cAMP-dependent protein kinase differentially regulates prestalk and prespore differentiation during Dictyostelium development

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SUMMARY

We and others have previously shown that cAMP-dependent protein kinase (PKA) activity is essential for aggregation, induction of prespore gene expression and multicellular development in Dictyostelium. In this manuscript, we further examine this regulatory role. We have overexpressed the Dictyostelium PKA catalytic subunit (PKAcat) in specific cell types during the multicellular stages, using prestalk and prespore cell-type-specific promoters to make PKA activity constitutive in these cells (independent of cAMP concentration). To examine the effects on cell-type differentiation, we co-transformed the PKAcat-expressing vectors with reporter constructs expressing lacZ from four cell-type-specific promoters: ecmA (specific for prestalk A cells); ecmB (specific for prestalk B and anterior-like cells in the slug); ecmBΔ89 (specific for stalk cells); and SP60 (prespore-cell-specific). By staining for β-galactosidase expression histologically at various stages of development in individual strains, we were able to dissect the morphological changes in these strains, examine the spatial localization of the individual cell types, and understand the possible roles of PKA during multicellular development. Expression of PKAcat from either the ecmA or ecmB prestalk promoters resulted in abnormal development that arrested shortly after the mound stage, producing a mound with a round apical protrusion at the time of tip formation. Prestalk A and prestalk B cells were localized in the central region and the apical mound in the terminal differentiated aggregate, while prespore cells showed an aberrant spatial localization. Consistent with a developmental arrest, these mounds did not form either mature spores or stalk cells and very few cells expressed a stalk-cell-specific marker.

Expression of PKAcat from the prespore promoter resulted in abnormal morphogenesis and accelerated spore cell differentiation. When cells were plated on agar, a fruiting body was formed with a very large basal region, containing predominantly spores, and a small, abnormal sorocarp. Mature spore cells were first detected by 14 hours, with maximal levels reached by 18-20 hours, in contrast to 24-26 hours in wild-type strains. When cells were plated on filters, they produced an elongated tip from a large basal region, which continued to elongate as a tubular structure and produce a ‘slug-like’ structure at the end. The slug was composed predominantly of prestalk cells with a few prespore cells restricted to the junction between the ‘slug’ and tube. As the slug migrated, these prespore cells were found in the tube, while new prespore cells appeared at the slug/tube junction, suggesting a continual differentiation of new prespore cells at the slug’s posterior. The slug eventually produced a fruiting body-like structure that in many cases was abnormal and showed only a low level of SP60 expression.

Models are presented of the roles cAMP-dependent protein kinase plays in regulating various aspects of Dictyostelium differentiation.

Key words: Dictyostelium, cAMP-dependent protein kinase, prestalk, multicellular development

INTRODUCTION

Dictyostelium grows as single-celled amoebae. Upon starvation, ~10^5 cells aggregate and undergo a series of morphogenetic changes to form a mature fruiting body containing a spore mass (sorus) atop a vacuolated stalk. The multicellular developmental program is mediated through extracellular signals (for reviews, see Firtel et al., 1989; Devreotes, 1989; Firtel, 1991; Kimmel and Firtel, 1991). In particular, extracellular cAMP plays several roles in directing development, via cell surface receptors and G-proteins. Early in the developmental cycle, cAMP pulses act as both the signal to direct aggregation and the chemottractant. During morphogenesis, higher levels of extracellular cAMP regulate expression of both prestalk and prespore genes. At present, little is known about the signal transduction pathways downstream of the second messengers; however, it is likely that a variety of kinases are involved,
as in other metazoans. One likely candidate is cAMP-dependent protein kinase (PKA), which has diverse regulatory roles and is thought to mediate all known pathways activated by intracellular cAMP in eukaryotes (Taylor et al., 1988, 1990). In mammalian systems, the holoenzyme, which is inactive, consists of two regulatory (R) subunits and two catalytic (C) subunits (Rc2c2); the catalytic subunits are released when cAMP binds to the regulatory subunits. In Dictyostelium, PKA is a simple heterodimer (RC; de Gunzburg et al., 1984).

The roles that intracellular cAMP and PKA play in controlling cellular differentiation in Dictyostelium are not well understood. Synag mutants are blocked in activation of adenyl cyclase, and thus do not produce intracellular cAMP or relay the pulsatile cAMP signal, but they develop properly when allowed to aggregate with wild-type cells (Darmon et al., 1975; Theibert and Devreotes, 1986). This would suggest that intracellular cAMP is not required for aggregation or cellular differentiation. However, several lines of evidence indicate that cAMP-dependent protein kinase plays essential roles in regulating Dictyostelium development. These are derived from two sets of experiments: (1) those in which a dominant negative regulatory subunit was used to inhibit cAMP-mediated activation of PKA and (2) analysis of PKA catalytic subunit null mutants (see below for references). We have shown that the catalytic subunit of Dictyostelium PKA is encoded by DdPK3, renamed PKAcat (Mann and Firtel, 1991; Mann et al., 1992), a gene also cloned and examined by Burki et al. (1991) and designated PK2 by that group.

