Glycogen synthase kinase-3 (GSK-3) is regulated during Dictyostelium
development via the serpentine receptor cAR3

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SUMMARY

Glycogen synthase kinase-3 (GSK-3) is required during metazoan development to mediate the effects of the extracellular signal wingless/Wnt-1 and hence is necessary for correct cell type specification. GSK-3 also regulates cell fate during Dictyostelium development, but in this case it appears to mediate the effects of extracellular cAMP. By direct measurement of GSK-3 kinase activity during Dictyostelium development, we find that there is a rise in activity at the initiation of multicellular development which can be induced by cAMP. The timing of the rise correlates with the requirement for the Dictyostelium homologue of GSK-3, GSKA, to specify cell fate. We show that loss of the cAMP receptor cAR3 almost completely abolishes the rise in kinase activity and causes a mis-specification of cell fate that is equivalent to that seen in a gskA− mutant. The phenotype of a cAR3− mutant however is less severe than loss of gskA and ultimately gives rise to an apparently wild-type fruiting body. These results indicate that in Dictyostelium extracellular cAMP acts via cAR3 to cause a rise in GSKA kinase activity which regulates cell type patterning during the initial stages of multicellularity.

Key words: GSK-3 regulation, Pattern formation, Dictyostelium, cAMP, Glycogen synthase kinase-3

INTRODUCTION

The development of multicellular organisms requires the regulated spatial and temporal patterning of cell differentiation. GSK-3, a serine/threonine protein kinase, is essential for this process. This kinase appears to be present in all eukaryotes and shows strong sequence conservation within its catalytic core (Plyte et al., 1992). GSK-3 is multifunctional but during development is required for cell fate specification in organisms as diverse as Drosophila, Xenopus and Dictyostelium (Siegfried et al., 1992; Bourious et al., 1990; He et al., 1995; Harwood et al., 1995).

The developmental role of GSK-3 was first investigated in Drosophila where it is encoded by the Zeste-white3/shaggy (zw3/shg) gene (Siegfried et al., 1992; Bourious et al., 1990). The name shaggy refers to a phenotype seen in the zygote where all cells of the proneural cluster adopt a neural fate and the consequent increase in neurosensory bristles leads to a ‘shaggy’ appearance (Simpson et al., 1993). In the embryo zw3/shg plays a role in wingless (wg) signalling and hence its loss affects segment polarity and cell specification within the imaginal discs (Siegfried et al., 1994). This signalling pathway is conserved in nematodes and vertebrates (Cox and Peifer, 1998). The complex mixture of cell types that arise during metazoan development has made it impossible to directly measure GSK-3 kinase activity. Genetic evidence however suggests that wingless, and its Wnt-1 homologues, antagonise the effects of GSK-3. This hypothesis is supported by the observation that treatment of mouse fibroblasts with Wg protein leads to inactivation of GSK-3β (Cook et al., 1996). Although this suggests an inhibition of kinase activity in response to Wg/Wnt-1, it does not preclude other regulatory mechanisms that act during embryogenesis. In this paper we present evidence that an increase in GSK-3 activity occurs during Dictyostelium development and this is required for correct pattern formation.

Dictyostelium cells exhibit a very simple developmental program. Cells grow as a homogeneous population of unicellular amoebae which when starved aggregate to form a multicellular mass. The ultimate product of the developmental process is the fruiting body. This structure has three major elements: a large, single spore head; a stalk that supports the spore head and a basal disc that anchors the stalk to the substratum. Each element derives from a specific precursor cell population; spores derive from prespore, the stalk derives from prestalkA and prestalkO (pstA and pstO) and the basal disc derives from prestalkB cells (pstB) (Jermyn et al., 1989; Williams et al., 1989; Jermyn and Williams, 1991). Specification of these cell types occurs during the initial stages of multicellular development, as cells form a loosely packed
cellular aggregate known as the mound (Williams et al., 1989). GSKA, the Dictyostelium homologue of GSK-3, is required at the mound stage to regulate the formation of both prespore and pstB cells. In a mutant that lacks gskA, the pstB cell population within the mound is expanded and the prespore cell population reduced and hence the mutant ultimately gives rise to a fruiting body with a greatly enlarged basal disc and a tiny spore head (Harwood et al., 1995).

