Development 128, 2569-2579 (2001)
Printed in Great Britain © The Company of Biologists Limited
DEV3402

CRTF is a novel transcription factor that regulates multiple stages of *Dictyostelium* development

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Accepted 20 April 2001

SUMMARY

During aggregation, *Dictyostelium* establish nanomolar oscillation waves of extracellular cAMP, but as development progresses, cells become responsive to higher, non-fluctuating concentrations of cAMP. The regulation of the promoter responsible for expression of cAMP receptor subtype 1, CAR1, during aggregation reflects these signaling variations. Transcription of *CAR1* from the early, aggregation promoter is activated by cAMP pulsing, but is repressed by continuous exposure to micromolar concentrations of cAMP. Deletion and mutation analyses of this promoter had defined an element essential for cAMP-regulated expression, and mobility shift assay, DNA crosslinking and DNase I footprinting experiments had identified a nuclear protein (CRTF) with zinc-dependent sequence binding specificity. In our study, CRTF was purified to homogeneity, peptides were sequenced and full-length cDNAs were obtained. The deduced CRTF protein is ~100 kDa with a C-terminal, zinc finger-like motif required for DNA binding; CRTF purified from cells, however, represents only a 40 kDa C-terminal fragment that retains DNA-binding activity.

As might have been predicted if CRTF were essential for the regulation of CAR1, crtf-null strains fail to develop under standard conditions or to exhibit induced expression of *CAR1* or other cAMP-regulated genes. Furthermore, crtf-nulls also fail to sporulate, even under conditions that bypass the dependence on early cAMP signaling pathways. In addition, early developmental events of crtf-null strains could be rescued with exogenous cAMP treatment, constitutive expression of CAR1 or co-development with wild-type cells; however, these treatments were insufficient to promote sporulation. This suggests a cell-autonomous role for CRTF during late development that is separate from its capacity to control CAR1 expression. Finally, ablation of CRTF promotes a precocious induction of certain cAMP-dependent gene expression pathways. We suggest that CRTF may function to help insulate distinct pathways from simultaneous and universal activation by cAMP. CRTF, thus, exhibits multiple complex and independent regulatory functions during *Dictyostelium* development.

Key words: Transcription factor, Development, Gene regulation, *Dictyostelium*

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 adenylyl 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.,

1991; Saxe et al., 1993; Johnson et al., 1993; Louis et al., 1994). CAR1 is expressed earliest and exhibits the highest affinity for cAMP (Saxe et al., 1991; Johnson et al., 1992). CAR1 is also the major cAMP receptor that mediates and relays signaling during aggregation. More downstream, cAMP signaling through CAR1 is required for chemotactic movement and developmentally regulated gene expression. In the absence of CAR1, development is arrested prior to aggregation and cAMP-induced gene expression (Klein et al., 1988; Sun and Devreotes, 1991), but this early developmental block can be bypassed by exposure to exogenous cAMP and stimulation of CAR3 (Soede et al., 1994).

Of the plethora of genes induced by the pulsatile cAMP signal during early *Dictyostelium* development, *CAR1* is among the first. Its expression is regulated through multiple promoters (Saxe et al., 1991; Louis et al., 1993); however, *CAR1* is not expressed during logarithmic growth, and as nutrients become depleted and cell density increases, *CAR1* is induced at a low level (Mann and Firtel, 1989; Rathi at al., 1991; Saxe et al., 1991; Louis et al., 1993). This regulation is likely to be the result of response to the secreted glycoprotein PSF that accumulates maximally as cells exit log phase growth (Saxe et al., 1991; Louis et al., 1993; Mu et al., 1998). *CAR1* expression via the early promoter is significantly activated (20- to 50-fold) by pulsatile cAMP signaling at nanomolar levels (Louis et al., 1993). As the extracellular cAMP level increases in conjunction with multicellular mound formation, the early *CAR1* promoter becomes de-activated; however, *CAR1* is not fully repressed. At high, continuous cAMP concentrations, the late-specific promoter is induced and a second *CAR1* mRNA form is expressed at a low level throughout the remainder of development (Saxe et al., 1991; Louis et al., 1993).

We have previously identified a sequence element termed GAC-DR within the *CAR1* early promoter that is essential for cAMP pulse-mediated activation (Mu et al., 1998). This short element conveyed inducibility to a heterologous minimal promoter, and mutation of the element in an otherwise full-length *CAR1* early promoter construct abolished cAMP-induced expression. We had further identified a nuclear factor with binding specificity for the *CAR1* early element (Mu et al., 1998). We now report the purification of the CAR1 transcription factor CRTF using sequence-specific DNA affinity chromatography. We have isolated *CRTF* cDNAs and created crtf-null strains by homologous recombination. Surprisingly, CRTF is required not only for *CAR1* expression and early developmental events, but also throughout later developmental stages. crtf-nulls have a conditional aggregation-minus phenotype that can be rescued by mimicking CAR1 function. Nonetheless, mutant cells possess severe developmental defects that are independent of *CAR1*, cAMP signal transduction, and response to additional factors secreted by wild-type cells. Finally, CRTF seems to be absolutely required in a cell-autonomous manner for spore cell differentiation, underlying its complex role though *Dictyostelium* development.

