Induction of terminal differentiation of *Dictyostelium* by cAMP-dependent protein kinase and opposing effects of intracellular and extracellular cAMP on stalk cell differentiation

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SUMMARY

Expression of the catalytic (C) subunit of the cAMP-dependent protein kinase (PKA) of *Dictyostelium* under the control of heterologous, cell-type-specific promoters causes ectopic terminal differentiation. When expressed under the control of a prespore-specific promoter, development is accelerated, to yield highly aberrant fruiting bodies that contain a basal mass of spore cells surrounding a central stalk-like structure. When expressed under the control of a prestalk-specific promoter, development arrests much earlier, at the tight mound stage. Prestalk cells move to the apices of these mounds, apparently normally, but no tip is formed. Most of the prestalk cells remain arrested in their development but there are a few isolated stalk cells scattered within such mounds.

We show that extracellular cAMP represses stalk cell-specific gene expression in cells where the kinase is constitutively active, suggesting that inhibition of stalk cell differentiation by cAMP in normal cells (Berks and Kay, 1988) occurs because of an effect of extracellular cAMP on an intracellular signalling pathway independent of PKA. We propose a scheme whereby two separate events, a rise in intracellular cAMP levels and a fall in extracellular cAMP concentration, are required to induce stalk cell differentiation.

Key words: *Dictyostelium*, stalk cell, cAMP, protein kinase, terminal differentiation

INTRODUCTION

In order that a *Dictyostelium* fruiting body be formed, cells within the migratory slug must sense their environmental conditions and adjust their behaviour accordingly. Moreover, the stalk cell and spore cell precursors must do so in perfect synchrony. Prestalk cells are the first cells to show a response to the changes in environmental conditions that trigger culmination: the presence of overhead light, a drying atmosphere and a reduction in ionic strength. They arrest their forward movement, the entire prestalk region lifts up towards the light and formation of the stalk is initiated. The most anterior of the prestalk cells form the slug tip, a region with some of the properties of an embryonic organiser (Raper, 1940; Rubin and Robertson, 1975). It may be that the prestalk cells also orchestrate the process of culmination, with the prespore cells falling under their ultimate control. Alternatively, prestalk and prespore cells may respond independently, but synchronously, to the culmination stimulus. In order to distinguish these possibilities, we must first understand the signalling pathways that control terminal differentiation.

Ammonia is produced in copious amounts during development, as the result of catabolism, and the extracellular concentration of ammonia appears to regulate entry into culmination: if the ambient ammonia concentration within the aggregate is artificially reduced, by exposure to an enzymatic mixture that uses ammonia as a substrate, fruiting body formation is initiated (Schindler and Sussman, 1977). During normal development, the rate of loss of ammonia presumably increases as the aggregate lifts up towards overhead light, and this acts to induce culmination (Bonner et al., 1982).

A number of pieces of evidence suggest that ammonia regulates entry into culmination by modulating the intracellular cAMP concentration. Extracellular cAMP is the chemoattractant, that draws cells together during aggregation, and it also acts to control gene expression at several different stages of development (reviewed by Schaap, 1986; Firtel, 1991). Cyclic AMP is released from cells in a pulsatile fashion and diffuses through the aggregation territory. It binds to a membrane receptor and activates a number of intracellular enzymatic activities, including adenylate cyclase (reviewed by Gerisch, 1987; Janssens and Van Haastert, 1987). Ammonia represses the accumulation of cAMP in response to an extracellular cAMP signal (Schindler and Sussman, 1979), so that a drop in ammonia concentration would be expected to increase the intracellular...
lar concentration of cAMP. Such a rise is observed at culmi-

nation (Abe and Yanagisawa, 1983; Merkle et al., 1984).

A number of studies show that elevation of the intracel-

lular cAMP concentration is necessary for spore and stalk
cell differentiation. When cells are deprived of an air-water
interface, by incubation in a Petri dish under buffer, terminal
differentiation to form spore cells is repressed but addition
of 8-bromo cAMP, a membrane permeant cAMP analogue,
will induce spore cell differentiation (Kay, 1989). 8-bromo
cAMP also induces prestalk cells to differentiate into stalk
cells and acts to overcome the inhibitory effects of caffeine
on stalk cell differentiation (Kwong et al., 1988; Maeda,
1988).

