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# The Phosphorylated C-Terminus of cAR1 Plays a Role in Cell-Type-Specific Gene Expression and STATa Tyrosine Phosphorylation

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**cAMP receptors mediate some signaling pathways via coupled heterotrimeric G proteins, while others are G-protein-independent. This latter class includes the activation of the transcription factors GBF and STATa. Within the cellular mounds formed by aggregation of *Dictyostelium*, micromolar levels of cAMP activate GBF function, thereby inducing the transcription of postaggregative genes and initiating multicellular differentiation. Activation of STATa, a regulator of culmination and *ecmB* expression, results from cAMP receptor-dependent tyrosine phosphorylation and nuclear localization, also in mound-stage cells. During mound development, the cAMP receptor cAR1 is in a low-affinity state and is phosphorylated on multiple serine residues in its C-terminus. This paper addresses possible roles of cAMP receptor phosphorylation in the cAMP-mediated stimulation of GBF activity, STATa tyrosine phosphorylation, and cell-type-specific gene expression. To accomplish this, we have expressed cAR1 mutants in a strain in which the endogenous cAMP receptors that mediate postaggregative gene expression *in vivo* are deleted. We then examined the ability of these cells to undergo morphogenesis and induce postaggregative and cell-type-specific gene expression and STATa tyrosine phosphorylation. Analysis of cAR1 mutants in which the C-terminal tail is deleted or the ligand-mediated phosphorylation sites are mutated suggests that the cAR1 C-terminus is not essential for GBF-mediated postaggregative gene expression or STATa tyrosine phosphorylation, but may play a role in regulating cell-type-specific gene expression and morphogenesis. A mutant receptor, in which the C-terminal tail is constitutively phosphorylated, exhibits constitutive activation of STATa tyrosine phosphorylation in pulsed cells in suspension and a significantly impaired ability to induce cell-type-specific gene expression. The constitutively phosphorylated receptor also exerts a partial dominant negative effect on multicellular development when expressed in wild-type cells. These findings suggest that the phosphorylated C-terminus of cAR1 may be involved in regulating aspects of receptor-mediated processes, is not essential for GBF function, and may play a role in mediating subsequent development.** 

***Key Words: Dictyostelium;* cAMP; receptor; GBF; gene expression; STAT.**

## INTRODUCTION

*Dictyostelium* development can be separated into two distinct phases (Chen *et al.*, 1996; Firtel, 1995; Loomis, 1996; Williams, 1995). In the first stage, ~$10^5$ cells chemotactically aggregate to form a mound in response to nanomolar pulses of cAMP. During the second stage, rising levels of cAMP within the mound induce cells to express a series of postaggregative genes (Brown and Firtel, 1999).

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Products of some of these genes, in combination with high extracellular cAMP and other morphogens such as DIF, are thought to lead to differentiation of prespore cells and several subclasses of prestalk cells, followed by morphogenesis (Aubry and Firtel, 1999; Early *et al.*, 1993). The cellular response to cAMP is mediated by a family of four cell-surface, seven-span, G-protein-coupled receptors (cARs), which have distinct temporal and spatial patterns of expression and different binding affinities (Kim *et al.*, 1998; Verkerke-van Wijk *et al.*, 1998; Rogers *et al.*, 1997).

Aggregation-stage signaling pathways are mediated predominantly through cAR1, the highest-affinity cAMP receptor, which acts through the heterotrimeric G protein containing the G$\alpha$2 subunit (Chen *et al.*, 1996; Devreotes, 1994; Firtel, 1995; Rogers *et al.*, 1997). Nanomolar cAMP pulses stimulate the activation of adenylyl and guanylyl cyclases, which function in pathways that control the propagation of the cAMP signal (signal relay) and chemotaxis, respectively. Furthermore, the pulsatile, nanomolar cAMP signal induces the expression of aggregation-stage genes (e.g., genes encoding cAR1, G$\alpha$2, and the cell adhesion molecule CsA), as well as stimulating cAMP-mediated $Ca^{2+}$ influx, activation of the MAP kinase ERK, and phosphorylation of the receptor on serine residues in its C-terminal tail (Chen *et al.*, 1996; Devreotes, 1994; Firtel, 1995; Hereld *et al.*, 1994). Although the heterotrimeric G protein containing the G$\alpha$2 subunit is essential for aggregation and the activation of adenylyl and guanylyl cyclases, other receptor-regulated pathways, which include cAMP-mediated $Ca^{2+}$ influx, ERK2 activation, ligand-mediated receptor phosphorylation, and activation of the GBF and STATa transcription factors, are G-protein-independent (Araki *et al.*, 1998; Maeda *et al.*, 1996; Milne *et al.*, 1995; Schnitzler *et al.*, 1995). Thus, aggregation is controlled by cAR1 through both G-protein-dependent and -independent effector pathways.

One of the key features that controls the directed chemotaxis of cells toward an aggregation center is the cycling of the receptor and coupled signaling pathways between adapted and de-adapted states. Ligand binding to the cARs rapidly activates the downstream signaling pathways and leads to desensitization of the receptor and adaptation of effector pathways with kinetics that are pathway-specific. Examination of receptor mutants, in which the C-terminal tail is deleted or all potential C-terminal phosphorylation sites are mutated, revealed that receptor phosphorylation is associated with a reduction in the receptor's affinity (e.g., loss of ligand binding; Caterina *et al.*, 1995a,b; Kim *et al.*, 1997), but is not required for adaptation of the effector pathways; the pathways remain adapted as long as cAMP is present. The extracellular cAMP ligand is rapidly degraded by an extracellular phosphodiesterase, leading to dephosphorylation, resensitization of the receptors, and de-adaptation of effector pathways within 6 min. As the mound forms, the level of extracellular cAMP is thought to rise to micromolar levels (Abe and Yanagisawa, 1983). High extracellular cAMP represses the expression of aggregation-stage genes and induces the expression of postaggregative-stage and cell-type-specific genes (Firtel, 1995). This response is mediated through the transcription factor GBF, whose function is required for postaggregative and cell-type-specific gene expression and subsequent morphogenesis (Schnitzler *et al.*, 1994). Constitutive expression of GBF is sufficient to enable cAMP to induce the postaggregative/cell-type-specific genes in vegetative cells in a receptor-dependent fashion. This experimental technique, therefore, temporally and morphologically separates induction of postaggregative gene expression from aggregation and multicellular development. GBF activation employs the same cAMP receptors required for aggregation but functions via a G-protein-independent pathway (Schnitzler *et al.*, 1995). Thus, one of the key features of *Dictyostelium* development is that the presentation of the ligand in the form of a low pulsatile signal (normally during aggregation) or at a high, sustained level (in the mound) can induce different signaling pathways within the same cell, resulting in distinct biological responses.

An additional cAMP-receptor-dependent and G-protein-independent pathway stimulated by the high levels of cAMP present during mound formation is activation of the transcription factor Dd-STATa (Araki *et al.*, 1998; Mohanty *et al.*, 1999). Dd-STATa is rapidly tyrosine phosphorylated and translocated to the nucleus in all mound-stage cells, although during the slug stage, nuclear enrichment is observed at high levels only in cells present in the anterior prestalk A domain. Dd-STATa binds to activator elements in the *ecmA* promoter and two repressor elements in the *ecmB* promoter; *in vivo* it acts as a repressor for *ecmB* (Kawata *et al.*, 1996; Mohanty *et al.*, 1999). *Dd-STATa*-null cells show defects in chemotaxis and in the negative regulation of *ecmB* expression (*ecmB* is expressed throughout the prestalk region), with little effect on *ecmA* expression apart from alterations in spatial patterning (Mohanty *et al.*, 1999). Dd-STATa is rapidly tyrosine phosphorylated in response to cAMP in cells starved or pulsed for 4–6 h in *in vitro* suspension assays, allowing the kinetics of Dd-STATa activation to be compared between wild-type cells and cells expressing mutations in cAR1 or the downstream signaling pathway.

