Cytokinins induce sporulation in Dictyostelium

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The social amoeba Dictyostelium discoideum diverged from the line leading to animals shortly after the separation of plants and animals but it retained characteristics of both kingdoms. A GABA<sub>B</sub>-like receptor and a peptide, SDF-2, with homologs found only in animals, control sporulation, while cytokinins, which act as hormones in plants, keep spores dormant. When SDF-2 binds its receptor DhkA, it reduces the activity of the cAMP phosphodiesterase RegA such that cAMP levels can increase. It has been proposed that the cytokinin discadenine also produces in an increase in cAMP but acts through a different histidine kinase, DhkB. We have found that discadenine and its precursor, isopentenyl adenine, not only maintain spore dormancy but also initiate rapid encapsulation independently of the SDF-2 signal transduction pathway. DhkB and the adenylyl cyclase of late development, AcrA, are members of two component signal transduction families and both are required to transduce the cytokinin signal. As expected, strains lacking the isopentenyl-transferase enzyme chiefly responsible for cytokinin synthesis are defective in sporulation. It appears that SDF-2 and cytokinins are secreted during late development to trigger signal transduction pathways that lead to an increase in the activity of the camp-dependent protein kinase, PKA, which triggers rapid encapsulation as well as ensuring spore dormancy.

KEY WORDS: Discadenine, Isopentenyl adenine, Zeatin, Histidine kinase, SDF-2, Sporulation

INTRODUCTION

Development of Dictyostelium discoideum leads to a fruiting body where the mass of spores is held up on a tapering stalk that can be several millimeters high. At the beginning of culmination, migrating slugs become upright and prestalk cells at the tip construct a cellulosic stalk tube. Cells enter the tube and vacuolize to give it added strength. Once the stalk has extended down through the underlying prespore cells to the substratum, further expansion of cells within the stalk extends it upwards. Prestalk cells continue to climb the stalk and enter at the top before vacuolizing. Prespore cells follow behind until the stalk is almost complete, at which point they rapidly encapsulate into dormant spores. The whole process takes about 24 hours (Raper, 1940; Loomis, 1975). As each spore is surrounded by a cellulose reinforced protein coat, prespore cells cannot move once they are encapsulated. Premature encapsulation results in spores that cannot reach the top of the stalk. Therefore, the timing of sporulation must be carefully controlled.

Sporulation of dispersed cells of a strain (KP) with partially constitutive PKA activity has been shown to be density dependent when they are developed on the bottoms of multi-test wells (Anjard et al., 1997). At densities higher than 10<sup>3</sup> cells/cm<sup>2</sup>, a phosphopeptide of about 1.2 kDa accumulates in the buffer. When this peptide, SDF-1, is purified and added back to KP cells developing at lower density, it induces sporulation 90 minutes later in a process that requires protein synthesis (Anjard et al., 1997). SDF-1 accumulates in fruiting bodies of wild-type cells where it is found together with several other factors that can also induce encapsulation of KP cells developed at low density. GABA<sub>B</sub>, produced by the enzyme glutamate decarboxylase (GadA) induces sporulation at 1 nM concentration (Anjard and Loomis, 2006). When GABA<sub>B</sub> binds its receptor GrlE, a G-protein-coupled receptor of the GABA<sub>B</sub> metabotropic receptor-like family, it triggers the rapid release of the precursor of a second peptide factor, SDF-2, that can also induce encapsulation in test cells. SDF-2 is a 34 amino acid peptide cleaved from the secreted precursor AcbA (acyl-CoA binding protein) (Anjard and Loomis, 2005). SDF-2 binds the receptor histidine kinase DhkA and inhibits phosphorelay to the internal cAMP phosphodiesterase RegA, resulting in a decrease in its activity. The internal concentration of cAMP can then increase and activate PKA, which leads to rapid encapsulation of prespore cells (Anjard and Loomis, 2005).

