Genetic Interactions of the E3 Ubiquitin Ligase Component FbxA with Cyclic AMP Metabolism and a Histidine Kinase Signaling Pathway during Dictyostelium discoideum Development

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Upon exhaustion of the bacterial food supply, amoebae of Dictyostelium discoideum undergo a multicellular developmental program that results in the formation of fruiting bodies consisting of viable spores held atop a dead, vacuolated stalk. Typically, 80% of the amoebae differentiate into spores and the remainder differentiate into the stalk. Because only spores are able to propagate, a variant strain which formed spores to the exclusion of prestalk cells. Because FbxA cells are developmentally deficient in pure culture, we were able to select suppressor mutations that promote sporulation of the original mutant. One suppressor mutation resides within the gene regA, which encodes a cyclic AMP (cAMP) phosphodiesterase linked to an activating response regulator domain. In another suppressor, there has been a disruption of dhkA, a gene encoding a two-component histidine kinase known to influence Dictyostelium development. RegA appears precociously and in greater amounts in the fbxA mutant than in the wild type, but in an fbxA/dhkA double mutant, RegA is restored to wild-type levels. Because the basis of regA suppression might involve alterations in cAMP levels during development, the concentrations of cAMP in all strains were determined. The levels of cAMP in the double mutant dhkA/fbxA is relatively normal. The levels of cAMP in the various mutants do not correlate with spore formation, as would be expected on the basis of our present understanding of the signaling pathway leading to the induction of spores. Altered amounts of RegA and cAMP early in the development of the mutants suggest that both fbxA and dhkA genes act earlier than previously thought.

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family of signal transducing receptors. When the gene is mutated, a severe phenotype, characterized by overproduction of stalk cells and relatively few spores, results (42). Whether phosphorylation of RegA by DhkA or other histidine kinases inhibits or activates its phosphodiesterase activity is controversial (39, 42).

Because of the effects of fbxA mutation on cell type proportioning and on the developmental fate of cells in chimeras with the wild type, we asked how this gene is regulated and searched for proteins with which FbxA interacts. Because, without wild-type cells, fbxA mutants do not develop beyond the slug stage and make few spores, we were able to select suppressor mutations that allow the formation of fruiting bodies containing detergent-resistant spores. The suppressor mutations affect regA and dhlkA. The interplay between fbxA, regA, and dhlkA during development has been characterized. Our measurements of cAMP in these strains were not as we had expected. We observe a profound dependence of cAMP accumulation on the DhkA histidine kinase.

**MATERIALS AND METHODS**

**Strains.** All strains and their origins are described in Table 1. Amoebae were grown on bacterial lawns with *Klebsiella pneumoniae* or in the axenic medium HLS, as described by Sussman (36).

**Recovery of the fbxa promoter.** The promoter of *fbxa* was obtained from genomic DNA of REMI (restriction enzyme-mediated integration) (18) mutant 82. This mutant arose from the insertion of plasmid pBNI near the 3′ end of the gene to create the fbxa mutation (25). The original *chtA/fbxa* mutant arose from an insertion of a different plasmid at the same site in strain AX3-1 (12). The genomic DNA of mutant 82 was digested with ClaI, yielding the plasmid pCtr-Cla, which consisted of most of the *fbxa* coding sequence, 1.4 kb of upstream sequence, and phiBluescript.

The transcriptional start site was determined by using 5′ rapid amplification of cDNA ends as described in the Ver.2 kit from Gibco/BRL. Comparison of the 5′ extended cDNA sequence with the genomic sequence recovered from the rescue of the REMI insertion defined the 5′ untranslated region (UTR).

The complete fbxa cDNA was inserted into pCR2.1-Topo (Invitrogen). A *BamHI-EcoRI* fragment from pCS2-Mt6 containing the coding sequence for the six-myc epitope was inserted at the 5′ end of fbxa in the pCR2.1-Topo plasmid by using the *BamHI* and *EcoRI* sites. A *HindIII-XbaI* fragment of the pCR2.1-Topo-fbxa plasmid containing the *fbxa* cDNA was cloned into the *HindIII-XbaI* sites of plasmid pDXA-HC (22) (GenBank accession no. X85123). A full map of the locus was constructed by combining sequence data obtained in this work with information from the various *Dictyostelium* genome databases accessible via the DictyBase World Wide Web server (http://dictybase.org/dicty.html). A map of the *fbxa* locus is presented in Fig. 1.