MATERIALS AND METHODS

Growth, development and DNA-mediated transformation of *Dictyostelium*

Wild-type (Ax-3), and blasticidin- and G418-resistant cell lines were 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 concatamerized the double-stranded oligomer of the *CAR1* early promoter element (5′-TTTATAGACTTT-TTGACCTATAGGAGTGTAAA-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 (Morio 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 λZAP-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).

crtf-null strains

The targeting construct for disruption of the *CRTF* gene was made by inserting the blasticidin resistance gene cassette into the *Cla*I site within the DNA-binding domain of the CRTF 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 *CRTF* gene (with Demerec

nomenclature of *crtF*) disruption by homologous recombination and the absence of random insertions of the targeting construct in the genome,

β-galactosidase staining

Nitrocellulose filters with developed structures were fixed with 1% glutaraldehyde and stained as described (Richardson et al., 1994).

Isolation and hybridization of RNA

Total RNA was prepared, size separated on formaldehyde/agarose gels and transferred to nitrocellulose (Kimmel, 1987). Probes were radiolabeled by random priming using [α-^{32}P]dCTP and hybridized to RNA blots at 37°C in 0.8 M Na^+ and 50% formamide (Wahl et al., 1987).

Expression of HA-tagged CRTF and immunoblot analysis

The full-length CRTF sequence was extended at its C-terminal end by addition of the influenza hemagglutin (HA) epitope YPYDVDYA. The construct was confirmed by sequence analysis, fused to the *act15* promoter in an integrating transformation vector, and transformed into crtf-null cells. Multiple positive crtf-null::*act15*/*CRTF-HA* clones were selected by resistance to G418 and developed for phenotypic analysis.

Nuclear proteins were prepared from cells and separated by gel electrophoresis in 10% Tricine (NOVEX) and blotted onto PVDF membrane. The membrane was blocked with 5% non-fat milk for 2 hours at room temperature, incubated for 1 hour with rabbit anti-HA polyclonal IgG (Y-11, Santa Cruz), rinsed with Tris-buffered saline (TBS) and incubated for 1 hour with HRP-conjugated anti-rabbit IgG. After another wash with TBS, the membrane was developed by ECL (Pierce).

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.

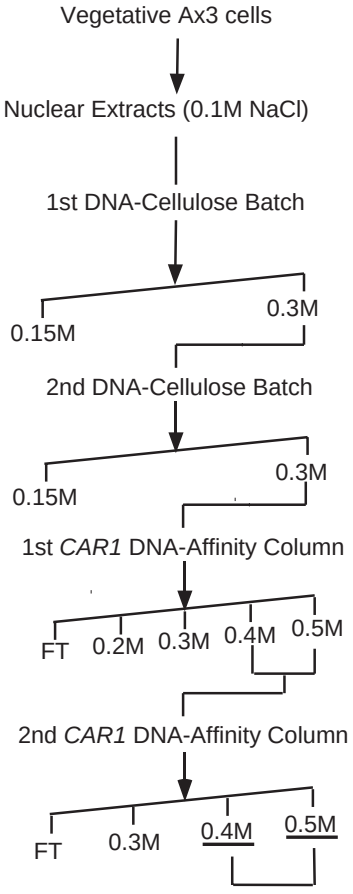

Fig. 1. Purification scheme for CRTF. Final pools of 0.4 M and 0.5 M NaCl eluates from the *CAR1* DNA affinity column represent an activity enrichment of 10,000-fold (see Materials and Methods).

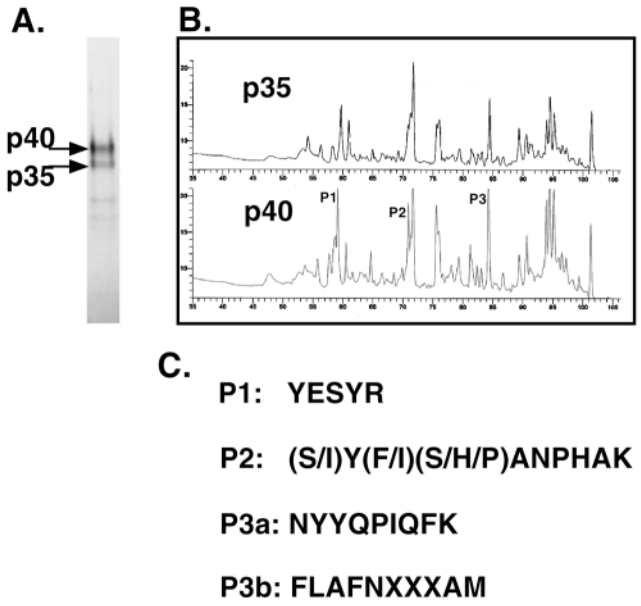

Fig. 2. Analysis of purified CRTF. (A) Purified CRTF was separated by SDS-polyacrylamide gel electrophoresis and detected by silver staining. (B) HPLC analysis of tryptic peptides for the p35 and p40 forms of CRTF. (C) Deduced peptide sequences of P1, P2 and P3. The P3 fraction is a mixture of peptides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