Strains in which the genes encoding either AcbA or DhkA are disrupted sporulate poorly but can sometimes reach 60% of the wild-type level of spores, suggesting that there may be other sporulation signals. Disruption of the gene encoding another histidine kinase, DhkB, reduces the proportion of spores to about one-third when culmination is first completed (Zinda and Singleton, 1998). The number of viable spores in dhkB<sup>–</sup> strains decreases after 25 hours of development such that it is only 3% of wild-type levels by 72 hours, apparently because the spores germinate while still on top of the stalk. Zinda and Singleton (Zinda and Singleton, 1998) suggested that dhkB<sup>–</sup> mutant cells were unable to respond to the germination inhibitor, discadenine, to maintain dormancy. DhkB may also play a role in initiating sporulation as double mutants lacking both DhkA and DhkB are much more impaired in spore formation than either of the single mutants lacking only one of these histidine kinases (Wang et al., 1999). The almost complete lack of sporulation in dhkA<sup>–</sup> dhkB<sup>–</sup> strains suggests that sporulation may result from the combined activity of both histidine kinases. The adenylyl cyclase AcrA is also required for sporulation and spore dormancy as the null strain is impaired in spore formation (Soderbom et al., 1999). Most of the spores that form in acrA<sup>–</sup> null strains fail to remain dormant and rapidly germinate. AcrA is a membrane associated adenylyl cyclase that carries a degenerate histidine kinase domain of unknown function. The region of the domain involved in phospho-transfer in other histidine kinases has multiple variations in key amino acids in AcrA and is probably not functional, whereas the two receiver domains in AcrA are well conserved and could accept a phosphate group transferred by one of the 13 functional histidine kinases existing in Dictyostelium (Anjard and Loomis, 2002).
Cytokinins are N6 substituted adenine derivatives that affect growth and development of plants by activating two-component phosphorelay pathways (Mok and Mok, 2001; Kakimoto, 2001; Kakimoto, 2003; Rashotte et al., 2006). There are three different receptor histidine kinases in the mustard Arabidopsis thaliana: AHK2, AHK3 and AHK4/CRE1 (Inoue et al., 2001; Nishimura et al., 2004; Suzuki et al., 2001; Yamada et al., 2001). Cytokinins bind to a conserved extracellular loop of about 200 amino acids found in each of these receptors, referred to as the CHASE domain (Anantharaman and Aravind, 2001; Heyl et al., 2007). Discadencine is a derivative of the cytokinin isopentenyl adenine, which is synthesized by condensation of isopenentylene phosphoryphosphate and 5’AMP followed by removal of the ribose phosphate group (Abe et al., 1976; Taya et al., 1978). Both isopentenyl adenine and discadencine can be extracted from Dictyostelium fruiting bodies and shown to inhibit germination when added to washed spores at levels above 1 μM (Tanaka et al., 1978; Ibara et al., 1980) (D. Cotter, personal communication). Moreover, discadencine shows cytokinin activity in an assay using tobacco callus cells (Nomura et al., 1977). We have found that both discadencine and isopentenyl adenine, as well as other cytokinins, induce rapid sporulation in Dictyostelium in a process that is dependent on DhkB and AcrA. The cytokinin signaling pathway is independent of the SDF-2 pathway but both converge at the level of activation of DhkB through an increase of intracellular cAMP.

MATERIALS AND METHODS

Chemicals

Synthetic SDF-1 and SDF-2 have previously been described (Anjard and Loomis, 2005). Zeatin and isopentenyl adenosine were purchased from Acros Organics (Geel, Belgium). Isopentenyl adenine, other cytokinins and adenine were purchased from Sigma (St Louis, MO). Synthetic DL-discadencine was a kind gift from Dr David Cotter (van Es et al., 1996). To generate the dhkB/K strain, KP cells were transformed with the construct used in the original disruption of dhkB (Zinda and Singleton, 1998). After selection with blasticidin and sub-cloning, the dhkB fragment was amplified by PCR and cloned in the pGEMT vector (Promega -/K strain, KP cells were cloned into the unique EcoRI site located at codon 35 of the dhkB/Zinda and Singleton, 1998). After selection with blasticidin and sub-cloning, dhkB disruptants were identified using specific primers. More than 90% of the clones presented the expected pattern for dhkB disruption.

In order to disrupt iptA (DDB0233672; GenBank XP 642693), a 1.5 kb fragment was amplified by PCR and cloned in the pGEMT vector (Promega A1360). The BSR cassette from pBSR519 (Puta and Zeng, 1998) was cloned into the unique EcoRI site located at codon 35 of the iptA. For gene disruption, 10 μg of the plasmid was linearized with NoI before electroporation into 107 AX4 cells. Disruption of the endogenous gene in transformants was confirmed by PCR using primers located outside the cloned sequences.