**Reporter constructs.** To construct an *fbxa* gene with an epitope tag coding sequence under the control of the endogenous promoter, the actin-15 promoter (*ClaI-BamHI*) fragment of pDXA-fbxa/*fbxa* was replaced with the 1.4-kb *ClaI-BglII* fragment of pCtr-A-Cla (see above; the sequence, −1375 to −1, can be found under GenBank accession no. AF151111). This fragment contains about one-half of the 5′ UTR of *fbxa*, in addition to the putative upstream promoter sequence.

A green fluorescent protein (GFP)-expressing plasmid under the control of the 1.4-kb sequence was created by removing the Sul-NcoI fragment from the pCtr-A-Cla plasmid, which carries the *fbxa* promoter, the 5′ UTR and 84 nucleotides of the coding sequence of *fbxa*. This fragment was cloned into the vector pTX-GFP digested with SulI and KpnI, which removed the actin-15 promoter (19). A linker sequence carrying an NcoI recognition sequence on one end and a KpnI sequence on the other end was used to ligate these two fragments. The resulting plasmid contained the *fbxa* promoter in front of the GFP coding sequence in pTX-GFP (HR108).

**Isolation of suppressors.** A population of strain 82 (25) was mutagenized by REMI (18) with *BamHI*-restricted pBSR1 in the presence of DprII (2). We collected approximately 83,000 transformants from 46 REMI plates. These were divided into four subpopulations. Each was grown in HLS supplemented with 5 μg of blastidicin S/ml. Each batch was plated for development on 4.2-cm-diameter SorC plates containing 50 μg/ml carbenicillin (SM plates) (17 mM KH₂PO₄, Na₂HPO₄ [pH 6.0] containing 50 μg/ml carbenicillin (SM plates) (36). Four filters were allowed to develop for 36 h, and four filters were allowed to develop for 46 h. The filters were harvested, and the cells were resuspended in SorC containing 0.3% Triton X-100 and placed in an ice bath for 10 min. Triton X-100 at this concentration inhibits or activates its phosphodiesterase activity is controver-

**Measurement of spore formation.** Amoebae (2.5 × 10⁵) were grown in association with *K. pneumoniae* on SM agar petri dishes (100 mm in diameter) at 22°C. After 6 or 7 days the entire plate was harvested with 20 ml of SorC buffer. Triton X-100 was added to 0.3% (vol/vol) final concentration, and the mixture was placed in an ice bath for 5 min. The mixture was sedimented at 10,000 × g for 10 min at 20°C. The pellet was washed once in 10 ml of SorC and suspended in 10 ml of SorC containing 0.3% (final concentration) Triton X-100. The mixture was incubated for 10 min in an ice bath, sedimented, and washed once more with 10 ml of SorC. The pellet was suspended in SorC, the spores present in each sample were counted, and appropriate dilutions were plated on SM/5 plates in association with *K. pneumoniae* to determine the number of viable spores.
spores. The spore count of the DH1 sample, the control, was set at 100. The results are the averages of three separate experiments, except those for HR52 and HR45, which were determined in two experiments.

**Recovery of suppressor genes.** Suppressor strains were characterized by developmental phenotype and genomic Southern blotting. DNA from clones that represented different REMI events, as judged by phenotypic and Southern blotting criteria, was digested with appropriate restriction enzymes and circularized by overnight ligation at 2 μg of DNA/ml. Transformation of bacteria and recapitulation of the phenotypes of disruptants were based upon standard methods (18). While some REMI clones readily yielded plasmids containing the flanking DNA, other suppressor clones (including the reg4 clones) never did. In such cases it proved possible to recover several kilobases of flanking DNA by means of inverse PCR (IPCR).