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MSSIEQLYSTTQFPSLSSCNTNINNTNNNNNINNINNNTNI    41
NTGNLLKNTFFKNNIIYNTNNNSINNNSNNNIINNNNNNLL    82
NSNNQNSFQNQNNLNSIHNHNNNNNNNNSNNNNTSNSENLD   123
SLKSALLSSNIFNGSSSSIAHQNLINLIISSQNNNNNNGNS   164
NSNTPDQKSTMADILLNSSVPDYFEW---CSDIINMPKDGN   202
EKSNNSNGGNNNGNNNGNNNNNHNNNNNNNSNNNNNNNSDS   243
VPSPQNNYQSPQSDNQSSMVPSFNFSNQNSANSSNNNNNNN   284
GSSNNNNNVRSEPTTPNDESNNNSNNTNNNNNNNNNNNNNQ   325
QQQLSSNQNSSPLIYSVPSYYVSNYGVVLSAPMKGNSSSSI   366
LPPQAWPQNNNNNNGNSNSNNGNNNASGFSDFMFNGSNIDY   407
NSIMNNNNNNNNNNNNNNNNNNNNNSSNNNNNNGNNNNGN   448
NGGMFNGGFVNNNNHNNNNNNNNNGNLNFHNFLQFQNQLQ   489
NSNQQQQQQQLQQQQQLQQHQQQQQQLQQQQLQQQFNIGKP   530
CNINQKKGRGANNNNNNMPIGNQASLVISHPSLKMEGHHHL   571
QQQQQQQHHHQHQQHQQNGNGNNTQIVNSAFLDLSSPDSHN   622
QMSPSSPICLPESPMGAQHWESGLDPSSLQKQNEINPPISV   653

RSNVVFFEPYLAGEPGRHKACWNLLEPMHRNYYQPIQFKLP   694
SFPDTSLPITQI^DDKTGIFDSQRFLAFNNPQAMSKYESYR   734
IYIHPSLGYSGNAKRFKQQPDVNEKALILDGNVYDGHLNPI   775
YNCKICTEYYQTKSYFSANPHAKGKVLLVKNNILTRVKDGG   816
FTLSLKPMCCSGHNSHIPLYFHFTLTNPLTNEVVLQSLINV   857
NVKQWKKSVPNKSKKQRFE                         876
 *     *     *
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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 asparagine (N)- and poly glutamine (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 form an atypical zinc-finger motif are in bold. The asterisks denote positions for C-terminal deletions to amino acids 870, 865 and 859 (see Fig. 4). A putative nuclear localization sequence at the C terminus is in green. ^ indicates cloning position of BLAST gene within the DBD for the gene disruption construct.

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 an effective N-terminal truncation. This form of CRTF retains the DBD, but is deleted of the runs of polyasparagine and polygutamine. 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).

In shaking culture, crtf-null cells grow with the same doubling time as wild-type cells and on bacterial lawns crtf-nulls form normal size growth plaques; however, development within these plaques is very poor. This is seen more clearly when cells are developed directly on solid substrata at various cell densities. On agar plates at low density (0.7×10^5 cells/cm^2), crtf-null development is completely blocked prior to aggregation (Fig. 6A). However, this is a conditional aggregation-minus phenotype. At higher cell density (4.5×10^5 cells/cm^2) on agar plates or on nitrocellulose filter matrices, there is a partial rescue of the aggregation-minus phenotype, but later stages of development are delayed significantly and are inefficient compared with wild type (Fig. 6B). Wild-type cells complete aggregation by 10 hours, form slugs and culminate in fruiting body formation at 24 hours. Here, the crtf-null cells did not complete aggregation before 48 hours. Their aggregation territories and migration streams are much larger than wild-type, consistent with a primary defect in CAR1-dependent cell-cell signaling (Klein et al., 1988; Sun and Devreotes, 1991). A small portion of the slugs (<25%) eventually (72 hours) form fruiting bodies, but with sorocarps of a glassy appearance that is characteristic of defective spore differentiation (Loomis, 1998). While the aggregation defects of crtf-nulls are correlated with the loss of normal *CAR1* expression (see below), compromised development at post-aggregation stages may suggest an additional role for CRTF during late development.

