INTRODUCTION

In Dictyostelium, development is initiated upon starvation, promoting a G protein-coupled receptor signal/response cascade (see Aubry and Firtel, 1999; Soderbom and Loomis, 1998; Parent and Devreotes, 1996; Parent and Devreotes, 1999; Brzostowski and Kimmel, 2001); single amoeboid cells aggregate to form multicellular structures that undergo cytodifferentiation and morphogenetic change, leading to terminally differentiated fruiting bodies comprising mature spore and stalk cells. While many of the molecular signaling events that regulate early Dictyostelium development have been described in detail, little is known of their relationship to transcriptional control of gene expression.

Aggregation of Dictyostelium is mediated by chemotactic response to extracellular cAMP. cAMP stimulates specific G-protein-coupled, seven transmembrane (7-TM) receptors that, by positive feedback, activate adenyl cyclase (AC) to relay the cAMP signal; oscillatory pulses of cAMP are created through receptor sensitization/desensitization and the actions of secreted phosphodiesterase and phosphodiesterase inhibitor (see Aubry and Firtel, 1999; Soderbom and Loomis, 1998; Brzostowski and Kimmel, 2001). As development proceeds, cAMP accumulates to higher levels and the cAMP receptors expressed during aggregation become constitutively adapted (desensitized) for AC activation. Nonetheless, during late development, these cAMP receptors remain active, but signal primarily through G-protein-independent pathways (Parent and Devreotes, 1996; Aubry and Firtel, 1999; Brzostowski and Kimmel, 2001).

Four 7-TM cAMP receptor subtypes (CAR1, CAR2, CAR3 and CAR4) have been identified in Dictyostelium (Saxe et al.,...
grown and developed on solid substrata (Kim et al., 1999; Louis et al., 1993; Williams et al., 1989) or differentiated in suspension culture in the absence or presence of exogenous cAMP (Ginsburg and Kimmel, 1997; Kimmel, 1987). Cells were transformed and selected for G418-resistance using lacZ expression vectors (Louis et al., 1993; Williams et al., 1989). Transformations were confirmed by Southern and/or PCR analyses. Multiple, independent transformants were used to confirm consistency of results.

Purification and peptide analysis of CRTF
CRTF activity was enriched from nuclear extracts (Mu et al., 1998) through two rounds of batch purification with double-stranded DNA cellulose (see Fig. 1), as monitored by mobility shift assay (Mu et al., 1998). CRTF activity was eluted at 0.3 M NaCl, diluted to 0.1 M NaCl and loaded onto a sequence-specific, CAR1 DNA affinity column, that was constructed by coupling concanavalized the double-stranded oligomer of the CAR1 early promoter element (5′-TTTATAGACATT-TTGACCTATAAGAGTGTAAA-3′; Mu et al., 1998) to CNBr activated CL-4B sepharose (Sigma). CRTF activity was purified by stepwise elution with increased NaCl concentration. After two rounds of CAR1 DNA affinity purification, CRTF activity was enriched by ~10,000 fold. The 0.4 M and 0.5 M fractions contained the predominant CRTF activity and were separately analyzed by SDS gel electrophoresis. Each fraction contained two protein bands (40 kDa and 35 kDa), in similar proportions (see Fig. 2A).

The 0.4 M and 0.5 M fractions were pooled and the p40 and p35 bands were excised separately and digested in-gel with trypsin. The resulting peptides eluted and analyzed by reverse-phase HPLC. The peptide profiles of the p40 and p35 were almost identical (see Fig. 2B). Three peptide peaks were collected and sequenced by Edman degradation. We obtained four peptide sequences, P1, P2, P3a and P3b. The latter two were a mixture within the P3 fraction (see Fig. 2C).

cDNA isolation
Sense and antisense degenerate primers were designed from the peptide sequences. The sense primer from P3a and antisense primer from P2 amplified an ~350 bp DNA fragment from genomic DNA. Sequencing confirmed the additional presence of P1 and P3a. We performed a BLAST search against the Japanese Dictyostelium EST database (Mori et al., 1998) to obtain additional sequences. We identified two cDNAs containing the 350 bp CRTF sequence but these were significantly shorter than full-length CRTF mRNA. We amplified a directional AZAP-II Dictyostelium CDNA library to obtain full-length CRTF cDNA. Genomic sequences were confirmed by BLAST search against combined partial Dictyostelium sequences.