The bioassay was carried out on KP cells and their derivatives after 18 hours development in monolayers as previously described (Anjard et al., 1998a). The wild-type strain AX4, the Strains and bioassay

The wild-type strain AX4, the pkac overexpressing strain KP and its derivative dhkA/K have been previously described (Anjard et al., 1992; Anjard et al., 1998a). The ucaG strain was a kind gift from Pauline Schaap (van Es et al., 1996). To generate the dhkB/K strain, KP cells were transformed with the construct used in the original disruption of dhkB. After selection with blasticidin and sub-cloning, dhkB disruptants were identified using specific primers.

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Development and spor viability

Cell growth was assayed using electronic microscopy (EM) (Sussman, 1987). Development was initiated by harvesting exponentially growing cells at a density of 2-5×107/ml. Cells were washed with PBS buffer [20 mM Na/K phosphate (pH 6.5), 20 mM KCl, 1.2 mM MgSO4], centrifuged again at 1000 rpm for 5 minutes and resuspended at a density of 1×107 cells/ml in PBS buffer before being deposited on nitrocellulose filters placed on pads saturated with PDF (Anjard and Loomis, 2005).

For spore viability assays, 107 washed cells were deposited on a small filter and developed for 24 hours. Spores were collected by placing the filter in an Eppendorf tube with 1 ml PDF containing 0.5% Triton X-100 and briefly vortexed. Spores were incubated for at least 5 minutes in the buffer containing 0.5% Triton X-100 and then centrifuged at 6000 rpm for 1 minute and resuspended in 1 ml PDF. After counting and dilution, 50 spores were plated on triplicate on SM plates with a K. aerogenes suspension (Anjard and Loomis, 2005). The number of plaques, corresponding to the number of viable spores, was scored after 4-5 days of incubation at 22°C. Spore viability assays were repeated at least three times.

Isopentenyl adenine binding assay

Vegetative cells were centrifuged at 1200 rpm for 5 minutes and washed twice in 10 ml binding buffer [50 mM phosphate buffer (pH 7.5), 200 mM NaCl] per 108 cells. Developed cells were generated by depositing 5×107
to $10^6$ cells on 4.5 cm diameter filters and incubating at 20°C until the fruiting bodies reached the early-mid-culmination stage (~22 hours). Fruiting bodies were collected on a spatula and washed three times in binding buffer before the cells were resuspended at density of 2.5 x $10^7$/ml in binding buffer containing 1.25 mM adenine. Protein concentration of the suspension was determined using the BioRad reagent.

Aliquots of 400 μl of the cells suspension were incubated for 5 minutes at room temperature with the indicated concentrations of $^3$H-isopentenyl adenine ($^3$H-IP) with and without a 10,000 fold excess cold iP, the final volume being 500 μl. Suspension (400 μl) was then filtered under vacuum through a GF/C filter (Whatman, Maidstone, England) on a manifold unit and washed three times with 2 ml binding buffer. The filters were counted in scintillation liquid, together with a standard for the amount of $^3$H-iP used. Each point was carried out in duplicate (two tubes with $^3$H-IP alone, two tubes with $^3$H-IP and a 10,000 fold excess unlabelled iP) and repeated three independent times. Specific binding was taken as the difference between cell-associated counts in the absence and presence of unlabelled iP and was normalized to fmol $^3$H-IP per mg of protein.

Isopentenyl adenine and discadenine quantitation

Isopentenyl adenine and discadenine were purified from developed Dictyostelium cells using a simplification of the protocol of Taya et al. (Taya et al., 1980). Cells (10^9) of each strain were developed on filters for the indicated times, harvested with a spatula and resuspended in 10 ml water. Cells were pelleted by centrifugation at 2000 rpm for 5 minutes and the supernatants were incubated for 10 minutes with 1 ml of Amberlite XAD-2 resin. The resin was collected by centrifugation at 1000 rpm for 5 minutes and washed twice in 10 ml water. Cytokinins were eluted from the resin by three successive additions of 1 ml 30% ethanol which were pooled and dried under vacuum before being resuspended in 100 μl of methanol. Between 2.5 and 25 μl of the samples were loaded on a Majic C-18 Column (ID 1 mm × 150 mm) column using an ultrafast HPLC apparatus (Microm BioResources) coupled to a LCQdeca-Mass spectrometer with electrospray ionization source (ESI) under positive ion mode (ThermoFinnigan). The LC mobile phase A was 2.5% methanol in water and the LC mobile phase B was pure methanol. The LC flow rate was 50 μl/minute, and the LC gradient was 10% B to 95% B in 20 minutes then held at 95% B for three minutes. Isopentenyl adenine gives a characteristic [M+H+] peak at m/z 204 and is eluted after about 17 minutes in this gradient. Discadenine gives a [M+H+] peak at m/z 305 and is eluted after about 3 minutes. The product identities were further confirmed by ESI-MS/MS analysis. Upon ESI-MS/MS fragmentation, isopentenyl adenine gives characteristic daughter peaks at m/z 136 and m/z 148 respectively, while discadenine gives a daughter peak at m/z 204 (data not shown). Known amounts of isopentenyl adenine and discadenine standards were run under identical conditions for quantitation of the samples using select ion monitoring.

