Eukaryotic Cell, Dec. 2006, p. 2024–2032 Vol. 5, No. 12
1535-9778/06/$08.00+0 doi:10.1128/EC.00287-05
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Genetic Evidence that the Acyl Coenzyme A Binding Protein AcbA and the Serine Protease/ABC Transporter TagA Function Together in *Dictyostelium discoideum* Cell Differentiation[▽]

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Received 7 September 2006/Accepted 10 October 2006

The acyl coenzyme A (CoA) binding protein AcbA is cleaved to form a peptide (SDF-2) that coordinates spore encapsulation during the morphogenesis of *Dictyostelium discoideum* fruiting bodies. We present genetic evidence that the misspecification of cell types seen in mutants of the serine protease/ABC transporter TagA results from the loss of normal interactions with AcbA. Developmental phenotypes resulting from aberrant expression of the TagA protease domain, such as the formation of supernumerary tips on aggregates and the production of excess prestalk cells, are suppressed by null mutations in the *acbA* gene. Phenotypes resulting from the deletion of *tagA*, such as overexpression of the prestalk gene *ecmB* and the misexpression of the prespore gene *cotB* in stalk cells, are also observed in *acbA* mutants. Moreover, *tagA*$^-$ mutants fail to produce SDF-2 during fruiting body morphogenesis but are able to do so if they are stimulated with exogenous SDF-2. These results indicate that the developmental program depends on TagA and AcbA working in concert with each other during cell type differentiation and suggest that TagA is required for normal SDF-2 signaling during spore encapsulation.

We previously described TagA, a predicted serine protease/ABC transporter, and showed that it is required for proper cell differentiation during the development of *Dictyostelium discoideum* (7). Disruption of the ATPase domain of TagA by an insertion mutation in the *tagA* gene resulted in a mutant that produced an excess of prestalk cells and supernumerary prestalk tips on aggregates midway through development. Transcriptional profiling of development indicated that *tagA* insertion mutant (*tagA1209*; see Fig. 1A) cells have defects during early development, as well as a muted developmental program, compared to wild-type cells. Furthermore, cell type-specific genes failed to show cell type specificity in fruiting bodies of this *tagA* mutant. These data led to the conclusion that *tagA* is involved in specifying cell fate (7). Based on TagA's domain structure, an N-terminal protease attached to an ABC transporter, one attractive model is that TagA acts in a manner analogous to the processing and transport of peptide antigens into the endoplasmic reticulum of T cells through the combined action of the proteosome and the TAP1/TAP2 transporter (1). Support for this hypothesis should come from the identification of relevant TagA substrates, and that would provide a starting point for understanding the molecular mechanism of TagA function in cell type determination. TagA mRNA is enriched in prespore cells, so its potential substrates should also be found in prespore cells, but to date there are no obvious candidate proteins or peptides upon which TagA might act.

Sporulation in *Dictyostelium* is a highly regulated process coordinated with the completion of fruiting body formation. At least two signaling peptides, known as spore differentiation factors 1 and 2 (SDF-1 and SDF-2), coordinate spore encapsulation with morphogenesis. SDF-1 is a cationic peptide released at the onset of culmination that induces encapsulation approximately 90 min later in a process that requires intervening protein synthesis (2). SDF-2 is an anionic peptide that induces rapid spore encapsulation by a process that does not depend upon subsequent protein synthesis (5). The protein precursor of SDF-2 was recently shown to be the acyl coenzyme A (CoA) binding protein AcbA (4). Interestingly, the human homolog of AcbA is the precursor of neuropeptides that modulate $GABA_A$ receptor function in central nervous system neurons (6). Although *acbA* is expressed in prespore cells during culmination, its product, AcbA, must be processed by the prestalk-specific serine protease/ABC transporter TagC (4, 5). We explored the possibility that TagA and AcbA might function together to specify cell fate, since TagA has a structure very similar to that of TagC and *tagA* is expressed in prespore cells, where *acbA* is also expressed.

We found that some of the previously described phenotypes resulting from disruption of *tagA* were the consequence of loss of the ABC transporter function while leaving the serine protease domain intact. These phenotypes, which appear to have been caused by unregulated protease activity, were suppressed by null mutations in *acbA*. On the other hand, misexpression of cell type-specific genes that result from the deletion of both the protease and ABC transporter domains of *tagA* were mimicked rather than suppressed by mutations in *acbA*. In addition, SDF-2 signaling is abrogated in *tagA* mutants. Together, these results support a direct interaction between TagA and AcbA that specifies cell fate.

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▽ Published ahead of print on 20 October 2006.

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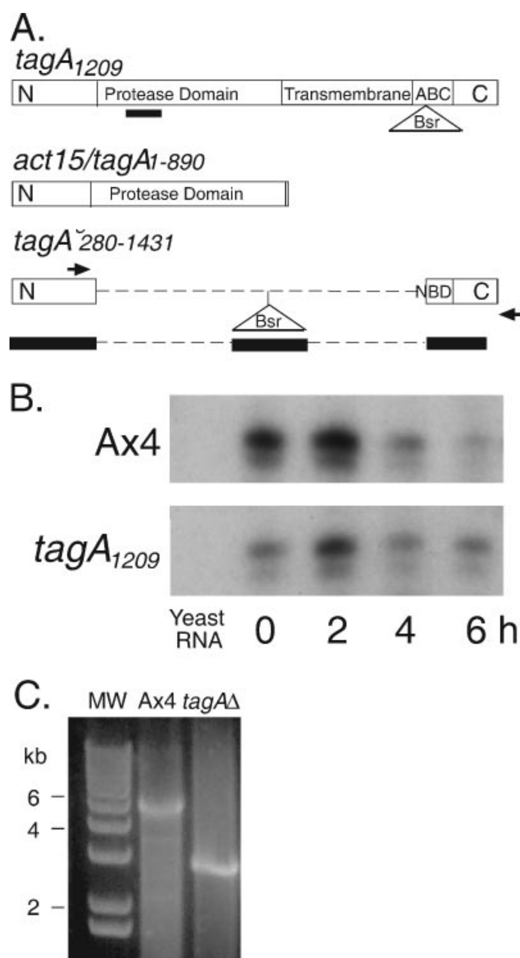

FIG. 1. *tagA* structure, expression, and deletion. (A) The top diagram shows the structure of the *tagA1209* insertion mutation shown within the *tagA*-coding region (open rectangles) (7). The parts of the gene that encode the protease, transmembrane, and ATP binding cassette (ABC) domains are indicated. The positions of the RNase protection probe (black bar) and the blasticidin resistance cassette (Bsr) are also shown. The middle diagram shows the portion of the *tagA*-coding region included in the TagA protease domain expression construct that is driven by an actin gene promoter. The bottom diagram shows the deduced structure of the partial deletion of the *tagA* gene. Dashed lines indicate the absence of the corresponding *tagA*-genic DNA. Black bars indicate the segments of DNA that are included in the knockout construct. The arrows indicate the position of the diagnostic PCR primers (not to scale). (B) RNase protection assays (RPA) of the wild type and *tagA1209* using a probe located 5′ of the 1209 insertion site, indicated by a black bar in panel A. (C) PCR confirmation of the *tagA* partial deletion strain (*tagA*Δ), using genomic DNA of the mutant and wild type amplified with one oligonucleotide primer contained within the deletion construct described above for panel A and one primer corresponding to the genomic DNA on the 3′ side of the *tagA*-coding region. MW, molecular size marker.