Expression of recombinant CRTF in Escherichia coli
Different CRTF truncation fragments were amplified by PCR and cloned in frame into pET28a (Novagen) and transformed into BL21 (DE3). The BL21 (DE3) transformants were grown to log phase and induced for protein expression with 0.4 mM isopropyl-β-D-thiogalacto pyranoside for 2 hours at room temperature. The cells were harvested and sonicated in 10 mM Tris (pH 8.0), 1 mM EDTA, 100 mM NaCl, and 1 mM DTT and cleared by centrifugation. Soluble extracts were analyzed by SDS gel electrophoresis. Extracts containing equivalent amounts of recombinant protein (~10ng) were used for gel retardation assay with labeled GAC-DR probe (Mu et al., 1998).

crft-null strains
The targeting construct for disruption of the CRFT gene was made by inserting the blasticidin resistance gene cassette into the CiaI site within the DNA-binding domain of the CRFT cDNA (see Fig. 3). The construct was linearized and electroporated into log phase vegetative Ax3 cells and blasticidin-resistant colonies were picked and screened by PCR. Southern analysis confirmed CRFT gene (with Demerec
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RESULTS

Purification and cDNA isolation of the novel transcription factor CRTF

Mobility shift assays have identified the nuclear factor CRTF that binds to the CAR1 early promoter element in a zinc-dependent manner (Mu et al., 1998). In combination with DNA-crosslinking analyses, it has been suggested that CRTF was ~40 kDa (Mu et al., 1998). Although we proposed that in vivo promoter binding of CRTF was essential for the activation of CAR1 expression during aggregation (Saxe et al., 1991; Louis et al., 1993), the 40 kDa CRTF is present at all stages of development, regardless of the transcriptional state of CAR1 (Mu et al., 1998). To examine CRTF function further, we purified CRTF as a step towards cDNA isolation and characterization.

CRTF was purified to homogeneity from nuclear extracts prepared from growing cells (Fig. 1). The final step involved affinity binding to the CAR1 early promoter element coupled to a sepharose column. After two rounds of CAR1 DNA-affinity purification, CRTF activity was enriched ~10,000-fold compared with nuclear extracts. Only two protein bands (40 kDa and 35 kDa) were observed by SDS gel electrophoresis (Fig. 2A) and both were independently capable of binding to the CAR1 promoter element.

The p40 and p35 bands were excised and digested in-gel with trypsin, and the resulting peptides resolved by reverse-phase HPLC. The peptide profiles of p40 and p35 were nearly identical (Fig. 2B), suggesting that both derive from a single protein type. Four peptide sequences, p1, p2, p3a and p3b, were obtained that were common to p40 and p35 (Fig. 2C). Sense and antisense degenerate primers were designed using the peptide sequences of p3a and p2 as template. The sense primer for p3a and antisense primer for p2 amplified an ~350 bp DNA fragment; this fragment encoded CRTF peptides p1 and p3b, in addition to p2 and p3a (see Fig. 3).

Full-length cDNAs were obtained by amplification from a cDNA library and indicate that the CRTF protein is 876 amino acid residues (Fig. 3), ~100 kDa, considerably larger than purified (40 kDa) CRTF. We have been unable to identify larger CRTF forms in vivo by analyses of extracts from whole cells or various developmental stages. The N-terminal region of the predicted protein is enriched in homopolymeric runs of glutamine and asparagine residues, that derive, respectively, from CAA and AAT trinucleotide repeats, a feature not uncommon in developmentally regulated genes in Dictyostelium (see Kimmel and Firtel, 1985). This region, which could potentially serve as a transcriptional activation domain in a full-length form, is absent from the purified 40 kDa form. A putative nuclear localization signal is located at the C terminus. No significant sequence alignments to other proteins could be found through BLAST search of GenBank.

