tope standard (PeeDee belemnite) according to

$\delta^{13}C = [(^{13}C/^{12}C_{sample} \div {}^{13}C/^{12}C_{standard}) - 1] \times 1000$

Isotope ratios were determined with a Europa 20-20 continuous-flow mass spectrometer with reproducibility of 0.2 per mil.

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19. We thank K. Cottingham, J. Hodgson, D. Thomas, and N. Voichick for assistance with experiments and sampling; S. Blumenshine, N. Caraco, K. Cottingham, T. Kratz, D. Lodge, Y. Vadeboncoeur, and K. Webster for helpful discussions; and T. Frost and R. Hellenthal for logistical support. N. Haubenstock and K. Tholke performed the stable isotope analyses. This manuscript benefited from reviews by P. Leavitt and K. Webster. Financial support was provided by the National Science Foundation–Ecosystems Program and Doctoral Dissertation Improvement Program. This is a contribution to the University of Wisconsin, Center for Limnology, the Institute of Ecosystem Studies, and the University of Notre Dame Environmental Research Center.

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Dictyostelium Development in the Absence of cAMP

Bin Wang and Adam Kuspa*

Adenosine 3′,5′-monophosphate (cAMP) and cAMP-dependent protein kinase (PKA) are regulators of development in many organisms. *Dictyostelium* uses cAMP as an extracellular chemoattractant and as an intracellular signal for differentiation. Cells that are mutant in adenylyl cyclase do not develop. Moderate expression of the catalytic subunit of PKA in adenylyl cyclase–null cells led to near-normal development without detectable accumulation of cAMP. These results suggest that all intracellular cAMP signaling is effected through PKA and that signals other than extracellular cAMP coordinate morphogenesis in *Dictyostelium*.

In both vertebrates and invertebrates, the control of cAMP synthesis and its detection by cellular targets such as PKA are essential for many developmental processes (*1*). During *Dictyostelium* development, cAMP is used as a signal both inside and outside of cells (*2*). Pulses of extracellular cAMP are generated by the cells and are used for chemotaxis during aggregation, a process that brings 10^5 cells into a mound. Extracellular cAMP is detected by G protein–coupled cell surface receptors (*3*). This results in the chemotaxis of cells toward increased cAMP as well as propagation of the signal through the activation of ACA, the adenylyl cyclase that produces cAMP during development (*4*). PKA, the major downstream effector of the cAMP-ACA signaling pathway inside the cell, is activated when its regulatory subunit (PKA-R) binds cAMP and dissociates from the catalytic subunit (PKA-C) (*5*). Cells in which PKA-R is inactivated (*6*) or in which PKA-C is constitutively active (*7*, *8*) develop rapidly, which suggests that PKA regulates the timing of development.

After aggregation, extracellular cAMP has been proposed to direct a number of processes, including the sorting of cell types into distinct tissues, slug migration, terminal cell differentiation, and the morphogenesis of the final fruiting body (*9*–*11*). To test the role of extracellular cAMP in development, we obviated the requirement for cAMP as a second messenger by rendering PKA constitutively active. By doing this in an ACA-null mutant, we produced cells that do not make cAMP but have the key intracellular cAMP pathway activated. Because PKA activation promotes many developmental events (*7*, *8*, *11*, *12*), these cells allowed an assessment of the processes that are independent of cAMP.

We introduced an expression plasmid with an epitope-tagged PKA-C coding region under the transcriptional control of an *actin15* promoter into *acaA* mutant cells by transformation (*13*). The *actin15* promoter directs relatively constant amounts of transcription throughout development in all cells (*14*). After the primary transformants were cloned on growth plates, cells that had exhausted the bacterial food supply within some of the colonies were clearly undergoing development. The epitope-tagged PKA-C protein was produced by these developing transformants [*acaA*(PKA-C) cells] but was not detected in transformants that did not develop (*13*). Analysis of the DNA and RNA of several isolates of *acaA*(PKA-C) cells confirmed that the *acaA* locus remained disrupted and that *acaA* mRNA did not accumulate at any time during development (*13*). We also measured PKA-C activity in cells that had been developing for 8 hours, as measured by phosphorylation of the peptide substrate Kemptide (*15*). The *acaA*(PKA-C) cells exhibited 4.3 times the activity (1.4 nmol min^{-1} mg^{-1}) of the parental *acaA* cells and 1.6 times the activity of wild-type cells.