In this article, we investigate the effect of cAR1 mutations on multicellular development, the induction of GBF-mediated gene expression, and STATa tyrosine phosphorylation in response to cAMP. Our results show that although cAMP-mediated GBF function is not affected, the postaggregative stage of the developmental program is impaired in cells expressing mutant receptors, with STATa tyrosine phosphorylation being affected by a constitutively phosphorylated receptor. Furthermore, the constitutively phosphorylated receptor mutant exhibits a dominant phenotype when expressed in wild-type cells.

## MATERIALS AND METHODS

### *Cell Culture and Molecular Biological Procedures*

All cell culture procedures and molecular techniques used have been described previously (Schnitzler *et al.,* 1994). Individual details are described in the figure legends.

### *Construction of Strains Constitutively Expressing GBF*

The Act15-GBF expression vector has been previously described (Schnitzler *et al.,* 1994). Individual strains were transformed with this vector by electroporation, selecting for G418 resistance.

### *Construction of Strains Constitutively Expressing cAR1 Mutants*

*car1/3*-null cells and KAx-3 cells were transformed with the extrachromosomal vector (Ddp1 origin of replication) by electroporation, selecting for G418 resistance (40 $\mu$g/ml for transformations in *car1/3*-null cells and 30 $\mu$g/ml for the transformations in KAx-3 cells). Individual clones were picked from colonies which were able to grow on a lawn of *Escherichia coli* B/r containing 80 $\mu$g/ml G418, on which cells from the whole population of transformants had been plated.

### *STATa Immunoprecipitations*

Exponentially growing cells were pulsed in potassium phosphate buffer (pH 6.2) with 30 nM cAMP every 6 min for 4 h, before being treated with 300 $\mu$M cAMP for the times indicated. Cells ($2 \times 10^7$) were pelleted and resuspended in 1 ml NP-40 cell lysis buffer (1× PBS, pH 7.4, 50 mM NaF, 1 mM vanadate, 1% NP-40, 2 mM EDTA, pH 7.2, 1 mM sodium pyrophosphate, 1.6 $\mu$g/ml leupeptin, 4 $\mu$g/ml aprotinin). Cells were allowed to lyse on ice for 5 min before centrifugation at 15K rpm for 10 min at 4°C. Monoclonal anti-STATa (2.5 $\mu$l) was added to the supernatant and incubated on a slow rocker for 1 h at 4°C before addition of 40 $\mu$l of a 50% slurry of protein A beads and incubation for a further 45 min. Beads were washed four times in NP-40 buffer, 50 $\mu$l SDS sample buffer was added, and the mixture was boiled for 3 min. Western blots of immunoprecipitated STATa proteins were performed using anti-phosphotyrosine antibody. Blots were then stripped and reprobed with the anti-STATa antibody. Results were visualized by ECL.

## RESULTS

### *The C-Terminus of cAR1 Is Important for Cell-Type-Specific Gene Expression but Is Not Essential for the Activation of GBF*

Three mutant cAR1 cAMP receptors that have altered patterns of ligand-mediated phosphorylation of the C-terminal cytoplasmic tail have been previously described (Caterina *et al.,* 1995a,b; Kim and Devreotes, 1994). Two of these do not exhibit C-terminal tail phosphorylation: cAR1-289T, in which the C-terminal cytoplasmic tail has been deleted at residue 289, and cAR1-cm1234 (Caterina *et al.,* 1995a,b), in which all the serine residues of cAR1 that are normally phosphorylated in response to cAMP have been deleted or mutated to glycine or alanine. The third mutant, cAR1-106C, is a chimera in which the N-terminal 40% of the protein is from cAR2, with the remaining C-terminal part from cAR1. cAR1-106C is constitutively phosphorylated, presumably because its conformation mimics that of ligand-occupied cAR1 (Kim and Devreotes, 1994). In previous work, all three receptors were constitutively expressed from the *Actin 15* (*Act15*) promoter in cells in which the genes encoding cAR1 and cAR3, the two cARs expressed during aggregation, had been disrupted. Cells lacking both cAR1 and cAR3 (*car1/3*-null cells) do not aggregate and exhibit no cAMP-mediated responses. Expression of cAR1-289T and cAR1-cm1234 in *car1/3*-null cells complements the *car1/3*-null cells' aggregation defect, but the cells lack agonist-induced loss-of-ligand binding. In contrast, *car1/3*-null cells transformed with the constitutively phosphorylated cAR1 mutant 106C were unable to aggregate. Figure 1 shows a cartoon of the structure of these receptors.

Figure 2 depicts the developmental phenotypes of *car1/3*-null cells expressing either wild-type cAR1 or the mutant receptors and the expression levels of the postaggregative gene *LagC*, the prestalk-specific gene *ecmA*, and the prespore-specific gene *SP60/CotC* during development on nonnutrient $NaPO_4$-buffered agar. *car1/3*-null cells expressing wild-type cAR1 aggregate, form slugs, and culminate with normal developmental timing. Consistent with published results (Insall *et al.,* 1994; Soede *et al.,* 1994), the timing of induction and the level of expression of the assayed genes in these cells were indistinguishable from those which have been observed in wild-type cells (data not shown; Fig. 2; Dynes *et al.,* 1994; Insall *et al.,* 1994; Soede *et al.,* 1994; Williams *et al.,* 1987).

*car1/3*-null:*Act15-cm1234* cells aggregate normally as previously described and form mounds (Kim *et al.,* 1997). As shown in Fig. 2, many of the aggregates are delayed at the mound stage at 24 h, but eventually all mutants form fruiting bodies by 48 h (data not shown). In contrast to wild-type cells or *car1/3*-null:*Act15-cAR1* cells, development of *cAR1/3*-null:*Act15-cm1234* cells beyond the mound stage is very asynchronous. *LagC* and the cell-type-specific genes *SP60/cotC* and *ecmA* are expressed, although the level of expression of *ecmA* is reduced and *LagC* mRNA was not detected after 16 h of development. These results suggest that the cAR1 C-terminal serine residues, normally phosphorylated in response to cAMP and constitutively phosphorylated in the multicellular stages (Vaughan and Devreotes, 1988), are not essential for GBF activation or cell-type-specific gene expression, though receptor phosphorylation may be involved in controlling some aspects of multicellular differentiation or the timing of development. Whereas most clonal isolates that were examined had a similar phenotype, the morphological phenotype ranged from complete mound arrest to almost wild-type (data not shown).

To further examine the possible role of the cAR1 C-terminus in postaggregative gene expression, we exam-

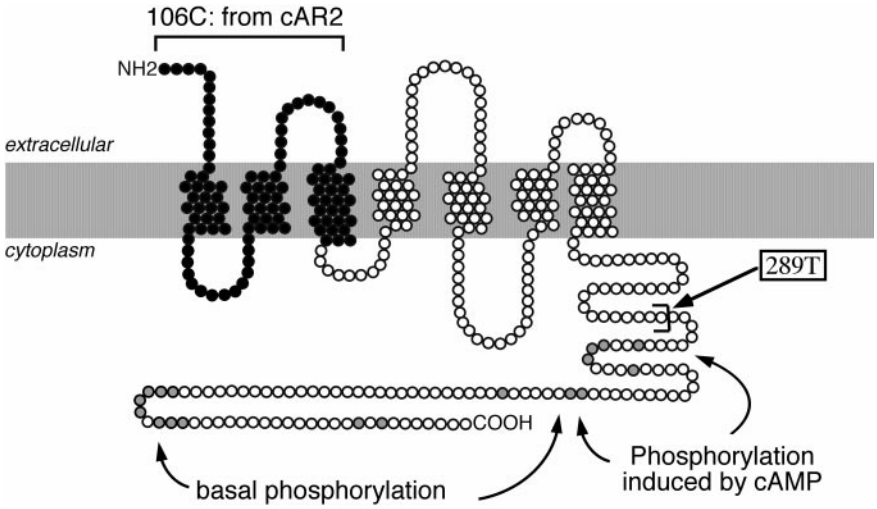


**FIG. 1.** Diagram depicting the cAR1 receptor and the mutants 289T and 106C. A schematic diagram of cAR1 is shown. All 18 serine residues in the C-terminal domain are shown as gray-filled circles. Serine clusters containing sites of basal and cAMP-induced phosphorylation are indicated. Mutant 289T is a deletion of the C-terminal tail from residue 289 (Caterina *et al.*, 1995a). Mutant 106C is a chimera between cAR2 and cAR1, with the first three N-terminal transmembrane regions being from cAR2 and the remainder of the receptor from cAR1 (Kim and Devreotes, 1994). The cAR2-derived sequences of 106C are blackened. The 106C mutant is constitutively phosphorylated when expressed in cells.