MATERIALS AND METHODS

Strain construction, cell growth, and development. The *Dictyostelium discoideum* strains used in this study are described in Table 1. Ax4 cells were grown in HL-5 liquid medium supplemented with 50 μg/ml streptomycin and 50 U/ml penicillin (16). Neomycin-resistant strains (Neo^r) and all of their derivatives were grown in HL-5 liquid medium supplemented with 20 μg/ml G418 (Geneticin). All strains were removed from drug-containing media 36 h prior to assay. Cells were plated for synchronous development on nitrocellulose filters as described previously (16). Transformation of *Dictyostelium* cells was performed according to previous guidelines (10) using a BTX 600 electroporation device (Genetronics, San Diego, CA). Inactivation of the *tagA* gene was achieved by homologous recombination at the genomic locus after electroporation of linearized DNA fragments. The *tagA* deletion construct was created such that the bulk of the *tagA* locus, including the ATP binding domain of the ABC transporter, the transmembrane helices, and the putative active residues of the serine protease domain, would be replaced by a 1.4-kb blasticidin S resistance cassette from pBsR503 (12). The DNA fragment containing the 5′ region of the *tagA* locus was synthesized by PCR and was placed into TA cloning vector pCR2.1 (Invitrogen). This fragment consists of bases 2 to 888 of the *tagA* coding region with a base pair inserted between 882 and 883 to create a ClaI site. The 3′ region of the *tagA* locus was similarly synthesized by PCR, and the product consisted of bases 4207 to 5088 of the coding region. These 5′ and 3′ fragments were then cloned into XbaI/HindIII-digested pGEM3 plasmid (Promega) using an XbaI site added to the 5′ *tagA* fragment, a HindIII site added to the 3′ *tagA* fragment, and the ClaI sites that border the *tagA* deletion on both the 5′ and 3′ fragments. The blasticidin S resistance cassette was then placed into the ClaI site. This construct was linearized prior to transformation using HindIII and XbaI (Fig. 1). Integration at the native locus was determined by PCR analyses.

SDF-1 and SDF-2 activity assays. SDF-1 and SDF-2 were separated on cation and anion exchange resins, respectively (2). Serial dilutions were added to KP cells that had been incubated at low cell density overnight, and spores were visually counted after 2 h (2). Units are given as the reciprocal of the highest dilution giving maximal activity. Cells dissociated from culminants were primed with 10 pM synthetic SDF-2. After 5 min of incubation, the cells were washed and 1 pmol recombinant AcbA added as indicated (3). SDF-2 in the supernatant was assayed after an additional 1-h incubation.

β-Galactosidase staining. In all cases, images are of representative fingers or fruiting bodies. Experiments were performed multiple times on at least two independent strains. In the codevelopment experiments, the *cotB*/*lacZ*-marked strains were mixed with wild-type cells, marked with *act15*/*GFP*, at a ratio of 20:1 and allowed to form fruiting bodies. The pattern of green fluorescent protein (GFP) expression was used to assess admixture of the two strains and to ensure that mutant cells were present in the stalk before staining for β-galactosidase activity. Staining of developmental structures and spores for β-galactosidase was carried out as described previously (15). Cells were developed on white filters for the specified time, and the under-pad was removed. Cells were fixed for 10 min by gently dropping from a pipette onto the filter, 3.7% formaldehyde in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0). The solution was wicked off from the edge of filter, and the filter was washed one time with Z buffer. Cells were permeablized for 20 min with 0.1% NP-40 in Z buffer, and the filter was washed with Z buffer. Staining was carried out with 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-Gal) staining solution {0.5 ml 100 mM $K_3[Fe(CN)_6]$, 0.5 ml 100 mM $K_4[Fe(CN)_6]$, 0.5 ml 20 mM X-Gal, 1 ml 10 mM EGTA, 7.5 ml Z buffer} at 37°C for up to 24 h, and staining was stopped by rinsing with Z buffer. Structures were sometimes counterstained with 0.1% eosin Y in Z buffer for 1 min and rinsed with Z buffer.

RESULTS

Creation of a *tagA* deletion mutant. The previously characterized *tagA1209* mutation was generated by an insertion that interrupted the nucleotide binding domain of the ABC transporter (7). We previously showed that *tagA1209* failed to accumulate detectable mRNA throughout development using an RNase protection assay that detected coding sequences downstream of the insertion site. We suspected that the *tagA1209* mutant might still be able to express the protease domain encoded in the 5′ half of the *tagA* gene. Probing for mRNA upstream of the 1209 insertion showed that, indeed, this portion of the *tagA* mRNA was present during early development, although at somewhat lower levels than in wild-type cells (Fig. 1A and B). Thus, it is possible that the protease domain of TagA is expressed in the *tagA1209* mutant. Therefore, we generated a *tagA* deletion mutant that replaced the serine protease domain and most of the ABC transporter domain with a blasticidin resistance cassette (*tagA*Δ; see Fig. 1A). A new deletion strain was isolated by PCR-based identification of the

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TABLE 1. *Dictyostelium* strains used in this study