When *acaA*(PKA-C) cells were axenically grown, washed, and placed on Millipore filters, they underwent relatively normal development. They began to aggregate by 10 hours, forming broad "streams" of cells that persisted for several hours, such that mound formation was delayed by about 2 hours (Fig. 1A). The mounds produced by the *acaA*(PKA-C) cells progressed through a normal sequence of developmental structures and formed fruiting bodies by 30 hours (Fig. 1, B and C).

To determine whether constitutive PKA activity restored cAMP signaling in the *acaA*(PKA-C) cells, we measured adenylyl cyclase activity and cAMP production in several ways. After cells were pulsed with cAMP for 5 hours, *acaA*(PKA-C) cells had <0.4% of the adenylyl cyclase activity of wild-type cells (*16*). During development on filters, adenylyl cyclase activity in wild-type cells increased to a maximum at 8 hours and then declined, as previously reported (*17*). The activity in *acaA*(PKA-C) cells and *acaA* mutant cells never rose above the background of the assay (*16*). We also directly measured the accumulation of cAMP in intact aggregation-competent cells (*18*). When cells were stimulated with 2′-deoxy-cAMP, cAMP rapidly accumulated in wild-type cells but remained at background in the *acaA*$^-$ and *acaA*(PKA-C) cells (Fig. 2A). During the development of

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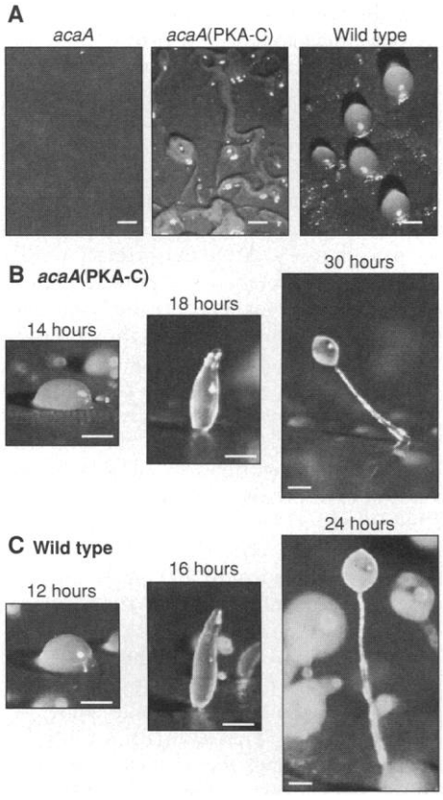

Fig. 1. Development of wild-type and PKA-C–rescued *acaA* cells. (**A**) Typical fields of *acaA* mutant, *acaA*(PKA-C), and wild-type cells, 12 hours after they were spread on a Millipore filter to initiate development (*27*). (**B** and **C**) Multicellular structures of *acaA*(PKA-C) cells (B) and wild-type cells (C) are shown with the time of development above each panel. The development of the *acaA*(PKA-C) cells is delayed relative to the wild-type cells, so similar morphological stages are shown for comparison. Scale bars, 250 μm.

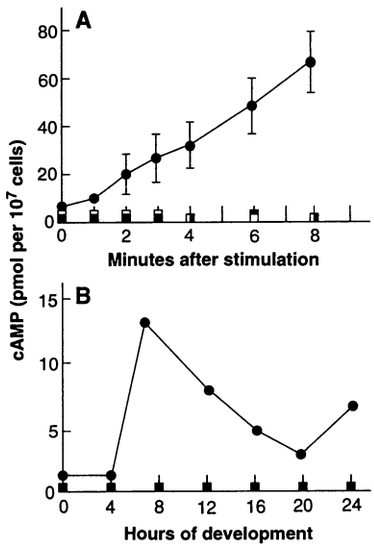

Fig. 2. cAMP produced by cAMP-stimulated cells and by cells developing on filters. (**A**) Production of cAMP from wild-type cells (solid circles), *acaA* mutant cells (open squares), or *acaA*(PKA-C) cells (solid squares) after stimulation with 2′-deoxy-cAMP (*18*). Means and SEs are shown for three experiments performed in triplicate. Absence of error bars indicates that the error was smaller than the symbol. (**B**) Total concentration of cAMP present at various times for wild-type cells (solid circles) or *acaA*(PKA-C) cells (solid squares) developing on filters (*18*). One representative experiment is shown.

