Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 10701–10705, November 1992
Developmental Biology

DdPK3, which plays essential roles during *Dictyostelium* development, encodes the catalytic subunit of cAMP-dependent protein kinase

SANDRA K. O. MANN*, WES M. YONEMOTO†, SUSAN S. TAYLOR†, AND RICHARD A. FIRTEL*‡

Departments of *Biology and †Chemistry, Center for Molecular Genetics, University of California, San Diego, La Jolla, CA 92093-0634

Communicated by Donald R. Helinski, July 31, 1992 (received for review May 21, 1992)

ABSTRACT **We have previously reported the analysis of DdPK3, a developmentally regulated putative serine/threonine kinase that shares ≈50% amino acid sequence identity with metazoan cAMP-dependent protein kinase A (PKA) and protein kinase C, within their catalytic domains. Cells in which the DdPK3 gene has been disrupted do not aggregate but they are able to induce aggregation-stage genes in response to cAMP pulses and the prestalk-specific *ras* gene *DdrasD* in response to high continuous levels of cAMP but will not induce prespore gene expression. In this report, we present conclusive evidence that *DdPK3* encodes the catalytic subunit of the *Dictyostelium* PKA. *DdPK3* null cells lack kinase activity that phosphorylates a PKA-specific substrate and is specifically inhibitable by recombinant cAMP-dependent protein kinase inhibitor. DdPK3 expressed in *Escherichia coli* has PKA activity that is inhibitable by protein kinase inhibitor. When *Ddpk3* null cells are complemented with DdPK3 expressed from an actin promoter on an extrachromosomal vector (low copy number), PKA activity is restored and the cells proceed to the slug stage but will not culminate, suggesting that properly regulated PKA activity is essential for culmination. Moreover, overexpressing DdPK3 in wild-type cells on integrating vectors (high copy number) from either an actin or prespore-specific promoter results in accelerated development and the ability to form mature spores in monolayer culture in the presence of high cAMP, a developmental potential lacking in wild-type cells.**

Development in *Dictyostelium* is mediated through cell–cell interactions that act via intracellular signal transduction pathways to affect gene regulation and cell function. The organism grows vegetatively as single-celled amoebae. Upon starvation, a multicellular developmental program is initiated during which $\approx 10^5$ cells aggregate and proceed through morphogenesis to form a mature fruiting body containing spores and a vacuolated stalk (1–3). Aggregation is mediated by a pulsatile cAMP signal originating from cells within an aggregation center. The cAMP binds to cell surface receptors that initiate an apparently branched G-protein-coupled signaling pathway, leading to the activation of adenylyl cyclase, guanylyl cyclase, and phospholipase C (1-4). This in turn leads to relay of the cAMP signal, chemotaxis toward the aggregation center, and regulation of gene expression, respectively, although phospholipase C function has not been definitively determined. High levels of extracellular cAMP and the morphogen DIF then regulate cell-type-specific gene expression during the multicellular stages (1, 5-8).

The role of intracellular cAMP during *Dictyostelium* development has not been defined. Analyses of *Synag* mutants, which lack receptor activation of adenylyl cyclase during early development, indicate that receptor-mediated increases in intracellular cAMP are not essential for chemotaxis or for pulse-induced gene regulation (2, 3, 9, 10); however, whether these *Synag* mutants are altered in adenylyl cyclase regulation during later development is unknown. At the time of culmination, a late prespore gene, *Dd31*, has been shown to be inducible by the membrane-permeable analog 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) but not by extracellular cAMP (11), suggesting that this gene is induced by increases in intracellular cAMP and not through cell surface receptors. Consistent with this is the observation that 8-Br-cAMP can potentiate spore cell differentiation from prespore cells (12).