RESULTS

Cytokinins induce rapid sporulation

Addition of discadenine or isopentenyl adenine results in rapid encapsulation of developing KP cells (Fig. 1A). Maximum activity was observed at concentrations of 10 nM, considerably less than the levels required for inhibition of germination of wild-type spores (Tanaka et al., 1978; Taya et al., 1980). It appears that discadenine and isopentenyl adenine not only function to maintain spore dormancy but also act earlier to induce sporulation. Zeatin, a plant cytokinin not found in Dictyostelium, induces rapid encapsulation but only when added to about 100-fold higher concentration (Fig. 1A). The artificial cytokinins, thidiazuron, kinetin and 6-benzyl-aminopurine, which are not found in either plants or Dictyostelium but act as effective as the natural cytokinins in plants, also induce rapid sporulation in Dictyostelium when present at more than 10 μM (Fig. 1B). Induction of sporulation by cytokinins appears to act through PKA as addition of either of the specific inhibitors, H89 or myristoylated PKI, blocks sporulation in response to isopentenyl adenine (Fig. 1A).

The number of spores started to increase within 10 minutes of addition of discadenine and reached maximum at 45 minutes (Fig. 2). There was no response to 1 mM adenine which is not a cytokinin. Simultaneous addition of discadenine and SDF-2 did not increase the proportion of spores (data not shown).

iptA encodes isopentenyl-transferase which generates cytokinin

The first step dedicated to the biosynthesis of cytokinins is catalyzed by isopentenyl-transferase and results in the condensation of isopentenyl pyrophosphate with AMP, ADP or ATP (Taya et al., 1978; Ihara et al., 1984). After dephosphorylation, the ribose moiety is removed to generate isopentenyl adenine. Isopentenyl adenine is also generated by post-transcriptional addition of an isopentenyl group to adenosine groups in tRNA. Most bacteria and
animals have a single gene encoding isopentenyl-transferase to modify their tRNAs. Plants, however, have multiple isopentenyl-transferases (Takei et al., 2001; Miyawaki et al., 2006). *Dictyostelium* has three isopentenyl-transferases genes, *iptA*, *iptB* and *iptC*. A phylogenetic analysis of the corresponding proteins showed that IptB and IptC are closely related to the eukaryotic and bacterial isopentenyl-transferases that modify tRNAs, while IptA clusters with enzymes involved in cytokinin synthesis (data not shown).

We constructed a plasmid for expression of *iptA* in *E. coli* and found that culture supernatants of bacteria transformed with this construct contain material that induces rapid sporulation in KP cells, whereas supernatants from cultures of untransformed cells have no effect (data not shown). Cytokinins were purified from supernatants of *iptA*-expressing bacteria by extraction with ethylacetate and absorption on the hydrophobic resin Amberlite XAD-2. The recovered material was analyzed by Mass Spectroscopy after HPLC on C-18 column and compared with authentic compounds. We found 210 pmol/ml isopentenyl adenine in the supernatants of

![Fig. 2. Time course of spore induction by discadenine.](image)

KP cells that had developed at low density in buffer containing cAMP were treated with 100 nM discadenine (circles) or 1 mM adenine (squares). The proportion of cells that became ellipsoid phase-bright spores was determined microscopically over the following hour. The experiments were repeated three times.