Table 1. Cell density dependence of aggregation. Means (±SD) of the total number of aggregates observed at 14 hours are shown at each cell density for three spots of cells developing on the same buffered-agar plate (*29*). Data from a typical experiment are shown. Most of the aggregates in every sample continued through development and formed fruiting bodies.

Cell density (10^5 cells/cm^2)	Multicellularity (aggregates/cm^2)	
	Wild type	*acaA*(PKA-C)
55	200 ± 7	150 ± 20
28	170 ± 20	140 ± 9
14	130 ± 22	120 ± 9
5.5	42 ± 13	0
2.8	25 ± 9	0
1.4	18 ± 9	0
0.55	0	0

wild-type cells, steady-state amounts of cAMP were highest during aggregation, declined until the time of culmination, and then increased during fruiting body formation (Fig. 2B). In the *aca*A(PKA-C) cells, cAMP was below detection limits at all times tested (*18*). These measurements of adenylyl cyclase activity and cAMP production are consistent with previous findings that ACA accounts for all of the detectable adenylyl cyclase activity during development (*4*). Thus, the development of *aca*A(PKA-C) cells is not attributable to the ACA-independent synthesis of cAMP.

Pulsatile cAMP signaling and chemotaxis is thought to be required for the recruitment of cells from a distance, which enables properly sized aggregates to form irrespective of the initial cell density (*19*). Because *aca*A(PKA-C) cells aggregate without producing detectable cAMP, we examined the cell density dependence of their aggregation. The *aca*A(PKA-C) cells aggregated at the cell density that is normally used for development on an agar surface (Table 1, second highest density). The aggregates were of normal size, and most went on to form fruiting bodies. However, at cell densities of 5.5×10^5 cells/cm^2 and lower, the *aca*A(PKA-C) cells failed to aggregate, whereas the wild-type cells formed aggregates of normal size and roughly in proportion to the total number of cells in the developing field (Table 1). These results directly confirm the requirement of cAMP signaling for the production of properly sized aggregates by cells at low density. Certain mutant cells have been shown to aggregate without pulsatile cAMP signaling in the presence of high, constant amounts of cAMP (*4*) or nonhydrolyzable cAMP derivatives (*20*). Our results show that cells at high density are able to aggregate without cAMP as long as PKA is active.

Within aggregates, randomly distributed prestalk cells move to the apex, where they form a tip that can lead prespore cells on extended migrations as slugs (*21*). It has been assumed that these cell movements result from chemotactic responses to extracellular cAMP (*9*); hence, we tested whether *aca*A(PKA-C) cells formed prestalk regions and migrating slugs. Neutral red preferentially stains prestalk cells and is not transferred between cells (*22*). As observed by neutral red staining, prestalk cells sorted to the apex of *aca*A(PKA-C) cell aggregates in a manner indistinguishable from that of wild-type cells (*23*). These aggregates formed slugs with normal proportions of prestalk cells in their tips, and they migrated at the normal rate (*23*). These results rule out any essential role for extracellular cAMP in cell sorting or slug migration.

We also examined several molecular and cellular markers of development. The expression of *pdsA* was restored in the *aca*A(PKA-C) cells; however, a delay in the transition from the 1.9-kb transcript to the 2.4-kb transcript was evident (*24*) (Fig. 3A). Expression of the *carA* gene in *aca*A(PKA-C) cells appeared normal. The *acaA* gene was expressed during aggregation of the wild-type cells starting at 6 hours and, as expected, was not expressed in either of the *acaA* mutants. The expression patterns of the cell type–specific genes *cotA*, *ecmA*, and *spiA* in *aca*A(PKA-C) cells were remarkably similar to those of wild-type cells (Fig. 3B). The correct temporal regulation of *cotA* and *ecmA* in the *aca*A(PKA-C) cells indicates that specific temporal or spatial regulation of PKA is not required for the timing of cell type–specific gene expression. Later in development, the onset of expression of the sporulation-specific gene *spiA* was delayed by about 4 hours in the *aca*A(PKA-C) cells, consistent with the delay in the completion of development in these cells (Fig. 3B). The *aca*A(PKA-C) cells produced the same number of spores per fruiting body as were produced by wild-type cells (*25*), and the *aca*A(PKA-C) spores were viable, as judged by their ability to germinate and form colonies on bacterial growth plates (*26*). The formation by *aca*A(PKA-C) cells of a stalk capable of