In eukaryotic cells, intracellular cAMP is thought to function as a second messenger through cAMP-dependent protein kinase A (PKA) (13). PKA controls a number of diverse regulatory pathways. The PKA holoenzyme consists of two regulatory (R) and two catalytic (C) subunits in yeast and mammals and one R and one C subunit in *Dictyostelium* (14-16). The holoenzyme is inactivate. The binding of two molecules of cAMP by the R subunit results in a release of C, which is now enzymatically active. Expression of the *Dictyostelium* regulatory subunit is low in vegetative cells and increases during development, corresponding to increases in cAMP-regulated kinase activity in crude extracts (15).

Wild-type and putative dominant negative mutant forms of the murine R_I and the *Dictyostelium* R subunit have been expressed in *Dictyostelium* cells from various promoters (17-19). The dominant negative mutant forms do not bind cAMP and thus do not release C subunit when intracellular levels of cAMP rise (20). Cells overexpressing either the mouse or *Dictyostelium* mutant R (R_m) subunits from an actin promoter that is active in vegetative cells and through early development show an impairment in aggregation (17, 18). When the *Dictyostelium* R_m is expressed from a promoter (*ecmA*) that is active in prestalk A cells, entry into culmination is repressed and the terminal morphology is aberrant (19). These results indicate that PKA plays an essential role during aggregation and in the decision-making process during culmination. In addition, overexpression of the wild-type *Dictyostelium* R subunit, but not that of mouse, results in an inhibition of aggregation (17, 21).

We have previously reported the cloning and initial analysis of a putative serine/threonine protein kinase designated DdPK3 (22), which was also cloned by Burki *et al.* (23). The gene is developmentally regulated and encodes a 648-base-pair open reading frame with the putative kinase domain in the C-terminal half. Expression is low in vegetative cells and is induced during early development in response to cAMP pulses (22). *Ddpk3* null cells, created by gene disruption, do not aggregate but will synergize with wild-type cells to form

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Abbreviations: PKA, protein kinase A; PKC, protein kinase C; PKI, protein kinase inhibitor; 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate.

‡To whom reprint requests should be addressed at: Center for Molecular Genetics, Room 225, 9500 Gilman Drive, University of California, San Diego, La Jolla, CA 92093-0634.

coaggregates. By marking the *Ddpk3* null cells with an *Act15/lacZ* reporter gene, we mapped their fate and showed that all null cells in the coaggregate are subsequently lost as a discrete mass on the side as the wild-type cells form a "first finger" structure and continue through morphogenesis. Similar results were obtained by Harwood *et al.* (18) using cells cotransformed with a vector expressing the mutant PKA R_m subunit. The *Ddpk3* null cells activate cAMP-pulsed-induced aggregation-stage genes and the prestalk-specific gene *DdrasD* in response to high extracellular levels of cAMP, but not prespore-specific genes (22).

In this report, we present evidence that *DdPK3* encodes the catalytic subunit for cAMP-dependent protein kinase. We also present evidence that overexpression of DdPK3 from either actin or prespore promoters leads to rapid development and the ability of single cells in culture to differentiate into spores in response to cAMP. Expression of DdPK3 from an actin promoter complements the aggregation-deficient phenotype of *Ddpk3* null cells but does not allow the cells to proceed past the slug stage, suggesting that proper spatial as well as temporal regulation of PKA during later development is essential for culmination.

MATERIALS AND METHODS

Strains, *Dictyostelium* Development, and Assay for Spore Formation. Wild-type KAx-3 strains and the *Ddpk3* null strains, which are isogenic to the parents except for the *DdPK3* locus, have been described (22). Cells were grown and developed as described (22). The assay for spore formation in submerged culture was performed as described (12).

PKA Assay. The assay for PKA activity was performed in a volume of 20 μl for 10–15 min at 30°C using as a substrate a recombinant bovine type I regulatory subunit (R_I) containing an engineered alanine-to-serine change at position 97 to produce a PKA recognition sequence (24). Recombinant mouse catalytic subunit was purified according to Yonemoto *et al.* (25). Protein kinase inhibitor (PKI) was purified as described (26). *Dictyostelium* cells were developed on buffered agar plates, harvested at the times indicated, and resuspended in assay buffer (24). Cells were lysed by forcing them through Nucleopore filters (3.0-μm pore size), the crude extracts were centrifuged for 10 min at 10,000 × *g*, and the supernatant was collected. Unless noted, all assays were performed in the presence of 60 μM cAMP to assay total PKA catalytic activity. The assay reaction mixture was fractionated by SDS/PAGE on a 10% gel, which was fixed, dried, and autoradiographed to determine the level of R_I phosphorylation.