Genomic DNA of problematic REMI clones was first examined by conventional PCR with ExTag DNA polymerase (TaKaRa) and primers spanning various regions of the REMI plasmid. This analysis revealed that the integrating plasmid had suffered a deletion of at least 1 kb and was incapable of ampicillin expression. Primers for IPCR were selected from within surviving plasmid DNA and used to recover the flanking *Dictyostelium* regions from circularized genomic DNA. If the interval between the inverse primers used was sufficiently large (1 kb), it was unnecessary to open or nick the circular template molecules between priming sites either by restriction digestion or prolonged heating. An extension temperature of 68 °C was used because of the AT bias of *Dictyostelium* DNA. IPCR products were sequenced directly, without cloning. A search of the databases identified the gene that had been disrupted by REMI, and targeted gene disruption with an appropriate plasmid confirmed the basis of suppression.

**Determination of cAMP levels in *Dictyostelium* cells.** Cells growing in HL5 were harvested, washed in SorC, and counted. Two hundred microliters of cells at 8 × 10^7/ml were plated onto 2.5-cm-diameter nitrocellulose filters layered over a filter paper pad saturated with SorC. The cell pellet was suspended in 150 μl of 3.5% (vol/vol) perchloric acid and frozen. Before the assay, the suspension was thawed, the cellular debris was sedimented, and the supernatant was collected. Pellets were retained for protein assay. Twenty-one microliters of 15% (vol/vol) KHCO₃ was added to the supernatant, which was centrifuged at high speed in the cold. The pellet was discarded, and the supernatant was collected for the cAMP assay. cAMP in the supernatant was determined with the Apbiotech (Amersham Pharmacia, Piscataway, N.J.) RPN225 cAMP enzyme immunoassay kit. The pellet was dissolved in 150 μl of 1 M KOH, and the amount of protein in each sample was determined by the Bradford assay. cAMP values were normalized to the protein in each sample. The data are the averages of six independent determinations for each strain and time point.

**Nucleotide sequence accession number.** The sequence of the complete 8,548-bp *fbxA* locus, including some of the 3′ flanking gene, has been assigned GenBank accession no. AF151111.

**RESULTS**

**The *fbxA* locus.** To study the function and regulation of the *fbxA* gene, we recovered the full coding sequence of the gene, as well as 1.4 kb of upstream and nearly 4 kb of downstream flanking regions. The outline of the locus is shown in Fig. 1. Plasmids pBSR3 and pJB1 integrated at a common *Dpn*I site during REMI mutagenesis to yield *fbxA* derivatives of AX3 (12) and DH1 (25). A second insertion, into the F-box coding domain of the gene (Fig. 1), conferred the same phenotype as the more 3′ insertion (data not shown). The promoter contains 1,375 nucleotides and is typically AT rich. With the transcription start (CAP) site designated +1, there is a 230-bp 5′ UTR. The sequence observed corresponds in size to that estimated from Southern blots and therefore is unlikely to have been grossly rearranged. At the 3′ end of the gene is another open reading frame, which we have named *lysA* and which codes for a putative enzyme involved in bacterial cell wall degradation. An AT-rich region of about 440 bp between the two sequences presumably acts to terminate both transcripts. Three polyadenylation signals (AATAAA) occur after the termination codon of *fbxA*. The first of these follows the stop codon by 30 bp.

**Complementation of the *fbxA* mutation.** Nelson et al. (25) reported that *fbxA* expression occurs predominantly within the prestalk population. When we transformed the *fbxA* mutant with constructs that expressed the myc-tagged *fbxA* structural gene either from a constitutive actin promoter (HR42) or from

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**FIG. 1.** Map of the *fbxA* locus. The full sequence can be found under GenBank accession no. AF151111. CAP, transcription start.
the prestalk ecmA promoter (HR43) (11, 13), the phenotype was not significantly improved (data not shown). The aggregates remained long slugs and did not culminate to form fruiting bodies, even after several days. We tested for expression of the transgene by Western blotting using anti-myc antibodies; in all cases the protein was expressed (data not shown), despite its lack of phenotypic effect.