Strain	Relevant genotype	Parental strain	Drug marker(s)	Reference
Ax4	Ax4[a]	Ax3		9
	Ax4 [*ecmA/GFP*]	Ax4	Neo^{rb}	8
	Ax4 [*cotB/lacZ*]	Ax4	Neo^{r}	7
	Ax4 [*act15/GFP*]	Ax4	Neo^{r}	7
AK1142	Ax4 [*ecmB/lacZ*]	Ax4	Neo^{r}	7
AK1200	*tagA*$^{-}$	Ax4	bs^{rb}	This work
AK1201	*tagA*$^{-}$ [*ecmA/GFP*]	Ax4 [*ecmA/GFP*]	Neo^{r}, bs^{r}	This work
AK1202	*tagA*$^{-}$ [*ecmB/lacZ*]	Ax4 [*ecmB/lacZ*]	Neo^{r}, bs^{r}	This work
AK1203	*tagA*$^{-}$ [*act15/GFP*]	Ax4 [*act15/GFP*]	Neo^{r}, bs^{r}	This work
AK1204	*tagA*$^{-}$ [*cotB/lacZ*]	Ax4 [*cotB/lacZ*]	Neo^{r}, bs^{r}	This work
AK859	*tagA1209*	Ax4	bs^{r}	7
AK1215	*tagA1209* [*cotB/lacZ*]	Ax4 [*cotB/lacZ*]	Neo^{r}, bs^{r}	7
AK1216	*tagA1209* [*act15/GFP*]	Ax4 [*act15/GFP*]	Neo^{r}, bs^{r}	This work
	acbA^{-}	Ax4	bs^{r}	4
AK1205	*acbA*$^{-}$ [*ecmA/GFP*]	Ax4 [*ecmA/GFP*]	Neo^{r}, bs^{r}	This work
AK1206	*acbA*$^{-}$ [*cotB/lacZ*]	Ax4 [*cotB/lacZ*]	neo^{r}, bs^{r}	This work
AK1207	*acbA*$^{-}$ [*ecmB/lacZ*]	Ax4 [*ecmB/lacZ*]	Neo^{r}, bs^{r}	This work
AK1208	*acbA*$^{-}$ [*act15/GFP*]	Ax4 [*act15/GFP*]	Neo^{r}, bs^{r}	This work
AK1209	Ax4 [*act15/tagA1-890*]	Ax4	Neo^{r}	This work
AK1210	*acbA*$^{-}$ [*act15/tagA1-890*]	Ax4 [*act15/tagA1-890*]	Neo^{r}, bs^{r}	This work
AK1211	*tagA*$^{-}$/*acbA*$^{-}$	HL328	bs^{r}	This work
AK1212	*tagA*$^{-}$/*acbA*$^{-}$ [*ecmA/GFP*]	*tagA*$^{-}$/*acbA*$^{-}$	Neo^{r}, bs^{r}	This work
AK1213	*tagA*$^{-}$/*acbA*$^{-}$ [*ecmB/lacZ*]	*tagA*$^{-}$/*acbA*$^{-}$	Neo^{r}, bs^{r}	This work
AK1214	*tagA*$^{-}$/*acbA*$^{-}$ [*cotB/lacZ*]	*tagA*$^{-}$/*acbA*$^{-}$	Neo^{r}, bs^{r}	This work

[a] Ax4 is used as the wild-type laboratory strain. Ax4 is an axenic derivative of NC4 derived from Ax3 (9).
[b] Neo^{r}, neomycin resistance; bs^{r}, blasticidin S resistance.

appropriate homologous recombinant among a population of *Dictyostelium* transformants (Fig. 1C).

The insertion mutant *tagA1209* forms characteristic multitipped fingers following aggregation that are not seen in wild-type strains (7). Aggregation of the *tagA* deletion mutant produced a single gnarled finger, suggesting the multitipped phenotype of the insertion mutant results from alterations in the activity of the serine protease when it is not associated with the ABC transporter (Fig. 2A). To directly test this possibility, we placed the protease-coding sequence from *tagA* in an expression cassette under control of the constitutive actin 15 promoter (*act15/tagA1-890*; see Fig. 1A). When this construct was expressed in the wild-type strain Ax4, the cells formed multitipped fingers following aggregation that were similar to those seen in the *tagA1209* insertion mutant (Fig. 2A).

The *tagA1209* insertion mutant also produces about twice the normal number of *ecmA*-expressing cells, some of which localize to the upper and lower cups of the sorus during culmination (7). When we introduced an *ecmA/GFP* construct into the *tagA* deletion strain, there was no obvious overproduction of *ecmA*-positive cells (Fig. 2B). This suggests that the overexpression of *ecmA*-positive cells also results from alterations in the regulation of the serine protease rather than loss of the ABC transporter activity of TagA.

Evidence for the interaction of TagA and AcbA. The substrate for the protease activity of TagA is not known, but that of a related serine protease/ABC transporter, TagC, is likely to be the acyl-CoA binding protein AcbA, which is the precursor of the culmination signaling peptide SDF-2 (4). Therefore, we explored the possibility that the TagA protease acts on AcbA, perhaps during early development, before TagC is expressed. If this is the case, then multitipped aggregates should not be observed in *acbA* mutants. In fact, the multitipped phenotype was not observed in the *acbA* mutant or the *acbA*$^{-}$/*tagA1209* double mutant (Fig. 3A). Moreover, expression of the TagA protease in an *acbA*-null strain did not lead to multitipped aggregates as it does in wild-type cells (Fig. 3B). Likewise, a null mutation in *acbA* suppresses the overproduction of *ecmA*-positive cells in the *tagA1209* insertion mutant (compare Fig. 2B with 3C). These genetic interactions suggest that the TagA protease functions with AcbA to control the number of tips on aggregates and the number of *ecmA*-positive cells in fruiting bodies.

***tagA* mutants do not produce SDF-2.** AcbA is found only in prespore cells before it is released during culmination and processed by TagC into the 34-amino-acid peptide SDF-2 that triggers spore encapsulation (4, 5). TagA protein is present in all cells in the early stages of development, and the expression of its mRNA becomes restricted to prespore cells and spores, thus, it may be acting on AcbA within cells during earlier stages of development. To test whether there might be further connections between TagA and AcbA, we determined whether *tagA1209* and *tagA*Δ strains could generate SDF-2 activity. Using a bioassay in which less than 1 pM SDF-2 can be detected by its ability to induce rapid spore encapsulation in a strain overexpressing the cyclic AMP-dependent protein kinase PkaC, we found that neither of the *tagA* mutant strains produced any measurable SDF-2 activity (Table 2). Extracellular SDF-2 signaling displays a form of positive feedback that probably serves to propagate the signal for spore encapsulation during culmination (3). Thus, it is possible to "prime" signaling-competent cells with exogenous SDF-2 and monitor the production of endogenous SDF-2. To further test for the production of SDF-2 in the mutants, we primed cells with 10 pM