Expression Systems. To express DdPK3 in *Dictyostelium*, the coding region was amplified by PCR and then cloned into the Actin15 expression vector BS18 (27) or downstream from the *SP60* promoter by replacing the *lacZ* gene in *SP60/lacZ* (28) with the DdPK3 coding region. These vectors were transformed into KAx-3 cells and G418-resistant transformants were selected as described (27, 28). *Ddpk3* null cells were transformed with an *Act15/DdPK3* fusion on a pATANB43-based extrachromosomal vector (29).

To express the DdPK3 catalytic subunit in *Escherichia coli*, the coding region from a DdPK3 cDNA clone starting at amino acid 287 (22) was cloned in-frame in pGEX-KG (30), which was confirmed by direct sequence analysis. The vector was transformed into *E. coli* and grown at 30°C, and expression of the fusion protein was induced with isopropyl β-D-thiogalactoside. SDS/PAGE analysis confirmed the presence of a 68-kDa fusion protein in the induced cells. The fusion protein was purified from a 10,000 × *g* supernatant over a glutathione affinity column as described (31).

RESULTS

Analysis of the DdPK3 Kinase Domain. Initial analysis indicated that DdPK3 shares ≈50% amino acid sequence identity with metazoan protein kinases C (PKCs) and PKA catalytic subunits (22). We have further analyzed the sequence data using information about the interaction of mam-