In eukaryotic cells, intracellular cAMP exerts its effects by
regulating the activity of cAMP-dependent protein kinase
(PKA). The Dictyostelium PKA contains a single R and a
single C subunit (de Gunzburg and Veron, 1982; Mutzel et
al., 1987). If either the Dictyostelium or the mammalian R
subunit is mutated, such that it cannot bind cAMP, a
dominant inhibitor of the Dictyostelium C subunit is formed.
These will arrest cells prior to aggregation when expressed
under the control of a non cell-type-specific, actin promoter
(Firtel and Chapman, 1990; Harwood et al., 1992a). Selective
expression in prestalk cells of the dominant inhibitor derived
from the Dictyostelium R subunit (Rm) greatly favours prolonged slug migration and arrests culmi-
nation at a very early stage, prior to stalk tube formation
(Harwood et al., 1992b). When expressed under the control
of a prespore-specific promoter, Rm prevents spore cell
differentiation but a normally shaped and proportioned fruit
is formed (Hopper et al., 1993). If the C subunit gene is ina-
tivated by homologous gene disruption then development is
arrested prior to aggregation and prespore-specific genes are
not expressed (Mann and Firtel, 1991).

The above analyses show that an active C subunit is
necessary for progression along both the terminal pathways
differentiation and analysis of the rdeC rapid develop-
ment mutants, which have an inactive R subunit, shows that
constitutive activation of the C subunit is also a sufficient
stimulus to induce premature terminal differentiation (Abe
and Yanagisawa, 1983; Simon et al., 1992). In addition to
displaying precocious stalk and spore cell differentiation,
the rdeC mutants are sporogenous, i.e. they will form spores
in submerged conditions without addition of 8-bromo cAMP
(Kay, 1989).

It is also possible to generate constitutively active C
subunit by over-expressing the C subunit gene to a level suf-
ficient to exceed the amount of R subunit present within
the cell. The C subunit of Dictyostelium (PkaC) is a 70×10³ M⁻¹
protein, which differs from its mammalian counterpart in
that it contains a long N-terminal region, upstream of the
kinase domain, which is of unknown function (Burki et al.,
1991; Mann and Firtel, 1991; Mann et al., 1992). When the
pkaC gene is over-expressed, an aberrant fruiting body is
formed, with a short, thick stalk and the strain is rendered
sporogenous (Anjard et al., 1992; Mann et al., 1992).

We have now fused the pkaC gene to cell-type-specific
promoters to determine the consequences of its over-
expression in prestalk or prespore cells. We used two
different genes for this purpose. The pspA gene encodes
PsA, a cell surface protein of unknown function, and is specif-
cally expressed in prespore cells (Kreft et al., 1983; Early
et al., 1988). The ecmA gene encodes EcmA, an extracel-
ular matrix protein, and it is specifically expressed in prestalk
cells (Jerryn et al., 1987). We find that selective expression
of the C subunit in prestalk cells has markedly different
effects from selective expression in prespore cells, with
over-expression in prestalk cells causing a much earlier and
more dramatic block to development.

Analysis of transformant clones where the C subunit is
over-expressed in prestalk cells also helps to resolve a
conundrum concerning the effect of extracellular cAMP
upon stalk cell differentiation. The studies described above
argue very strongly that a rise in intracellular cAMP is
necessary for stalk cell differentiation. During aggregation,
an increase in the extracellular cAMP concentration leads,
via activation of adenylate cyclase, to a rise in intracellular
CAMP. If this same signalling pathway were to function
during later development exogenously added extracellular
CAMP would be expected to elevate intracellular CAMP and
so stimulate stalk cell differentiation. However, extracellular
CAMP represses stalk cell differentiation (Berks and
Kay, 1988; Berks and Kay, 1990). Our data suggest a
potential resolution to this apparent paradox, by showing
that CAMP represses stalk-specific gene expression even in
strains where the C subunit is rendered constitutively active.
This suggests that extracellular CAMP acts through an intra-
cellular signalling component other than adenylate cyclase
to repress stalk cell differentiation.

MATERIALS AND METHODS

Dictyostelium transformation and culture

Dictyostelium Ax2 cells were grown in axenic medium (Watts
and Ashworth, 1970), plasmid DNA was introduced by calcium
phosphate precipitation (Nellen et al., 1984) and clones of cells
resistant to G418 (GIBCO Ltd.) were isolated. For development,
cells were grown to 3-6×10⁶/ml and harvested by centrifugation
at 1000 × g. After resuspension in an equal volume of either PDE
(20 mM KCl, 1.2 mM MgCl₂, 8.7 mM K₂HPO₄, 13.3 mM KClPO₄)
or K₂ (16.5 mM KH₂PO₄, 3.8 mM K₂HPO₄, pH 6.2) buffer
the cells were resuspended in the same buffer at 3-5×10⁶/ml. The cell
suspension was deposited on black 0.45µm pore size nitrocellulose
filters (Schleicher & Schuell AG) on a filter pad saturated with PDF
or K₂ containing 500 µg/ml streptomycin and incubated at 22°C
in a humid chamber.