During early development, PKAcat activity is required for aggregation. pkacat null mutants, created by gene disruption, do not aggregate (Mann and Firtel, 1991). Overexpression of a dominant negative mutant of the mammalian or Dictyostelium regulatory subunit (Rm) during vegetative growth and early development also prevents aggregation (Firtel and Chapman, 1990; Harwood et al., 1992a). In this case, Rm cannot bind cAMP and apparently sequesters the PKAcat in inert complexes. In addition, high-copy-number overexpression of the wild-type regulatory subunit inhibits aggregation (Simon et al., 1989) (see Discussion).

Once aggregation has occurred, PKAcat activity is required for the early stages of morphogenesis. pkacat null cells marked with the lacZ gene driven from an actin promoter form chimeric aggregates when mixed with wild-type cells (Mann and Firtel, 1991). However, as development proceeds, the null cells are lost from the multicellular structure and form a discrete clump at the base of the wild-type fruiting body. Similar results are observed using cells that overexpress Rm from an actin promoter and that are marked with the lacZ gene (Harwood et al., 1992b).

Culmination also requires proper PKAcat activity. pkacat null cells that have been ‘complemented’ with PKAcat driven from an Actin15 promoter will aggregate and proceed through the early stages of development apparently normally, forming a first-finger-type structure (Mann et al., 1992). However, they remain in this structure and do not culminate or form spores, even after >48 hours. Williams and co-workers have shown that when Rm is expressed from the prestalk-specific ecmA promoter, thereby blocking PKAcat activity in prestalkA cells, development is blocked at the slug stage (Harwood et al., 1992a). They have suggested that stalk cell differentiation during culmination is controlled by the concentration of intracellular cAMP and the concomitant activation of PKA. Moreover, several groups have suggested that the differentiation of prespore cells to spores is also mediated via increases in intracellular cAMP, presumably acting through PKA (Kay, 1989; Riley et al., 1989; Richardson et al., 1991). The rdeC rapidly developing strains (Abe and Yanagisawa, 1983) have been found to contain mutations within the gene for the PKA regulatory subunit that either create null mutants or result in the production of inactive R (Simon et al., 1992). These rapidly developing strains are sporogenous (they can be induced to form spores in single-cell culture by cAMP). Rapid development and sporogeny are also properties of cell lines that overexpress PKAcat and presumably have constitutively active PKA (Anjard et al., 1992; Mann et al., 1992).

To better understand the roles of PKA during development, we have expressed PKAcat from prestalk A (ecmA), prestalk B (ecmB) and prespore (SP60) promoters in a wild-type background. To examine cellular differentiation and morphogenesis in these overexpression mutants, appropriate lacZ expression constructs were introduced into each of the cell lines, enabling us to localize individual cell types within the developing organism. Our results indicate that PKAcat plays differential roles in regulating the prestalk and prespore pathways.

MATERIALS AND METHODS

Cell growth and development and lacZ expression

Dictyostelium wild-type axenic strain KA-3 was grown in HL5 axenic medium and development was examined on 12 mM Na/K phosphate buffer (pH 6.1) (PB)-containing agar plates, on Millipore filters placed on agar containing PB, or on Whatman 50 filters saturated with PB or MES-PDF (Mann and Firtel, 1987; Haberstroh and Firtel, 1990).

Cells were stained for β-galactosidase expression from lacZ reporter gene constructs as previously described (Haberstroh and Firtel, 1990; Esch and Firtel, 1991).

Cells were transformed with single vectors conferring G418 resistance by electroporation as previously described (Howard et al., 1988; Dynes and Firtel, 1985). For co-transformations of a PKAcat-expressing vector and a lacZ-expressing vector, the vector expressing the PKAcat construct carried the G418 selectable marker, and the vector carrying the lacZ reporter construct had no selectable marker. Cells were selected for G418 resistance and screened by β-gal staining for those carrying the lacZ vector. In all cases, we examined both transformed populations and several clonal isolates. Clonal strains were isolated as described previously (Firtel and Chapman, 1990).

To assay for viable spores, we quantitated the ability of putative spore cells to form clones on bacterial plates after treatment with detergent and heat as described previously (Hadwiger and Firtel, 1992). Under these conditions, only mature spores are viable and capable of forming clones.