The morphology of crtf-null fruiting bodies suggests that

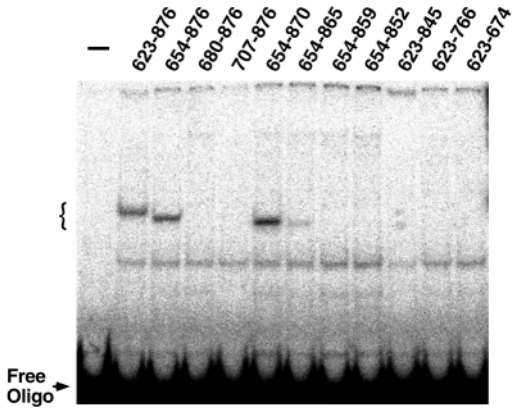

Fig. 4. Deletion mapping of the DNA-binding domain of CRTF. A series of truncated CRTF forms were expressed in *E. coli* and equal amounts of protein assayed for ability to complex (bracket) with the *CAR1* element by electrophoretic mobility shift.

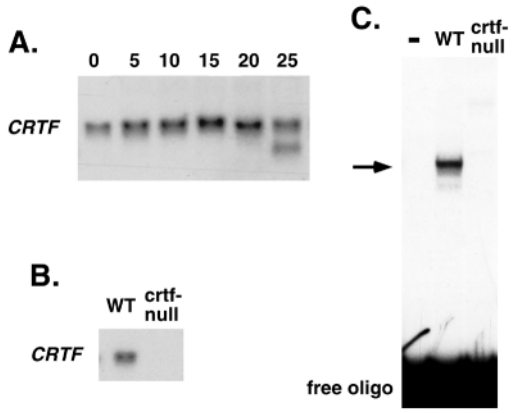

Fig. 5. CRTF expression in WT and crtf-null strains. (A) *CRTF* mRNA expression at various hours of development. (B) *CRTF* mRNA expression in wild-type (WT) and crtf-null strains. (C) CRTF DNA-binding activity (arrow) from nuclear extracts of wild-type (WT) and crtf-null strains treated with 20 nM cAMP at 6 minutes intervals for 5 hours. Control (–) had no added extract (see Fig. 7).

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 ctrtf-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 (Saxe et al., 1991; Louis et al., 1993). In crtf-null cells (see Fig. 8), we observe only a very low level of *CAR1* expression that reflects the minimal gene activation seen at the transition from growth in response to starvation (Mann and Firtel, 1989; Rathi et al., 1991; Saxe et al., 1991; Louis et al., 1993). *CAR1* expression remained low with no subsequent induction. We also failed to see the transition from early to late promoter expression as development progressed. The low level of CAR1 and resulting defects in cAMP signaling explain the impaired aggregation of crtf-null cells.

We investigated whether exogenous cAMP could rescue gene expression of the crtf-nulls (Fig. 8). Wild-type and crtf-null cells were developed in shaking culture in the absence or presence of 20 nM pulses of cAMP added at 6 minute intervals. Wild-type cells will endogenously secrete pulsatile cAMP and induce CAR1 expression to high levels by 6 hours of development, a process accelerated by treatment with exogenous cAMP (Fig. 8). crtf-null cells expressed only a low level of *CAR1* mRNA in untreated shaking cultures, similar to that observed for cells developed on solid substrates. Exogenous cAMP pulses, however, were able to rescue *CAR1* expression patterns (Fig. 8). Similar transcriptional results were observed for the early genes *csA* and *Gα2*, the expression of which parallels that of *CAR1*. Nonetheless, we still did not detect any factors that could interact with the *CAR1* promoter element in extracts of cells that had been activated for *CAR1* expression by exogenous cAMP stimulation (see Fig. 5C). Thus, although CRTF is essential to initiate cAMP pulse-induced *CAR1*, *csA* or *Gα2* expression, this defect can be bypassed. Transcription factors acting at sites other than those centered proximate to the GAC-DR element may participate.

CRTF is not essential for cell differentiation and cell sorting

We have also examined the expression of prespore- and prestalk-specific genes in the crtf-nulls. As these cells develop asynchronously, we monitored gene expression in shaking culture, where cell differentiation can be induced by selective treatment with cAMP. Cells were pulsed with 20 nM cAMP