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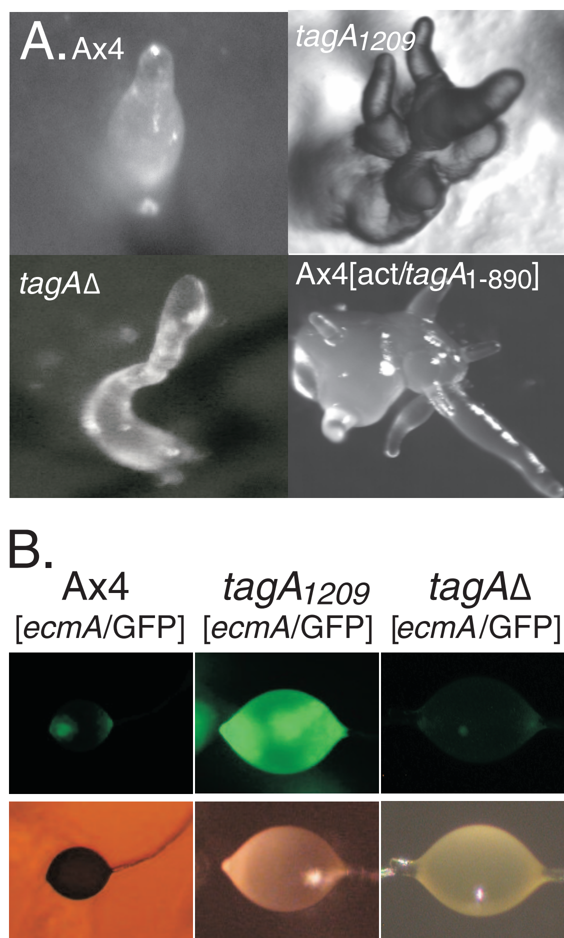

FIG. 2. Multitipped phenotype and the overproduction of *ecmA*-positive cells. (A) The developmental morphology of mutants after 16 h of development. In the wild-type strain (Ax4), a single finger emanates from an aggregate that had formed a single tip earlier. Both the *tagA* insertion mutant (*tagA1209*) and the Ax4 strain expressing the protease domain of TagA (Ax4[*act*/*tagA1-890*]) form multiple fingers from a single aggregate. (B) Localization of cells expressing *ecmA*/*GFP* in the upper and lower cups of sori after 24 h of development.

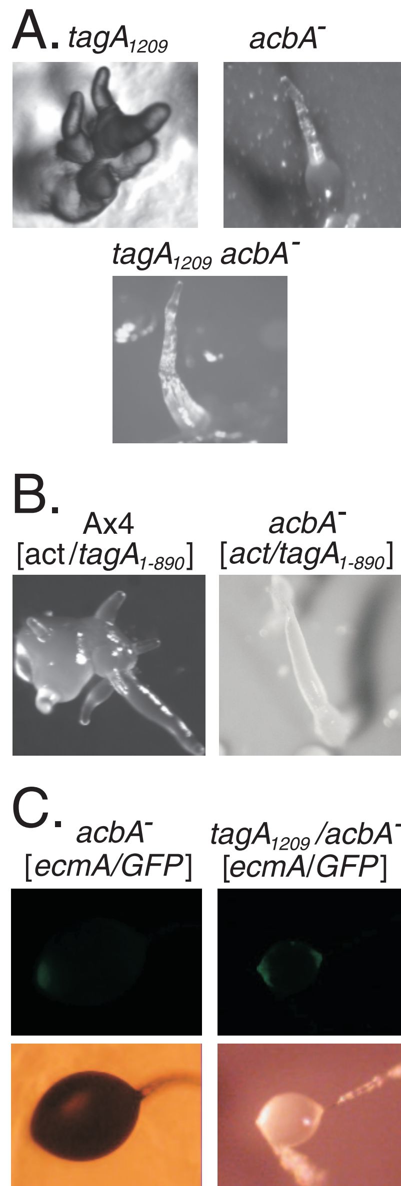

FIG. 3. Phenotypic suppression by *acbA*-null mutations. (A) Morphology of *acbA*$^-$ strains after 16 h of development. The multiple fingers that emanate from multitipped aggregates of the *tagA1209* insertion mutant (left panel; this is the same image as that in Fig. 2A) are not observed in the *acbA* mutant or the *acbA tagA1209* double mutant. (B) Expression of the TagA protease domain (*act15*/*tagA1-890*) does not result in the multitipped phenotype in the *acbA*$^-$ genetic background (the left panel is the same image as that in Fig. 2A). (C) Localization of cells expressing *ecmA*/*GFP* in the upper and lower cups of sori after 24 h of development. In comparison to the TagA insertion mutant shown in Fig. 2B, the excess *ecmA*-positive cells were not observed in the sori of the *tagA1209 acbA* double mutant.

SDF-2 peptide for 5 min, washed out the peptide, and then measured the amount of SDF-2 released after a further 60 min of incubation. Again, the *tagA* deletion mutant failed to produce detectable SDF-2 (Table 2). The failure to make SDF-2 is unlikely to be the result of a defect of a much earlier development event, since these strains did produce the other spore differentiation factor, SDF-1 (Table 2), and strains lacking either TagA or AcbA are still able to sporulate, although they do so asynchronously and at reduced levels (3, 7). The apparent overproduction of SDF-1 activity in the *tagA* deletion mutant and the *acbA* mutant is suggestive of a negative feedback loop between TagA (or SDF-2) signaling and SDF-1 production.

The prestalk-specific serine protease/ABC transporter TagC is known to be required for the generation of SDF-2 peptides from AcbA protein (4, 5). Thus, the failure of *tagA*Δ mutants to secrete SDF-2 might be caused by compromised TagC function in these cells. The fact that cells can process exogenously provided AcbA provided a means to assess TagC activity in the prestalk cells of the *tagA* mutant (3, 4). We tested whether *tagA*Δ cells are able to process recombinant AcbA into SDF-2, as described previously, by dissociating *tagA*Δ cells from the mid-culminant stage of development (22 h), "priming" them with 10 pM SDF-2 peptide for 5 min, washing out the peptide, and finally adding 1 pmol of recombinant AcbA protein (3). After a 60-min incubation, the supernatant was found to con-

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TABLE 2. SDF-1 and SDF-2 production[a]

Strain	Amt of SDF-1 in sori ($U/10^3$ cells)	Amt of SDF-2 in sori ($U/10^3$ cells)	Amt of primed SDF-2 ($U/10^3$ cells)	Amt of primed plus AcbA SDF-2 ($U/10^3$ cells)
Ax4	2×10^3	10^4	1.5×10^4	$>10^4$
acbA$^-$	10^4	<0.02	<2	$>10^4$
tagA1209	2×10^3	<0.01	ND	ND
*tagA*Δ	10^4	<0.01	<2	$>2 \times 10^4$

[a] For priming, cells dissociated from 22-h developing cells (culminants) were exposed to 10 pM SDF-2 with or without the SDF-2 precursor protein AcbA (1 pmol) added (see the text for details). ND, not done.

tain over 10^4 U of SDF-2 activity, which represents much more SDF-2 peptide than was added for priming (Table 2). Thus, the deletion of *tagA* does not appear to interfere in an obvious way with the expression of TagC or the ability of prestalk cells to process AcbA or respond to low levels of SDF-2.