Constructs

PKAcat expression vectors were constructed by replacing the SP60 promoter cassette in SP60/PKAcat described in Mann et al. (1992) with a cassette carrying the ecmA or ecmB promoter. The ecmA
and *ecmB* promoters, generously supplied by J. Williams, have been previously described (Williams et al., 1989; Ceccarelli et al., 1991). The promoters from the Williams lab contained a polylinker located after the ATG protein initiation codon. To make constructs with the *ecmA* and *ecmB* promoters, the promoters were isolated from the above *lacZ* expression vectors (Williams et al., 1989a,b; Ceccarelli et al., 1991) and the DNA was treated with ExoIII to remove the coding region and the ATG initiation codon. Reactions in which the digestion proceeded into the 5′UT region were capped with an *SphI* linker and appropriately sized clones were sequenced. A clone in which the *SphI* linker was inserted in the 5′UT region downstream from the Cap site was selected for each promoter. Each was individually fused to the PKAcat coding cassette using the same strategy as used for the *SP60* promoter. The *SP60* promoter has been described by our lab (Haberstroh and Firtel, 1990; Haberstroh et al., 1991).

**RESULTS**

**Morphological phenotypes of strains overexpressing PKAcat**

To examine the effect of PKA overexpression during development, we placed the PKAcat coding region downstream from cell-type-specific promoters and expressed these in

Fig. 1. Phenotypes of PKAcat-overexpressing strains. Presented are photographs of the terminal morphology of strains overexpressing PKAcat from the *Actin 15* (Act), *ecmA* (eA), *ecmB* (eB) and *SP60* (60) promoters when strains are plated on non-nutrient agar. A wild-type (WT) fruiting body is also shown. Photographs of the wild-type and Act15 strains are from Mann et al. (1992).
KAx-3 wild-type cells. The promoters used were *ecmA*, *ecmB* and *SP60*, which are preferentially expressed in prestalk A cells (*ecmA*), prestalk B and anterior-like cells (ALCs) (*ecmB*), and prespore cells (*SP60*) (see Materials and Methods; Williams et al., 1989; Haberstroh and Firtel, 1990; Haberstroh et al., 1991; Ceccarelli et al., 1991; Jermyn and Williams, 1991). *ecmA* expression is similar to that of *DdrasD*; it is first observed in ~10-15% of the cells scattered throughout the late aggregate (Williams et al., 1989; Esch and Firtel, 1991; Williams, 1991; Kimmel and Firtel, 1991). These cells then form the anterior prestalk domain and are designated as prestalk A cells. *ecmB* expression is first observed at the time of tip formation in cells at the base of the aggregate and in a small population of cells scattered throughout the aggregate with the pattern expected of ALCs. Later in development, *ecmB* is expressed in cells comprising a small cone within the anterior prestalk region, designated as prestalk B cells, and in ALCs. The prestalk B domain is thought to act as the initiation site for prestalk → stalk cell differentiation and stalk cell invagination during culmination; *ecmB* is then expressed in maturing stalk cells. *SP60*-expressing cells are first observed as scattered cells within the developing mound that become spatially restricted to a ring as the tip forms. They eventually constitute ~80% of the cells (Haberstroh and Firtel, 1990). In the slug, these cells constitute the majority of the posterior 85% of the slug. The Actin 15 (*Act15*) promoter, which we have used in these and earlier studies (Mann et al., 1992), is preferentially expressed during growth and early development, with expression con-

![Fig. 2. Spatial patterning of prestalk A, prestalk B and prespore cells in wild-type strains from *ecmA*/*lacZ* (eA), *ecmB*/*lacZ* (eB), *ecmBΔ89/lacZ* (Δ89), and *SP60/lacZ* (60) expression. Stable transformants were stained for β-gal expression as described in Materials and Methods. (eA left) *ecmA*/*lacZ* staining at the tipped aggregate stage; (eA middle) *ecmA*/*lacZ* staining at the slug stage; (eA right) *ecmA*/*lacZ* staining in the mature fruiting body; (eB left) *ecmB*/*lacZ* staining at the slug stage; (eB right) *ecmB*/*lacZ* staining in the mature fruiting body; (Δ89) *ecmBΔ89/lacZ* staining in the maturing fruiting body; (60 left) *SP60/lacZ* staining in the slug; (60 right) *SP60/lacZ* staining in the mature fruiting body. See text for details.](image)
Role of PKA during Dictyostelium development

continuing into the multicellular stages in a non-cell-type-specific manner.

Fig. 1 shows the terminal developmental phenotypes of the wild-type control (Fig. 1, panel WT) and the PKAcat overexpressing strains. Cells expressing PKAcat from the SP60 promoter and developed on non-nutrient agar have an abnormal fruiting body with a large base and a very small sorus on the end of a long, thin stalk (Fig. 1, panels 60). A normal tip forms during early development, but this extends abnormally and slugs are not formed. Development is complete by ~18 hours. When the lower portion of the SP60/PKAcat fruiting bodies and the stalk are examined by microscopy after squashing, most of the cells in the lower mass appear to be spores, as determined by their capsular shape and refractility under phase contrast (data not shown). As described below, we demonstrated these cells to be spores by assaying them for heat and detergent resistance. SP60/PKAcat strains developed on Millipore filters have a different morphology (see below). A small fraction (~1%) of the terminal fruiting bodies have the unusual structure shown in Fig. 1, panel 60 (lower).