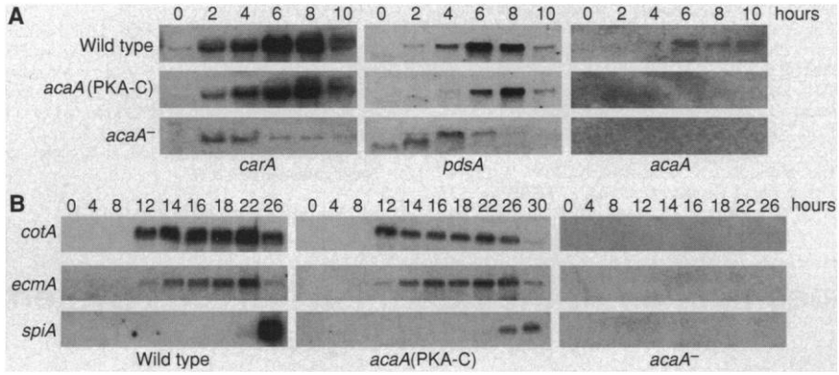

Fig. 3. Gene expression in the PKA-C–rescued *acaA* cells. Northern blots of mRNA purified from cells at the indicated times of development on filters are shown after hybridization with several gene probes and autoradiography (*13*). (**A**) Expression of aggregation-stage genes. *carA* encodes the major aggregation-stage cAMP receptor cAR1 (*3*), *pdsA* encodes an extracellular phosphodiesterase (*30*), and *acaA* encodes the adenylyl cyclase ACA (*4*), which is disrupted in the *acaA* mutant and *acaA*(PKA-C) cells. (**B**) Expression of cell type–specific genes. *cotA* is a prespore-specific gene (*31*), *ecmA* is a prestalk-specific gene (*32*), and *spiA* is a sporulation-specific gene (*33*). In this experiment, the wild-type cells and *acaA*(PKA-C) cells formed fruiting bodies by 26 and 30 hours, respectively, and the *acaA* mutant cells showed no visible signs of development.

carrying a normal-size sorus above the substratum (Fig. 1B) signified proper stalk cell production. Thus, because PKA-C activation overcomes the requirement for cAMP signaling after aggregation, all responses to extracellular cAMP later in development must be mediated by PKA.

How can our results be rationalized, given that work over the past 30 years has shown that extracellular cAMP is required for development? It is unlikely that adventitious regulatory mechanisms are induced by PKA-C overexpression, substitute for cAMP signaling, and recapitulate the wild-type developmental program. Instead, the *aca*A(PKA-C) cells reveal that other signaling systems can coordinate development in the absence of extracellular cAMP. These other signaling systems may provide essential regulation that is only observed to depend on cAMP-mediated temporal control of events in most experimental tests. Perhaps extracellular cAMP signals, transduced through PKA, are required to initiate events at critical points in development, gating the tempo of the cAMP-independent regulatory events. In this view, activation of PKA-C would have the effect of relieving a series of rate-limiting steps within a complex regulatory network, the downstream components of which remain largely unexplored.