Full-length and truncated forms of CRTF were expressed in E. coli and assayed for mobility shift activity (see Fig. 4). N-terminal deletion to residue 654 did not alter the ability of CRTF to interact with the CAR1 promoter element. However, N-terminal deletion of only an additional 26 amino acids, beyond the first H/C cluster of the putative zinc finger (see Fig. 2C).
A. p40

B. p35

C. P1: YESYR

P2: (S/I)Y(F/I)(S/H/P)ANPHAK

P3a: NYQPIQFK

P3b: FLAFNXXAM

Fig. 2. Analysis of purified CRTF. (A) Purified CRTF was separated with SDS-polyacrylamide gel electrophoresis and detected by silver staining. (B) HPLC analysis of tryptic peptides for the p35 and p40 forms of CRTF. (C) Deduced peptide sequences of P1, P2 and P3. The P3 fraction is a mixture of peptides P3a and P3b.

3), completely abolished interaction with this cognate DNA sequence. Deletion of six residues from the C terminus did not alter DNA binding, but deletion of five additional amino acids, to residue 865, decreased DNA affinity by ~10-fold. Further C-terminal truncation to amino acid 858 abolished all DNA binding. Thus, residues 654 to 870 are essential for interaction with the CAR1 promoter element. Our previous study had shown that zinc is required for CRTF binding to DNA (Mu et al., 1998) and there are several cysteine/histidine clusters in the DNA-binding domain (DBD), although their spacing does not match precisely with any standard consensus DNA-binding motif. The DNA-binding domain of purified CRTF may form a novel, atypical zinc-finger motif. The 40 kDa form of purified CRTF represents a novel N-terminal truncation. This form of CRTF retains the DBD, but is deleted of the runs of polyasparagine and polyglutamine. As expected, the four peptide sequences obtained from purified CRTF lie within the 40 kDa region (Fig. 3).

CRTF is required for normal development

The CRTF gene is single-copy with a lone 121 nt intron (see Fig. 3) within the N-terminal protein coding region. Consistent with the DNA binding data (Mu et al., 1998), northern hybridization for CRTF mRNA indicate expression throughout development (Fig. 5A). At most stages, CRTF mRNA is ~3 kb. An additional slightly smaller mRNA species is detected at late culmination that may result from an alternative transcriptional start. To study the function of CRTF, we created multiple (14) null strains by homologous recombination. The targeted strains do not express detectable levels of CRTF mRNA (Fig. 5B). Nuclear extracts from these null strains lack CRTF activity as monitored by mobility shift assay of the CAR1 promoter element, regardless of developmental stage or treatment (Fig. 5C).

Fig. 3. Amino acid sequence of full-length CRTF. Deduced amino acid sequence (with numbers indicated) from the full-length CRTF cDNA. --- represents the position of the single intron. The poly asparagin (N)- and poly glutam (Q)-rich runs present in the full-length but not the truncated form of CRTF are shown in red and blue, respectively. The DNA-binding domain (DBD) is boxed. The P1, P2, P3a and P3b peptides are underlined. The H/C residues that may alter DNA binding, but deletion of five additional amino acids, to residue 865, decreased DNA affinity by ~10-fold. Further C-terminal truncation to amino acid 858 abolished all DNA binding. Thus, residues 654 to 870 are essential for interaction with the CAR1 promoter element. Our previous study had shown that zinc is required for CRTF binding to DNA (Mu et al., 1998) and there are several cysteine/histidine clusters in the DNA-binding domain (DBD), although their spacing does not match precisely with any standard consensus DNA-binding motif. The DNA-binding domain of purified CRTF may form a novel, atypical zinc-finger motif. The 40 kDa form of purified CRTF represents a novel N-terminal truncation. This form of CRTF retains the DBD, but is deleted of the runs of polyasparagine and polyglutamine. As expected, the four peptide sequences obtained from purified CRTF lie within the 40 kDa region (Fig. 3).