Phenotypes found in both *tagA*$^-$ and *acbA*$^-$ mutants. We previously showed that the *tagA1209* insertion mutant formed slugs with an overabundance of cells expressing the PstB marker, *ecmB*. These cells ultimately accumulate in the lower cup and outer basal disk of fruiting bodies (18). The newly made *tagA* deletion mutant also produced an excess number of *ecmB*-positive cells, suggesting that this phenotype does not result from unregulated protease activity but rather from a complete lack of TagA function (Fig. 4). Interestingly, the *acbA*-null strain and the *tagA1209 acbA* double mutant also formed slugs with excess *ecmB*-positive cells (Fig. 4). That the *tagA* and *acbA* mutants share this unusual phenotype further suggests that TagA and AcbA function together in a process of cell specification.

Defects of the *tagA*Δ and *acbA* mutants in specifying the prestalk cells are also apparent in the PstA and PstO cell populations. We followed PstA and PstO cell development in these mutants with a GFP reporter construct driven by the *ecmA* promoter (18). In pure populations of migrating slugs, the level of expression of this reporter gene appears to be reduced in both the *tagA*Δ and *acbA* mutant relative to the wild type (Fig. 5A, upper panels). This is a consistent result that was observed in every slug examined and in multiple transformants of the reporter construct into the mutant parental strains. When these mutants were mixed 1:1 with unmarked wild-type cells, the mutants appear to be compromised in their ability to form *ecmA*-expressing cells (Fig. 5A, middle panels). With both mutants a range of chimeric slugs was observed, from those with fairly normal mutant contribution to the PstA and PstO cell populations (Fig. 5A, center panels) to those where only a few PstA cells appear to be produced by the mutant cell population (Fig. 5A, middle-right panels). This defect in the production of *ecmA*-expressing cells was not detected when the mutants were mixed with unmarked mutant cells (Fig. 5A, lower panels).

While the *tagA*Δ and *acbA* mutants are capable of making grossly normal stalks when developed as pure populations, they appear to be unable to contribute to the stalk cell population in the presence of wild-type cells. When mutant cells were mixed at a 1:1 ratio with wild-type cells and allowed to form fruiting bodies, no mutant stalk cells were observed within the entire length of the stalks. This is illustrated with high-power images of stalk sections that are shown in Fig. 5B (middle panels). This experiment was also carried out with mutant cells marked with a general cell marker, *actin/GFP*, and the same results were obtained (data not shown).

The *tagA* insertion mutant expresses the prespore cell marker *cotB/lacZ* in vacuolated stalk cells within the stalk tube, again suggesting imperfections in prestalk or stalk cell specification (7). At the time we reported this finding, such a phenotype had not been previously described. Surprisingly, we observed this phenotype in the *tagA* deletion mutant as well as in strains lacking AcbA (Fig. 6A). To test whether errors in cell fate specification in these mutants resulted from a lack of intercellular signaling at any time during development, we developed them together with wild-type cells and looked for the presence or absence of *lacZ*-positive stalk cells. Since *tagA* and *acbA* mutant cells do not form stalk cells when mixed 1:1 with wild-type cells (see above), we had to reduce the proportion of wild-type cells in the mixtures to ensure that some of the mutant cells contributed to the stalk of the chimera. We found that by adding 5% wild-type cells to the mixtures, some of the mutant cells differentiated as stalk cells. The wild-type partner in these experiments was tagged with the *actin/GFP* marker to assess their contribution to the stalk. Visual inspection by fluorescence microscopy confirmed that both wild-type and mu-

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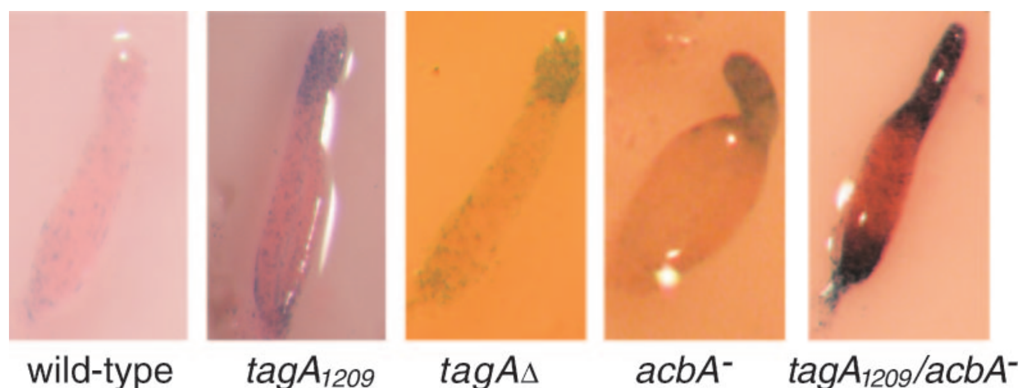

FIG. 4. *ecmB* expression in migrating slugs. EcmB-positive cells were visualized in migrating slugs using the *ecmB/lacZ* reporter construct. Slugs were fixed after 18 h of development, permeablized, and stained for β-galactosidase activity. An overabundance of *ecmB*-expressing cells are observed in the anterior of each mutant slug.