A consequence of the poor development of the fbxA mutant is its almost total inability to produce viable, detergent-resistant spores. Despite the apparent lack of phenotypic complementation, the myc-tagged FbxA protein expressed from the actin promoter achieved a significant increase in detergent-resistant spores (22.3% of the wild-type value). This increase occurred only if the developing structures were allowed to mature for 72 h; at earlier times, almost no detergent-resistant spores formed. The parental strain makes detergent-resistant spores on well-formed stalks as early as 24 h after the start of development. Expression of the myc-tagged FbxA protein from the prestalk ecmA promoter also resulted in increases in the number of spores over the background of the fbxA mutant, but not to the extent achieved by the actin promoter (0.016% for the fbxA mutant versus 0.34% for the ecmA promoter-driven construct (HR43). One way to explain both the lack of gross phenotypic complementation and the partial complementation of the sporulation deficiency is to suggest that the endogenous fbxA promoter controls a pattern of expression that is complex (3, 7, 17, 32).

Activity of the fbxA promoter. The promoter-containing fragment was inserted into a plasmid to drive the expression of the myc-tagged fbxA structural gene. In a strain transformed with this construct (HR47), in contrast to results with the actin and ecmA promoters, phenotypic complementation was successful, resulting in transformants with an almost wild-type morphology, as shown in Fig. 2. In addition, spores (32.2% of the wild-type level) were made. The success of this complementation makes it unlikely that the previous failure of the myc-tagged FbxA protein to complement when expressed from the actin or ecmA promoter is due to a deleterious effect of the peptide tag. The developmental profile of the endogenous promoter’s activity, with an activation by 4 to 8 h, is shown in the myc-probed Western blot in Fig. 2D.

Nelson et al., using in situ hybridization, showed that most of the fbxA mRNA was expressed in prestalk cells (25). To examine this pattern of expression in greater detail, we fused the fbxA promoter to the gene encoding GFP and transformed wild-type amoebae to give strain HR108. The results are shown in Fig. 3. For most of development, the fbxA promoter acts like a prestalk promoter. Fluorescence first appears in the mound stage (Fig. 3A) and rapidly segregates to a constricted area that is the forming tip of the mound (B and C). GFP preferentially stains the anterior, prestalk region of the slug as it forms (D and E). Culminants display fluorescence in the papilla, upper and lower cups, and maturing stalk (F and G). This pattern of expression resembles that of the prestalk promoter ecmA (43). We do not infer from these results that there is no expression in prespore cells, only that there is more in prestalk cells.

Late in development, when the spores have risen to the top of the fruiting body and undergone encapsulation, they too express GFP (Fig. 3H and I), as do the stalk cells. As a control, we examined cells expressing an ecmA-GFP construct (HR23) and found that there was no expression, even at late times, in spore cells (data not shown).

Isolation and characterization of suppressor mutants. To search for proteins that interact with FbxA, we isolated suppressors of the fbxA mutant. The mutant cells form long and misproportioned slugs which fail to culminate and which produce few spores. Suppressors of the fbxA mutant were isolated as described in Materials and Methods and the genes involved were identified.

One class of suppressor (HR48), examined by phenotype and Southern blotting, arose from disruption of regA, a gene encoding an intracellular cAMP phosphodiesterase that was discussed above. Another class (HR49) resulted from disruption of dhkA. DhkA is an autophosphorylating histidine kinase, homologous to members of two-component signal transduction pathways in other organisms (21, 42). Because the RegA phosphodiesterase is subject to phosphorylation mediated by one or more histidine kinases (29, 40), the means of suppression of the fbxA mutation by regA and dhkA disruption may be related.

The phenotypes of the suppressed strains are shown in Fig. 4. The original fbxA mutant (second panel from the left) forms, as a terminal structure, long, misshapen slugs which cover the agar surface and which can extend up to the plastic petri plate lid. The same phenotype occurs if development takes place on Millipore filters. Although the final fruiting bodies of each of the two suppressor strains, the fbxA/regA and fbxA/dhkA strains, clearly differ from the wild type and each other, both strains produce spore masses supported by a stalk. The fbxA/regA double mutant has a particularly thick stalk, and many spores fail to ascend the stalk. The phenotypic consequence of each disruption was confirmed by a targeted gene knockout. Thus, the disruption by homologous DNA recombination of regA in an fbxA mutant background (HR46) produced the
same phenotype as that of the original suppressor (HR48) isolate shown in the third panel. Targeted gene disruption of dhkA in the fbxA mutant background (HR51) recapitulated the developmental phenotype of the original dhkA suppressor (HR49) shown in the fifth panel.