Dictyostelium cells utilise cAMP as an extracellular signal during development. During the early stages, prior to formation of the mound, low concentration pulses of cAMP act as a chemoattractant to coordinate aggregation. In the mound, extracellular cAMP levels rise and are required for post-aggregative gene expression. Extracellular cAMP induces prespore differentiation but represses pstB cell formation (Berks and Kay, 1990). In a mutant that lacks gskA both cAMP responses are affected simultaneously, so that in cultures of isolated cells prespore cell differentiation is severely reduced and cAMP mediated repression of pstB cell differentiation is lost entirely (Harwood et al., 1995). This suggests that both regulatory effects of extracellular cAMP act through a single signal transduction pathway that requires the gskA gene. Such a pathway could respond to the external concentration of cAMP and specify cell fate accordingly. In the simplest case, extracellular cAMP could directly regulate GSKA kinase activity (Harwood et al., 1995; Ginsburg and Kimmel, 1997).

This hypothesis makes two predictions. First, GSKA activity should be regulated during development. Prespore induction requires gskA and, because 80% of cells differentiate into prespore cells, GSKA activity would be expected to rise during mound formation. Second, a cAMP receptor should act upstream of GSKA to specify the prespore:pstB cell ratio. In this paper we show that in wild-type cells GSKA kinase activity does increase at the mound stage. Furthermore we report that the cAMP receptor cAR3 has the properties expected of an upstream activator of GSKA.

MATERIALS AND METHODS

Cell culture and development

The D. discoideum strains were grown at 22°C in shaking suspension in HL5 medium. Cells were transformed either by electroporation (Howard et al., 1988) or the CaPO4 method (Early and Williams, 1987) and selected in HL5 medium supplemented with heat killed [B/r] and 20 μg/ml tetracycline; pH 6.2) supplemented with 5 mM cAMP. After 20 hours the medium was removed, the cells washed three times and fresh medium was added, supplemented with 100 nM DIF and 5 mM cAMP or 100 μM cAMP-S (adenosine monophosphothioate, Sp-isomer) as described in the text (Berks and Kay, 1988). The number of differentiated stalk cells was scored after a further 24 hours. To induce spore differentiation, wild-type and mutant cells were washed in KK2 and plated at a density of 10⁶ cells/ml on tissue culture plates (Falcon 3001) in stalk medium (10 mM MES, 2 mM NaCl, 10 mM KCl, 2 mM MgSO4, 2 mM NaH2PO4, 40 mM KH2PO4, 10 mM MgSO4, 2 mM MgCl2) for 15 minutes. After washing twice in Z buffer, they were incubated in staining solution (0.1% X-gal, 5 mM potassium ferrocyanide and 5 mM potassium ferricyanide; pH 6.2) plus 15 mM 8-bromo-cAMP (Kay, 1989). The spore number was scored after 48 hours. All experiments were done at least in triplicate and all errors are shown as ±s.e.m.

β-galactosidase staining

Structures were fixed with 1% gluteraldehyde in Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 2 mM MgCl2) for 15 minutes. After washing twice in Z buffer, they were incubated in staining solution (0.1% X-gal, 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide in Z buffer) at 37°C (Dingermann et al., 1989).

RESULTS

GSKA kinase activity increases prior to mound formation in the wild type

GSKA activity can be detected in growing cells and during early stages of development; however its activity increases during mound formation to peak at approximately twice the level of that seen in growing cells (Fig. 1A). This correlates with the beginning of prespore and prestalk cell differentiation and is the developmental stage at which genetic evidence suggests that gskA is required to establish the prespore:pstB ratio (Harwood et al., 1995). Kinase activity measured from extract were incubated with the 20 μg of peptide substrate (RRRPSAVPPPSLRSRHS(PQ4)HQRR) in assay buffer (50 mM HEPES, pH 7.5, 4 mM MgCl2, 0.5 mM EGTA, 2 mM DTT, 100 μM ATP, [γ-32P]ATP to 500-1000 cts/minute per pmole) at room temperature in a final volume of 20 μl. Reactions were stopped after 8 minutes by addition of an equal volume of 15 mM phosphoric acid. Incorporation of [γ-32P]ATP was measured by binding to P81 phosphocellulose paper (Whatman), washing extensively with 7.5 mM phosphoric acid and scintillation counting. A second sample was assayed in the presence of 50 mM LiCl, a GSK-3 inhibitor, and specific activity was calculated by subtraction of this sample (background) from the original. Specific activity is expressed as pmole of phosphate transferred/μg of total protein in sample. Each experiment was repeated in quintuplicate and all errors are shown as ±s.e.m.
AR3 regulates GSK-3 in Dictyostelium