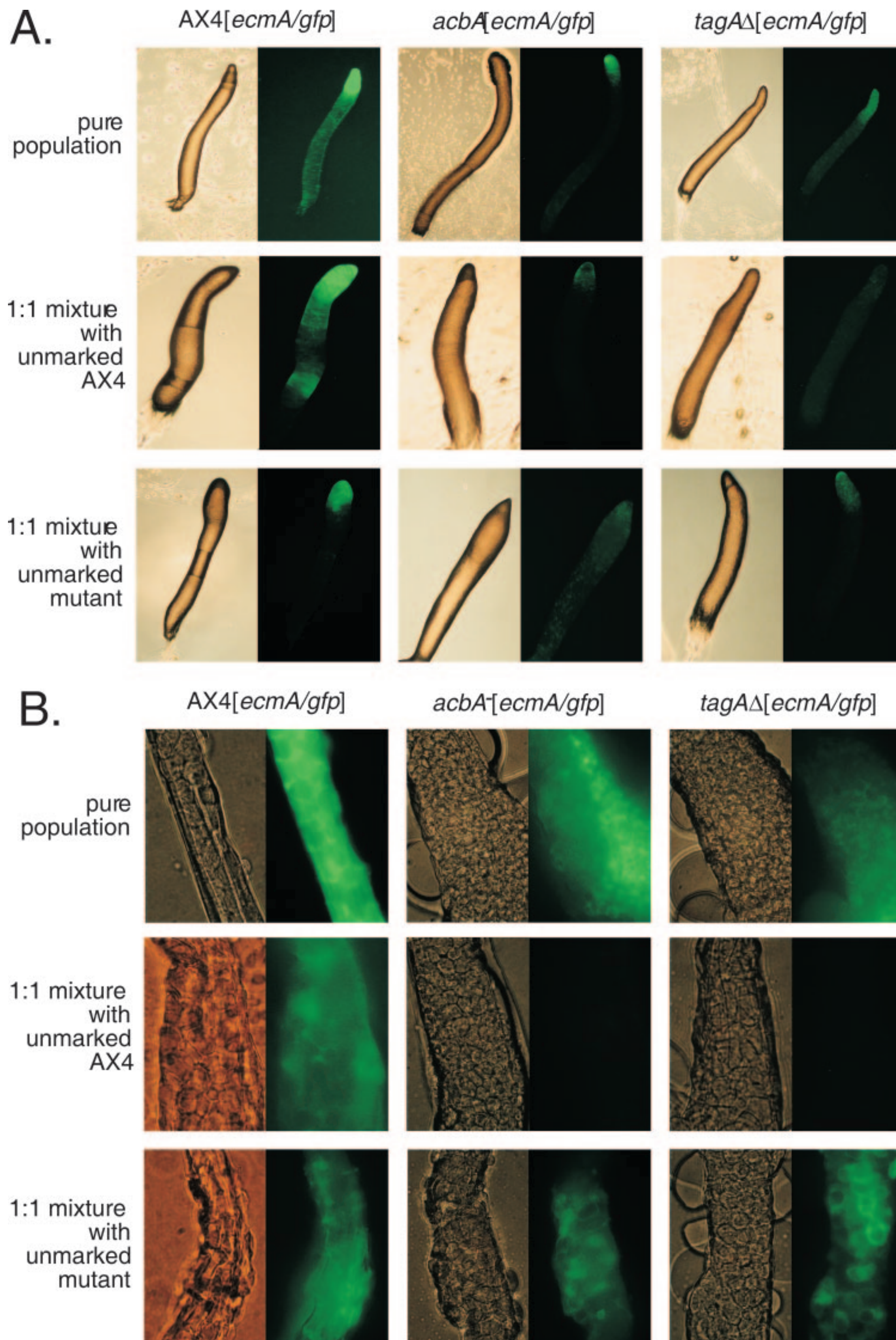

FIG. 5. Defective *tagA* and *acbA* mutant prestalk cells in chimeras. (A) Ax4 (wild type), *acbA* mutant cells, and *tagA* deletion (*tagA*Δ) cells expressing GFP under the control of the prestalk promoter *ecmA* were allowed to develop to the slug stage as pure populations (top panels), as 1:1 mixtures with unmarked wild-type AX4 cells (middle panels), or with unmarked mutant cells (bottom panels). Only the AX4 mixture with unmarked *acbA* mutant cells is shown, and the mixture with unmarked *tagA* mutant cells gave similar results (data not shown). Only the marked mutants mixed with unmarked mutants of the other genotype are shown. Mixtures of marked mutants with unmarked mutants of the same genotype gave similar results (data not shown). The fluorescence panels were all imaged with the same exposure times, and representative slugs are shown. (B) The mixtures described in panel A were allowed to develop into fruiting bodies, and segments of the resulting stalks were imaged with the same exposure times. The GFP appears to be excluded from the stalk cell vacuoles, giving a reticulated pattern. The level of fluorescence observed in the middle panels is similar to that of GFP-negative slugs (data not shown). In the bottom-left panels, only the control mixture with *tagA*Δ mutant cells is shown, but the mixtures with *acbA* mutant cells were similar.

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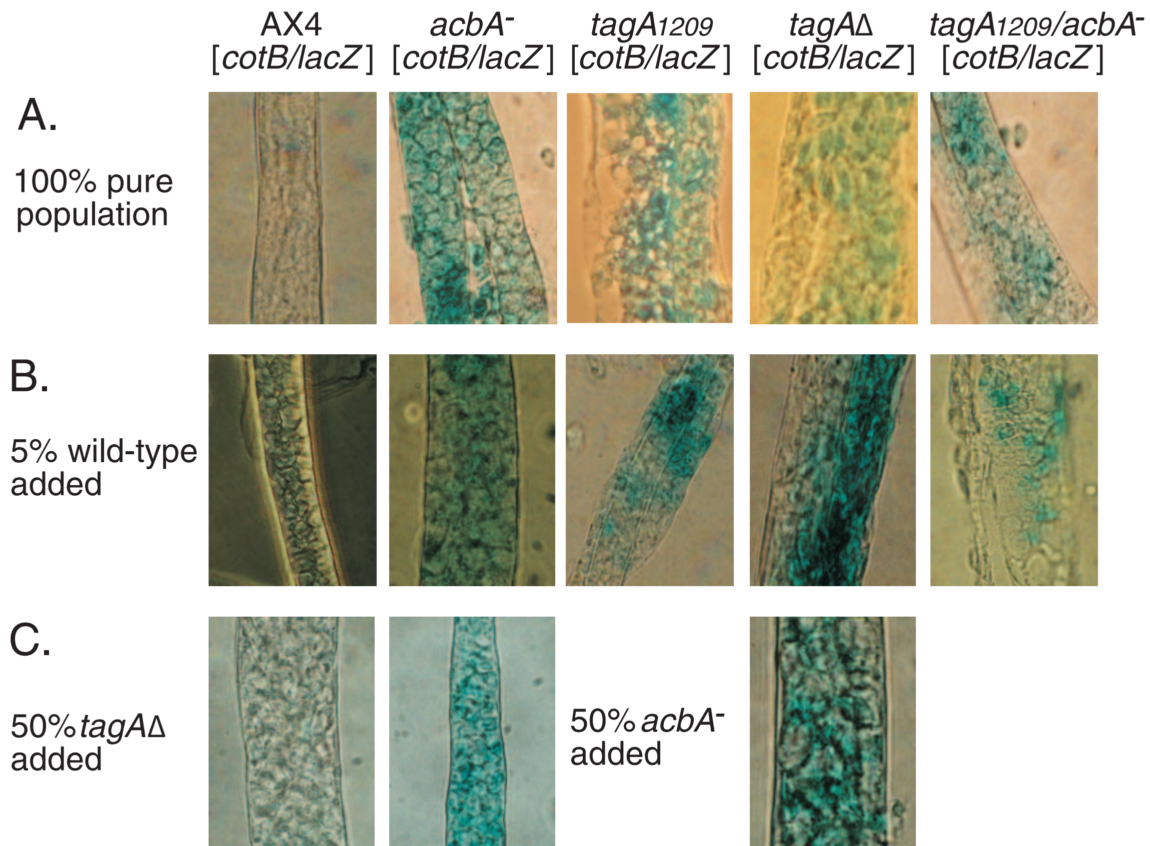