The efficiency of suppression of the sporulation defect in fbxA was high. Amoebae of strain DH1, of the derived fbxA mutant, and the other suppressors were grown on SM plates and allowed to develop. The number of viable, detergent-resistant spores was determined, and relative spore yield was tabulated. Table 2 shows that not only the gross phenotype of the fbxA mutant (Fig. 4) but also the mutant’s ability to form viable spores was rescued by these suppressors.

We also have shown that dhkA and regA mutations in the
DH1 background do not affect the ability of *fbxA* cells in chimeras to preferentially form spores. However, in chimeras with the wild type the suppressors are not recovered in spores, indicating that this aspect of the phenotype of *fbxA* mutants is suppressed. These and other data will be presented elsewhere.

**The influence of *fbxA*** **on RegA levels.** Why is it that inactivation of the *regA* or *dhkA* gene suppresses the *fbxA* developmental block? In the absence of the F-box protein, which connects the target(s) of ubiquitination with the ubiquitination machinery of an SCF complex, one might expect the target of that complex to accumulate in excess of normal, regulated levels. Mohanty et al. showed that RegA is a target of the *FbxA-SCF* complex (23). Figure 5 is a Western blot that shows that RegA is developmentally regulated in DH1 wild-type amoebae and is missing in the *regA* mutant (HR45) and in the *fbxA/regA* double mutant (HR48) but accumulates to unusually high levels in the *fbxA* mutant (strain 82). Note also that RegA appears prematurely in the *fbxA* mutant. This observation supports the idea that RegA is a target for degradation mediated by the F-box protein FbxA. Finally, although RegA accumulation in a *dhkA* mutant (HR52) and in the *fbxA/dhkA* double mutant (HR49) is relatively normal, its appearance is premature in the double mutant.

**cAMP levels.** Since the suppressors we identified affect a cAMP phosphodiesterase and a histidine kinase thought to regulate that phosphodiesterase, we measured the level of intracellular cAMP in our mutants, as shown in Fig. 6. The *fbxA* mutant, in which the level of RegA protein is high, would be expected to have a low cAMP level, but the level is actually about the same as that in the wild type. The *regA* null mutant would be expected to have large amounts of cAMP, but it too is similar to the wild type. These results differ from those of Abe and Yanagisawa (1), perhaps reflecting differences in the control strains or assay conditions. These assays measure only the accumulation of cAMP. There may be rapid oscillations of cAMP or local effects of cAMP that are regulated by RegA.

In contrast, there is a general effect on the cAMP levels of *dhkA* mutants. The *dhkA* mutant contained a low level of cAMP during several stages of development. It is worth noting that these mutants develop aberrantly, especially at the later time points. Nonetheless, the low level of cAMP in the *dhkA* mutant is surprising. The *fbxA/dhkA* suppressor strain exhibits higher levels of cAMP than the strain with mutant *dhkA* only, despite the excess of RegA phosphodiesterase (Fig. 5).

**DISCUSSION**

The *fbxA* mutation has pronounced effects on cell type proportioning and on cell fate choice in chimeras with wild-type cells (12). The cloned *fbxA* promoter rescued both the morphological phenotype and spore-forming capacity of the original mutant better than the actin or *ecmA* promoters. Nelson et al., using in situ hybridization, showed that *fbxA* mRNA accumulated in the prestalk cells of slugs (25); in agreement, we found GFP accumulation predominantly in the prestalk compartment throughout development. However, late in development, when spores have undergone encapsulation, they too expressed GFP, perhaps because of a role of cAMP in spore encapsulation.