later stages of development varies between experiments, however the general trend is a decrease in kinase activity after 16 hours of development. This variation may be due to the asynchronous onset of fruiting body formation (culmination). The same pattern of kinase activity was found in all wild-type strains examined (data not shown, see also Fig. 4A). A developmental time course of the gskA− mutant was also tested and no significant GSKA kinase activity detected. Immunoblotting showed no evidence of a change in GSKA protein expression during development, suggesting that the observed increase in kinase activity is due to an increase of specific activity rather than a change in GSKA protein expression (Fig. 1B).

Identification of a cAMP receptor which represses stalk cell formation

To investigate the significance of the rise of GSKA kinase activity, we sought a mutant in which the rise failed to occur. As previous results suggested that gskA mediates the effects of extracellular cAMP on prespore and pstB cell-specific gene expression, we concentrated our search on the existing cAMP receptor mutants. Four cell surface cAMP receptors (cAR1-4) have been identified in Dictyostelium and are all closely related members of the serpentine receptor superfamily (Klein et al., 1987; Saxe et al., 1991). As an initial screen we used a stalk cell monolayer assay to test mutants in which individual receptors have been completely removed by gene disruption (Sun and Devreotes, 1991; Saxe et al., 1993; Johnson et al., 1993; Louis et al., 1994). In this assay, cells are plated as low density monolayers (10⁴ cells/cm²) and are incapable of differentiation without addition of cAMP and DIF (Berks and Kay, 1988). cAMP is added in the initial 20 hours of development, removed and the cells washed prior to addition of DIF, a potent inducer of stalk cell differentiation (Kay and Jermyn, 1983). After a further 24 hours in the presence of DIF, cells differentiate into stalk cells. If cAMP is included with DIF during this second period, stalk cell differentiation in the wild type is repressed. The gskA− mutant shows no cAMP repression under these conditions (Harwood et al., 1995). This assay requires cells to be competent for stalk cell differentiation and therefore mutants that arrest in very early stages of development or do not respond to DIF are eliminated from the analysis. This is the case for cells lacking the cAR1 receptor which is required for the cellular response to cAMP during early development (Fig. 2A).

Fig. 1. (A) A developmental time course of GSKA kinase activity for wild type (AX2) and a gskA− mutant. The wild-type developmental stages are indicated, the mound stage corresponds to 12 hours of development. The background activity seen in the gskA− mutant corresponds to non-specific kinase activity and the same level of activity is seen in the absence of GSK-3 substrate (Ryves et al., 1998). Kinase activity is shown as specific activity expressed as pmol of phosphate transferred/mg of total protein. (B) GSKA protein level examined by immunoblotting with anti-sgg antibody. GSKA protein is present throughout development of the wild type, but absent from a gskA− mutant.

Fig. 2. (A) Wild type, gskA− mutant and all cAR− mutants were tested for their ability to differentiate into stalk cells in low density monolayer culture in the absence or presence of 5 mM cAMP. (B) Wild type, cAR2− and cAR3− mutants were further tested for their ability to differentiate into stalk cells in monolayer culture in the absence or presence of 100 μM cAMP-S.
Loss of either cAR2 or cAR4 had no effect on the induction of stalk cell differentiation by DIF or repression by cAMP. The degree of cAMP repression seen in the cAR2\textsuperscript{−} mutant appeared to be greater than in wild type, but was not investigated further. Loss of cAR3 also had no effect on stalk cell differentiation in the absence of cAMP, but exhibited a complete loss of cAMP repression (Fig. 2A). The monolayer assay was repeated with a non-hydrolysable analogue of cAMP, cAMP-S. Stalk cell differentiation of both wild-type and cAR2\textsuperscript{−} mutant cells was repressed by cAMP-S, but under the same conditions cAR3\textsuperscript{−} mutant cells were able to differentiate (Fig. 2B). This indicates that the cAR3\textsuperscript{−} mutant does not escape cAMP repression by elevated degradation of extracellular cAMP.