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16. Adenylyl cyclase (AC) activity was measured as described previously [(*28*) and references therein]. Exponentially growing cells were washed and suspended at 2×10^7cells/ml in phosphate buffer [50 mM KH_2PO_4 and 50 mM Na_2HPO_4 (pH 6.1)] and pulsed at 6-min intervals for 5 hours with 50 nM cAMP. Cells were washed and concentrated to 10^8 cells/ml, and samples were rapidly lysed and assayed for the production of ^{32}P-labeled cAMP in a standard assay cocktail with no additions, with 5 mM $MnSO_4$ added, or with 40 μM guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S) added. Triplicate assays were performed for each sample prepared from three independent experiments. The AC activity in *acaA*(PKA-C) cells and in the *acaA*-null cells was $\leq 0.08 \pm 0.07$ pmol min^{-1} mg^{-1} under all conditions. The AC activity in wild-type cells was 25 ± 3 pmol min^{-1} mg^{-1} in the presence of GTP-γ-S. AC activity was also measured during development by collecting filter-developed cells at various times, concentrating them to 10^8 cells/ml, and assaying lysates for the presence and absence of $MnSO_4$ or GTP-γ-S. In one typical experiment, the AC activity of wild-type cells peaked at 17 pmol min^{-1} mg^{-1} in the presence of GTP-γ-S, whereas the AC activity of *acaA*(PKA-C) cells ranged from −0.25 to +0.54 pmol min^{-1} mg^{-1}.
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Specification of the Zebrafish Nervous System by Nonaxial Signals

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The organizer of the amphibian gastrula provides the neurectoderm with both neuralizing and posteriorizing (transforming) signals. In zebrafish, transplantations show that a spatially distinct transformer signal emanates from tissues other than the organizer. Cells of the germring (nonaxial mesendoderm) posteriorized forebrain progenitors when grafted nearby, resulting in an ectopic hindbrain-like structure; in contrast, cells of the organizer (axial mesendoderm) caused no posterior transformation. Local application of basic fibroblast growth factor, a candidate transformer in *Xenopus*, caused malformation but not hindbrain transformation in the forebrain. Thus, the zebrafish gastrula may integrate spatially distinct signals from the organizer and the germring to pattern the neural axis.

The developing vertebrate central nervous system is patterned by inductive interactions (*1*). The gastrula organizer (referred to as the "dorsal lip" in amphibians, "node" in amniotes, and "shield" in fish) is thought to be the source of patterning information (*2*). Analyses using amphibian embryos have indicated temporally distinct signals within the organizer (*3, 4*): An activator signal from the anterior axial mesoderm defines the anterior neurectoderm, and a subsequent transformer signal from the chordamesoderm (notochord) repatterns nearby neural tissue into more posterior types. In mouse, chick, and fish embryos, the elimination of the organizer does not abolish anteroposterior (AP) patterning in the neurectoderm; hence, there is a source of pattern information in non-organizer tissues (*5, 6*).

The neural fate map of zebrafish (*7*) shows patterning by 6 hours of development, when gastrulation has only advanced to the formation of a thickened blastoderm margin (germring) and an embryonic shield at its dorsal side. Forebrain progenitors are located far from the germring, spanning the dorsal midline (Fig. 1A). In contrast, hindbrain progenitors lie close to the germring, lateral to the embryonic shield, with midbrain progenitors in between (Fig. 1A). The early regionalization of anterior (forebrain) and posterior (hindbrain) neural progenitors within the neurectoderm allowed us to investigate signals that may differentially pattern the neuraxis. We hypothesized that proximity to the germring might specify more posterior neural fates. Indeed, labeled presumptive forebrain progenitors (Fig. 1B), transplanted (*8*) at shield stage to the position of the presumptive hindbrain, adopt the hindbrain fate (Fig. 1C). Moreover, presumptive hindbrain cells are not committed to a specific fate at this stage (*9*). Thus, the signals that normally instruct or permit cells to adopt the hindbrain fate are still active in vivo at 6 hours.

Because deletion of the shield disrupts notochord but not hindbrain development (*6*), the signals responsible for hindbrain patterning probably do not come exclusively from the shield. Germring tissue may be a source of such a posteriorizing signal. The shield contributes to axial mesoderm, notochord, and ventral neural tissues (*10*) (Fig. 1D); the germring gives rise to somitic mesoderm, posterior mesoderm, and endoderm (Fig. 1, E and F) (*11*). To investigate patterning by nonaxial germring tissue, we transplanted sectors of the shield (0°) and the germring at defined angular distances from the dorsal midline (45°, 90°, 135°, and 180°) to the animal pole, a region fated to become forebrain (Fig. 1, A and B), of shield-stage zebrafish. If the germring were the source of a patterning signal, such grafts

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