ined *car1/3*-null cells expressing cAR1-289T. *car1/3*-null:*Act15-289T* cells showed a moderate aggregation defect. Development was delayed and not all of the cells entered the mound (Fig. 2). Eventually, after about 30 h, the mounds started to dissipate and, after 2–3 days, a few small fruiting bodies were apparent among the dispersed cells (data not shown). *LagC* was induced to a level similar to that of *car1/3*null:*Act15-cAR1* cells, but the appearance of the transcript was delayed, consistent with delayed aggregation. The level of *LagC* expression remained high through 24 h, which is consistent with a developmental arrest at the mound stage. *SP60/cotC* expression was also significantly delayed and the level of expression was very low. No *ecmA* expression was observed. These data indicate that *car1/3*-null:*Act15-289T* cells have a deficiency in cell-type-specific gene expression. As the expression of *LagC,* which depends on cAR signaling (Firtel, 1995), extends through 24 h, we expect that the inability of these cells to express cell-type-specific genes is not due to an inability to induce earlier, postaggregative genes such as *LagC*. cAR1-289T and cAR1-cm1234 thus produce a pattern of developmental defects that indicates a role for the C-terminal tail, and possibly its phosphorylation, in morphogenesis and the expression of cell-type-specific genes. These defects are manifested primarily after aggregation, at the transition between the mound and the formation of the tipped aggregate.

As previously described, *car1/3*-null cells constitutively expressing cAR1-106C from the *Act15* promoter (*car1/3*-null:*Act1-5106C* cells) do not aggregate, although some very loose mounds are observed (Kim and Devreotes, 1994). Consistent with this phenotype, expression of postaggregative or cell-type-specific genes is not detected.

### *Effect of Exogenous cAMP on car1/3-Null Cells Expressing Mutant Receptors*

As multicellular development is compromised in some of the mutant strains, we examined the ability of exogenous cAMP to rescue gene expression in these cells. During development, aggregation-stage genes such as *csA* [encoding the cell adhesion molecule contact sites A (gp80)] are induced in response to the pulses of cAMP produced during aggregation and repressed in the multicellular stages by high, continuous cAMP (Jermyn *et al.*, 1987; Mann and Firtel, 1987, 1989). Postaggregative genes are induced as the mound forms in response to high, continuous cAMP (Firtel, 1995; Mehdy and Firtel, 1985). The cell-type-specific genes are induced subsequently and require both high cAMP and the products of some postaggregative genes. In the case of the prestalk gene *ecmA,* DIF is required for maximal expression (Williams *et al.*, 1987). These conditions can be mimicked in suspension culture, allowing one to examine the effects of exogenous cAMP on gene expression and bypass certain developmental mutants (Jermyn *et al.*, 1987; Mann and Firtel, 1987, 1989). Under such conditions, *car1/3*-null:*Act15-cAR1* cells exhibit a pattern of gene expression similar to that observed previously for wild-type cells (Insall *et al.*, 1994; Soede *et al.*, 1994). As shown in Fig. 3, *csA* is induced in suspension culture with or without exogenous cAMP pulses, the latter presumably due to the

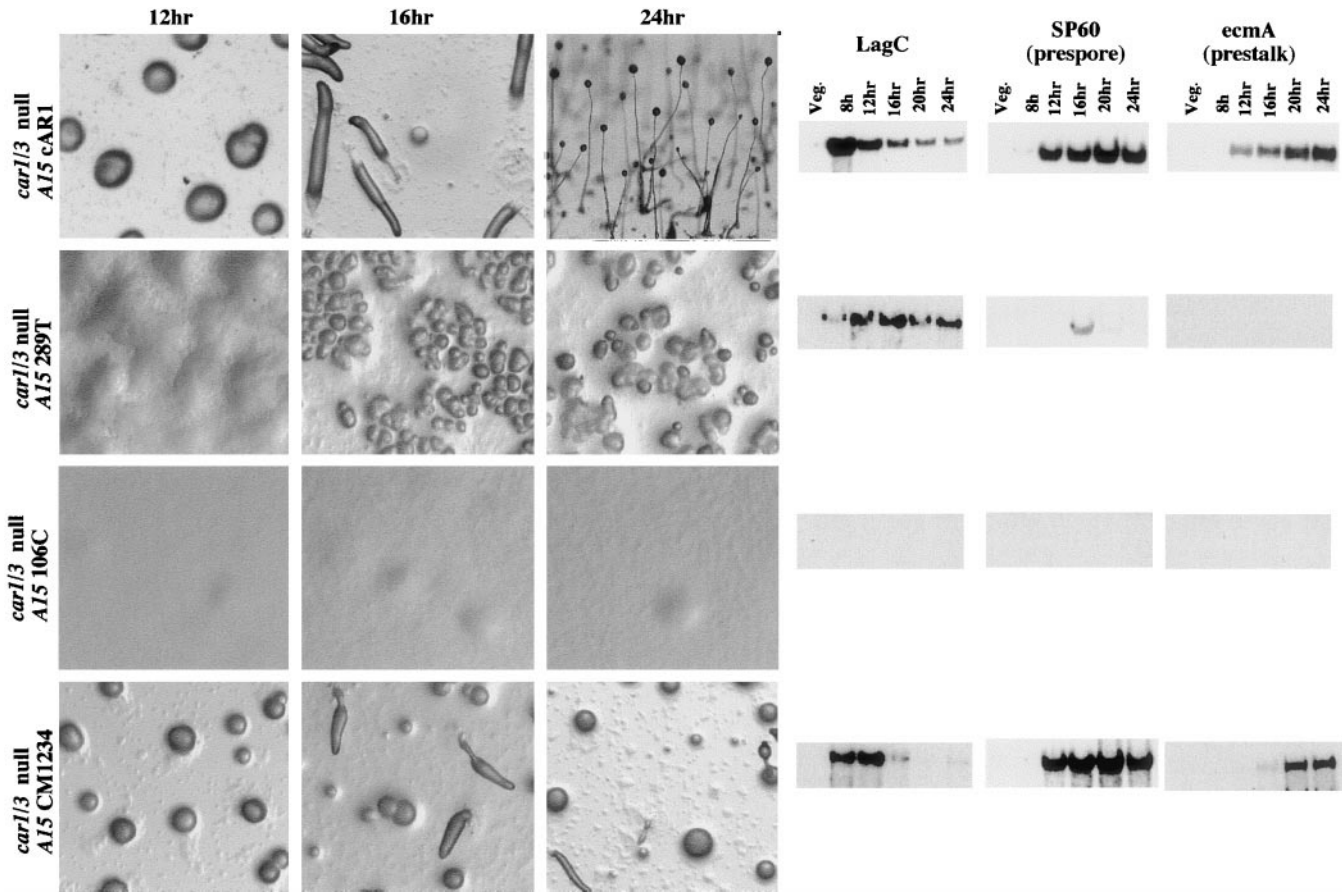


**FIG. 2.** Phenotypes and developmental gene expression in *car1/3*-null cells expressing the cAR1 receptor mutants. Vegetatively growing *car1/3*-null cells expressing the wild-type cAR1 or the cAR1 receptor mutants, 289T, cm1234, or 106C, from the *Actin 15* promoter, were washed in 12 mM $NaKPO_4$ (Mann and Firtel, 1987) and plated on $NaKPO_4$-buffered agar for development at the same concentrations. Photographs were taken and RNA was collected after 12, 16, and 24 h, as indicated. All photographs were taken at the same magnification (1.5× on a Nikon SMZ-U Zoom dissecting microscope). RNA was size-fractionated on denaturing gels and probed with DNA from *LagC*, *SP60/cotC*, and *ecmA*.

endogenous cAMP oscillations. As expected, the addition of high, continuous cAMP represses *csA* expression. *GBF* gene expression is induced maximally in cells given high, continuous cAMP. *LagC* is induced by high, continuous cAMP and not by pulsing alone and is expressed higher after 3 h than after 6 h of treatment. *SP60/cotC* is maximally expressed after 6 h of cAMP treatment, whereas *ecmA* expression is highest with continual cAMP treatment in cells that have been previously pulsed. *car1/3*-null:*Act15-289T* cells exhibit a high level of *csA* expression only in cells that are pulsed with cAMP (Fig. 3). *GBF* expression is reduced in cells that are starved compared to that in cells that are first pulsed and then given high cAMP. As in *car1/3*-null:*Act15-cAR1* cells, expression of *GBF* is reduced after 6 h of continuous cAMP. *LagC* expression, which is required for the expression of the cell-type-specific genes (Dynes *et al.*, 1994), is also expressed at high levels only in cells that are first pulsed and then given high cAMP for 3 h. Neither of the cell-type-specific genes examined is expressed.