FIG. 6. *cotB* gene expression in *tagA* and *acbA* mutants. (A) A *cotB*/*lacZ* reporter construct was used to assess the aberrant expression of the prespore-specific *cotB* gene in stalk cells. Fruiting bodies were fixed and stained for β-galactosidase activity after 24 h of development. A single representative section of the stalk produced by each strain is shown. (B) The *cotB*/*lacZ*-marked strains shown in panel A were mixed with 5% wild-type cells, marked with *act15*/*GFP*, and allowed to form fruiting bodies. GFP expression was used to ensure that mutant cells were present in the stalk before staining for β-galactosidase activity. (C) The *cotB*/*lacZ*-marked strains shown in panel A were mixed 1:1 with indicated mutant cells, marked with *act15*/*GFP*, and allowed to form fruiting bodies. GFP expression was used to determine if the stalk contained a roughly equal proportion of the two cell types before staining for β-galactosidase activity. Images are of representative fruiting bodies. Experiments were performed multiple times on at least two independently obtained strains.

tant cells entered the stalk tubes of chimeric fruiting bodies (data not shown). Mutant cells in the mixed stalk were seen to have expressed the *cotB*/*lacZ* reporter (Fig. 6B). Within the limits of the use of only a small proportion of wild-type cells in the mixtures, these results suggest that mutations in either *tagA* or *acbA* result in a cell-autonomous defect in stalk cell differentiation.

The inability to form stalk cells in admixtures with wild-type cells is a defining phenotype of the *tagB* and *tagC* mutants (14). It is also well documented that TagC and AcbA are part of the spore encapsulation pathway (4, 5, 17), and the shared stalk cell production defect of *tagA*, *tagB*, *tagC*, and *acbA* mutants suggests some commonality of function in prestalk/stalk cell differentiation. To further explore the relationship between TagA and AcbA, we examined mixtures of the *tagA* and *acbA* mutants to test whether (i) either mutant could exclude the other from the stalk cell population and (ii) either mutant could rescue the other's defect in cell fate as revealed by misexpression of the *cotB* promoter. For each of these experiments, we carried out two control experiments where one or the other of the strains in each mixture was marked with an *actin*/*GFP* marker to ensure admixture. Except for the case of the exclusion of mutant cells from the stalk when mixed with wild type, all mutant/mutant mixtures produced evenly mixed cells within slugs and fruiting bodies, including the stalks (data not shown). The *acbA* and *tagA* mutant mixtures (1:1 ratio) in which one of the strains was labeled with *ecmA*/*GFP* produced stalks in which about half of the cells were GFP positive (Fig. 5B, lower panels). This indicates that neither mutant influences the other's terminal differentiation into stalk cells. In the mixtures (1:1) where one of the mutants was labeled with *cotB*/*lacZ* and the other with *act15*/*GFP*, inspection by fluorescence microscopy of the resulting stalks revealed that they were roughly even mixtures of the two mutants (data not shown). Stalks from the same mixtures were also stained with X-Gal for β-galactosidase activity and revealed roughly equal numbers of *lacZ*-positive and *lacZ*-negative cells (Fig. 6C). In addition, neither of the mutant/wild-type mixtures resulted in misexpression of *cotB* in the wild-type cells (one mixture is shown in Fig. 6C). This indicates that the misexpression of *cotB* could not be induced in wild-type cells by either mutant or rescued in either mutant by the presence of the other mutant.

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DISCUSSION

Our previous report of the consequences of disrupting the *tagA* gene described a complex set of phenotypes involving the overexpression and misexpression of cell type specific genes, cell type proportioning and morphogenesis (7). Here we have shown that mutations in the acyl-CoA binding protein gene, *acbA*, suppress some of the phenotypes caused by *tagA* mutations and mimic others. These genetic interactions and phenotypic similarities suggest that TagA and AcbA function together, either directly or indirectly, to control cell differentiation.

The multitipped phenotype previously described for the *tagA* insertion mutant is likely the consequence of the expression of a truncated version of TagA. TagA mRNA encoding the serine protease domain but lacking the ABC transporter domain was observed to accumulate during early development of the insertion mutant *tagA1209*. The multitipped phenotype was also observed when the TagA serine protease domain was expressed in wild-type cells. These results, combined with the observation that the *tagA* deletion mutant does not have a multitipped aggregate phenotype, indicates that the *tagA1209* allele is allomorphic, rather than null, for TagA function as we had previously supposed (7). The suppression of this multitipped phenotype in these strains by null mutations in *acbA* can be interpreted in several ways. The protease activity in *tagA1209* cells is likely to be unregulated or misregulated as the result of its separation from the ABC transporter domain of TagA, so it may hydrolyze its natural substrate constitutively or ectopically or may hydrolyze an entirely new substrate. AcbA may regulate TagA's access to its natural substrate or prevent access to abnormal substrates while carrying out its normal function as an acyl-CoA binding protein. The AcbA homolog in yeast is known to be involved in membrane trafficking, so the loss of AcbA in *Dictyostelium* might alter the cellular localization or disposition of many proteins (13). Alternatively, the multitipped phenotype could result from aberrant proteolysis of AcbA itself. This is plausible, since TagA and AcbA are both predicted to be associated with membranes and since the TagA homolog TagC is required to process AcbA into the SDF-2 peptide (4, 5). The lack of SDF-2 biogenesis from either the *tagA* insertion strain or the *tagA* deletion strain supports the hypothesis that AcbA is a substrate of TagA, at least during culmination. By the same argument, the excess of *ecmA*-positive cells in the upper and lower cups of *tagA1209* mutant sori that is suppressed by a null mutation in *acbA* also appears to be due to abnormal proteolysis, perhaps of AcbA itself.

On the other hand, the excess *ecmB*-positive cells at the slug stage, the inability to contribute to the PstA lineage (stalk) in chimera with wild-type cells, and the misexpression of *cotB* in the stalk cells of fruiting bodies was observed in both the *tagA* insertion and deletion mutants. These phenotypes appear to be due to the lack of TagA function. Consistent with this idea, these phenotypes were not suppressed by *acbA* mutations and so do not appear to result from the abnormal hydrolysis of proteins or abnormal processing of AcbA. However, the surprising finding that *acbA* mutants produce all these same phenotypes suggests that both AcbA and TagA are required for the same aspect of cell fate specification.

