibility is that SrfA and PKA function in parallel to control the expression of genes required for spore formation, including SpiA.

In yeasts, MCM1 is activated by the pheromone-signaling cascade via the well-characterized members of the MAP kinase pathway (Shore & Sharrocks 1995). A *Dictyostelium* serine/threonine kinase, MKCA, which has a kinase domain related to that of the Cdc42-regulated yeast kinase Ste20 but lacks a small-G-protein–interacting domain, was identified as a partial suppressor of the sporulation defect of *tagB*-null cells (Shaulsky et al 1996). The exact function of MCKA is not clear; however, analysis of its function during terminal differentiation may shed light on control of this pathway.

STATa Regulates Stalk Differentiation Although *STATa*-null cells exhibit defects during aggregation, the major function of STATa in *Dictyostelium* development is in spatial patterning and expression of the stalk marker *ecmB* (Mohanty et al 1999). STATa binds strongly in vitro to the activation domain sequence containing a direct repeat of the sequence TTGA found in the promoters of the prestalk-specific gene *ecmA*, which is expressed at high levels in pstA cells and lower levels in pstO cells and ALCs (Kawata et al 1997). STATa also binds strongly to a repressor element in *ecmB*. This repressor element is an inverted repeat of the same sequence. Expression of *ecmB* is controlled through proximal and distal regulatory elements directing expression in cells that have entered the stalk tube and cells that form the upper cup of the fruiting body, respectively (Ceccarelli et al 1991). In the slug, *ecmB* is expressed exclusively in the very anterior prestalk AB cells, which are thought to have already entered the stalk pathway, and to a lesser degree in rearguard cells and some ALCs (Jermyn et al 1989, Jermyn & Williams 1991). Deletion of this repressor element results in precocious expression of *ecmB* in the entire pstA and some of the pstO domains, which suggests that protein binding to this *cis*-acting region blocks *ecmB* in prestalk cells before culmination (Harwood et al 1993). During culmination, this repression is released via a mechanism that is thought be regulated by increases in PKA activity.

STATa-null organisms do not culminate and form structures that lack stalk cells and a stalk tube, although the cells differentiate into stalk cells in culture (Mohanty et al 1999). A detailed analysis indicated that *STATa*-null cells are hypersensitive to the inhibitory effects of extracellular cAMP, which suppresses stalk cell differentiation in vivo. Expression levels of the prestalk and prespore marker genes (*ecmA* and *SP60*, respectively) are normal, which indicates that STATa is not required for prestalk cell differentiation. However, *ecmB/lacZ*, a marker that is normally specific for cells on the stalk cell differentiation pathway, is expressed throughout the prestalk region, and *ecmB* is greatly overinduced by DIF in *STATa*-null cells in monolayer assays. These analyses suggest that complete stalk cell differentiation requires at least two events: a commitment step whereby the repression exerted by STATa is lifted and a second step that is blocked in a *STATa*-null organism. This latter step may involve inhibition by extracellular cAMP.

STATa-null organisms are able to express cell-type–specific genes including *ecmA*, which suggests that, even though STATa binds the activator element of the *ecmA* promoter in vitro, it is not the regulator that induces its activation in vivo. This result is inconsistent with the initially proposed model in which STATa acts as a transcriptional activator or repressor, depending on whether STAT dimers bind the DNA regulatory elements in a head-to-tail conformation or in a head-to-head orientation (Kawata et al 1996). On the other hand, the prestalk marker *ecmB* is precociously induced and expressed throughout the pstA region, whereas its expression in this domain is repressed in wild-type organisms. This observation is consistent with STATa acting as a negative regulator of *ecmB* expression and a repressor that prevents precocious commitment to stalk cell differentiation. It is expected that either STATb or STATc will be the activator of prestalk cell differentiation. A DNA-binding activity that interacts with the $TTG^{A}/_{T}$ direct repeat found in the *ecmA* activator region is present in *STATa*-null cells (Mohanty et al 1999). The presence of STATb and STATc and the high conservation of $TTG^{A}/_{T}$ as a binding site for STATa and mammalian STATs suggest that prestalk cell differentiation is regulated by one of the other two STATs. How DIF, the morphogen required for prestalk cell differentiation, functions will be of extreme interest. In mammalian cells, STAT proteins appear to function predominantly as transcription activators, except for some variants of STAT5, which can function as transcriptional repressors (D Wang et al 1996). Interestingly, these variant forms of STAT5 lack the C-terminal transcriptional transactivation domain present in most other STATs, which is also absent from *Dictyostelium* STATa.

The function of STATa in delaying stalk terminal differentiation as well as the hyperresponsiveness of *STATa*-null cells to DIF for stalk-specific gene expression are difficult to understand in terms of the development of *STATa*-null cells. The *STATa*-null strain lacks stalk cell differentiation, and a column of cells replaces the normally vacuolated stalk and supports a reduced spore mass that remains mostly undifferentiated. The reduced spore cell number might be a direct consequence of the absence of stalk cell differentiation and secretion of or the inability to respond to the appropriate spore maturation-inducing peptides (Anjard et al 1998b). A way

to interpret these results is to consider stalk differentiation as a two-step process, each step being under the control of a distinct STATa function: (*a*) a repression step suggested by the repression of *ecmB* expression and (*b*) an induction step that might also involve cAMP.

CONCLUSIONS AND PERSPECTIVE

Although *Dictyostelium* has a relatively simple developmental program compared with those exhibited by metazoans, the pathways are impressively complex, which suggests that we have only scratched the surface of understanding the development of organisms such as *C. elegans* and *Drosophila*, let alone mice and humans. Many of the developmental pathways used by *Dictyostelium* to control spatial patterning and cell fate decisions (e.g. STATs and GSK-3 to control patterning) have direct parallels in metazoans. Because *Dictyostelium* is a eukaryote with a relatively simple multicellular developmental program and with pathways that are conserved between *Dictyostelium* and humans, the further elucidation of pathways controlling *Dictyostelium* development should provide further insights into how these pathways function in metazoans and how they may have evolved.

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