The similarities between the phenotypes of cAR3\textsuperscript{−} and gskA\textsuperscript{−} mutants in monolayer culture suggested an interaction between these genes. To further investigate, we examined the expression of the cAR3 gene in the gskA\textsuperscript{−} mutant. cAR3 is expressed during late aggregation and multicellular stages of development in wild-type cells (Fig. 3A). Strong expression of cAR3 protein occurred slightly later in the gskA\textsuperscript{−} mutant, first seen at 10 hours after the initiation of development, but with longer film exposure cAR3 protein could be detected during late aggregation (Fig. 3A). To confirm that the small difference between wild-type and the gskA\textsuperscript{−} mutant is not significant, cAR3 expression was increased during the early stages of development. Ectopic expression of cAR3 from the actin15 promoter restores cAMP repression of stalk cell differentiation to the cAR3\textsuperscript{−} mutant. Expression in the gskA\textsuperscript{−} mutant had no effect on its phenotype (Fig. 3B,C). These observations indicate that the gskA\textsuperscript{−} mutant phenotype is not due to a failure to express cAR3 protein and suggest that cAR3 acts upstream of gskA.

**Loss of cAR3 attenuates GSKA kinase activation**

We examined developmental regulation of GSKA activity in the cAR3\textsuperscript{−} mutant (Fig. 4A). GSKA kinase activity in growing cells is indistinguishable from that in wild type. Kinase activity in the mutant continues to follow the wild-type pattern until 8 hours of development. At this point GSKA kinase activity has risen to a level 20% higher than that of growing cells. The cAR3\textsuperscript{−} mutant however does not increase kinase activity further and hence fails to show the 200% increase seen in the mound of wild-type cells. The failure to increase GSKA kinase activity beyond 8 hours of development corresponds to the time where we first detect cAR3 gene expression (Fig. 3A). Immunoblotting indicated wild-type levels of GSKA protein during development of the cAR3\textsuperscript{−} mutant and demonstrates that the failure to increase kinase activity is not due to a reduction in GSKA expression (Fig. 4B).

To further examine the regulation of GSKA, isolated wild-type cells were stimulated with cAMP (Fig. 4C). This caused a 60% increase in GSKA kinase activity. The slightly lower magnitude of the increase compared to that seen during normal development may be due to suboptimal induction conditions. cAMP induction of a cAR3\textsuperscript{−} mutant failed to increase GSKA kinase activity. This is consistent with our observations made during development and suggests that for isolated cells under these conditions the cAR3 receptor is the major cAMP receptor mediating GSKA stimulation.

**Increased expression of GSKA does not block stalk cell formation**

Our results suggest that a two-fold increase in GSKA kinase activity is sufficient to mediate the effects of extracellular cAMP on stalk cell development. To investigate whether this response could be mimicked by artificially raising the cellular
Fig 4. (A) A time course of GSKA kinase activity for wild type (AX3) and the cAR3- mutant developed on nitrocellulose. The wild-type developmental stages are indicated. The cAR3- mutant fails to increase GSKA activity after 8 hours of development. Kinase activity is shown as specific activity expressed as pmoles of phosphate transferred/ug of total protein. (B) Immunoblotting of samples from the cAR3- mutant with anti-sgg antibody show a constant level of GSK-A protein throughout development. Comparison is shown to wild-type levels at 12 hours of development. (C) Cells were developed for 6 hours on KK2-agar, disaggregated and plated at 10^7 cells/ml in stalk medium. Cells were treated with 5 mM cAMP for 15 minutes as indicated and GSKA kinase activity measured. Western blotting demonstrates no change in protein expression during stimulation. 0, initial kinase activity; +, cAMP stimulation for 15 minutes; −, no stimulation for 15 minutes.