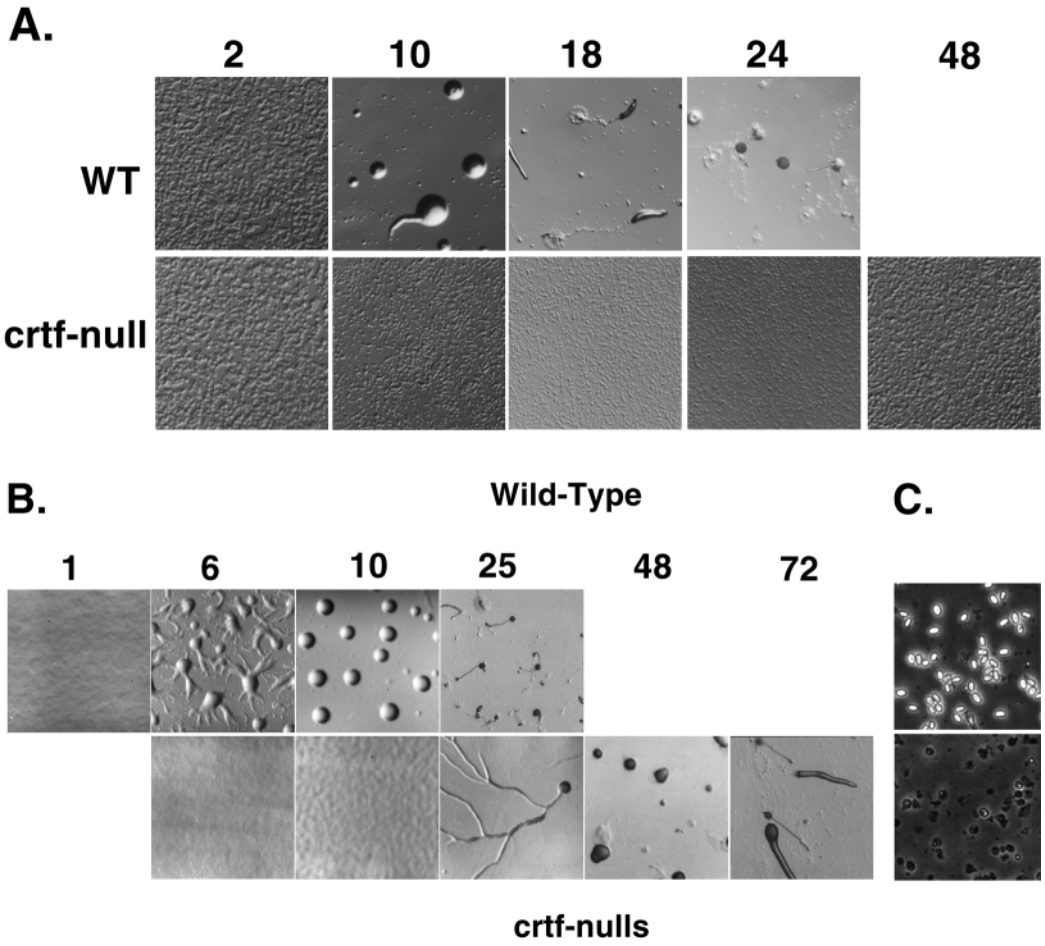

Fig. 6. Abnormal development of crtf nulls. (A) Wild-type and crtf-null strains were developed (time in hours) on agar at 0.7×10^5 cells/cm^2. (B) Wild-type and crtf-null strains were developed (time in hours) on agar at 4.5×10^5 cells/cm^2. **(**C) Wild-type and crtf-null strains were developed to fruiting bodies as in Fig. 6B and spores analyzed by phase microscopy.

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 sluges and to the spore mass of fruiting bodies. Prestalk *ecmA*/*lacZ* 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,