While the fruiting bodies of *tagA* and *acbA* mutants are not grossly abnormal morphologically, the presence of *cotB*-positive stalk cells within their stalks indicates that the transcriptional regulation within the cells is altered. There are two equally plausible explanations for this unprecedented phenotype. Given the several-hour half-life of the β-galactosidase expressed from the reporter that we used, it may be that the stalk cells of *tagA* and *acbA* mutants differentiate as prespore cells for some period of their developmental history. Alternatively, it could be that the suppression of *cotB* expression in PstA cells and/or stalk cells requires both TagA and AcbA. The fact that the *tagA* and *acbA* mutants are also excluded from the PstA cell lineage when they are mixed with wild-type cells argues they are defective in specifying this prestalk cell type. Indeed, the gnarled-finger morphology seen in the *acbA* and *tagA* null mutants is strikingly similar to that of the *ecmA* knockout (11). This further supports the evidence of lower *ecmA* expression in all the mutants (but not in the *tagA1209* mutant), as determined by *ecmA/GFP* expression (see Fig. 2B, 3C, and 5A). The inability of wild-type cells to correct the cell specification defects in the *tagA* and *acbA* mutants as measured by the presence of *cotB*-positive cells within stalk tubes suggests that the phenotype is cell autonomous. One caveat to this interpretation is that we could only examine the differentiation of the mutants' stalk cells when mixed with 5% wild-type cells, because the wild-type cells are better able to form stalk cells than either mutant and appear to "out-compete" them for stalk cell differentiation. However, 1:1 mixtures of *acbA* and *tagA*Δ mutants produced stalks that had equal representation of both mutants and where both mutants displayed *cotB* misexpression. The fact that neither mutant prevents the other mutant from forming stalk cells suggests that either the two mutants produce equally defective prestalk cells and so do not "compete" with each other or that the two mutants are defective in the same cell specification pathway, so that the mixed mutants make stalks as if they were a pure population of either mutant. The most favorable interpretation of the latter possibility is that TagA and AcbA function in the same intracellular signaling pathway, but other possibilities are not excluded by these experiments. However, these experiments do appear to formally rule out the possibility that TagA and AcbA are required for distinct extracellular signaling events required for proper cell fate specification and are consistent with a cell-autonomous cell fate defect in both mutants.

AcbA is found only in prespore cells during late development, and it must be secreted in order for it to be processed by the serine protease activity of TagC that is found on prestalk cells (4, 14). The proteolytic product, SDF-2, is the ligand of the receptor histidine kinase DhkA found on the surface of both prespore and prestalk cells (17). Binding of SDF-2 to DhkA inhibits phosphorelay, resulting in an increase in PKaC activity and rapid encapsulation of prespore cells (4). While the lack of release of SDF-2 in both the *tagA*Δ and *acbA*$^-$ strains may account for some of their developmental abnormalities, it cannot account for all the complex phenotypes seen in these strains, since release of SDF-2 only occurs during culmination (2, 5). It appears that TagA and AcbA function in aggregates such that a single finger is produced and also to ensure proper cell type-specific gene expression during the slug stage. The excess *ecmB*-positive cells seen in *tagA*Δ and *acbA*$^-$ strains may be the result of precocious developmental timing, since *ecmB* is normally expressed in only a few anterior cells at

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the slug stage but in a much larger number of cells in the basal disc and lower cup of the fruiting body (18).

At our current stage of understanding, we can consider two hypotheses: (i) TagA is required in all cells for proper cell fate specification and it sets the conditions for proper AcbA signaling later in development, and (ii) proper cell fate specification requires that TagA process AcbA into SDF-2 (or some other peptide) and transports the product(s) across a membrane. The first hypothesis provides a more general model but still suggests that AcbA functions earlier than has been previously documented. Thus, early roles for SDF-2 and its receptor, DhkA, would have to be considered as the simplest way to explain AcbA's involvement in cell fate specification. The second hypothesis leads to specific predictions that are readily testable. Whether or not TagA and AcbA proteins interact directly as a processing enzyme/transporter and substrate will have to be addressed by cell biological and biochemical experiments. In either model, the terminal event may be the control of PKA activity through SDF-2 attenuation of DhkA signaling. There are several lines of evidence suggesting this as a viable model. First, SDF-2 is thought to attenuate constitutive signaling through the DhkA histidine kinase pathway, leading to elevated cyclic AMP levels and subsequent activation of PKA (4, 5, 17). Second, DhkA mRNA is expressed in prespore cells at 16 h of development and in all prestalk/stalk cell types of culminants and fruiting bodies (17). DhkA may well be expressed in all cells within aggregates, since its mRNA is dramatically up-regulated at 12 h of development, but this issue has not been examined directly. Finally, SDF-2 overproduction might be expected to reproduce defects similar to those observed in *dhkA* mutants. If TagA protease normally cleaves AcbA into SDF-2, overexpression of this protease may result in overproduction of SDF-2. The fact that *acbA* mutations suppress the TagA protease overexpression phenotypes lends some credence to this notion. Thus, it is interesting to note that TagA protease overexpression and DhkA null mutations both lead to excessive expression of *ecmA* in a cell-autonomous manner (7, 17). If the SDF-2/DhkA system is operating early in development we would expect the signaling to be intracellular, since wild-type cells do not rescue the cell specification phenotypes of *tagA* or *acbA* mutants.

The roles of AcbA, its subcellular localization, and the involvement of TagA are yet to be clearly delineated in biochemical terms at the finger and slug stages, but they appear to be fundamental to subsequent cell differentiation and morphogenesis. Although it seems likely that TagA and AcbA interact directly, there is currently no biochemical data to suggest the nature of this interaction. Based on the data presented, however, we can only speculate on the nature of the active factor that acts to specify cell fate. The misspecification of cell fate seen in the *tagA1209* strain indicates that TagA transport function is vital to this process. We speculate that the multiple tips on aggregates and the excess of prestalk cells as judged by the *ecmA/GFP* marker in the *tagA1209* mutant relative to the *tagA* deletion and $acbA^{-}$/*tagA1209* mutants is caused by proteolytic processing of AcbA by TagA in the absence of transport, resulting in a prestalk-like cell fate. However, it is not obvious that the processed form of AcbA required for cell fate specification early in development should be identical to SDF-2. Indeed, we have been unable to detect SDF-2 activity in cell lysates of developing cell aggregates or slugs (unpublished observations). Biochemical experiments aimed at directly addressing the issue of the potential enzymatic role of TagA in AcbA processing are the current focus of research.

ACKNOWLEDGMENTS

We thank Danny Fuller for superb technical assistance.

This work was supported by USPHS grant GM52359 (to A.K.) and GM078175 (to W.F.L.) from the National Institutes of Health. M.C. was supported, in part, by an NIH Minority Student Development Grant (R25GM56929; G. Slaughter, P.I.).

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