Expression of PKAcat from the two prestalk promoters ecmA and ecmB results in phenotypes that are similar to each other (see Fig. 1, panels eA and eB). In both cases, a mound is formed that starts to develop a tip. However, a rounded mass usually develops in place of a true apical tip and morphological differentiation terminates at this point.

As described previously, PKAcat expressed from the Act15 promoter, shown in Fig. 1, panel Act, results in substantially accelerated development and fruiting bodies containing somewhat shorter, thicker stalks, larger sorocarps and enlarged basal plates (Mann et al., 1992). These results are shown for comparison with the other phenotypes.

Spatial development within wild-type aggregates

To examine cellular differentiation and distribution of the various cell types within the abnormal structures described above, lacZ gene fusions expressing β-galactosidase from the ecmA, ecmB, ecmBΔ89 or SP60 promoters were individually co-transformed into KAx-3 wild-type cells with the PKAcat expression vectors described above (see Materials and Methods). Analyses of β-gal expression patterns throughout development, therefore, allow us to examine the timing and spatial patterning of prestalk A, prestalk B and prespore cells. Fig. 2 shows these staining patterns in wild-type cells.

Spatial development within aggregates expressing PKAcat from the ecmA or ecmB promoters

Transformants that overexpress PKAcat from either of the

![ecmA/lacZ](image1)
![ecmB/lacZ](image2)
![SP60/lacZ](image3)

![ecmA/lacZ](image4)
![ecmB/lacZ](image5)
![SP60/lacZ](image6)

Fig. 3. Spatial patterning in strains expressing PKAcat from the ecmA or ecmB promoter. Cells were co-transformed with (1) a vector carrying a selectable marker (G418 resistance) and either the ecmA/PKAcat or ecmB/PKAcat fusion gene and (2) a vector carrying one of the following lacZ fusion genes: SP60/lacZ, ecmA/lacZ, or ecmB/lacZ. Stable transformed populations from at least two independent transformations were selected and then at least two independent clonal isolates that carried and expressed the lacZ fusion gene were analyzed. Cells were stained for β-gal expression as described in Materials and Methods. Shown are representative photographs of clonal isolates. The upper panels show ecmA/PKAcat-expressing clones, the lower panels show ecmB/PKAcat-expressing clones. The left panels show aggregates stained for ecmA/lacZ expression, the center panels show ecmB/lacZ staining and the right panels show SP60/lacZ staining.

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- **ecmA/lacZ**
- **ecmB/lacZ**
- **SP60/lacZ**

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prestalk promoters, ecmA or ecmB; are blocked early in development. In these strains, β-gal expression from either prestalk promoter is initially observed in cells scattered through the mass of the late aggregate, with many cells near the tip and a ring of cells around the base. In the terminal differentiated state, most of the staining cells are found in the bulbous protrusion and as a ring around the base of the mound (Fig. 3, left and center panels). More ecmB-expressing cells than ecmA-expressing cells are found in the basal ring. In these structures, SP60/lacZ expression, as observed by β-gal staining, is first detected as a ring of cells forming in the middle of the developing aggregate, very similar to the pattern in wild-type cells (Haberstroh and Firtel, 1990) (data not shown). In the terminally differentiated structure, SP60-expressing cells are found in a narrow double ring at the periphery and in the bulbous protrusion, the same region in which ecmA-expressing cells are found. This is in contrast to the distinct spatial partitioning of prestalk and prespore cells in the wild-type organism. In addition, some scattered cells stain within the body of the mound (Fig. 3, right panels). We note that a significant proportion of the cells in ecmA/PKAcat and ecmB/PKAcat transformants show little staining with any of the cell-type-specific markers tested.

In contrast to wild-type development, prestalk cells do not differentiate into a stalk tube in the ecmA and ecmB/PKAcat transformants. To determine whether mature stalk cells are formed, two approaches were used. We analyzed expression of the ecmBΔ89 promoter, a deletion of the ecmB promoter that is expressed in maturing stalk cells and not during the early stages (Ceccarelli et al., 1991), and found that very few ecmBΔ89/lacZ-expressing cells are present (data not shown). Many of these are located at the periphery of the mound. When these aggregates are squashed, we confirm that the body of the mound contains very few (10-20) staining cells. To examine for mature stalk cells, we stained aggregates with calciphlor, which stains the cellulose wall matrix in mature stalk cells. No staining cells were observed under fluorescence (data not shown). These results, therefore, suggest that overexpression of PKAcat in prestalk cells blocks prestalk → stalk cell differentiation either directly or by arresting development at an earlier stage.