In shaking culture, crtf-null cells grow with the same doubling time as wild-type cells and on bacterial lawns crtf-null cells do not complete aggregation before 48 hours. Their cultivation that may result from an alternative transcriptional start. To study the function of CRTF, we created multiple (14) null strains by homologous recombination. The targeted strains do not express detectable levels of CRTF mRNA (Fig. 5B). Nuclear extracts from these null strains lack CRTF activity as monitored by mobility shift assay of the CAR1 promoter element, regardless of developmental stage or treatment (Fig. 5C).

The morphology of crtf-null fruiting bodies suggests that
CRTF is required for terminal spore differentiation. Mature Dictyostelium spores are oval in shape and are bright under phase microscopy. crtf-null cells do not form mature spores and, indeed, all cells of the crtf-null spore mass are dark in phase with round or irregular shape (Fig. 6C). These cells are fragile and easily disrupted under a glass cover slip. The severe developmental defects in the crtf-null cells suggest that CRTF might have a broader role in Dictyostelium development than just in regulating gene expression during the earliest stages of development.

**HA-tagged CRTF rescues crtf-null development**

To investigate the relationship between CRTF function and protein size, we expressed full-length CRTF carrying a C-terminal HA tag in crtf-null cells. Constitutive expression of CRTF-HA was able to rescue all aspects of development in crtf-nulls; aggregation and fruity body formation appeared normal (Fig. 7A) as did spore cell differentiation in these (crtf-null::act15/CRTF-HA) cells (Fig. 7B).

To determine whether processing of CRTF was related to its function, we prepared nuclear extracts from growing and differentiated crtf-null::act15/CRTF-HA cells and from wild-type and crtf-null controls (Fig. 7C). The relative size of the CRTF-HA proteins were determined by immunoblot analysis. Despite the rescue of crtf-null development by full-length CRTF fused to HA, we detect only a ‘processed’, 40 kDa form of CRTF-HA in crtf-null::act15/CRTF-HA cells, irrespective of developmental stage; a full-length CRTF form at a predicted ~100 kDa was not detected. A nonspecific crossreacting species of ~60 kDa is detected in all cell lines and stages of differentiation.

**Early gene expression defects in crtf-null cells**

The CAR1 gene is regulated by two distinct promoters that exhibit stage-specific activities during development. The early 2.0 kb CAR1 mRNA is induced by nanomolar cAMP pulses and reaches a peak of expression at ~5 hours of development. Activity of this early promoter is repressed at aggregation by continuous exposure to high levels of extracellular cAMP, which coordinately induces expression from the late CAR1 promoter and accumulation of the 2.2 kb late CAR1 mRNA.
for 5 hours to induce early developmental events. Cells were then incubated in the absence of further cAMP treatment or were maintained at 500 μM cAMP for an additional 4 hours to induce cell differentiation (Fig. 9A). Under these conditions, prestalk gene *ecmA* and prespore genes *psA* and *cotB* will be induced in wild-type cells after incubation with 500 μM cAMP following cAMP pulsing (Ginsburg and Kimmel, 1997). For crtf-null cells, however, significant levels of prestalk and prespore expression were detected in cells cultured in the absence of high level cAMP, although 500 μM cAMP promoted significant upregulation of both prestalk and prespore gene expression beyond that of wild type. Under these conditions, the crtf-nulls form agglomerates in a manner similar to wild type. Thus, the precocious activation of these genes in crtf-nulls would not seem to reflect an accelerated developmental program (see below) or an increase in endogenous levels of cAMP. CRTF may participate in a repressive pathway for cell-specific gene expression during early stages of development.

Expression of the sporulation maker *spiA* was studied using cells developed on filters (Fig. 9B). In wild-type, *spiA* was expressed at 23 hours of development as spores begin to mature. *spiA* was also expressed in crtf-nulls, although at a later and broader time range because of developmental delay and asynchrony.

Spatial patterning of prestalk and prepore cells was studied in wild-type and crtf-null strains marked with cell-specific promoters fused to the *lacZ* reporter (Fig. 9C). As in wild-type cells, expression of prespore *psA/lacZ* was localized to the posterior region of the crtf-null slugs and to the spore mass of fruiting bodies. Prestalk *ecmAllacZ* was expressed in the anterior region in the slugs and in stalk structures of fruiting bodies in both wild-type and crtf-nulls. Although in the absence of CRTF, development is impaired and the major markers of cell differentiation and cell sorting are de-repressed, CRTF does not seem to be required for normal spatial expression of these markers.