To determine whether exogenous cAMP could help restore the developmental potential of these strains, cells were treated with extracellular cAMP in suspension and then plated for multicellular development on $PO_4$-buffered agar. As shown in Fig. 4, *car1/3*-null:*Act15-289T* cells that were starved for 4 h before being given high, continuous cAMP for an additional 6 h developed only to the loose mound stage by 15 h after plating, whereas similarly treated *car1/3*-null:*Act15-cAR1* cells formed fruiting bodies. *car1/3*-null:*Act15-289T* cells that were pulsed with nanomolar cAMP for 4 h prior treatment with micromolar cAMP for 6 h formed loose mounds with small tip-like early culminant structures 15 h after plating; some of these formed very small fruiting bodies by 20 h. Thus, although the cAR1 C-terminus is not essential for the activation of GBF

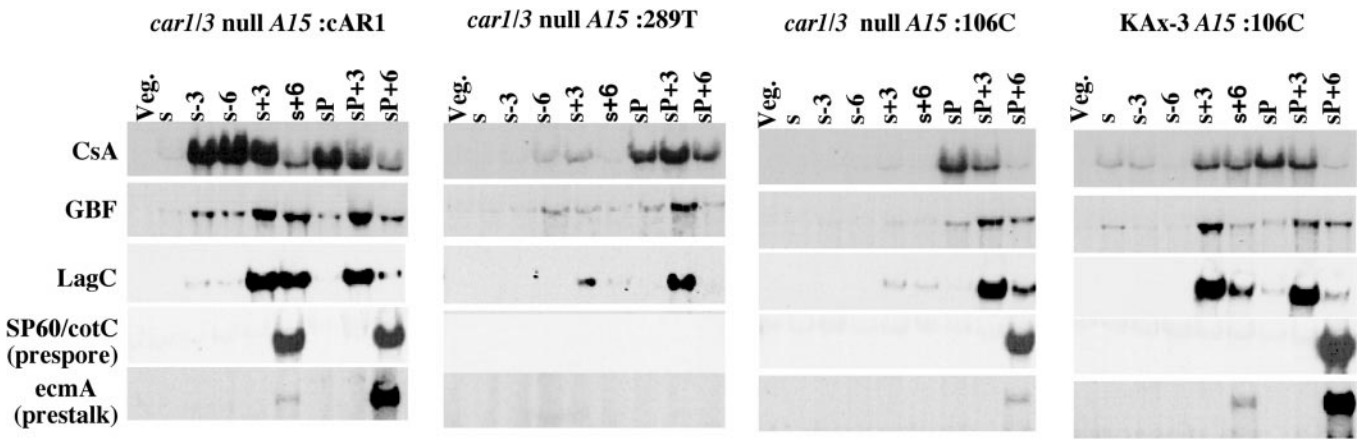


**FIG. 3.** Effect of exogenous cAMP addition on gene expression in cells expressing cAR1 mutants. *car1/3*-null cells or KAx-3 cells expressing wild-type cAR1 or the cAR1 receptor mutants 289T or 106C were grown axenically, washed, and resuspended in 12 mM $NaKPO_4$ at $5 \times 10^6$ cells/ml. Cells were either shaken (s) or pulsed (sP) with 30 nM cAMP at 10-min intervals for 4 h at 230 rpm. 300 $\mu$M cAMP was added to pulsed (sP) or nonpulsed (s) cells as indicated and cells were shaken for additional times with cAMP added to a final concentration of 150 $\mu$M cAMP every 2 h. Samples were taken at 3 and 6 h (labeled +3 or +6, indicating 3 or 6 h of cAMP treatment). Some cells received no cAMP for the duration of the experiment (−3 and −6). RNA was collected after 4 h of shaking (with and without cAMP pulsing) and after a further 3 and 6 h (with and without 300 $\mu$M cAMP) as indicated. RNA was size separated and probed with DNA from *CsA, GBF, LagC, SP60/cotC,* and *ecmA.*

activity, *car1/3*-null:*Act15-289T* cells are clearly defective in cell-type-specific expression and morphogenesis. *car1/3*-null:*Act15-106C* cells, which are unable to aggregate, express *csA* in response to cAMP pulsing. As with *car1/3*-null:*Act15-289T* cells, *GBF* and *LagC* are induced by high cAMP only in cells that were previously pulsed for 4 h. *SP60/cotC* is expressed at almost wild-type levels, whereas *ecmA* expression is very low, even in cells that were first pulsed before being given high cAMP. Thus, cells expressing a receptor lacking a C-terminal tail or the 106C chimeric receptor are unable to properly induce cell-type-specific gene expression, even in response to exogenous signals. Interesting, *car1/3*-null:*Act15-106C* cells given a combination of pulsing and continuous cAMP are able to produce multicellular aggregates. However, only very small fruiting bodies are produced from the center of the loose aggregate, with most of the cells not participating in morphogenesis past the mound stage. This phenotype is consistent with the inability of these cells to effectively induce prestalk gene expression, which is thought to be required for tip formation (Aubry and Firtel, 1999; Williams, 1995).

### *A Constitutively Phosphorylated Receptor Has a Dominant Negative Effect in Wild-Type Cells*

Wild-type cAR1 and the three mutant receptors were transformed into wild-type KAx-3 cells. As previously reported (Insall *et al.*, 1994), we find that constitutive expression of the full-length wild-type receptor in KAx-3 cells has no effect on development. Of the mutant receptors, only 106C produced an aberrant phenotype when expressed in wild-type cells (Fig. 5). Although the KAx-3:*Act15-106C* cells aggregated normally and expressed *LagC* at wild-type levels, the mounds started to dissipate by 16 h of development while simultaneously forming multiple tiny tips on the surface of the mound. By 24 h, a few of the tips had developed into small fruiting bodies with abnormally long stalks, while other tips did not develop further. Cell-type-specific gene expression in KAx-3:*Act15-106C* cells was significantly attenuated compared to that in wild-type cells, with low levels of *ecmA* detected only after 20 h of starvation. Notably, *SP60/cotC* expression oscillated, with moderate levels of the transcript present at 12 and 20 h and very low levels (observed upon a much longer exposure) occurring at 16 and 24 h. Probing with casein kinase 2 (CK2), which is constitutively present throughout development (Kikkawa *et al.*, 1992), demonstrated that all lanes of the blot contained roughly equal quantities of RNA. Presumably, the *SP60/cotC* expression is a reflection of the developmental state of the cells, the very low level of expression at 16 h corresponding to the time of mound dispersion.