The effects of loss of FbxA on cell proportioning and cell fate suggest that the protein is active by the time of mound formation, when amoebae undergo their initial differentiation into prespore and prestalk cells. Our Western blot experiments revealed the accumulation of FbxA protein by 4 h of development, and other results here argue for even earlier expression: the cAMP phosphodiesterase RegA accumulates to high levels in the *fbxA* background, and this accumulation can be seen even in vegetative amoebae. It follows that FbxA itself must be active in wild-type vegetative cells so that its elimination can result in the RegA excess.

Expression of the FbxA protein may occur at low levels in cells other than prestalk cells. This may explain the developmental advantage which *fbxA* amoebae exhibit when mixed with wild-type cells (12). If prespore cells did not normally express the FbxA protein, mutating them should make no difference. Since *fbxA* prespore cells affect their wild-type neighbors in chimeras, there must be some expression of FbxA. This may be at levels too low to be detected by Western blotting or by expression of GFP from the isolated promoter at the time of establishment of the cell type proportions.

**FIG. 5.** RegA expression in the presence and absence of FbxA. Blots of extracts of the various strains developed on filters for the indicated times were prepared and probed with an anti-RegA antibody. The blots were stained with Ponceau S prior to antibody treatment to ensure that equal amounts of protein were applied to each lane. WT, wild type.
Mutations in genes regA and dhkA were selected by their ability to suppress the spore-forming defect of fbxA cells, and both suppressors also rescued substantially the culmination deficiency of the fbxA strain. RegA is a target of SCF-mediated degradation, as shown by Mohanty et al. (23). RegA has two important domains, a response regulator region and a cAMP phosphodiesterase domain. The site of integration of the disrupting plasmid in the regA suppressor was at nucleotide 686 in the response regulator domain. The second suppressor, DhkA, is a receptor histidine kinase. The DhkA molecule has several domains including a CHASE domain, which was identified as a receptor for plant cytokinins, and a histidine kinase domain (4). The disrupting plasmid inserted at nucleotide 5799 of the coding sequence, which is at the 3’ side of the coding sequence for the histidine kinase domain (see references 4 and 24 for reviews of the domain structure of DhkA and similar molecules). The fact that mutation of dhkA suppresses a mutation in fbxA (which in turn regulates RegA) provides a connection from the cell surface to the cAMP regulatory apparatus of the cell (37, 39).

FbxA is an F-box protein component of an SCF ubiquitin ligase complex, and such complexes target proteins for degradation by proteasomes. We found RegA expression to be turned on dramatically at about 12 h in development of wild-type amoebae, although the protein is present at low levels throughout the process (see also reference 39). The level of RegA in the fbxA mutant is high, a finding that is consistent with RegA being the target for degradation by the FbxA-SCF complex (23). RegA also appears earlier in development in this mutant than in the wild type, suggesting that RegA levels are normally kept low very early in development, and even in vegetative amoebae, through SCF-mediated proteolysis. RegA levels appear to be depressed in the presence of the dhkA mutation, as seen either by comparing the dhkA mutant to the wild type or by comparing the fbxA/dhkA strain to the fbxA strain.

**FIG. 6.** cAMP levels during development of various mutants. Intracellular cAMP concentrations were determined as described in Materials and Methods.
RegA is itself a regulated molecule, controlled by degradation but also by phosphorylation through a two-component histidine kinase-signaling pathway (37, 39). Since dhkA mutants, like regA mutants, suppress the effect of the fbxA mutation, this observation might be explained by a mechanism whereby DhkA activates RegA. Thus, in the absence of FbxA, RegA levels would be high and that of cAMP would be low, but since RegA’s cAMP phosphodiesterase activity could depend on DhkA, eliminating DhkA would allow cAMP accumulation and restore development. Such a model is consistent with that proposed by Thomason et al. (38) but not that of Wang et al., who suggest that DhkA inhibits the phosphodiesterase activity of RegA (42). Perhaps the effect of the dhkA mutation on RegA at early developmental times differs from that at late development times, in response to changes in other (unknown) signaling components. Alternatively, as various two-component histidine kinases also function, under particular circumstances, as protein phosphatases, RegA may be activated by another histidine kinase, and yet DhkA could counter that effect.