**Post-aggregation and sporulation defects in crtf-nulls are cell autonomous and independent of CAR1 expression**

Some of the developmental defects described for the crtf-nulls are similar to those of car1-nulls (Klein et al., 1988; Sun and Devreotes, 1991). As CRTF is required for CAR1 expression, potentially mere rescue of CAR1 expression in crtf-nulls would bypass these defects. We addressed this in two ways. However,
we ultimately concluded that CRTF was required for more pathways than just those that lead to the induction of CAR1 and other early cAMP pulse-regulated genes.

First, we differentiated cells in shaking culture with exogenous cAMP pulses and then maintained the cells at 500 μM cAMP. As shown previously, these conditions promote early (see Fig. 8) and cell-specific (see Fig. 9) gene expression. The cAMP-treated cells were then deposited on filters for further development. Wild-type cells form slugs within 3 hours and fruiting bodies with mature spores by 10 hours (Fig. 10A). For crtf-nulls, development was slower and more asynchronous. Slugs did not form until 10 hours after plating and fruiting body formation took an additional 12 hours. Even after 24 hours of cAMP treatment, many structures had not completed development and only few mature spores were detected in those that did culminate (Fig. 10B).

We also attempted to rescue crtf-null development by constitutive expression of CAR1. Although these cells progressed through aggregation more efficiently than in the absence of ectopic CAR1, late development and sporulation remained aberrant (Fig. 10C,D). Thus, late developmental defects in crtf-nulls were not due to the compromised expression of CAR1.

To determine if the developmental defects observed in crtf-nulls are cell autonomous or non-cell autonomous, we performed chimeric development in the presence of wild type, in order to provide essential secreted morphogens and chemoattractants. crtf-null aggregate develops normally in the presence of a wild-type strain. Here, wild-type cells initiate normal cAMP signal/relay that mediates chemotaxis and induces early gene expression in the mutants. The presence of wild type, however, cannot rescue later development defects in the crtf-nulls. Relatively, development is improved with an increasing ratio of wild-type to crtf-null cells; however, even chimeras with 80% wild-type cells still developed more asynchronously and significantly slower than did wild type alone. The sporulation defects characteristic of crtf-nulls (see Fig. 6C) are not rescued by chimeric development (Fig. 11A). Under phase microscopy, the ratio of dark-phased, round cells to mature bright spores reflected that of the input strains. This was confirmed using null strains marked with a reporter that is preferentially expressed during spore cell differentiation. Viability of crtf-null (BLAST-resistant) spores also did not improve when developed in chimeras. These results clearly indicate that late developmental defects of crtf-null cells are primarily cell autonomous in nature.

A major pathway essential for spore differentiation requires signaling through cAMP-dependant protein kinase, PKA (Loomis, 1998; Thomason et al., 1999). Indeed, direct activation of PKA using the membrane-permeable cAMP
analog 8-Br cAMP can rescue sporulation of many defective cell lines and even promote sporulation in the absence of aggregative development (Loomis, 1998). As the sporulation marker spIA requires activation by PKA (Richardson et al., 1994; Mann et al., 1994) and, yet, is efficiently expressed in crtf-null cells (see Fig. 9B), it would appear that impaired signaling though PKA is not the primary cause of the sporulation defects in crtf-nulls. Nonetheless, we examined the effect of 8-Br cAMP on sporulation of crtf-nulls. In the presence of 8-Br cAMP, ~70% wild-type cells differentiated into phase bright, oval-shaped spores within 40 hours. In contrast, 8-Br cAMP had no affect on spore differentiation of crtf-nulls (Fig. 11B). Thus, CRTF may represent an essential sporulation transcription factor that functions downstream of PKA activation or in a requisite parallel pathway.