β-Galactosidase staining

Aggregates were fixed whilst still on the nitrocellulose filter in Z
buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 2 mM
MgSO₄, pH 7.0) containing 1% glutaraldehyde (Dingermann et al.,
1989). Fixed cells were washed with three changes of Z buffer then
incubated in staining solution (Z buffer supplemented with 5 mM
K₃(Fe(CN)₆)₃, 5 mM K₃(Fe(CN)₆), 1 mM X-gal and 1 mM EGTA)
for periods of between 4 and 20 hours.

Stalk cell induction

Cells were harvested from shaken suspension at densities between
1-5×10⁹/ml, washed three times in stalk salts (10 mM MES, 10
mM KCl, 2 mM NaCl, 1 mM CaCl₂, 15 mg/ml tetracycline, pH
6.2) and then incubated in tissue culture dishes at 8×10⁵ cells/cm²
in stalk salts. After eight hours, 5 mM cAMP was added and the
cells were incubated for a further 16 hours. They were then washed
twice with stalk salts and incubated for a further 24 hours in stalk
salts that was either supplemented with 5 mM cAMP or from which cAMP was omitted.

**Enzymatic assay for β-galactosidase**

Cells were harvested from the plate and washed twice in KK$_2$ buffer. 5x10$^7$ cells were solubilized in lysis buffer (100 mM phosphate buffer, pH 7.8, 8 mM MgCl$_2$, 1 mM EDTA, 1 mM DTT, 1% Triton X-100, 15% glycerol) and β-galactosidase activity was assayed using o-nitrophenyl-β-D-galactopyranoside as a substrate (Dingermann et al., 1989). Assays were performed in triplicate and enzymatic activity is presented as Katal/mg where one katal is defined as the amount of catalytic activity forming one mole of product per second.

**Northern transfer analysis**

RNA extraction and northern transfer analysis were performed as described previously (Hopper et al., 1993), using the pDd26 cDNA clone as a probe.

**RESULTS**

(1) Over-expression of PKA under the control of a prespore-specific promoter leads to the formation of aberrant structures and accelerates spore cell differentiation

The pspA-C construct contains the coding region of the C subunit gene under the transcriptional control of the pspA, prespore-specific promoter (Fig. 1). This fusion gene was introduced into cells by calcium phosphate precipitation. The vector contains a gene encoding resistance to the neomycin analogue G418 and, after selection of a population of cells resistant to a drug concentration of 50 µg/ml, three individual clones were analysed. All three clones differ radically from control, wild-type cells (Fig. 2A) and from transformants containing multiple copies of the C subunit expressed under control of its own promoter (Fig. 2B). When allowed to develop on nitrocellulose filters, early development appears quite normal with first fingers being formed by about 13 hours of development. Therefore, development is greatly accelerated, to form a mass of spores surrounding a central column of cells by about 16 hours of development (Fig. 2C-E). Aside from the extreme tip, which appears to be composed of undifferentiated cells, amoebae within the central column have the vacuolated appearance typical of stalk cells and stain with the cellulose-specific reagent calcofluor. Such structures remain indefinitely arrested in this form.

Morphologically identifiable, detergent-resistant spores can be detected in these structures by 16 hours of development, whereas normally spores are not detected until 24 hours of development. Analysis by northern transfer (data not shown) shows that the C subunit gene is over-expressed by a factor of approximately 100-fold relative to control cells. We assume, therefore, that the developmental defects in these strains result from the presence of an excess of C subunit over the amount of available R subunit.

(2) Expression of the C subunit under the control of a prestalk-specific promoter blocks development prior to tip formation but accelerates stalk cell differentiation

The construct ecmA-C contains the C subunit under the control of the ecmA, prestalk-specific promoter (Fig. 1). When this construct is introduced into Dictyostelium cells, and selection performed at a low G418 concentration (20 µg/ml), the isolated clones develop into a variety of structures. Some aggregates develop relatively normally, some form spore masses supported by a tiny stalk and some arrest as tight mounds with no apical tip (data not shown). When transformants are selected at a drug concentration of 100 µg/ml, all of the aggregates arrest at the tight mound stage (Fig. 3A).