We examined the ability of KAx-3:*Act15-106C* cells to induce gene expression in suspension culture in response to exogenous cAMP. Interestingly, KAx-3:*Act15-106C* cells did not express the pulse-induced gene *csA* without exogenous pulsing. Even with pulsing, the level of expression was very reduced compared to *car1/3*-null cells expressing cAR1 or 106C alone (Fig. 3). When KAx-3:*Act15-106C* cells in suspension were treated with 300 $\mu$M cAMP after 4 h of starvation, *LagC* and *GBF* were expressed to wild-type levels, whereas *SP60/cotC* was not detectably expressed and *ecmA* was induced to very low levels. When these cells were subsequently plated for development, most arrested at the mound stage. Aggregates that continued through development produced small fruiting bodies with abnormally

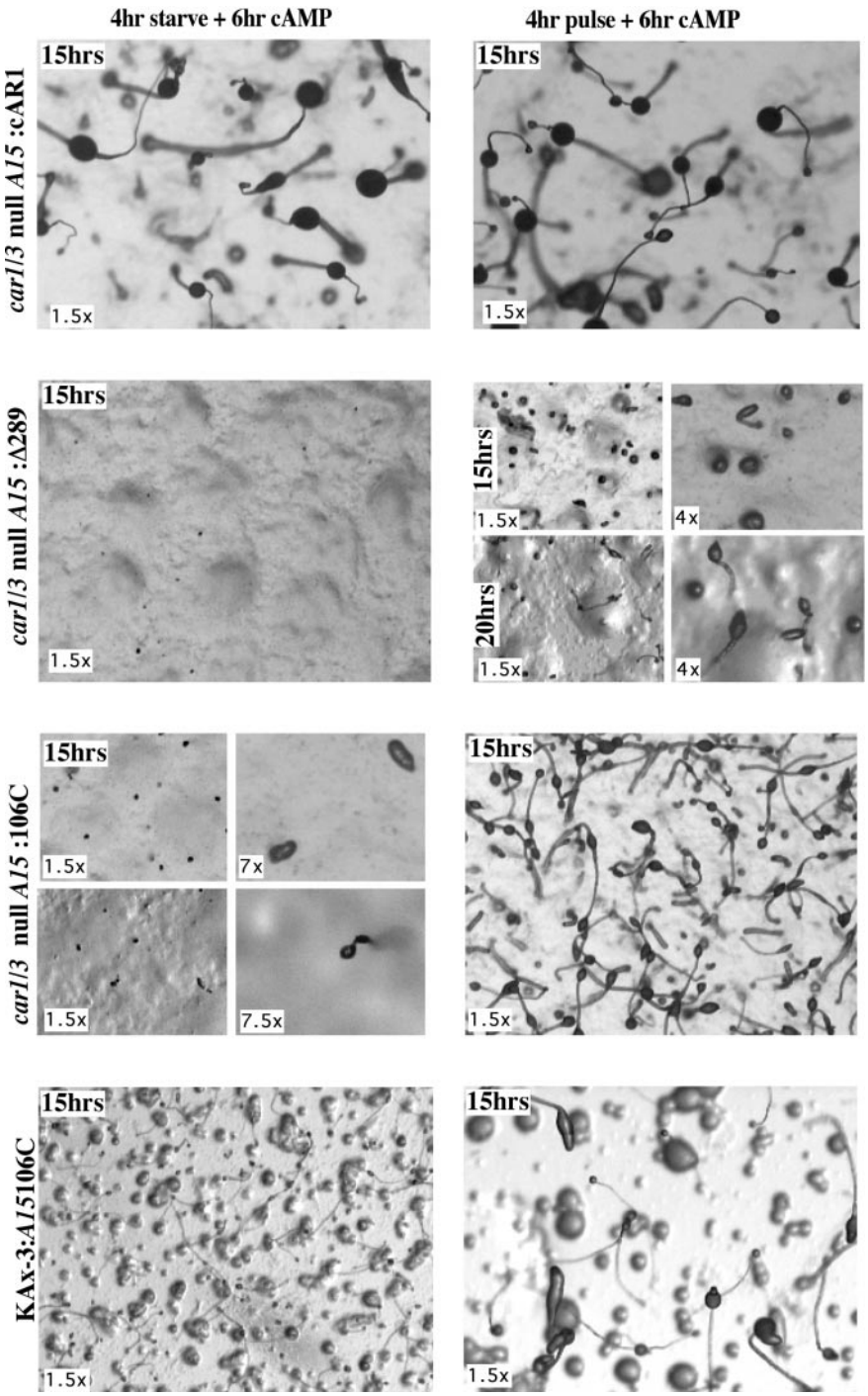


**FIG. 4.** Effect of pretreatment of strains with cAMP on developmental potential. $1 \times 10^7$ *car1/3*-null cells expressing wild-type cAR1, 289T, or 106C, or KAx-3 cells expressing the 106C cAR1 receptor from the experiment described in Fig. 3, were collected after 4 h of shaking with or without cAMP pulses and a further 6 h of treatment with 300 $\mu$M cAMP. The cells were washed in sodium phosphate buffer before being plated for development on $NaKPO_4$-buffered agar. Photographs were taken at 15 and 20 h after plating, as indicated. The original magnifications of each photograph are as shown.

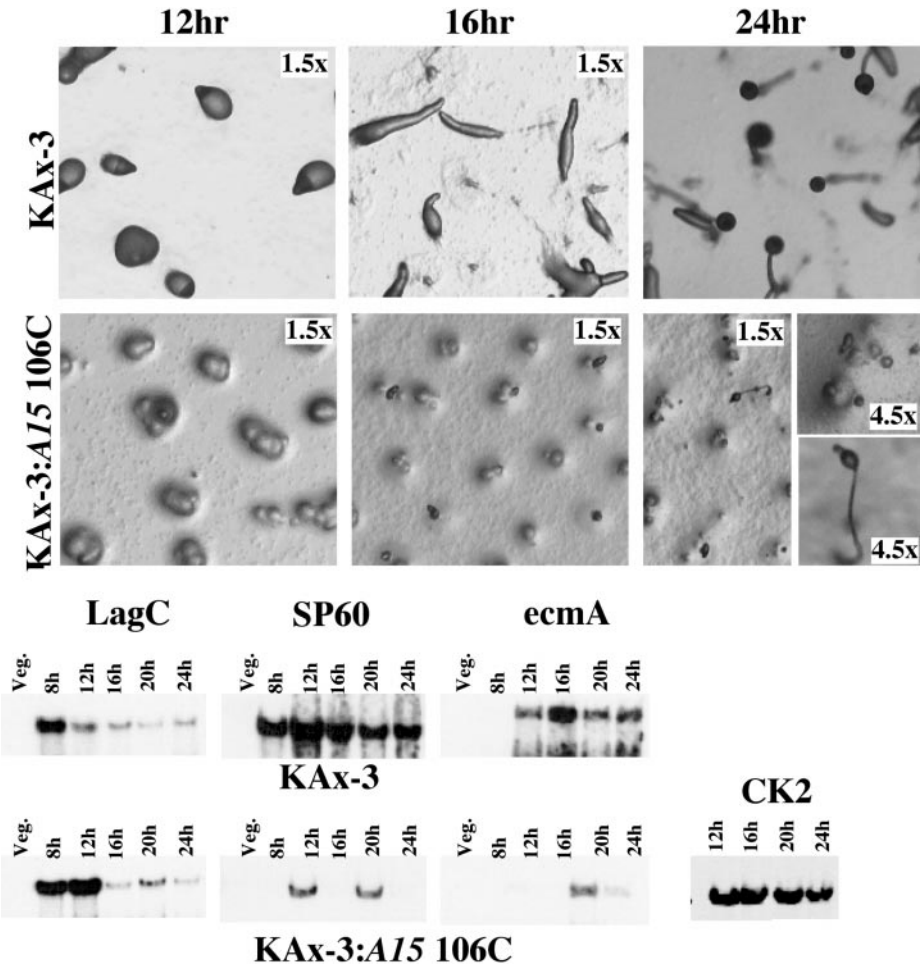


**FIG. 5.** Phenotypes and developmental gene expression in KAx-3 cells expressing the 106C receptor mutant. Axenically growing KAx-3 cells expressing the receptor 106C from the *Actin 15* promoter were washed and plated on $NaKPO_4$-buffered agar (pH 6.2) for development. Photographs and RNA were collected after plating at the times indicated. RNA was size separated and probed with DNA from *LagC*, *SP60*, *ecmA*, and *CK2*. CK2 is constitutively expressed throughout development (Kikkawa *et al.*, 1992) and is used as a standard for RNA loading.

long, thin stalks (Fig. 4). When the cells were first pulsed with cAMP prior to addition of high cAMP, all genes tested were expressed at near-wild-type levels, although the fruiting bodies formed upon plating remained fairly small, with elongated stalks. These results suggest that the 106C receptor functions as a dominant negative in wild-type cells and blocks both pulse-induced and cell-type-specific gene expression, which are regulated by independent pathways.