Expression of SP60/PKAcat results in severely abnormal morphogenesis and spatial patterning

SP60/PKAcat strains developed on Millipore filters, a procedure that we use when staining for β-gal expression (see Materials and Methods), show a different developmental morphology than when these cells are developed on non-nutrient agar (see Fig. 1, panel 60). The initial differentiation through the formation and elongation of the apical tip is similar. On Millipore filters, however, all SP60/PKAcat-transformed populations and clonal isolates show prolonged elongation of the apical region, which falls over to the sub-stratum. (Photographs of the developmental stages are presented in Figs 4 and 5; the staining patterns are discussed below.) This forms a slug-like structure, which migrates away from the basal mound but remains connected to it via a thin tubular structure (designated ‘tube’), which is usually 1 cm or more in length. The ‘slug’ finally differentiates into a small, abnormal ‘fruiting body-like’ structure that appears to have a fairly normal stalk with a basal disc. However, the sorus has an unusual morphology in that the spore mass is generally reduced in size relative to the stalk (see Figs 4B, 5E). The thin tube that connects the slug or ‘fruiting body’ to the basal region is distinct in structure and function from the stalk of the ‘fruiting body’. This tube contains cells (see below), but is not exclusively cellular since the observed cells are found in patches. The apparent size of the basal mound does not diminish during the migration process. Presumably, the tube-like structure arises from the slug and may be similar to the slime sheath formed during normal slug migration, but is unusual in that it maintains a very tubular form through the staining process and does not appear to collapse upon itself. The bulk of the cells remain in the large basal mound, in which mature spores are found by 16 hours. However, the slugs continue to migrate for an extended period, with ‘culmination’ not occurring until 24 hours or later.

Spatial patterning in SP60/PKAcat strains

Fig. 4 presents the staining patterns of ecmA/lacZ and ecmB/lacZ in SP60/PKAcat strains. ecmA and ecmB/lacZ-expressing cells are first observed scattered throughout the aggregate, similar to the pattern seen in wild-type strains (data not shown). As the tip forms and extends into a slug, ecmB/lacZ expression is found primarily in the anterior half of the slug, with some staining of the basal mound and the tube also observed (Fig. 4A). Fig. 4B shows an enlarged view of a ‘fruiting-body-like’ structure that arises from the slug. The stalk of the fruiting body stains intensely, while the sorus shows staining in the cap regions and in some cases throughout the structure. The ecmA/lacZ expression pattern (Fig. 4C-E) is similar, except that no staining of the basal mound is observed, and staining of the anterior of the slug is more intense. In this strain, ecmBΔ89/lacZ staining, which is thought to be specific to stalk cells, is observed in the stalk of the small ‘fruiting body’ (Fig. 4F). No staining is observed in the sorus, the tube, or the basal mound.

In these SP60/PKAcat-expressing strains, SP60/lacZ-expressing cells are first observed as a ring of cells forming in the center of the aggregate, as in wild-type strains (data not shown). As the tip emerges and extends, it is predominately unstained, while the basal mound stains more uniformly (Fig. 5A). During the ‘slug’ migration stage, the basal mound of the aggregate stains densely, indicating the presence of prespore or spor cells (Fig. 5A,B). Little or no SP60-directed β-gal expression is observed in the bulk of the ‘slug’; however, some staining is seen in the very posterior of the slug and within the tube, especially where it attaches to the basal mound and to the slug. As the tube extends during slug migration, the SP60/lacZ-staining cells are seen as patches within the tube that become more disperse, suggesting that some of the tube is non-cellularized at this point (since few cells in the tube stain with any of the other marker genes). Fig. 5C is a lower-magnification photograph that provides a view of the relative sizes of these structures. Fig. 5D shows a late developmental stage of a dense population. In the terminal stage ‘fruiting body-like’ structure that forms at the end of the tube (Fig. 5E), no SP60-driven staining is observed in the stalk, and little is seen in the basal disc, as expected. The central region of the sorus, where spore cells are normally localized, shows
Effect of PKAcat expression on spore differentiation

In order to examine the effects of PKAcat expression from the ecmA, ecmB and SP60 promoters on spore maturation, we assayed for mature spores at 27 and 36 hours after starvation by quantitating the number of viable cells that are heat- and detergent-resistant and can form plaques on bacterial lawns (Hadwiger and Firtel, 1992) (data not shown). Our analysis indicated that no detectable spores are produced (<0.001% of the level produced with wild-type fruiting bodies) by the ecmA/PKAcat- and ecmB/PKAcat-expressing strains. However, SP60/PKAcat-expressing cells produce viable spores in numbers similar to those for an untransformed wild-type control. Because development is more rapid in this strain, we also assayed for spores present at 18 hours of development. The proportion of heat- and detergent-resistant cells was approximately half that observed in structures developed for 27 hours.