Fig 5. (A) gskA- mutant cells expressing the gskA cDNA from an actin15 promoter (gskA+GSKA) were tested for their ability to differentiate into stalk cells in monolayer culture in the absence or presence of 5 mM cAMP. The untransformed gskA- mutant was used as a control. (B) Samples from growth phase wild-type, gskA- mutant and gskA+GSKA cells were immunoblotted with anti-sgg antibody. The expressed GSKA protein is larger than wild type due to the inclusion of a 6xHistidine tag. (C) Samples from growth phase wild-type, gskA- and the transformants, wild-type+GSKA and cAR3+GSKA cells were immunoblotted with anti-sgg antibody. Both endogenous and expressed proteins are present in wildtype+GSKA and cAR3+GSKA cells. In both cases the total amount exceeds protein expression in untransformed wild-type cells. (D) GSKA kinase activity of wild-type+GSKA and cAR3+GSKA cells measured in growing cells and those developed to the mound stage. The levels of activity are similar to those seen for untransformed cells (Figs 1A, 4A). (E) Wild-type and cAR3- mutant cells expressing a gskA cDNA from an actin15 promoter (wild-type+GSKA and cAR3+GSKA) were tested for their ability to differentiate into stalk cells in monolayer.
level of GS KA protein, the gskA cDNA was expressed from the actin 15 promoter. When expressed in the gskA mutant, the gskA cDNA was sufficient to restore cAMP repression in monolayer culture (Fig. 5A, B) and results in normal multicellular development (data not shown). Expression of GS KA had no effect on stalk cell formation in the absence of cAMP. Expression of gskA cDNA in wild-type cells more than doubled the level of GS KA protein (Fig. 5C). If the cellular concentration of GS KA protein was a major determinant of kinase activity, doubling GS KA expression would be expected to give elevated kinase activity and hence repress stalk cell differentiation even in the absence of cAMP. Measurements show that the level of GS KA kinase activity of these transformant cells is no higher than in the wild type (Figs 1A, 4A) and that again a two-fold increase in kinase activity occurs as cells enter the multicellular stages of development (Fig. 5D). In addition these cells show wild-type development. When tested in low density cultures, stalk cell differentiation is neither reduced in the absence of cAMP nor is it hypersensitive to addition of cAMP (Fig. 5E). This demonstrates that elevation of GS KA expression does not affect regulation of GS KA kinase activity and hence stalk cell formation, suggesting that in Dictyostelium GS KA-3 activity is tightly regulated.

Elevation of GS KA activity requires extracellular cAMP and hence ectopic expression of GS KA does not rescue the cAR3 mutant (Fig. 5C). Again ectopic expression during growth had no effect on the level of GS KA kinase activity in the cAR3 mutant. When the same cells were developed to mound stage however a much smaller increase of GS KA activity occurs. This fits the pattern observed for untransformed cAR3 mutant cells (Fig. 5D). In addition ectopic expression of gskA does not restore cAMP repression of stalk cell differentiation in low cell density monolayer assays (Fig. 5E). In this situation although the intracellular level of GS KA protein is increased, the ability to respond to extracellular cAMP is impaired and stalk cell repression cannot occur. It has not been possible to directly measure kinase activity in these low density cultures, but extrapolating from our other measurements of kinase activity we infer that extracellular cAMP regulates stalk cell formation by controlling GS KA activity and not its level of expression.

The cAR3 mutant has the same characteristics as the gskA mutant

Our initial investigation of the cAR3 mutant focused on stalk cell differentiation in monolayer culture. We therefore examined the cAR3 mutant for other similarities to the gskA mutant. The gskA mutant phenotype has two other characteristics; few spores differentiate in monolayer culture and pstB and prespore cell fates are mis-specified in the mound (Harwood et al., 1995). When wild-type or cAR2 mutant cells are incubated with 8-bromo-cAMP for 24 hours more than 25% of cells differentiate into spores (Fig. 6A). In the gskA mutant this figure is reduced to less than 5%. A substantial reduction in spore differentiation is also seen with the cAR3 mutant cells (Fig. 6A), although in this case approximately 10% of cells differentiated into spores.