### *Effect of cAR1 Mutants on Tyrosine Phosphorylation of STATa*

As with cAMP-mediated activation of GBF function, cAMP-mediated STATa activation is receptor-dependent but does not require heterotrimeric G proteins (Araki *et al.*, 1998). To investigate the effect of the cAR1 mutants on STATa activation, *cAR1/3*-null cells expressing the receptor mutants were pulsed for 4 h before being treated with high, continuous cAMP. As described by Araki *et al.* (1998), two forms of tyrosine-phosphorylated STATa are detected in wild-type cells and in *car1/3*-null:*Act15-cAR1* cells following cAMP addition (Fig. 6). The higher-mobility form is tyrosine phosphorylated after 30 s, an event which has been reported to correlate with nuclear translocation. The more slowly migrating form, designated Dd-STAT:pTyr+ by Araki *et al.* (1998), becomes tyrosine phosphorylated after 5 min stimulation by cAMP. This shift in mobility has been proposed to be due to posttranslational modifications occurring in the nucleus. Notably, *car1/3*-null:*Act15-106C* exhibited constitutive tyrosine phosphorylation of STAT5a in 4-h pulsed cells. Moreover, no increase in tyrosine phosphorylation above this level nor the appearance of Dd-STAT:pTyr+ was observed following cAMP treatment. In contrast, KAx-3:*Act15-106C* cells exhibited a

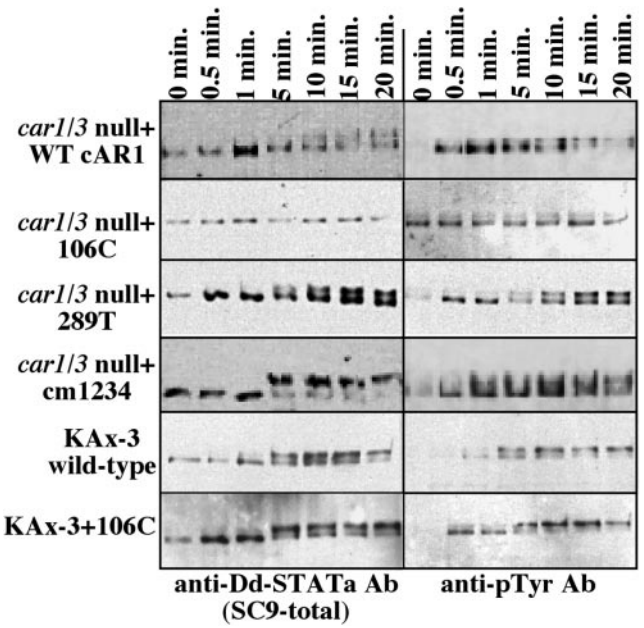


**FIG. 6.** cAMP induction of STATa tyrosine phosphorylation. Exponentially growing Ax3, Ax3:*Act15-106C*, *car1/3*-null:*Act15-car1*, *car1/3*-null:*Act15-106C*, *car1/3*-null:*Act15-289T*, and *car1/3*-null:*Act15-CM1234* cells in shaking suspension in $NaKPO_4$ buffer were pulsed with 30 nM cAMP for 4 h before addition of 300 $\mu$M cAMP for the times indicated. Western blots using either anti-STATa or anti-phosphotyrosine antibody were performed on STATa immunoprecipitates as described previously (Araki *et al.*, 1998).

normal STATa activation response. *car1/3*-null:*Act15-289T* and *car1/3*-null:*Act15-cm1234* stimulate tyrosine phosphorylation of STATa in response to cAMP with kinetics similar to those observed in *car1/3*-null:*Act15-cAR1* cells. However, in these strains, the relative level of the slower-migrating, hyperphosphorylated band is reduced compared to wild-type cells (KAx-3). These results suggest two alternate models: that the phosphorylated C-terminus of cAR1 may play a role in some aspects of STATa phosphorylation or that the first intracellular loop of cAR1 may be specifically required for this process. However, as cAR2 can complement many of the aggregation-stage functions of cAR1 when expressed in *car1/3*-null cells (Verkerke-van Wijk *et al.*, 1998), it is also possible that the structure of the chimera is sufficiently different from that of the wild-type receptors to cause a conformational change that results in a partially activated form sufficient to activate pathways in the absence of a ligand.

## DISCUSSION

### *Role of the C-Terminal Tail in Receptor-Mediated Responses*

In order for cells to progress past the loose mound stage, GBF must be induced and activated to stimulate the expression of downstream genes. The micromolar levels of cAMP thought to be present at the mound stage (Abe and Yanagisawa, 1983) activate GBF by signaling through cAR1 and cAR3, via a pathway that is distinct from that which regulates aggregation. Aggregation is mediated by nanomolar oscillatory pulses of cAMP that allow the receptor and downstream effector to alternate between active and inactive states. Signal relay (activation of adenylyl cyclase), chemotaxis, and pulse-induced gene expression require receptor-mediated, G-protein-dependent and -independent pathways. In contrast, STATa activation and postaggregative gene expression mediated through GBF are activated by high, continuous levels of cAMP acting through the same receptors but via a G-protein-independent pathway. One of the major differences between the state of the receptor during aggregation and mound-stage development is that cAR1 in mound-stage cells is constitutively phosphorylated on C-terminal serine residues in response to the high cAMP levels and thus should be in a lower-affinity conformation.

As previously described, cells expressing cAR1 receptors that are defective in C-terminal tail phosphorylation are able to aggregate essentially normally. In this article, we describe the effects of mutations that affect cAR1 tail phosphorylation on pathways required for multicellular development, including morphogenesis and cell-type-specific gene expression. Our data suggest that, although phosphorylation of the tail is not essential for GBF-mediated postaggregative gene expression, alteration of the C-terminal tail renders some receptor mutants unable to fully replace wild-type cAR1 and does not allow efficient differentiation beyond the mound stage. *car1/3*-null cells expressing the cAR1-289T receptor, in which the C-terminal tail is deleted, show a significant impairment in development: these cells arrest at the mound stage and lack cell-type-specific gene expression in the absence or presence of exogenous cAMP when tested in suspension cultures. Cells expressing cAR1-cm1234 in lieu of cAR1 and cAR3 also exhibit delayed, asynchronous development and many aggregates arrest at the mound stage. These cells express prespore-specific genes almost normally, but the expression of the prestalk-specific *ecmA* gene is reduced. As prestalk cell differentiation is needed for tip formation (Firtel, 1995; Loomis, 1996; Williams, 1995), this may account for the morphological defect observed in these cells. Whereas cAR1-289T has reduced stability (Kim *et al.*, 1997), it is unlikely that this is the reason for the defects observed in these cells. A reduced level of cAR1 in wild-type cells exhibits normal GBF-mediated activation of gene expression and the expression of the cAR-dependent post-aggregative gene *LagC* in *car1/3*-null:*cAR1-289T* cells is at normal levels, although it is delayed. Pulsing these cells for several hours restores some of the ability of the cells to induce postaggregative but not cell-type-specific genes.

These results present a convincing picture of a role for the C-terminal tail and possibly its phosphorylation in mediating these responses in the multicellular stages. Consistent with this conclusion, cells expressing cAR1-289T, in which

the C-terminal tail is deleted, exhibit significantly stronger phenotypes than cells expressing a receptor in which the phosphorylation sites in the tail are mutated. It is possible that the C-terminal tail may not be important in directly mediating these pathways (e.g., possibly by interacting with downstream components or stabilizing the interaction of such factors with other receptor domains), but it may play a role in ligand-mediated structural changes of the receptor that may be required for these functions. Similarly, the effect of the serine mutations may be due not to the loss of phosphorylation, but rather to changes in the structure of the tail resulting from the substitutions and short internal deletions. Our results suggest that the C-terminal tail is involved in controlling cell-type-specific gene expression that is separate from the activation of GBF function.