DISCUSSION

Expression of PKAcat in wild-type KAx-3 cells from cell-type-specific promoters results in abnormal morphological differentiation. In analyzing these experiments, we assume that PKAcat is being overexpressed relative to the regulatory subunit and that PKA activity is therefore constitutive in specific cells once the promoter has been activated. Even if a feedback mechanism provides for increased expression of the PKA regulatory subunit in these cells to compensate for the overexpression of PKAcat, it is likely that the overall level of free PKAcat is increased. We previously showed that overexpression of PKAcat from the Act15 promoter, which is expressed in all cells during early development, does not produce a very abnormal phenotype (Mann et al., 1992). In contrast, Anjard et al. (1992) showed that overexpression of PKAcat from the Act15 promoter carrying an 800-bp deletion between −180 and −1 kb upstream from the start site yields aggregates with a more abnormal phenotype. We expect that the differences are due to differential spatial expression of the PKAcat promoter. Whether the complete PKAcat promoter would give a similar morphology is not known.

Overexpression of PKAcat in prestalk cells blocks proper tip formation

Work in the Williams and Veron laboratories has shown that expression of the dominant negative PKA regulatory subunit (Rab) from the ecmA promoter inhibits culmination and blocks ecmB expression in stalk cells (see Introduction). From the results of these experiments and the observation that intracellular cAMP may activate the ecmB promoter during culmination, Williams, Veron and co-workers proposed a model in which cAMP, via the activation of PKA, mediates prestalk-to-stalk cell differentiation. In such a model, prestalk A cells that overexpress PKAcat might be expected to differentiate rapidly into stalk cells, a step that involves activation of the ecmBΔ89 promoter. We tested this model by examining the staining pattern of ecmBΔ89/lacZ in ecmA/PKAcat cells. In such experiments, we detect very few β-gal-staining cells, although we observe the expected number of ecmA/lacZ-staining cells (15-20%), which suggests that the overexpression of PKAcat in prestalk cells is not sufficient to induce stalk cell differentiation. One or more external signals may also be required, or stalk cell differentiation may be coupled to morphogenesis. By blocking morphogenesis at an early stage, overexpression of PKAcat from the ecmA promoter appears to inhibit prespore differentiation and/or spore maturation. Interestingly, the anterior bulbous region contains a mixture of prestalk and prespore cells whereas, in the wild-type, the cells have sorted by this time in development.

Our results suggest that overexpression of PKA in prestalk A or prestalk B cells, which arrests development, may do so by blocking the formation of a proper tip. The apical region of an aggregate, which consists of prestalk cells, is thought to act as a cAMP oscillator that participates in directing the multicellular stages of development (Siegert and Weijer, 1991, 1992). Reymond and co-workers have reported higher levels of cAMP in aggregates overexpressing PKAcat from a deleted PKAcat promoter (Anjard et al., 1992). Overexpression of PKAcat in prestalk cells may affect the cAMP signaling pattern in aggregates sufficiently to alter morphogenesis.

PKAcat drives spore cell differentiation

Expression of PKAcat from the SP60 prespore promoter results in accelerated development and abnormal morphogenesis. Our results suggest that overexpression of PKAcat in prespore cells is sufficient to accelerate spore differentiation. In the SP60/PKAcat strains, most of the spore cells differentiate in the basal mass of the aggregate. A possible explanation is that, in these aggregates, the prespore → spore differentiation initiates before the prestalk → stalk transition, as evidenced by the very precocious formation of mature spores relative to the elongation of the stalk tube. This early differentiation of the spore cells may prevent the ‘invagination’ of the stalk tube through the prespore mass, or it may prevent participation of these cells in morphogenesis, thus interfering with normal development. The accelerated spore differentiation that we observe is consistent with the sporogenous phenotype of this strain (Mann et al., 1992) and the fact that rdeC mutants lack active PKA regulatory subunit (Simon et al., 1992).