![Fig. 3. Isopentenyl adenine and discadenine production in wild-type and *iptA*-null strains.](image)

Isopentenyl adenine (squares) and discadenine (circles) recovered from wild-type cells (filled symbols) and mutant cells (open symbols) at the indicated times of development. The horizontal line indicates the approximate level of cytokinin required to induce spore formation fully.

![Fig. 4. Response of sporogenous strains to spore inducers.](image)

Mutant strains overexpressing the catalytic subunit of PKA as the result of being transformed with the KP construct were developed as monolayers at 2 x 10^5 cells/cm^2 for 20 hours. Cells of the indicated mutant strains were then treated with no addition (none), 100 nM discadenine (Disc), 100 nM isopentenyl adenine (iP), 1 μM zeatin, 10 pM synthetic SDF-1, 10 pM synthetic SDF-2 or 1 μM GABA. Spores were counted after 1 hour. Cells treated with SDF-1 were scored after 90 minutes. Each experiment was repeated three times. Error bars correspond to one standard deviation.
transformed bacteria and less than 10 pmol/ml in the supernatants of mock transformed cells (data not shown). It appears that iptA encodes the isopentenyl-transferase responsible for synthesis of isopentenyl adenosine.

Reduction of cytokinin production impairs spore viability

We disrupted iptA in wild-type AX4 cells by introducing a construct in which a blasticidin resistance cassette was inserted near the start of the gene and selected for blasticid resistant transformants. Strains in which iptA was disrupted by homologous recombination were found to grow and develop well, but to form many round wrinkled spores rather than the normal ellipsoid shaped spores. Cytokinins were purified from wild-type and mutant fruiting bodies at different times in development and quantitated following separation by HPLC. No isopentenyl adenine or discadenine could be detected before 20 hours of development. Thereafter, the cytokinins accumulated rapidly although isopentenyl adenine leveled off for 2 hours at 24 hours of development in wild-type cells (Fig. 3). The rate of accumulation of the cytokinins was reduced perhaps as the result of rapid conversion to discadenine at this time (see Table S1 in the supplementary material). However, developing them together with an equal number of wild-type cells resulted in improved sporulation (see Table S1 in the supplementary material). However, developing them together with an equal number of acbA– cells, which cannot make the SDF-2 precursor, resulted in only a modest increase in the number of spores. All of these strains except acbA– accumulated comparable levels of SDF-2 in their sori (see Table S1 in the supplementary material).

Induction of sporulation by cytokinins depends on DhkB and AcrA

When spores are collected from wild-type fruiting bodies and incubated at high density, they are inhibited from germinating by the presence of the germination inhibitor (Nomura et al., 1977). Disruption of the gene encoding the histidine kinase DhkB was found to result in spores that germinate in the presence of discadenine (Zinda and Singleton, 1998). We found that dhkB–K cells, developing at low density, fail to differentiate into spores in response to discadenine or other cytokinins but are still able to respond to SDF-2, SDF-2 and GABA just as well as wild-type KP cells (Fig. 4).

Histidine kinases relay phosphate to proteins carrying response regulator regions resulting in modulation of activity. The adenyl cyclase of late development, AcrA, carries two response regulatory regions that might be targets for DhkB (Anjard et al., 2001). In support of this notion, we found that cells lacking AcrA failed to respond to discadenine, isopentenyl adenine or zeatin, although they responded normally to the other sporulation inducers (Fig. 4). If the adenyl cyclase activity of AcrA is stimulated when its response
regulatory regions are phosphorylated, the resulting increase in cAMP would lead to high levels of PKA activity necessary for rapid encapsulation. We also determined whether the other two adenylyl cyclases, ACA and ACG, were essential for cytokinin induction of sporulation. As cells lacking ACA are unable to aggregate or proceed through development (Pitt et al., 1992), we introduced a construct that results in partially constitutive PKA activity into an acaA– strain. The resulting acaA–/K strain is able to make fruiting bodies but does not have a sufficiently sporogenous phenotype that we could test cells of this strain developing at low cell density for responses to cytokinins. Therefore, we dissociated cells from early culminants that had developed on filter supports and stimulated them with different spore inducing factors. Cells lacking ACA responded to discadenine, zeatin, SDF-2 and GABA just as well as cells dissociated from early culminants of wild-type AX4 cells (Fig. 5). Likewise, cells of the acgA– strain dissociated from early culminants responded well to cytokinins (Fig. 5). It appears that DhkB-dependent induction of encapsulation by cytokinins acts exclusively through the specific adenylyl cyclase that carries response regulatory regions, AcrA, while SDF-2 induction of encapsulation is not dependent on any specific source of cAMP. The response to SDF-1, however, is dependent on ACG (Fig. 5).