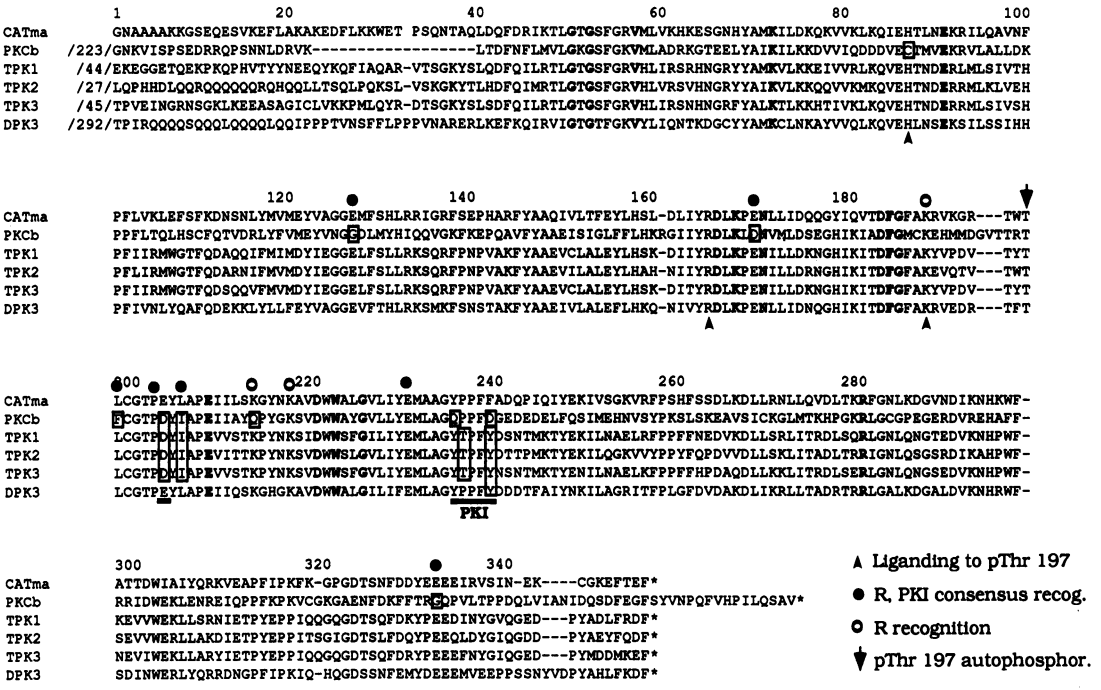

FIG. 1. Protein kinase catalytic domain alignment. CATma, the murine α catalytic domain; TPK1–TPK3, yeast catalytic domains (36); DPK3, *Dictyostelium* DdPK3 catalytic domain; PKCb, murine PKC (22). The position of the amino acid at the beginning of each region is indicated. The regulatory (R) and PKI consensus recognition sequences are described in refs. 32–34 as are the amino acids liganding to pThr 197 (marked with an arrowhead). Residues that are boxed indicate changes in highly conserved residues or in residues involved in regulatory subunit or PKI interaction compared to the murine PKA catalytic subunit. PKC shows changes in a number of these residues in the C terminus. Residues required for high-affinity interaction with PKI are underlined with a solid bar (see text).

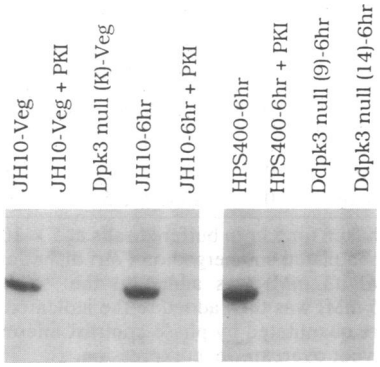

FIG. 2. PKA assays in crude extracts from wild-type and mutant cell lines. JH10 is the parental strain for *Ddpk3* null strain K. HPS400 is the parental strain for *Ddpk3* null strains 9 and 14. "Veg" refers to extracts from logarithmic-phase vegetatively growing cells, and "6hr" refers to cells harvested after developing for 6 hr on buffered agar. PKI refers to the addition of PKI (2 μg per assay).

malian and yeast PKA catalytic subunits with their respective regulatory subunits (32) and with PKI, which is specific for PKA (33-35). Structural analyses have identified residues that are essential for these interactions and for interaction with the autophosphorylated threonine at position 197. Fig. 1 provides a sequence comparison of the mouse PKA catalytic subunit, mouse protein kinase C, the three yeast PKA catalytic subunits (36), and the *Dictyostelium DdPK3* gene product (22). Residues known to be required for PKI and R recognition are indicated by circles. There is complete conservation of these residues among the mammalian and yeast PKA subunits and DdPK3. In contrast, PKC shows amino acid changes at a number of these positions. There is also a short acidic domain centered at residues 332–333 in the PKAs and DdPK3 that is not conserved in PKC.

Structural analysis identified two additional sites in the murine PKA catalytic subunit (33) that are required for the high-affinity binding of PKI: a glutamic acid residue at 203 and a five-amino acid hydrophobic region (residues 236–240). These sites are conserved in DdPK3 (except for a highly conserved F → Y change at residue 240) but not in PKC or in the yeast PKA catalytic subunits, which may account for the reduced affinity of PKI for the yeast catalytic subunit (37).

***DdPK3* Encodes a Protein Kinase that Is Inhibited by PKI.** We have made use of the *Ddpk3* null mutant to investigate whether *DdPK3* encodes the *Dictyostelium* PKA catalytic subunit. Crude 10,000 × *g* supernatants of cell extracts were prepared from wild-type and *Ddpk3* null cells (see *Materials and Methods*) and then assayed for their ability to phosphorylate a PKA substrate (the altered murine R_I subunit) in the presence and absence of PKI. Fig. 2 shows that kinase activity is present in crude extracts from the two different wild-type parental strains (JH10 and HPS400) but not in the respective null mutants, which show only background bands. This activity is >95% inhibitable by PKI.