One of the most interesting phenotypes, and potentially the most illuminating with respect to mechanisms controlling spore-cell differentiation, is the slug-like extension produced when SP60/PKAcat strains are plated on Millipore filters over non-nutrient agar, rather than directly on the agar. (The factors that cause this phenotypic difference are unknown, but the moistness of the surface on which the cell mass is resting may play a role.) Our results suggest that most of the cells in this slug-like structure are prestalk A, prestalk B or anterior-like cells. This structure is
therefore reminiscent of the anterior slug tips originally isolated by dissection (Raper, 1940). Fig. 6 presents a model of prespore and prestalk cell differentiation in these strains. As the slug-like structure migrates, some of the ecmA- and ecmB-expressing cells appear to redifferentiate into prespore cells; this occurs at the very posterior of the slug. These ‘new’ prespore cells appear to then be lost from the slug into the tube as the slug continues to migrate, possibly because they rapidly differentiate into spore cells. Many cells in the posterior half of the slug apparently do not express any of the cell-type markers and may be cells that are in the process of redifferentiating from prestalk cells to prespore cells. These observations are consistent with data from Raper (1940) showing that Dictyostelium slugs are regulative. Thus, at all stages of ‘slug’ migration, the pattern of staining in this structure remains the same.

Fig. 4. Spatial patterning of ‘prestalk’ cells in strains expressing PKAcat from the SP60 promoter. Experiments were performed as described in the Legend to Fig. 3. (A) ecmB/lacZ staining pattern in early ‘slugs’. Staining is also observed in the basal mound, though far lighter than for SP60/lacZ (compare to Fig. 5). (B) ecmB/lacZ in the small fruiting body that forms at the end of the tube. Arrowheads indicate small patches of staining cells within a tube structure. (C) ecmA/lacZ at the early ‘slug’ stage. (D) ecmA/lacZ staining pattern in an early ‘slug’. (E) ecmA/lacZ at terminal differentiation. (F) ecmBΔ89/lacZ at terminal differentiation. Small arrowheads indicate an unstained tube structure; the large arrowhead indicates an unstained sorus.
143Role of PKA during Dictyostelium development

The role and mechanisms of regulation of development by PKA

It is clear from experiments of the Maeda (1988), Kay (1989) and Barclay (Riley et al., 1989) laboratories that increases in intracellular cAMP can induce prespore cells to differentiate into spore cells. Since this can be achieved experimentally by the addition of high concentrations of the membrane-permeable cAMP analogue 8Br-cAMP, but not by the addition of cAMP, we expect that this is mediated via the activation of PKA. 8Br-cAMP binds to the PKA regulatory subunit, releasing PKAcat and thus increasing the intracellular concentration of free PKAcat. However, previous experiments with synag strains, which cannot activate adenyl cyclase but can synergize and participate in fruiting body and spore formation, have suggested that increases in intracellular cAMP are not necessary for induction of prespore gene expression (see Introduction). In addition, cells lacking adenyl cyclase due to gene disruption proceed through development under certain conditions (Pitt et al., 1992; P. Devreotes, G. Pitt, and P. Schaap, personal communication). These results suggest that increases in intracellular cAMP are not essential for prespore cell differentiation or development in general and present a paradox with our conclusion that cAMP-dependent protein kinase, the generic mediator of intracellular cAMP-controlled second messenger pathways, is essential for prespore differentiation. We suggest, therefore, that PKA has an essential regulatory function during Dictyostelium development, but that, under some circumstances, its catalytic activity is not necessarily regulated by the concentration of intracellular cAMP.

In Dictyostelium, the PKA holoenzyme readily dissociates into the R and C subunits in crude lysates and, as a result, it has been difficult to purify (de Gunzberg et al., 1984; Majerfeld et al., 1984). This suggests that the relative affinity of the R and C subunits for each other may not be as high as in mammals. We propose that, as in mammalian cells, PKA activity can be rapidly induced by increases in the intracellular cAMP concentration for certain cellular functions; however, for some functions or under some conditions, PKA activity may be mediated by non-cAMP-controlled changes in the relative level of free catalytic subunit. This could be achieved by an equilibrium-controlled dissociation of the holoenzyme regulated independently of cAMP concentrations, as shown in Fig. 7A, or by a relative decrease in the level of the regulatory subunit or a relative
increase in the level of catalytic subunit. Even if there were similar increases in both R and C, the absolute level of free C (and thus PKA activity) would increase due to an equilibrium-driven dissociation of holoenzyme to free R and C. Such a model can explain the observations of Simon et al. (1989) that overexpression of a wild-type regulatory subunit blocks development. Since it is unlikely that sufficient regulatory subunit is present to bind all free intracellular cAMP, we expect that, by increasing R relative to C in these cells, the equilibrium is driven towards holoenzyme, reducing the

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**Fig. 6.** Model of prespore and prestalk cell differentiation in SP60/PKAcat slugs. In the top figure, the blackened areas represent regions of the organism that express ecmA/lacZ at high levels, indicating the presence of prestalk A cells. In the lower two figures, the blackened areas represent regions of the organism that express SP60/lacZ at high levels and that contain prespore and spore cells. The middle figure depicts an aggregate after the small 'slug-like' structure has formed, showing SP60-driven β-gal expression in the posterior of the 'slug'. The lower figure shows a slightly later stage after further migration of the 'slug'. The 'older' prespore cells are now further into the tube, while newer prespore cells are now located in the new posterior of the slug. We propose that the new prespore cells arise from either a redifferentiation of cells within the 'slug' or possibly the continued differentiation down the prespore pathway of 'undetermined' cells within the 'slug'.