If a population selected at low drug concentration is subsequently exposed to a high drug concentration, then the proportion of arrested tight mounds increases to approach a value of 100%. The copy number of Dictyostelium G418 resistance vectors increases with increasing drug concentration (Nellen and Firtel, 1985). Hence we interpret the above results to mean that a high level of expression of the ecmA-C fusion gene arrests development at the tight mound stage while an intermediate level of expression produces a less severe block to development. Because the prestalk cells are greatly out-numbered by the prespore cells, it is not possible...
to obtain a reliable estimate of the degree of over-expression of the C subunit gene.

(3) In ecmA-C-arrested structures pstA cell differentiation and movement occur apparently normally

The differentiation of prestalk cells was investigated in co-transformant clones containing the ecmA-C and the ecmA-gal fusion genes. During normal development, the ecmA-gal gene is first expressed in randomly scattered cells in the loose aggregate (Williams et al., 1989). Cells expressing the ecmA gene, pstA cells, then accumulate in the apex. Once a tip is formed, ecmA-expressing cells are also found in the base where they are concentrated as a ring at the margin of the aggregate. If a culminant forms in situ, the latter population forms part of the basal disc but, if the first finger forms a migratory slug and moves away, they are left behind in the slime trail. In ecmA-C tight mounds, the ecmA-expressing cells arise at random and then accumulate at the apex and in a basal ring (Fig. 3B,C).

(4) The ecmA-C-arrested structures contain a small number of widely scattered pstAB cells and mature stalk cells

PstAB cells are found in the tip of the migrating slug at the position where the stalk tube will form during culmination (Jermyn et al., 1989). They co-express the ecmA and ecmB genes (Gaskell et al., 1992) and also are able to express ST-gal, a lacZ fusion gene containing a subfragment of the promoter of the ecmB gene that extends from nucleotide −877 to +112, numbered relative to the cap site (Ceccarelli et al., 1991). During normal development expression of ST-gal appears to occur concomitantly with a commitment to stalk cell differentiation, because cells outside the stalk tube do not express the fusion gene detectably while cells within the stalk tube express it very strongly (Ceccarelli et al., 1991).

In ecmA-C tight mounds, stained ST-gal-expressing cells are observed scattered throughout the aggregate. The proportion of such cells increases with time, so that an occasional staining cell is detected in the loose aggregate stage.
and in newly formed tight aggregates but after overnight development there are significant numbers of stained cells (Fig. 3D,E). Most of these are found within the skirt of scattered cells that surround the mound. Some of the ST-gal-expressing cells have the vacuolated appearance (Fig. 3F) typical of stalk cells and stain with calcofluor, a dye that gives a blue fluorescence when it interacts with cellulose (data not shown).

(5) The effect of extracellular cAMP on ST-gal expression

Comparison of the staining pattern observed for ecmA-C-expressing tight mounds using the ecmA-gal and ST-gal markers shows that the vast majority of cells that become pstA cells never differentiate into stalk cells. This is in marked contrast to pspA-C transformant strains, in which spore formation is accelerated and which are sporogenous.

Because expression of a dominant inhibitor of the C subunit in prestalk cells arrests their differentiation into stalk cells (Harwood et al., 1992b), we expected that prestalk cells derived from ecmA-C transformants might differentiate into stalk cells in an uncontrolled, precipitate manner.

The major difference between the pspA-C and ecmA-C transformants is that, for reasons that we do not yet understand, ecmA-C cells arrest at the tight mound stage. This suggested that conditions within the apex of the tight mound, the position where most of the prestalk cells are located, are inappropriate for stalk cell differentiation and that the failure to differentiate into stalk cells might therefore be an indirect consequence of the block to tip formation and elongation. We reasoned that one of the requirements for stalk cell differentiation might be a reduction in the extracellular cAMP concentration, because exogenous cAMP is known to repress the process (Berks and Kay, 1988, 1990). We
therefore determined the effect of exogenous cAMP upon the expression of ST-gal in ecmA-C:ST-gal co-transformant cells.

Cells were incubated in submerged culture on tissue culture plates for 8 hours and cAMP was then added to 5 mM. After a further 16 hours, during which time the cAMP acts to induce competence to undergo stalk cell differentiation (Berk and Kay, 1988), the incubation solution was replaced with fresh solution either containing or not containing 5 mM cAMP. Finally, 24 hours later, the level of expression of ST-gal was determined by enzymatic assay. The presence of cAMP during the final incubation period repressed the expression of ST-gal by a factor of 8-fold relative to the control (Table 1).