To confirm that the PKA activity observed in wild-type cells is due to the presence of an intact *DdPK3* gene, the DdPK3 coding region was inserted downstream from the Actin 15 (*Act15*) promoter on an extrachromosomal expression vector and transformed into *Ddpk3* null cells. Stable, G418-resistant transformants were assayed for kinase activity. The *Act15* promoter is expressed in vegetative cells through early development, with promoter activity decreasing in the multicellular stages (38). The results are presented in Fig. 3 *Upper*. The null mutant transformed with a control vector shows no activity at any developmental stage. The complemented null mutant exhibits a similar level of activity as the wild-type parental strain at 6 hr of development, which remains consistent throughout development. JH10 wild-type

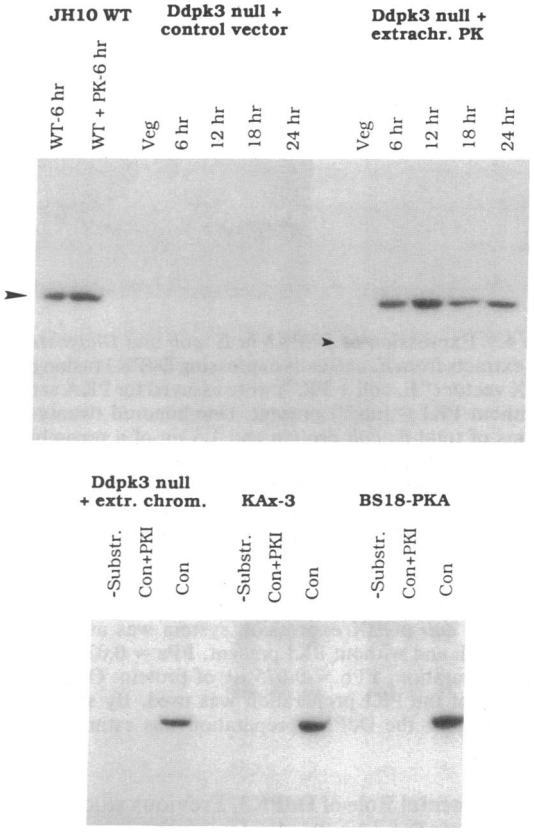

FIG. 3. PKA assays in crude extracts from wild-type (WT) and mutant cell lines. (*Upper*) PKA assays using extracts from JH10 wild-type cells and *Ddpk3* null cells (strain K) transformed with an extrachromosomal vector either containing ("extrachr. PK") or not containing ("control vector") the *DdPK3* coding region expressed from an actin promoter. Cells were developed for the times indicated. Crude extracts were then made and assayed for PKA activity in the presence of cAMP. Lanes with JH10 extracts are included as a control. "WT + PK-6 hr" uses extracts from JH10 cells expressing DdPK3 from the *Act15* promoter on an extrachromosomal vector. In one vegetative extract there is partial cleavage of the substrate due to endogenous protease activity (small arrowhead). Extracts were normalized for protein concentration. (*Lower*) Extracts from KAx-3 control cells (KAx-3 is the parental strain of JH10; JH10 is a thymidine auxotroph derived from KAx-3 by gene disruption of the *Thy1* gene; see ref. 22), *Ddpk3* null cells complemented with the extrachromosomal, *Act15/DdPK3* expression vector, and KAx-3 wild-type cells expressing DdPK3 from the *Act15* promoter on an integrating vector (BS18). All cells were developed for 6 hr. Assays were performed with substrate (Con), without substrate (−Substr.) to show the absence of the labeled band, and with substrate and PKI (Con+PKI).

cells transformed with the extrachromosomal vector expressing DdPK3 exhibit a slightly elevated level of PKA activity compared to cells transformed with a control vector (data not shown). As shown in Fig. 3 *Lower*, the activity observed in cells carrying the expression vectors is inhibitable by PKI.