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**Fig. 7.** Developmental pathways regulated by PKA in Dictyostelium. (A) The equilibrium and cAMP-driven changes in the relative ratios of free and regulatory subunit-associated PKAcat. (B) The developmental pathways proposed to be affected by levels of free PKAcat. Aggregation (cAMP relay) is blocked in pkacat null cells and in cells overexpressing a mutant mammalian or Dictyostelium regulatory subunit (Rm) that cannot bind cAMP. pkacat null cells are also blocked in cAMP induction of prespore genes. Further into the prespore pathway, overexpression of PKAcat generally from an actin promoter or from the prespore-specific SP60 promoter results in accelerated overall development and potentiates spore cell differentiation. In the prestalk pathway, overexpression of PKAcat at the onset of prestalk A or prestalk B differentiation appears to inhibit prestalk → stalk cell differentiation. However, expression of Rm in prestalk A cells also prevents stalk formation and culmination. Overexpression of PKAcat may inhibit this pathway at an earlier step than does Rm. This may account for the apparent contradiction that both overexpression of PKAcat and inhibition of PKAcat using Rm inhibit stalk cell differentiation. (See text for details and references.)
concentration of free C. Overexpressing wild-type mammalian R in *Dictyostelium* does not block development (Firtel and Chapman, 1990), which could be due to a lower affinity of the mammalian and *Dictyostelium* subunits for each other in the presence of free cAMP. How the required changes in PKAcat levels are regulated during the various stages of development is not known.

An additional possibility is that during normal development, PKAcat activity is essential, but that specific increases in activity are not regulated per se. In this model, some constitutive level of PKAcat must be present to phosphorylate specific, essential substrates; regulation may occur by a change in the rate at which these substrates are dephosphorylated. During culmination, the addition of 8Br-cAMP would cause release of more PKAcat, and increase the rate of the phosphorylation reaction, thus accelerating the kinetics of development, inducing the expression of prespore and other genes, and allowing sporulation to proceed precociously. Since prespore cell induction and prespore → spore differentiation can occur, under some conditions, in mutants unable to activate adenyl cyclase or in adenyl cyclase null mutants, we presume that during culmination the activity of PKAcat is not necessarily controlled by changes in the intracellular concentration of cAMP. A possible explanation for this is that PKA activity may be mediated by some as-yet-uncharacterized pathway, e.g. by increases in intracellular Ca\(^{2+}\) or cGMP, known to result from cAMP binding to cell surface receptors late in development. However, we think the latter possibility is unlikely, since R subunits have an ~10\(^3\) lower affinity for cGMP than for cAMP (Shabb et al., 1991). Notwithstanding all of the above possibilities, it is clear that changes in the relative level of PKAcat in various cell types alters development (see model presented in Fig. 7B), suggesting that feedback mechanisms must exist to control the development of stalk and spore cells differentially.

We know that the expression of both the regulatory subunit and PKAcat are developmentally regulated (de Gunzburg and Veron, 1982; Leichtling et al., 1984; de Gunzburg et al., 1986; Mann and Firtel, 1991; Burki et al., 1991; Mann et al., 1992). For PKAcat, our results suggest that two promoters are functional (Mann and Firtel, 1991; Burki et al., 1991; S. Mann and R. Firtel, unpublished data). One is preferential for growing cells and is repressed during aggregation by pulses of cAMP, which concurrently induces the late promoter. Analysis of the PKAcat promoter, isolated in our laboratory, will allow us to examine the spatial as well as transcriptional regulation of this gene. These studies, combined with a similar analysis of regulatory subunit expression, should allow us to examine the mechanisms by which the expression of each subunit is regulated. It is possible that differential levels of the two subunits are present in various cell types, which would then influence the effective concentration of free PKAcat.

Our results also suggest that PKAcat may directly regulate the induction of spore differentiation. This is consistent with the previously described induction of spore differentiation by the membrane-permeable analog 8-Br-cAMP, but not by exogenous cAMP, which only interacts with cell surface receptors (see Introduction). We expect that PKAcat must be activating genes that are essential for spore differentiation. At present, the trigger that initiates culmination and spore differentiation is unknown. In cells developed on agar, overhead light is capable of inducing culmination. We have recently shown that a major increase in the level of 1,2-diacylglycerol (DG) occurs during culmination and that there is a very rapid induction of DG production immediately upon light stimulation (Cubitt et al., 1993). Whether this represents a potential parallel regulatory pathway or may somehow couple to PKA is not known.

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