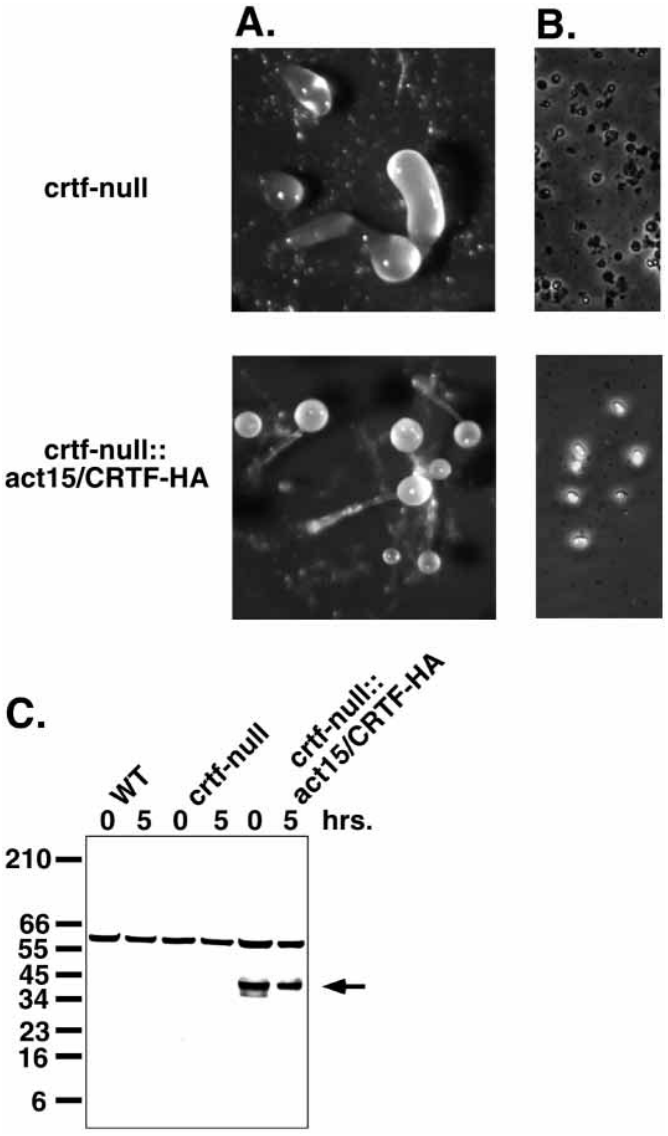

Fig. 7. Expression of CRTF-HA protein rescues crtf-null development. (A) crtf-null strain and crtf-null strain expressing the CRTF-HA tagged protein (crtf-null::*act15*/*CRTF-HA*) were developed on agar for 24 hours. (B) crtf-null and crtf-null::*act15*/*CRTF-HA* strains were developed to fruiting bodies as in Fig. 7A and spores analyzed by phase microscopy. (C) Wild-type, crtf-null and crtf-null::*act15*/*CRTF-HA* cells were differentiated in shaking culture in the presence of exogenous pulses of cAMP. Nuclear extracts were prepared from cells differentiated for 0 and 5 hours, and analyzed by immunoblot using anti-HA sera. Migration positions of molecular weight markers are indicated. Arrow indicates presence of the 40 kDa-Ha form of CRTF. A nonspecific band of ~60 kDa is detected in all cells.

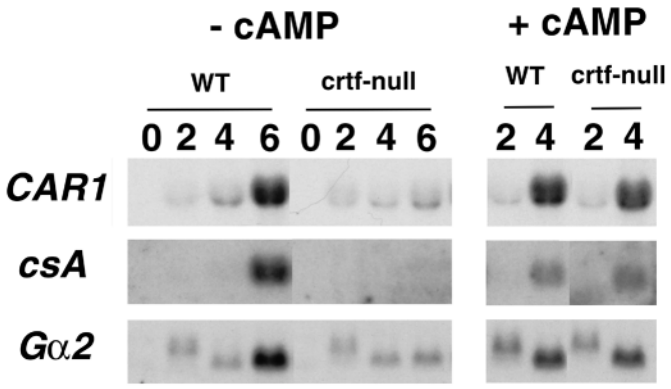

Fig. 8. Aggregation stage gene expression defects in crtf-nulls. WT and crtf-null strains were differentiated in shaking culture in the presence or absence of exogenous pulses of cAMP. RNA expression was examined after various hours of differentiation.

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 500μM 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 crft-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.

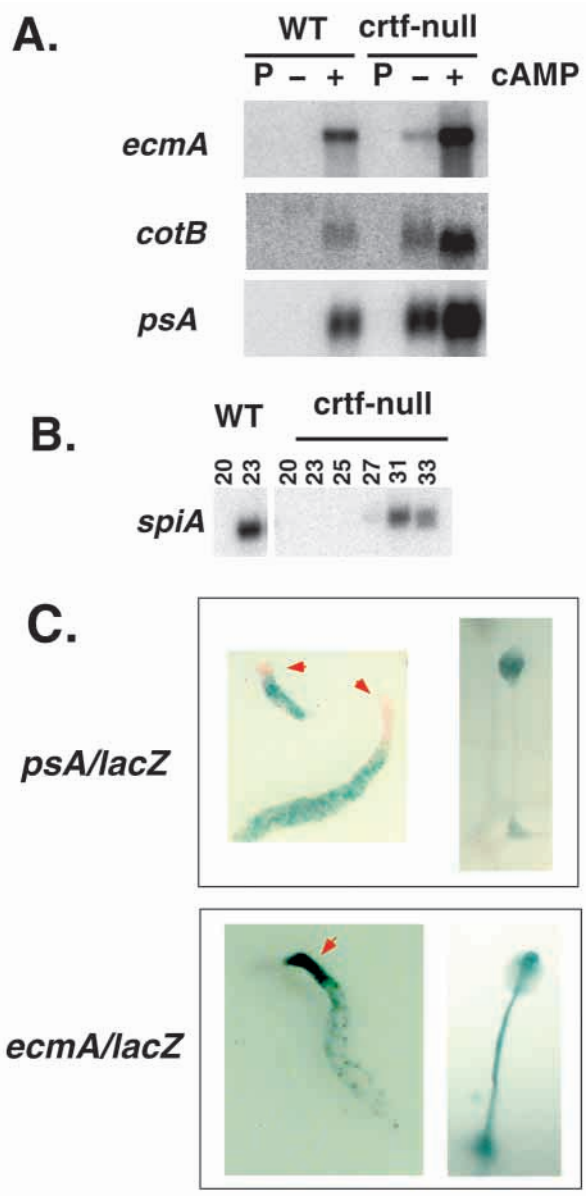

Fig. 9. Cell-specific gene expression patterns in crtf-nulls. (A) Expression of prestalk (*ecmA*) and prespore (c*otB*, *psA*) genes in wild-type and crtf-null strains differentiated in shaking culture in the presence of exogenous pulses of cAMP (P) followed by treatment without (–) or with (+) 500 μM 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.