We next examined the numbers of pstB and prespore cells in the cAR3 mutant at the mound stage using lacZ reporter genes. cAR3 mutant cells were transformed with either ecmB-lacZ, which at this stage is expressed only in pstB cells, or the prespore-specific marker psA-lacZ. (Williams et al., 1989). Cells were developed to the mound stage, disaggregated and then stained for β-galactosidase expression. When examined in

Fig. 6. (A) Wild-type, gskA-, cAR2- and cAR3- mutant cells were tested for their ability to differentiate into spores when treated with 8-bromo-cAMP in low density monolayer culture. (B) Cells were developed to the mound stage, disaggregated and then stained for β-galactosidase expression. Stained cells were counted and expressed as a percentage of the total number ± s.e.m.

Fig. 7. Wild-type (AX3) and cAR3- mutant cells transformed with ecmB-lacZ, a pstB cell marker, or psA-lacZ, a prespore cell marker, were developed to mound stage on nitrocellulose filters (reached approx. 12 hours after plating). All photographs show planar view of whole mounts. (A) ecmB-lacZ in wild type. (B) ecmB-lacZ in the cAR3- mutant. (C) psA-lacZ in wild type. (D) psA-lacZ in the cAR3- mutant.
this way the cAR3\(^{-}\) mutant shows a substantial increase in the number of cells that express ecmB-lacZ and a reduction in those that express psA-lacZ. (Fig. 6B). Again loss of cAR3 has a less severe effect on prespore differentiation than loss of gskA; cAR3\(^{-}\) and gskA\(^{-}\) mutants have 50% and 5% of the wild-type number of prespore cells respectively (Harwood et al., 1995). Stained whole mounts of the cAR3\(^{-}\) mutant transformed with ecmB-lacZ have an expanded population of pstB cells scattered throughout the mound (Fig. 7A,B). In contrast, prespore cells in the cAR3\(^{-}\) mutant are restricted to the central region of the mound and an expanded region of unstained cells is present at the base of mound. In planar view this appears as a ring around the stained cells (Fig. 7C,D). This unstained region is often present in wild-type cells, however it is enlarged in the cAR3\(^{-}\) mutant. A similar clustering of prespore cells is also seen in the gskA\(^{-}\) mutant (Harwood et al., 1995). These observations suggest that although cell specification is altered in both cAR3\(^{-}\) and gskA\(^{-}\) mutants, cell sorting still occurs. The cAR3\(^{-}\) mutant phenotype therefore resembles that of the gskA\(^{-}\) mutant by every known criterion (Harwood et al., 1995). Some aspects of the cAR3\(^{-}\) mutant phenotype, however, appear to be weaker than in the gskA\(^{-}\) mutant.

**The cAR3\(^{-}\) mutant has a prolonged mound stage but forms a wild-type fruiting body**

The finding that cAR3 regulates GSKA was surprising as the cAR3\(^{-}\) mutant had previously been assigned no phenotype. Consistent with these earlier observations we also found that the morphology of the fruiting body appeared identical to that of wild-type cells. We therefore examined the morphological changes that occur in cAR3\(^{-}\) mutant prior to culmination. We discovered that when developed on nitrocellulose, a commonly used substratum, cAR3\(^{-}\) mutant cells spend a prolonged period at the mound stage before proceeding with development (Fig. 8). This is exactly

![Fig. 8](image-url)  
**Fig. 8.** Wild-type (AX3) and cAR3\(^{-}\) mutant cells were observed during development on nitrocellulose. Mounds form at the same time (12 hours) in both the wild-type and cAR3\(^{-}\) mutant, but the next stage (tip formation) is delayed in the cAR3\(^{-}\) mutant by approximately 3 hours. All photographs are shown at the same magnification.

