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

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INTRODUCTION

Dictyostelium development can be separated into two distinct phases (Chen et al., 1996; Firtel, 1995; Loomis, 1996; Williams, 1995). In the first stage, \( \sim 10^5 \) cells chemo-tactically 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).
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 Ga2 subunit (Chen et al., 1996; Devreotes, 1994; Firtel, 1995; Rogers et al., 1997). Nanomolar cAMP pulses stimulate the activation of adenyllyl 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, Ga2, 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 Ga2 subunit is essential for aggregation and the activation of adenyllyl 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 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 mutants 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 \( \times \)10^9) were pelleted and resuspended in 1 ml NP-40 cell lysis buffer (13 mM sodium pyrophosphate, 1.6 mM 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 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 NaPO₄-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-
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 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
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 PO₄-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 culminating 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.
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 KA3 cells. As previously reported (Insall et al., 1994), we find that constitutive expression of the full-length wild-type receptor in KA3 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 KA3: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 KA3: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 KA3:Act15-106C cells to induce gene expression in suspension culture in response to exogenous cAMP. Interestingly, KA3: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 carl/3-null cells expressing cAR1 or 106C alone (Fig. 3). When KA3:Act15-106C cells in suspension were treated with 300 μM cAMP after 4 h of starvation, LagC and GFB 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. 3. Effect of exogenous cAMP addition on gene expression in cells expressing cAR1 mutants.

<table>
<thead>
<tr>
<th>carl/3 null Act15-289T</th>
<th>carl/3 null Act15-106C</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsA</td>
<td>GFB</td>
</tr>
<tr>
<td>LagC</td>
<td>SP60/cotC (prespore)</td>
</tr>
<tr>
<td>ecmA</td>
<td>(prestalk)</td>
</tr>
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</table>

Wild-type cAR1 and the three mutant receptors were grown axenically, washed, and resuspended in 12 mM NaKPO4 at 5 × 10^5 cells/ml. Cells were either shaken (s) or pulsed (sP) with 300 μM cAMP at 10-min intervals for 4 h at 230 rpm. 300 μM 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 μM 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 μM cAMP) as indicated. RNA was size separated and probed with DNA from CsA, GFB, LagC, SP60/cotC, and ecmA.

activity, carl/3-null:Act15-289T cells are clearly defective in cell-type-specific expression and morphogenesis, carl/3-null:Act15-106C cells, which are unable to aggregate, express CsA in response to cAMP pulsing. As with carl/3-null: Act15-289T cells, GFB 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. Interestingly, carl/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).
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 KA×·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 μM 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.
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 STATa 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. 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 NaKPO4-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.
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 adenyl cyclase), chemotaxis, and pulse-induced gene expression require receptor-mediated, G-protein-dependent and -independent pathways. In contrast, STATa activation and postaggregate gene expression mediated through GFB 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 GFB-mediated postaggregate 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 GFB-mediated activation of gene expression and the expression of the cAR-dependent postaggregate 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 postaggregate 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 mutants 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 Gβ 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 GβF 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; Rodriguez-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:Act15-106C 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 GβF 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 over-expressing 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 gø-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,
STATα is not an activator of ecmA in vivo. STATα 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 STATα 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 STATα 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 is 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


Received for publication July 5, 2000
Revised February 5, 2001
Accepted February 7, 2001
Published online March 29, 2001