As an alternative method of determining the effect of extracellular cAMP on stalk cell differentiation in a strain where PKA is rendered constitutively active, we used the rdeC mutant HTY217. This is an important experiment because for the ecmA-C strain we can only infer, from its aberrant acts to induce competence to undergo stalk cell differentiation (Sonneborn et al., 1963; Abe and Yanagisawa, 1983). The precise defect in development, that the kinase is rendered constitutive by its over-expression. In HTY217, where the R subunit is replaced with fresh solution either containing or not containing 5 mM cAMP. Finally, 24 hours later, the level of expression of ST-gal was determined by enzymatic assay. The presence of cAMP during the final incubation period repressed the expression of ST-gal by a factor of 8-fold relative to the control (Table 1).

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phosphorylation of a protein required for tip formation. One candidate target is some component in the cAMP signalling system, since stimulation of adenyl cyclase by extracellular cAMP is inhibited when Rm, the dominant inhibitor of PKA, is expressed under the control of the Actin 15 promoter (Harwood et al., 1992a).

When PKA is inactivated in prestalk cells, by expression of Rm under control of the ecmA promoter, stalk formation is prevented and the ST-gal gene is not inducible by DIF (Harwood et al., 1992b). These observations led to the formulation of a model where activation of PKA acts as the switch that induces stalk cell differentiation (Harwood et al., 1992b). In its simplest form it predicts that rendering the C cell maturation. Stalk cell differentiation in wild-type cells is inhibited by extracellular cAMP (Berks and Kay, 1988, 1989) and presumably one of these other signalling candidates is some component in the cAMP signalling system, since stimulation of adenylyl cyclase by extracellular cAMP is inhibited when Rm, the dominant inhibitor of PKA, is expressed under the control of the Actin 15 promoter (Harwood et al., 1992a).

In its simplest form it predicts that rendering the C subunit constitutively active in prestalk cells will cause them to differentiate into stalk cells in an uncontrolled, precipitate manner. This prediction is only partly fulfilled. Cells within the ecmA-C aggregates differentiate to become pstAB cells, some of which have the appearance of vacuolated stalk cells. In contrast, during normal development expression of the ST-gal construct and subsequent stalk cell differentiation occur only within the stalk tube (Ceccarelli et al., 1991). However in ecmA-C aggregates, fewer than 0.1% of cells become stalk cells, while 10-15% of cells express the ecmA gene.

Clearly, some other condition must be fulfilled for stalk cell maturation. Stalk cell differentiation in wild-type cells is inhibited by extracellular cAMP (Berks and Kay, 1988, 1990) and we show here that extracellular cAMP also represses ST-gal expression in ecmA-C cells and in rdeC cells. This suggests very strongly that extracellular cAMP exerts its inhibitory effect on stalk cell differentiation via a signalling pathway other than the cAMP receptor:G-protein:adenyl cyclase pathway. Binding of cAMP to the surface receptor triggers several intracellular responses (reviewed by Janssens and Van Haastert, 1987; Firtel et al., 1989) and presumably one of these other signalling pathways is responsible for the cAMP inhibition of stalk cell differentiation.

We assume then that the bulk of prestalk cells within the tip of the aggregate fail to differentiate into stalk cells because extracellular cAMP levels never decrease; perhaps because the decrease is linked in some way to tip formation and/or elongation, processes that do not occur in ecmA-C mounds. One observation which is consistent with this notion is that ST-gal-expressing cells in ecmA-C mounds are primarily located outside of the aggregate (Fig. 3E) where cAMP signalling is presumably reduced.

How might the reduction in extracellular cAMP levels necessary for stalk cell differentiation be achieved during normal development? During aggregation, cAMP phosphodiesterase (PDE) is secreted in order to degrade the extracellular cAMP between signalling pulses. The single PDE gene utilizes three different promoters to regulate expression at different stages of development and one of these is specifically active in prestalk cells (Podgorski et al., 1989). If there were localised activation of PDE at the entrance to the stalk tube, this would be expected to produce a reduction in cAMP levels at this position. In support of this notion, microdissection experiments show that, during culmination, PDE is highly enriched within the stalk (Brown and Rutherford, 1980). Acting in combination with the increase in intracellular cAMP that is triggered by the drop in ammonia levels, the fall in extracellular cAMP concentration at the stalk tube entrance might then be expected to induce stalk cell differentiation.

If this scheme is correct then pattern formation during culmination involves both a diffusible signalling molecule, ammonia, and a non-diffusible signalling molecule, PDE.

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