Cells expressing 106C in a *car1/3*-null background are unable to aggregate. These cells can activate many postaggregative pathways if given exogenous cAMP, but are defective in prestalk-specific gene expression. Although cells constitutively expressing either cAR2 (data not shown) or cAR1 with GBF in *car1/3*-null cells can activate postaggregative gene expression, it is not *a priori* clear whether a chimeric receptor composed of parts of cAR1 and cAR2 should affect receptor-mediated gene expression during multicellular development. It is possible that the chimeric receptor may have a sufficiently different structure than either of the very homologous parental receptors to affect downstream responses. These experiments show that cAR2 mediates postaggregative gene expression in response to high cAMP with the same efficiency as cAR1. In addition, expression of 106C in wild-type cells impairs multicellular developmental but not aggregation. This result, while more difficult to interpret, is consistent with a role for either the C-terminal tail in receptor-mediated pathways involved in multicellular development or the state of the C-terminal tail in the overall conformation of the receptor. It has been proposed that G-protein-coupled receptors, like receptor tyrosine kinases, may oligomerize and such oligomers might reflect a more "active" receptor conformation (Cvejic and Devi, 1997; Overton and Blumer, 2000). Dimerization has been proposed for several receptors in mammalian systems including the adrenergic receptor, opioid receptors, and chemokine receptors (Cvejic and Devi, 1997; Hebert *et al.*, 1996; Rodríguez-Frade *et al.*, 1999), as well as the yeast pheromone receptor (Overton and Blumer, 2000). This may also occur with cAR1 (P.N.D., unpublished observations), although this remains to be confirmed. If these models are correct, expression of 106C in wild-type cells may inhibit the formation of functional wild-type:wild-type receptor "dimers," thus preventing efficient multicellular development. In support of this possibility, expression of a signaling-defective chemokine receptor CCR2b in HEK293 cells with the wild-type receptor exhibits a dominant negative effect arising from their interaction (Rodríguez-Frade *et al.*, 1999). However, in either scenario, the dominant negative effect of 106C is probably affected by competition between wild-type signaling by the endogenous receptor and an interfering effect of 106C, the balance of which is reflected in the phenotype. Sufficient exposure to cAMP under suspension conditions that allow cell–cell contact may permit wild-type signaling. 106C is capable of mediating cAMP signals, as *car1/3*-null:*Act1-5106C* cells induce gene expression in slow-shake suspension assays in response to exogenous cAMP. In wild-type KAx-3 cells expressing *Act15-106C*, cell signaling probably occurs through both the endogenous receptor and 106C to differing extents.

### *Regulation of STATa Activation*

Activation of STATa, indicated by tyrosine phosphorylation and concomitant nuclear translocation, takes place at a time in development similar to that of GBF activation in mound-stage cells when levels of cAMP are high and cAR1 is in a constitutively phosphorylated state. Analysis of STATa tyrosine phosphorylation in *car1/3*-null cells overexpressing the 106C receptor shows that the STATa protein is constitutively tyrosine phosphorylated, with no further phosphorylation occurring upon cAMP stimulation. Interestingly, wild-type cells expressing the 106C receptor as well as endogenous cAR1 exhibit a normal STATa response. One possible explanation is that the cAR1 wild-type/106C receptor dimers, if they form, may function as a wild-type receptor with respect to STATa activation.

To account for receptor-mediated processes that are G-protein-independent, we previously proposed that the cARs may act as docking sites for effectors in addition to heterotrimeric G proteins and thus may function in a manner analogous to that of receptor tyrosine kinases in binding signaling components when activated, leading to stimulation of downstream pathways (Hereld *et al.*, 1994; Schnitzler *et al.*, 1994, 1995). Our results with the 106C receptor suggest that the phosphorylated C-terminus may function as part of a docking site for STATa and/or the as-yet-unidentified tyrosine kinase that phosphorylates STATa. However, the phosphorylated C-terminus is not essential for STATa tyrosine phosphorylation, as *car1/3*-null cells expressing either 289T or cm1234 exhibit normal cAMP-mediated STATa tyrosine phosphorylation. Interestingly, the amount of the hyperphosphorylated, slower-migrating STATa form in *cAR1/3*-null:*Act15-106C* is reduced compared to that in wild-type cells and is not stimulated by cAMP. This observation is reminiscent of *gβ*-null cells, in which cAMP stimulates the tyrosine phosphorylation and nuclear translocation of STATa, but the amount of hyperphosphorylated, slower-migrating STATa is reduced (Araki *et al.*, 1998). This finding led to the suggestion that this second phosphorylation event is G-protein-mediated. It is possible that the modified 106C receptor may not be able to interact efficiently with the G protein subunits and/or other proteins necessary to affect the second modification.

STATa is a repressor of *ecmB* expression and, although it binds to activator sequences in the *ecmA* promoter *in vitro*,

STATa is not an activator of *ecmA in vivo*. STATa is required for the proper regulation of other developmental responses, including aggregation and culmination (Mohanty *et al.*, 1999). There is obviously a defect in the ability of cAMP to activate *ecmA* expression in *cAR1/3*-null: *Act15-106C* cells when placed in shaking culture; however, this is unlikely to be due to the defects in cAMP-mediated STATa tyrosine phosphorylation. We also observed reduced expression of *SP60* and *ecmA* in *cAR1/3*-null:*Act15-289T* cells. Although these cells displayed normal activation of STATa in response to cAMP, it is also possible that the 289T receptor could affect activation of other STAT proteins which may play a role in expression of other cell-type-specific genes. It is probable that the receptor C-terminal tail mutations affect an interaction(s) with one or more as-yet-unidentified components of cAMP receptor-mediated pathways needed for cell-type-specific gene expression.

In conclusion, our analysis of receptor mutants suggests that regulation of morphogenesis and postaggregative gene expression are mediated by responses that occur via pathways distinct from those that regulate aggregation-stage pathways. The C-terminal tail of the receptor appears to play an important role in mediating these responses, through as-yet-unidentified components. Our observations provide new insights into the numerous responses that are regulated by cAMP and cAR1 and how the receptor might control such a diverse set of developmental functions.

## REFERENCES


Abe, K., and Yanagisawa, K. (1983). A new class of rapid developing mutants in *Dictyostelium discoideum:* Implications for cyclic AMP metabolism and cell differentiation. *Dev. Biol.* **95,** 200–210.

Araki, T., Gamper, M., Early, A., Fukuzawa, M., Abe, T., Kawata, T., Kim, E., Firtel, R. A., and Williams, J. G. (1998). Developmentally and spatially regulated activation of a *Dictyostelium* STAT protein by a serpentine receptor. *EMBO J.* **17,** 4018–4028.

Aubry, L., and Firtel, R. (1999). Integration of signaling networks that regulate *Dictyostelium* differentiation. *Annu. Rev. Cell Dev. Biol.* **15,** 469–517.

Brown, J. M., and Firtel, R. A. (1999). Regulation of cell-fate determination in *Dictyostelium*. *Dev. Biol.* **216,** 426–441.

Caterina, M. J., Devreotes, P. N., Borleis, J., and Hereld, D. (1995a). Agonist-induced loss of ligand binding is correlated with phosphorylation of cAR1, a G protein-coupled chemoattractant receptor from *Dictyostelium*. *J. Biol. Chem*. **270,** 8667–8672.

Caterina, M. J., Hereld, D., and Devreotes, P. N. (1995b). Occupancy of the *Dictyostelium* cAMP receptor, cAR1, induces a reduction in affinity which depends upon COOH-terminal serine residues. *J. Biol. Chem*. **270,** 4418–4423.

Chen, M. Y., Insall, R. H., and Devreotes, P. N. (1996). Signaling through chemoattractant receptors. *Trends Genet.* **12,** 52–57.