To determine whether the PKA activity is actually encoded by *DdPK3*, the DdPK3 catalytic domain was expressed in *E. coli* as a fusion protein with glutathione transferase, using the pGEX expression system (see *Materials and Methods*). The fusion protein was purified and showed predominantly a 68-kDa band on Coomassie-stained gels (data not shown), the expected size of the fusion protein. Fig. 4 demonstrates that the *E. coli*-expressed DdPK3 catalytic domain has kinase activity that can be inhibited >95% with PKI. *E. coli* transformed with a control pGEX vector (no DdPK3 insert) shows no activity. Extracts from wild-type JH10 cells were assayed as a positive control.

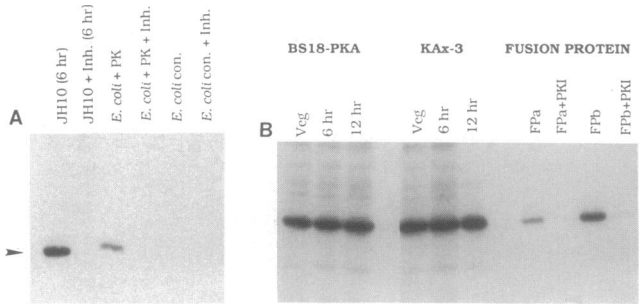

FIG. 4. Expression of DdPK3 in *E. coli* and *Dictyostelium*. (*A*) Crude extracts from *E. coli* cells expressing DdPK3 fusion protein on a pGEX vector ("E. coli + PK") were assayed for PKA activity with and without PKI ("Inh.") present. One-hundred twenty-three micrograms of total *E. coli* protein and 1.5 μg of a recombinant PKI preparation were used. An extract from *E. coli* cells carrying the pGEX vector with no insert ("E. coli con.") was also assayed. Assays using JH10 extracts are included as a control (66 μg of JH10 extract and 1.5 μg of the PKI preparation). (*B*) Wild-type KAx-3 cells and KAx-3 cells transformed with the BS18·DdPK3 integrating expression vector were developed for the times indicated. Extracts (22 μg) were assayed for PKA activity. On the right, fusion protein from the *E. coli* pGEX expression system was assayed for PKA activity, with and without PKI present. FPa = 0.025 μg of purified protein preparation; FPb = 0.075 μg of protein. One and one-half microgram of the PKI preparation was used. By staining of polyacrylamide gels, the DdPK3 preparation was estimated to be 50% pure.

Developmental Role of DdPK3. Previous studies have demonstrated that *Ddpk3* null cells do not aggregate; they activate cAMP-induced aggregation-stage and prestalk-specific genes but not prespore-specific genes (22). To further define the role of DdPK3 in development, we examined the developmental potential of the *Ddpk3* null cells complemented with *DdPK3* expressed from the *Act15* promoter on a low-copy-number extrachromosomal vector (39). These cells have a level of kinase activity similar to that of wild-type cells (Fig. 3), aggregate normally, and proceed through the initial stages of multicellular development to form first fingers or slugs (Fig. 5 *Upper*). Culmination, however, is not initiated and cells do not proceed past this point, even if development is allowed to continue for 48 hr.