**Fig. 6. Consequences of loss of components in the SDF-2 pathway.** Cells of the dhkA–/K, rdeA–/K strains were developed as monolayers at 2×10^5 cells/cm^2 for 20 hours. regA– cells were developed as monolayers at 5×10^5 cells/cm^2 for 20 hours. They were then treated with no addition (none), 100 nM discadenine (disc), 100 nM isopentenyl adenine (iP), 1 µM zeatin, 10 pM synthetic SDF-1, 10 pM synthetic SDF-2 or 1 µM GABA. Spores were counted after 1 hour. Cells treated with SDF-1 were scored after 90 minutes. Each experiment was repeated three times. Error bars correspond to one standard deviation.

For induction of sporulation by cytokinins is independent of DhkA, RdeA and RegA

SDF-2 induces rapid encapsulation by inhibiting the relay of phosphate from DhkA to the cAMP phosphodiesterase RegA via the H2 intermediate RdeA (Thomason et al., 1999; Anjard and Loomis, 2005). Cytokinins might be indirectly inducing sporulation by stimulating SDF-2 production, much as GABA induces sporulation by triggering the release of AcbA and its processing to SDF-2 (Anjard and Loomis, 2006). The resulting SDF-2-dependent inhibition of RegA, rather than activation of the GABA signal transduction pathway, is responsible for the rapid increase in PKA activity that leads to encapsulation. To determine whether cytokinins...
indicating that the receptor is developmentally controlled. However, adenine (Fig. 7B). Vegetative cells bound very little of the cytokinin, partially constitutive PKA activity. Cells of the (Fig. 1). We then tested vegetative and developed wild-type cells, as was very similar to the concentration dependence in the bioassay Kd of 6 nM (Fig. 7A). The concentration dependence for binding adenine. The cells bound 3H-isopentenyl adenine with an apparent absence or presence of 10,000 fold excess unlabelled isopentenyl domains (Zinda and Singleton, 1998). We determined the ability of found that which depend on phosphorelay through RdeA (Fig. 6). Likewise, we developed in monolayers were found to respond to addition of discadenine, isopentenyl adenine and zeatin were normal in cloned as wild-type culminants were incubated at 20°C for 5 minutes from wild-type culminants. However, DhkB has no predicted extracellular loops of more than 10 amino acids separating the transmembrane domains near the N terminus that might flanked by transmembrane domains. AcrA has two predicted extracellular loops of about 35 amino acids each as wild-type cells, indicating that neither DhkB nor AcrA is likely to be the isopentenyl adenine receptor.

### DISCUSSION

Spore differentiation is finely tuned to occur at the right time and place through a series of negative and positive signals. Two different signaling pathways involving the peptide SDF-2 and cytokinins converge to induce spore formation through the activation of PKA. Cytokinins are hormones that affect many different processes in plants but are not known to function in any animal. In plants, histidine kinases bind and mediate the responses to cytokinins. Although histidine kinases are prevalent in bacterial and plant signal transduction pathways, they are not found in metazoa (Anjard and Loomis, 2002). Nevertheless, Dictyostelium uses a histidine kinase, DhkB, in the cytokinin signal transduction pathway leading to encapsulation of spores. The use of cytokinins as hormones apparently predates the divergence between Dictyostelium and plants over a billion years ago.

The cyanobacteria Anaebena species genome includes two isopentenyl transferases, one being similar to IptA. Moreover, Anaebena encodes an adenylyl cyclase, cyaC, with the same unusual domain organization as AcrA, starting with a receiver domain followed by a histidine kinase domain and another receiver domain to finish with the adenylylate cyclase domain (Katayama and Ohmori, 1997). This suggests that components of the cytokinin pathway might have appeared in bacteria that predated eukaryotes but were lost in animal and fungal lineages.