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.

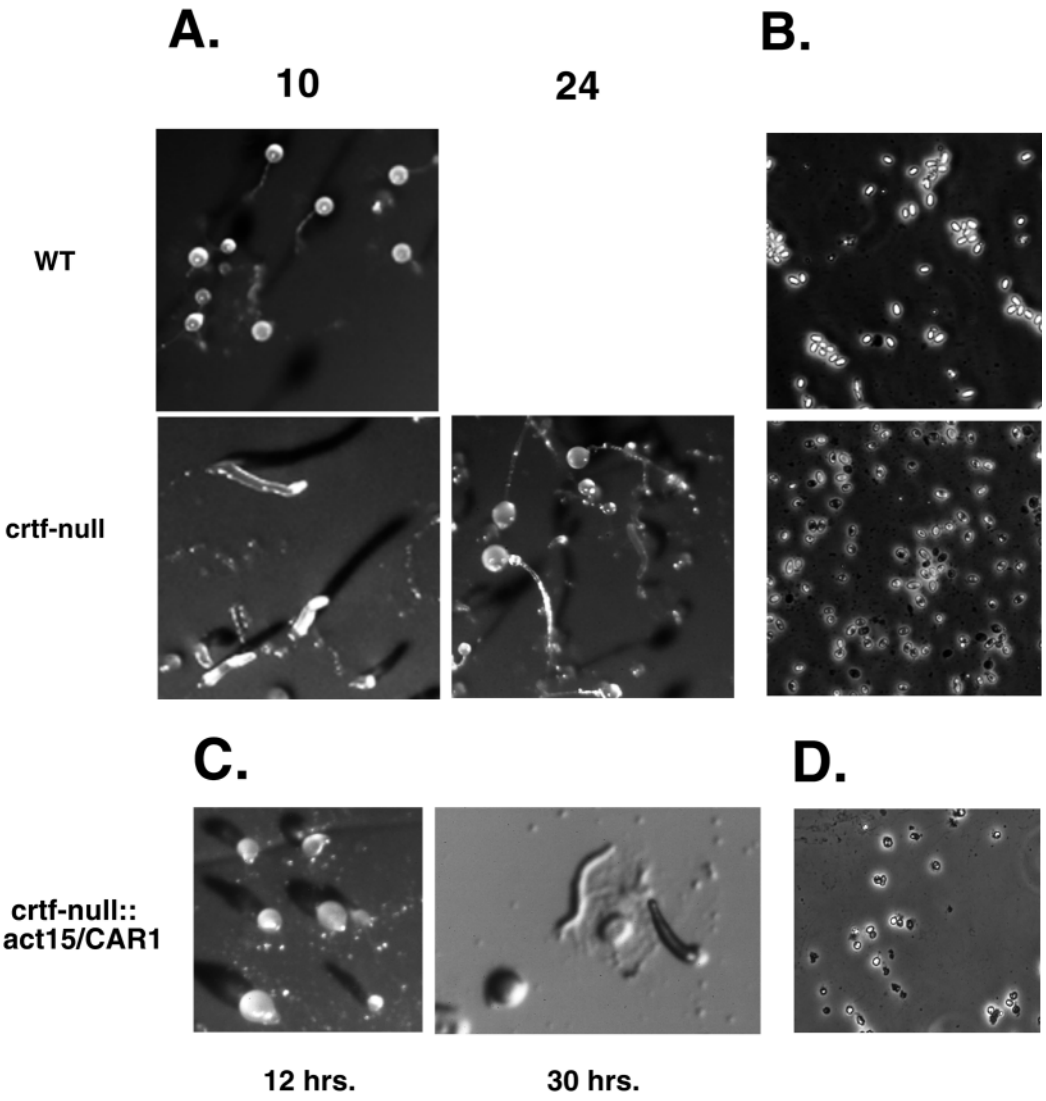

Fig. 10. CAR1-independent defects of crtf-nulls. (A) Wild-type and crtf-null strains differentiated in shaking culture for 5 hours in the presence of exogenous 30 nM pulses of cAMP for 5 hours followed by treatment with 500 μM cAMP for an additional 5 hours. Cells were then plated on filters and examined at 10 and 24 hours. (B) Spores obtained from cells developed to fruiting bodies in Fig. 8A were analyzed. (C,D) Constitutive expression of CAR1 in crtf-nulls will rescue aggregation but not late development or sporulation of crtf-nulls. crtf-nulls carrying *act15*/*CAR1* were developed (C) on filters for times shown and spores analyzed (D).