**DISCUSSION**

Starvation of *Dictyostelium* leads to growth arrest and the activation of gene sets required to initiate development. Adenylyl cyclase A (ACA) expression is crucial for cells to autonomously progress through development (see Aubry and Firtel, 1999; Soderbom and Loomis, 1998; Parent and Devreotes, 1996; Parent and Devreotes, 1999; Brzostowski and Kimmel, 2001). ACA is not expressed during logarithmic growth but is induced at high levels upon nutrient depletion. The resulting product of ACA action, secreted cAMP, serves both as an attractant to promote chemotactic aggregation and as a gene-activating morphogen. CAR1, the cell-surface target for cAMP during aggregation, is required for signal response and relay and is upregulated by a cAMP-activated positive transcriptional feedback loop (Louis et al., 1993; Mu et al., 1998). While recent analyses have dissected molecular mechanisms that regulate the progression from growth, little is understood about the post-starvation mechanisms that regulate cAMP-mediated gene induction. Thus, while ACA activity and resulting cAMP production are required for expression of *CAR1*, components, such as yakA, myb2 and PKA, which are fundamental to establishing cAMP signaling and aggregation, play no direct role in the transcriptional activation of *CAR1* during development (Sousa et al., 1998; Sousa et al., 1999; Otsuka and van Haastert, 1998).

We had previously shown that the GAC direct repeat (GAC-DR) sequence is an essential element of the *CAR1* promoter required for its induction during the early phases of *Dictyostelium* aggregation (Mu et al., 1998). We have now purified CRTF, the *CAR1* transcription factor that binds the GAC-DR element and isolated corresponding cDNA and genomic fragments. CRTF is an atypical zinc-finger protein without apparent dimerization or ligand binding motifs that plays a critical role in the regulation of *CAR1* expression at the early stages of developmental activation. Although crtf-nulls are severely compromised in *CAR1* expression, additional *CAR1* gene activating pathways appear to function in the absence of CRTF. Developmentally regulated gene expression in diverse systems involves the cooperative interaction of multiple transcription factors, and while ablation of any individual component of the system may restrict normal gene activation, transcription is often not completely abrogated. For *CAR1*, high level expression cannot be achieved in the absence of CRTF, but expression can be rescued by treatment with exogenous cAMP. Thus, CRTF is crucial for mediating the initial low-level cAMP signal at the onset of starvation that leads to *CAR1* induction and establishes the cAMP/CAR1 circuit. However, CRTF may be dispensable once cAMP signal/relay is established fully or is mimicked by exposure to an exogenous cAMP source. Additional factors that may regulate *CAR1* appear to act at sites separate from that of CRTF. We have not detected proteins that interact at or near the CRTF-binding motif using extracts from crtf-nulls that had been stimulated with cAMP and that exhibit activated *CAR1* expression.

![Fig. 9. Cell-specific gene expression patterns in crtf-nulls.](image-url)

(A) Expression of prestalk (*ecmA*) and prespore (*cotB, psA*) genes in wild-type and crtf-null strains differentiated in shaking culture in the presence of exogenous pulses of cAMP (P) followed by treatment without (−) or with (+) 500 μM cAMP. (B) spIA mRNA expression in wild-type and crtf-null strains at various stages of development (hours). (C) Spatial expression of prestalk *ecmA*/*lacZ* and prespore *psA*/*lacZ* in crtf-nulls; red arrows indicate prestalk regions of slugs.
Transcription factor CRTF of Dictyostelium

The developmental abnormalities observed in crtf-nulls are more severe than can be explained solely by defects in gene expression during early aggregation. In wild-type cells, early aggregation and post-aggregation genes are regulated differentially by response to cAMP stimulation. Exogenous cAMP presented in oscillating pulse waves will induce expression of the essential components of the cAMP signal transduction machinery (e.g. CAR1, Ga2), but not the cell type-specific genes (Kimmel, 1987; Aubry and Firtel, 1999). Maintenance of high non-fluctuating cAMP levels will repress the former gene set but induce post-aggregation and cell type-specific gene classes (Kimmel, 1987; Louis et al., 1993; Ginsburg and Kimmel 1997; Aubry and Firtel, 1999). While in crtf-nulls exogenous pulses rescue expression of CAR1 and Ga2, these cells also exhibit a precocious induction of cell-specific genes. Numerous signaling models have been postulated to explain how early and late cAMP-induction pathways for gene expression are insulated from one another (Aubry and Firtel, 1999; Brzostowski and Kimmel, 2001), but no data have addressed a molecular mechanism for such activity. Potentially, during early aggregation, CRTF serves to activate genes required for signal transduction and chemotaxis, yet simultaneously participates in the repression of post-aggregation, cell type-specific genes.