As the cytokinin response in Dictyostelium is mediated by the histidine kinase DhkB and the adenylyl cyclase AcrA to the exclusion of other histidine kinases or adenylyl cyclases, these proteins may have a close association. Moreover, considering that the only known H2 component, RdeA, is not required for the cytokinin response, DhkB may directly relay phosphate to the cytokinin and SDF-2. Cytokinins activate the histidine kinase DhkB and the adenylyl cyclase AcrA in prespore cells, while SDF-2 binds to the histidine kinase DhkA, such that the cAMP phosphodiesterase RegA is no longer activated. As a result, cAMP accumulates and activates PKA, which triggers encapsulation. PKA activity also leads to the release of the SDF-2 precursor AcbA which is processed by the prestalk specific protease TagC. Glutamate blocks the effect of PKA on AcbA. GABA competes with glutamate for the receptor GrlE. The kinases PI3K and PKBR 1 are essential components of the GABA signal transduction pathway leading to release of AcbA [adapted, with permission, from Anjard and Loomis (Anjard and Loomis, 2006)].
The cytokinin receptors are known in plants to be histidine kinases that bind cytokinins through an extracellular CHASE domain (Anantharaman and Aravind, 2001). This domain is found in only two Dictyostelium proteins, DhkA and ACG, but neither appears to be a cytokinin receptor as null mutants lacking these proteins respond normally to cytokinins (Figs 5, 6). Because neither DhkB nor AcrA appear to account for isopentenyl adenine binding, further studies will be required to recognize the Dictyostelium cytokinin receptor.

The cytokinin response could be an artifact of the bioassay as we used cells that overexpress PKA developed as monolayers or cells dissociated from culminants to assess rapid sporulation. However, iptA cells, in which synthesis of isopentenyl adenine is compromised in an otherwise wild-type background, are impaired in sporulation, indicating the importance of cytokinin signaling during normal development. Moreover, DhkB has been shown to play a direct role in determining the intracellular levels of cAMP during culmination as cAMP levels have been found to be significantly reduced in dhkb- spores (Zinda and Singleton, 1998).

Cytokinins can be produced either through a dedicated pathway or from the degradation products of tRNA (Kakimoto, 2003). Primitive plants like the moss Physcomitrella patens seem to use only the tRNA-IPT pathway for cytokinin production (Yedvakova and von Schwartzberg, 2007). In Dictyostelium, the inactivation of iptA results into a 5- to 10-fold reduction in isopentenyl adenine during culmination. The remaining isopentenyl adenine is probably generated from degraded tRNA. Discadenine levels are even lower in iptA cells (Fig. 3). However, when iptA cells are allowed to form fruiting bodies within clearings of a bacterial lawn, they can accumulate 25% as much discadenine as wild-type spores (data not shown). As these cells had been actively ingesting bacteria under these conditions, discadenine may have been derived from the degradation products of bacterial tRNA.

SDF-2 stimulates spore formation at about the same time as cytokinins (Anjard et al., 1999b). However, SDF-2 acts by removing the activating phosphates from RegA in the pathway involving RdeA and DhkA (Fig. 8). As the RegA cAMP phosphodiesterase activity drops, cAMP can accumulate and stimulate PKA. At the same time cytokinins activate an independent pathway such that cAMP is rapidly synthesized by AcrA. This dual control is similar to pushing the accelerator and releasing the brake on cAMP accumulation (Fig. 8).

As both SDF-2 and cytokinins are released at about the same time of development (t=22 hours), it is possible that their production is coordinated. However, cytokinins do not seem to play an essential role in SDF-2 production as iptA cells accumulate SDF-2 normally (see Table S1 in the supplementary material). Moreover, addition of cytokinins to developed KP cells does not result in the accumulation of SDF-2. Likewise, SDF-2 is not essential for cytokinin release as acbA cells produce as much cytokinin as wild-type cells (data not shown). Cytokinin production is dependent on the activity of isopentenyltransferase, which appears only during culmination (Ihara et al., 1980). Thus, the timing of cytokinin production may be mediated by the regulation of iptA. The timing of SDF-2 production may be set by the time of accumulation of GABA, which triggers release of the SDF-2 precursor AcbA (Anjard and Loomis, 2006). The enzyme that synthesizes GABA, Gada, accumulates only in prespore cells late in development and its regulation may determine the time of appearance of SDF-2. No matter how accumulation of SDF-2 and cytokinins are coordinated, both pathways need to be activated at about the same time to obtain maximal efficiency of sporulation.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/5/819/DC1

References


## Table S1. Summary of response to spore formation inducers

<table>
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<th>Response to cytokinin</th>
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### Additional References