![Fig. 9](image-url)  
**Fig. 9.** Wild-type (AX3) and cAR3\(^{-}\) mutant cells transformed with ecmB-lacZ or psA-lacZ were examined after prolonged development on nitrocellulose. (A) A cAR3\(^{-}\) mutant slug stained for psA-lacZ. * indicates the rearguard. (B) A wild-type slug stained for psA-lacZ. * indicates the rearguard. (C) A migrating cAR3\(^{-}\) mutant slug. A large deposit of cells remains at the initial aggregation position (arrow) and others are lost along the migration path (dotted line). (D) A cAR3\(^{-}\) mutant slug stained for ecmB-lacZ. Stained cells are discarded as the slug moves from its initial aggregation position (arrow). The anterior prestalk region (pst) is heavily stained and there are a large number of stained cells in the prespore region (psp). (E) A wild-type slug stained for ecmB-lacZ. Stained cells are concentrated into a ‘core’ within the anterior of the slug or scattered throughout the anterior region. Very few cells are lost at the rear (arrow). (F) A cAR3\(^{-}\) mutant during early culmination (a preculminant) stained for ecmB-lacZ. All prestalk regions (pst) are heavily stained and there are large number of stained cells in the prespore region (psp).
the developmental stage when gskA is required for cell patterning and where misproportioning of pstB and prespore cells is seen in the cAR3-mutant.

This raises the question of how the cAR3-mutant ultimately forms a correctly proportioned fruiting body. We found a number of unusual features of later development in the cAR3-mutant which could explain this process. After a prolonged period at the mound stage, the majority of mounds derived from cAR3-mutant cells give rise to slugs. Wild-type cells rarely form slugs under these developmental conditions. Slugs are motile structures which in the wild type have a distinctive pattern of cell types. Prespore cells are found in the posterior and prestalk cells in the anterior region of the slug. During slug migration prespore cells may trans-differentiate and enter the anterior. When marked by pslA-lacZ, these cells are visible as scattered cells in the anterior (Harwood et al., 1991). A similar anterior-posterior patterning is also present in the cAR3-mutant slugs, but there are fewer pslA-lacZ stained cells (Fig. 9A,B). The most striking feature is that prespore cells are excluded from a large region in the rear of the slug. Wild-type slugs often possess a small region that contains prestalk cells at their rear. This region known as the rearguard, is usually lost during slug migration. Slugs derived from cAR3-mutant cells shed large numbers cells from the rearguard as they migrate. These lost cells are visible on the substratum both at the beginning of the migration path and along its length (Fig. 9C). Furthermore there is an expanded zone of cells expressing ecmB-lacZ in the rear of the cAR3-mutant slug and in those cells deposited during migration (Fig. 9D,E). This suggests that cAR3-mutant slugs initially have an expanded rearguard which is lost as the slug migrates. Preferential loss of these prestalk cells during slug migration would readjust the cell type ratio towards wild-type proportions.

Despite loss of prestalk cells during migration, cAR3-mutant slugs and later developmental stages still contain increased numbers of ecmB-lacZ staining cells (Fig. 9D-F). In the wild type ecmB-lacZ staining is confined to a cone-shaped region within the tip of the slug and a small population of cells scattered throughout the prespore region. In the culminant these stained cells localise to the stalk tube, upper and lower cup regions and basal disc (Williams et al., 1989). In cAR3-mutant slugs and culminants staining is present in all prestalk and many prespore cells (Fig. 9D,F). This additional staining could reflect ectopic expression of ecmB in which case we are observing mis-specification of cell types during later stages of development. The β-galactosidase marker used in this study however is stable during Dictyostelium development and persists in cells that are no longer transcriptionally active; staining therefore marks the original specification of cell type and not necessarily the current set of expressed genes. As the morphology of the cAR3-mutant fruiting body appears wild type, we suggest that a more likely explanation for the additional ecmB-lacZ-stained cells seen in the cAR3-mutant is that they arose at the mound stage but then trans-differentiated into prespore and other types of prestalk cells. To summarise, we observe that the cAR3-mutant has an initial patterning defect which retards exit from the mound stage; eventually the wild-type pattern is restored and we propose that this occurs by the combined effects of preferential loss of prestalk cells from the rearguard and trans-differentiation of pstB cells to prespore and other prestalk cell types.