Studies of dhkA indicate that it is important at late stages of development, when the prespore cells encapsulate. A small peptide called SDF2 has been proposed as its ligand (5, 42). However, the fbxA gene acts much earlier (see above), and thus the corrective effects of dhkA mutation are likely to as well. In particular, the dhkA mutation suppresses the overaccumulation of RegA in the fbxA background at all times for which we have data, including data as early as after 0, 4, and 8 h of development. DhkA must be active (at least in the fbxA mutant background, but probably in wild-type cells also) at these early times.

The identification of regA and dhkA as the two suppressors of fbxA focused our attention on the developmental levels of cAMP in those strains. cAMP has long been recognized as a key controlling metabolite during Dictyostelium development: cAMP mediates many of the transitional steps in development, beginning with the transition from growth to development, which also depends on activation of PKA (34). Several hours later, secreted cAMP mediates aggregation, while intracellular cAMP increases are essential for the activation of new genes during the aggregation, mound, and culmination stages (for a review see reference 16). The encapsulation of the spores at the end of development will not proceed without a surge of intracellular cAMP (14, 38). Finally, the dormancy of spores appears to depend on high levels of intracellular cAMP (41). In contrast, cAMP is not necessary for the trophic phase of Dictyostelium’s life cycle; adenyllyl cyclase mutants grow well (26).

The importance of the recurrent activation of PKA during Dictyostelium development is evident from the properties of rapid-development mutants (rde). These mutants produce spores in a much shorter time than the wild type does, but at the expense of proper morphogenesis (1, 6, 9, 15, 33, 40). cAMP metabolism in most such mutants is affected. Mutations of the cAMP phosphodiesterase RegA or of a phosphotransfer protein called RdeA (which acts on RegA) all serve to drive up cAMP levels under particular circumstances to activate PKA and to accelerate the developmental program. Overexpression of the PKA catalytic subunit has a similar effect. Ax2 cells transformed with an actin-15-driven version of fbxA, selected at 100 μg of G-418/ml, proceed through development more rapidly than wild-type Ax2 cells (25; our unpublished observations). This is consistent with excessive degradation of the cAMP phosphodiesterase RegA and a consequent rise in intracellular cAMP.

The levels of intracellular cAMP that we found during the development of the fbxA, regA, and dhkA mutants are unexpected in light of the above considerations. Wild-type cells exhibited relatively constant levels of cAMP between 8 and 17 h of development. To our surprise, cAMP amounts were little changed from amounts in the wild type in either the regA null mutant or the fbxA null mutant (in which RegA accumulates to excess). The dhkA mutation does diminish cAMP levels, levels that are mostly restored to normal in the fbxA/dhkA double mutant.

The lack of effect of RegA mutations on cAMP levels can be explained in several ways. RegA is expressed predominantly by prestalk cells (roughly 20% of the total slug cell population) as shown previously (28). If so, changes in its phosphodiesterase activity might not influence the overall amount of cAMP that we measured. If RegA were to affect the rapid and fleeting rise of intracellular cAMP in response to an external cAMP pulse (as in aggregation), we would have missed this temporal effect. cAMP levels may be subject to regulation by phosphodiesterases other than RegA: a cAMP/cGMP activity, PdeE, has recently been described (8).

The effect of dhkA mutation on cAMP levels is noteworthy. cAMP levels are severalfold lower than wild-type levels throughout development of the dhkA mutant, even at a time (8 h) before the mutation has greatly affected overall morphology. Elimination of FbxA in addition to DhkA appreciably restores cAMP levels. Consistent with the effects on cAMP, the fbxA/dhkA double mutant sporulates 3- to 10-fold better than does the dhkA mutant and has improved terminal developmental morphology. The downstream target(s) or effector(s) of DhkA is not known, but the enzyme could affect adenyllyl cyclases (7, 17) or phosphodiesterases (7).

The present results define a new prestalk promoter, suggest an earlier time for dhkA activity than previously thought, connect this receptor histidine kinase to FbxA and RegA, and, most strikingly, show a dramatic dependence of cAMP accumulation on DhkA.

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