Cvejic, S., and Devi, L. (1997). Dimerization of the delta opioid receptor: Implication for a role in receptor internalization. *J. Biol. Chem*. **272,** 26959–26964.

Devreotes, P. N. (1994). G protein-linked signaling pathways control the developmental program of *Dictyostelium*. *Neuron* **12,** 235–241.

Dynes, J. L., Clark, A. M., Shaulsky, G., Kuspa, A., Loomis, W. F., and Firtel, R. A. (1994). LagC is required for cell–cell interactions that are essential for cell-type differentiation in *Dictyostelium*. *Genes Dev*. **8,** 948–958.

Early, A. E., Gaskell, M. J., Traynor, D., and Williams, J. G. (1993). Two distinct populations of prestalk cells within the tip of the migratory *Dictyostelium* slug with differing fates at culmination. *Development* **118,** 353–362.

Firtel, R. A. (1995). Integration of signaling information in controlling cell-fate decisions in *Dictyostelium*. *Genes Dev*. **9,** 1427–1444.

Hebert, T., Moffett, S., Morello, J., Loisel, T., Bichet, D., Barret, C., and Bouvier, M. (1996). A peptide derived from a beta2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. *J. Biol. Chem*. **271,** 16384–16392.

Hereld, D., Vaughan, R., Kim, J. Y., Borleis, J., and Devreotes, P. (1994). Localization of ligand-induced phosphorylation sites to serine clusters in the C-terminal domain of the *Dictyostelium* cAMP receptor, cAR1. *J. Biol. Chem*. **269,** 7036–7044.

Insall, R. H., Soede, R. D. M., Schaap, P., and Devreotes, P. N. (1994). Two cAMP receptors activate common signaling pathways in *Dictyostelium*. *Mol. Biol. Cell* **5,** 703–711.

Jermyn, K. A., Berks, M., Kay, R. R., and Williams, J. G. (1987). Two distinct classes of prestalk-enriched mRNA sequences in *Dictyostelium discoideum*. *Development* **100,** 745–755.

Kawata, T., Early, A., and Williams, J. (1996). Evidence that a combined activator–repressor protein regulates *Dictyostelium* stalk cell differentiation. *EMBO J.* **15,** 3085–3092.

Kikkawa, U., Mann, S. K. O., Firtel, R. A., and Hunter, T. (1992). Molecular cloning of casein kinase-II alpha-subunit from *Dictyostelium discoideum* and its expression in the life cycle. *Mol. Cell. Biol.* **12,** 5711–5723.

Kim, J. Y., and Devreotes, P. N. (1994). Random chimeragenesis of G-protein-coupled receptors: Mapping the affinity of the cAMP chemoattractant receptors in *Dictyostelium*. *J. Biol. Chem*. **269,** 28724–28731.

Kim, J. Y., Soede, R. D. M., Schaap, P., Valkema, R., Borleis, J. A., van Haastert, P. J. M., Devreotes, P. N., and Hereld, D. (1997). Phosphorylation of chemoattractant receptors is not essential for chemotaxis or termination of G-protein-mediated responses. *J. Biol. Chem*. **272,** 27313–27318.

Kim, J. Y., Borleis, J. A., and Devreotes, P. N. (1998). Switching of chemoattractant receptors programs development and morphogenesis in *Dictyostelium:* Receptor subtypes activate common responses at different agonist concentrations. *Dev. Biol.* **197,** 117–128.

Loomis, W. F. (1996). Genetic networks that regulate development in *Dictyostelium* cells. *Microbiol. Rev.* **60,** 135.

Maeda, M., Aubry, L., Insall, R., Gaskins, C., Devreotes, P. N., and Firtel, R. A. (1996). Seven helix chemoattractant receptors transiently stimulate mitogen-activated protein kinase in *Dictyostelium:* Role of heterotrimeric G proteins. *J. Biol. Chem*. **271,** 3351–3354.

Mann, S. K., and Firtel, R. A. (1989). Two-phase regulatory pathway controls cAMP receptor-mediated expression of early genes in *Dictyostelium*. *Proc. Natl. Acad. Sci. USA* **86,** 1924–1928.

Mann, S. K. O., and Firtel, R. A. (1987). Cyclic AMP regulation of early gene expression in *Dictyostelium discoideum:* Mediation

via the cell surface cyclic AMP receptor. *Mol. Cell. Biol.* **7,** 458–469.

Mehdy, M. C., and Firtel, R. A. (1985). A secreted factor and cyclic AMP jointly regulate cell-type-specific gene expression in *Dictyostelium discoideum*. *Mol. Cell. Biol.* **5,** 705–713.

Milne, J. L. S., Wu, L. J., Caterina, M. J., and Devreotes, P. N. (1995). Seven helix cAMP receptors stimulate $Ca^{2+}$ entry in the absence of functional G proteins in *Dictyostelium*. *J. Biol. Chem.* **270,** 5926–5931.

Mohanty, S., Jermyn, K. A., Early, A., Kawata, T., Aubry, L., Ceccarelli, A., Schaap, P., Williams, J. G., and Firtel, R. A. (1999). Evidence that the *Dictyostelium* Dd-STATa protein is a repressor that regulates commitment to stalk cell differentiation and is also required for efficient chemotaxis. *Development* **126,** 3391–3405.

Overton, M. C., and Blumer, K. J. (2000). G-protein-coupled receptors function as oligomers *in vivo*. *Curr. Biol.* **10,** 341–344.

Rodríguez-Frade, J., Vila-Coro, A., de Ana, A., Albar, J., Martínez-A., C., and Mellado, M. (1999). The chemokine monocyte chemoattractant protein-1 induces functional responses through dimerization of its receptor CCR2. *Proc. Natl. Acad. Sci. USA* **96,** 3628–3633.

Rogers, K. C., Ginsburg, G. T., Mu, X., Gollop, R., Balint-Kurti, P., Louis, J. M., and Kimmel, A. R. (1997). The cAMP receptor gene family of *Dictyostelium discoideum:* Expression, regulation, function. *In* "*Dictyostelium:* A Model System for Cell and Developmental Biology" (Y. Maeda, K. Inouye, and I. Takeuchi, Eds.), pp. 163–172. Universal Academy Press, Tokyo.

Schnitzler, G. R., Briscoe, C., Brown, J. M., and Firtel, R. A. (1995). Serpentine cAMP receptors may act through a G protein-independent pathway to induce postaggregative development in *Dictyostelium*. *Cell* **81,** 737–745.

Schnitzler, G. R., Fischer, W. H., and Firtel, R. A. (1994). Cloning and characterization of the G-box binding factor, an essential component of the developmental switch between early and late development in *Dictyostelium*. *Genes Dev.* **8,** 502–514.

Soede, R. D. M., Insall, R. H., Devreotes, P. N., and Schaap, P. (1994). Extracellular cAMP can restore development in *Dictyostelium* cells lacking one, but not two subtypes of early cAMP receptors (cARs). Evidence for involvement of cAR1 in aggregative gene expression. *Development* **120,** 1997–2002.

Vaughan, R. A., and Devreotes, P. N. (1988). Ligand-induced phosphorylation of the cAMP receptor from *Dictyostelium discoideum*. *J. Biol. Chem.* **263,** 14538–14543.

Verkerke-van Wijk, I., Kim, J. Y., Brandt, R., Devreotes, P. N., and Schaap, P. (1998). Functional promiscuity of gene regulation by serpentine receptors in *Dictyostelium discoideum*. *Mol. Cell. Biol.* **18,** 5744–5749.

Williams, J. (1995). Morphogenesis in *Dictyostelium:* New twists to a not-so-old tale. *Curr. Opin. Genet. Dev.* **5,** 426–431.

Williams, J. G., Ceccarelli, A., McRobbie, S., Mahbubani, H., Kay, R. R., Farly, A., Berks, M., and Jermyn, K. A. (1987). Direct induction of *Dictyostelium* prestalk gene expression by D1F provides evidence that DIF is a morphogen. *Cell* **49,** 185–192.

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