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*, *Gα2*), 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 *Gα2*, 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-

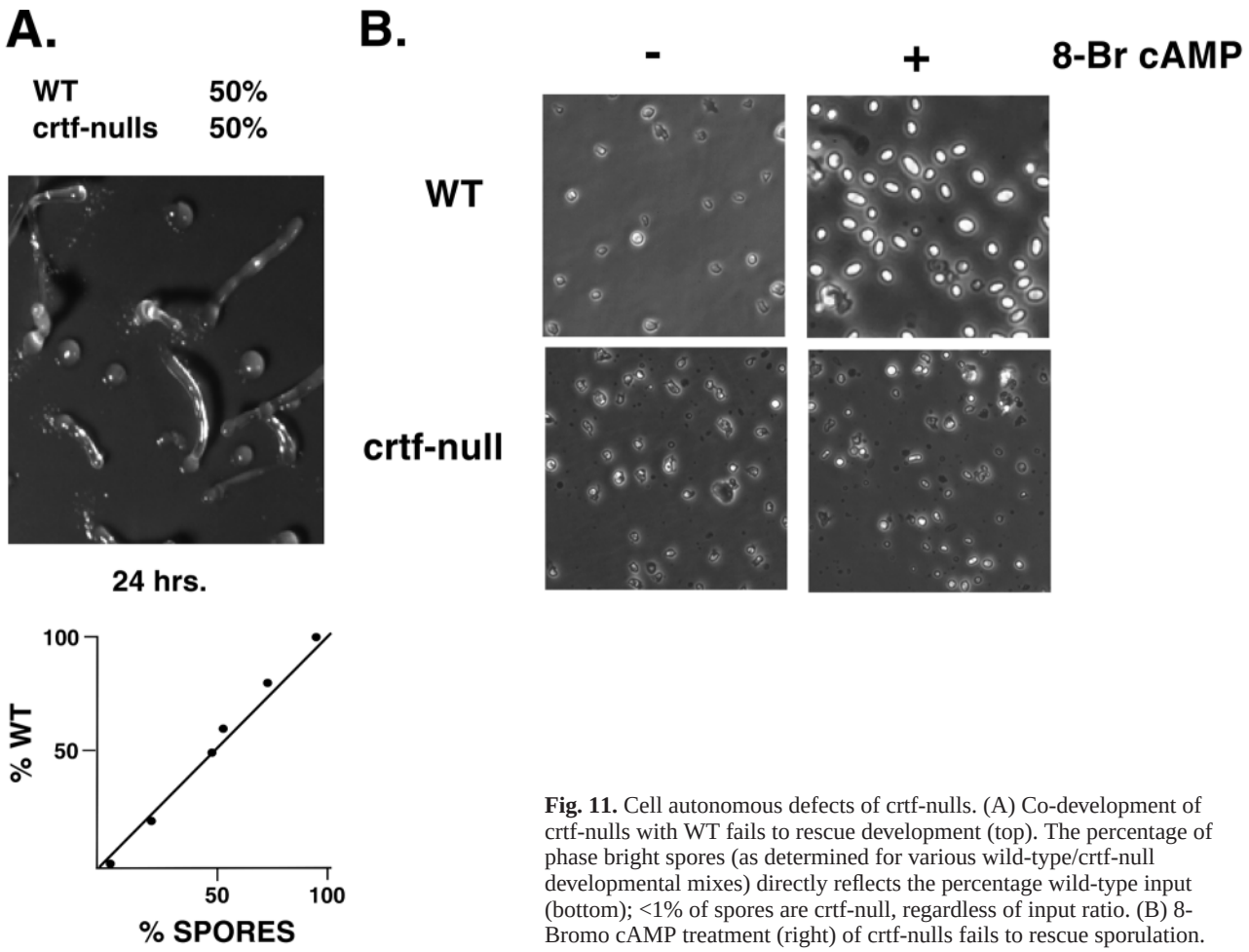

Fig. 11. Cell autonomous defects of crtf-nulls. (A) Co-development of crtf-nulls with WT fails to rescue development (top). The percentage of phase bright spores (as determined for various wild-type/crtf-null developmental mixes) directly reflects the percentage wild-type input (bottom); <1% of spores are crtf-null, regardless of input ratio. (B) 8-Bromo cAMP treatment (right) of crtf-nulls fails to rescue sporulation.

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.

We are very grateful to Loc Trinh for excellent technical assistance; to Dr R.A. Firtel for generously providing us with a *Dictyostelium* cDNA expression library; and to the Baylor, Jena, Sanger and Tsukuba Sequencing Centers for access to *Dictyostelium* genomic and cDNA sequencing data. We are very appreciative to the continued

discussions with Drs Leung Kim, Joseph Brzostowski, Lisa Kreppel, Brian Oliver and Pamela Schwartzberg.

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