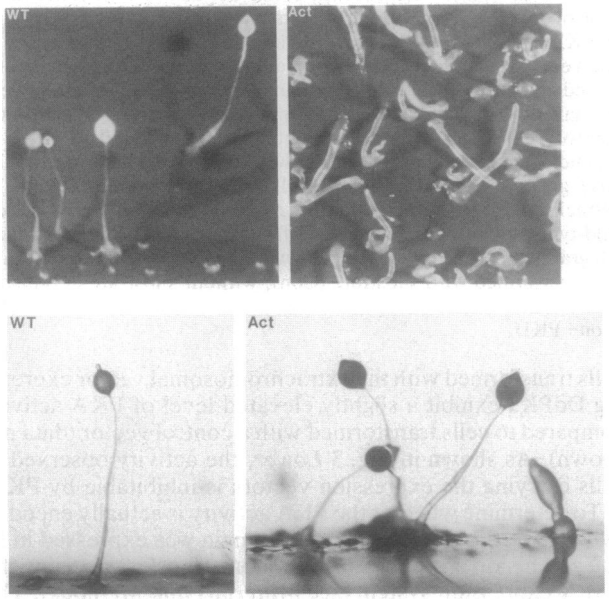

FIG. 5. Development of cells expressing DdPK3 from *Act15* promoter-expression vectors. (*Upper*) Control cells (WT) or *Ddpk3* null cells expressing *Act15/DdPK3* (Act) on the extrachromosomal vector developed for 24 hr. (*Lower*) Mature fruiting bodies of wild-type controls (WT) and wild-type cells expressing *Act15/DdPK3* on an integrating vector, which have a shorter, thicker stalk, larger base, and larger spore mass compared to the wild-type cells (wild-type fruiting bodies are ≈3 mm tall).

Table 1. Effect of overexpression of DdPK3 on spore formation of single cells

Strain	% spore formation of single cells		
	Control	cAMP	cAMP → 8Br-cAMP
KAx-3 (WT)	0	0	0
Act/PK3 overexp.	0	10.2 ± 2.2	13.4 ± 1.8
SP60/PK3 overexp.	0	15.0 ± 2.5	18.3 ± 3.1

Cells were shaken for 5 hr in buffered salts at 5×10^6 cells per ml, washed, and then plated submerged on Petri dishes at 7×10^2 cells per cm^2. cAMP (5 mM) was added to the "cAMP" cultures. 8-Br-cAMP (25 mM) was then added to the indicated cultures at 24 hr. Spores were quantitated by phase-contrast microscopy after 42 hr. WT, wild type; overexp., overexpression.

To determine the effect of DdPK3 overexpression on the development of wild-type cells, KAx-3 cells were transformed with a high-copy-number integrating vector expressing DdPK3 from the *Act15* promoter (27). When PKA activity is examined in these transformants, the levels are higher than in control wild-type cells, when extracts are normalized for protein content, and are inhibitable by PKI (Figs. 3 *Lower* and 4*B*; data not shown). When these cells are plated for development, the multicellular program is accelerated with aggregates forming by 8–9 hr and migrating slugs forming by 12 hr, in contrast to 10 hr and 16 hr, respectively, for control cells (data not shown). Formation of the mature fruiting body occurs at ≈18 hr rather than ≈24 hr and has a larger spore mass and a shorter, stockier stalk base than that of a wild-type structure (Fig. 5 *Lower*).

Increases in intracellular cAMP have been implicated in prespore-to-spore differentiation during the terminal stages of culmination (12). Moreover, mutations within the PKA regulatory subunit have been shown to be sporogenous—i.e., they allow cells to form spores in dilute monolayer culture in the presence of high extracellular cAMP (40). To examine whether overexpression of DdPK3 might lead to sporogeny, we used wild-type cells transformed with DdPK3 expressed from either the *Act15* promoter or the *SP60* prespore-specific promoter on a high-copy integrating expression vector (see *Materials and Methods*). Cells plated in dilute monolayer culture in the presence and absence of cAMP (see *Materials and Methods*) were assayed for their ability to form spores. The results (see Table 1) indicate that *Act/DdPK3*- and *SP60/DdPK3*-expressing cells can be induced to form spores in monolayer in the presence of high levels of extracellular cAMP. This ability is enhanced by 8-Br-cAMP in agreement with the results of Kay (12) on sporogenous mutants. Control wild-type cells do not form spores nor do *Act/DdPK3*- or *SP60/DdPK3*- expressing cells in the absence of exogenous cAMP.