Ablation of CRTF specifies additional paths for CRTF function during development. Apart from the aforementioned defects in gene expression, we observe gross abnormalities in development. The severe developmental delay and acute asynchrony are independent of CAR1 expression and cannot be rescued by treatment with exogenous cAMP or by co-development with wild-type cells. It is likely, that a collective and more global misregulation of gene expression at both temporal and quantitative levels during multi-cellularity contribute to the compromised development of the crtf-nulls.

CRTF is also required specifically in an activation pathway for the terminal differentiation of spore cells. Sporulation in Dictyostelium is complex and absolutely dependent upon PKA activation (Loomis, 1998; Thomason et al., 1999). Spore differentiation factors secreted by prestalk cells initiate a two component-mediated signaling cascade in prespore cells that leads to a rise in intracellular levels of cAMP, ultimately activating PKA (Richardson et al., 1994; Shaulsky et al., 1998; Thomason et al., 1998; Loomis, 1998; Thomason et al., 1999). Direct activation of PKA by treatment with cell-permeant 8Br-
cAMP promotes sporulation in the absence of aggregation and bypasses the requirements for normal developmental signaling. The autonomous role of CRTF in spore differentiation is underscored by the inability of crtf-nulls to sporulate in co-development with wild-type cells or when treated with 8Br-cAMP. CRTF must function either downstream in the PKA pathway or in an essential but independent parallel circuit.

The GSK3 protein kinase is also required for spore differentiation (Harwood et al., 1995; Thomason et al., 1999). cAMP stimulation of CAR3 leads to the activation of the tyrosine kinase ZAK1; this, in turn, will phosphorylate and consequently activate GSK3 in a required cascade for spore differentiation. 8Br-cAMP will not rescue sporulation defects in car3, zak1 or gsk3 nulls (Harwood et al., 1995; Plyte et al., 1999; Kim et al., 1999), and, thus, GSK3 functions either distally to or independently of PKA. CRTF has several consensus GSK3 phosphorylation sites, so potentially, CRTF may act as a target directly downstream of GSK3 or cooperatively with aarA, a β-catenin-related protein that is also a suggested substrate of GSK3 in the sporulation pathway (Grimson et al., 2000).

CRTF has complex and diverse functions throughout *Dictyostelium* development. CRTF is detected throughout development, yet CRTF-dependent *CAR1* expression is restricted to the early stages of aggregation. It is possible that the activity of CRTF is transiently and differentially regulated by a post-translational mechanism. The size of CRTF deduced from its cDNA sequence is ~100 kDa, but DNA-crosslinking studies had indicated that CRTF bound to the *CAR1* early promoter is ~40 kDa, regardless of developmental state. This size was confirmed by analysis of purified CRTF. In addition, only a 40 kDa CRTF species is detected in crtf-nulls expressing the full-length CRTF-HA fusion. The 40 kDa processed fragment of CRTF may represent the predominant form whose function is distinct from that of a less abundant full-length 100 kDa form, which would retain the N-terminal domain with possible N/Q-rich activating motifs. This type of mechanism would parallel that of Ci/Gli, where processed and unprocessed forms function, respectively, as transcriptional repressors or activators (Kalderon, 2000; Chuang and Kornberg, 2000). However, the distinct roles of CRTF in activating early gene expression and sporulation through different transduction circuits, yet also repressing cell-specific gene expression, punctuates the difficulty in analyzing any individual post-translational modification of CRTF presumed to be required for these varied functions. CRTF may be differentially activated in response to the context-specific signaling pathways that control *Dictyostelium* development.

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