DISCUSSION

In this paper, we present both genetic and biochemical evidence to demonstrate that GSKA kinase activity is upregulated during Dictyostelium development and is under the control of the cAMP receptor, cAR3. Direct measurement of GSKA kinase activity detects a two-fold increase in kinase activity as cells form the multicellular mound. The timing of this increase correlates with the requirement of gskA for cell type specification at the mound stage. We see no change in GSKA protein levels during development, and this suggests that the kinase is under post-translational control. In support of this conclusion we find that increased expression of gskA does not mimic cAMP repression of stalk cell differentiation. Although only a relatively small increase in activity is seen during multicellular development, this appears to be an important factor for cell fate determination within the mound as the attenuation of kinase activation in the cAR3-mutant results in mis-specification of cell fate. These results are consistent with our previous observations that extracellular cAMP regulates prespore and pstB cell gene expression via gskA (Harwood et al., 1995). We have further shown that in isolated cells extracellular cAMP will stimulate an increase in GSKA activity by a process which requires the cAMP receptor cAR3. This suggests a direct interaction between cAMP stimulation and GSKA activation, however at this point we cannot exclude the possibility that cAMP leads to the rapid release of a second factor which in turn regulates kinase activity. The pattern of cAR3 expression is consistent with its proposed role in the regulation of GSKA. cAR3 is expressed from late aggregation and appears to be the major cAR expressed in prespore cells (Yu and Saxe, 1996; Rogers et al., 1997). In our experiments increased kinase activity correlates with cAR3 expression. By immunoblotting we first detect cAR3 protein at 8 hours of development, the same time that we detect a rise in GSKA kinase activity. In the cAR3-mutant the profile of GSKA activity initially follows the wild type, but then shows no further increase in activity beyond 8 hours, resulting in a substantial reduction of the peak of activity seen at the mound stage. When combined with our genetic evidence, this argues that cAR3 acts upstream of GSKA to regulate kinase activity. The cAR3-mutant has been reported to have no distinct phenotype (Johnson et al., 1993); however our closer examination of differentiation both in monolayer culture and by use of cell-type specific markers indicates that by all known criteria it has a phenotype like that of the gskA-mutant. The cAR3-phenoype however is less severe than that seen in the gskA-mutant and the cAR3-mutant ultimately forms a fruiting body with an apparent wild-type morphology. This may be due to the presence of the residual GSKA activity seen during cAR3-mutant development, the effects of which could perhaps accumulate during the prolonged mound stage until development can proceed. The weaker phenotype of the cAR3-mutant suggests that there is a functional redundancy with other cAR proteins. A likely candidate is cAR1; the receptor that we could not test due to its requirement during the early stages of development. cAR3 has been shown to be redundant with cAR1 during aggregation (Insall et al., 1994) and as cAR1 is still expressed during multicellular development there is no reason to believe that this redundancy would not persist.
To our knowledge this is the first evidence for a ligand-stimulated elevation of GSK-3 activity and the first direct biochemical evidence of developmental regulation of GSK-3 kinase activity. Our findings contrast to those found for Xnt-1 signalling; here genetic evidence suggests that Xnt-1 stimulation may lead to a decrease in kinase activity. This difference could mean that Dictyostelium and metazoan GSK-3 proteins are regulated by different signalling pathways. A more interesting prospect is that these differences reflect different aspects of a conserved regulatory network. There are similarities between GSK-3 regulation in both groups of organisms; both pathways are regulated by an extracellular signal acting through serpentine membrane receptors (cARs and Frizzleds). Furthermore there is no evidence in metazoa to preclude the existence of an extracellular signal that increases kinase activity. Hence elevated kinase activity will counteract the effects of Xnt-1 stimulation may lead to a decrease in kinase activity. This precludes the existence of an extracellular signal that increases kinase activity. Our findings contrast to those found for Wnt-1 signalling in Xenopus embryos have demonstrated that injection of wild-type GSK-3 cDNA and hence elevated kinase activity will counteract the effects of Xnt-1 (He et al., 1995). In addition, an inhibitory signal transduction pathway could exist in Dictyostelium (Ginsburg and Kimmel, 1997) but it would not have been detected by the analysis presented in this paper. The simplicity of Dictyostelium development, its genetic tractability and amenability to biochemical analysis will enable further understanding of GSK-3 regulation.

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cAR3 regulates GSK-3 in Dictyostelium 333