DISCUSSION

***DdPK3* Encodes the DdPKA Catalytic Subunit.** A detailed sequence analysis of the DdPK3 catalytic domain has identified specific sequences conserved between murine PKA and DdPK3 that are virtually diagnostic for the PKA catalytic domain. In addition to the sequence comparison, we have used biochemical and molecular genetic evidence to show that *DdPK3* encodes the catalytic subunit for *Dictyostelium* PKA. *Ddpk3* null cells lack the PKA activity present in wild-type strains, and this activity is restored by the presence of an extrachromosomal vector carrying an *Act/DdPK3* fusion gene. In each case, this kinase activity is blocked by

the PKA-specific recombinant inhibitor PKI. That DdPK3 encodes the catalytic subunit is confirmed by our demonstration that an *E. coli*-expressed GST-DdPK3 fusion protein has PKA activity. No cross-hybridizing mRNAs or other DNA fragments are detectable by Northern or genomic Southern analyses (22). Therefore, the catalytic subunit of the *Dictyostelium* cAMP-dependent PKA appears to be encoded by a single gene, *DdPK3*, now designated *DdPKAcat*.

***DdPKAcat* Is Essential for Several Decision-Making Steps During *Dictyostelium* Development.** It is of interest that DdPKAcat activity is not required for vegetative growth in *Dictyostelium*; *Ddpk3* null cells grow well in axenic medium. However, it is apparent that the role of DdPKAcat during development is multifold. We have previously demonstrated that DdPKAcat is essential for aggregation (22). When *Ddpk3* null cells are mixed with wild-type cells, they coaggregate, but the *Ddpk3* null cells are lost as a discrete mass off to the side of the emerging first finger as development proceeds, suggesting that DdPKAcat activity is required for signal relay during aggregation and for the ability to participate in cell sorting and subsequent morphogenesis. The specific defect in aggregation is not known at present; however, null cells show proper expression of the pulse-induced genes (e.g., those encoding the cAMP receptor cAR1 and the cell adhesion molecule csA). Null cells also express the prestalk-specific *ras* gene *DdrasD* under our normal assay condition (high levels of exogenous cAMP) but do not express the prespore genes *SP60* and *14-E6* in the presence of cAMP under conditions that allow cell–cell contact.

Ddpk3 null cells expressing DdPKAcat from the *Act15* promoter on an extrachromosomal plasmid proceed through development to the first finger or slug stage but no further. Since the total level of PKA activity in extracts of these cells is similar to that in wild-type control extracts throughout development, we conclude that the arrested developmental phenotype is due to a misexpression of PKA activity relative to the spatial (cell-type) pattern of expression in wild-type aggregates using the *Act15* promoter. Since expression of PKAcat from the *Act15* promoter in wild-type cells leads to accelerated development, it is unlikely that the arrest is due to too high a level of expression in specific cell types. Rather, our results suggest that high levels of expression accelerate development and that proper spatial regulation of PKA activity is required for entry into culmination. Harwood *et al.* (19) have recently shown that expressing a dominant negative regulatory subunit gene from the prestalkA-specific *ecmA* promoter prevents cells from entering culmination. Combined, the results indicate that PKA plays multiple, essential roles at a number of crucial stages in *Dictyostelium* development.

Previous results have suggested that an increase in intracellular cAMP is required for terminal differentiation of prespore cells into spore cells. Expression of DdPKAcat from either an actin or *SP60* promoter on integrating vectors (high copy number) results in a sporogenous phenotype—i.e., cells are able to differentiate into spores in monolayer culture in the presence of high extracellular cAMP. This result is consistent with the analysis of the *rdeC* class of *Dictyostelium* rapidly developing mutants, which are sporogenous and include mutants with a defective DdPKA regulatory subunit gene, leading to a constitutively active PKAcat (34). This suggests PKAcat is required for prespore → spore differentiation and is consistent with 8-Br-cAMP, but not cAMP, inducing spore differentiation in wild-type cells (11). The complete requirements for prespore cell induction and prespore → spore differentiation are not yet known, although it is now clear that PKA is required for both. Our analyses, combined with those of other laboratories, indicate that DdPKA plays an essential role in various cell types at many stages of development. It will be of interest to examine the spatial pattern of DdPKAcat expression during development as well as determining the factors involved in its regulation.

We thank members of the Firtel and Taylor laboratories for helpful suggestions. This work was supported by U.S. Public Health Service grants to S.S.T. and R.A.F.

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