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An intersection of the cAMP/PKA and two-component signal transduction systems in *Dictyostelium*

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Terminal differentiation of both stalk and spore cells in *Dictyostelium* can be triggered by activation of cAMP-dependent protein kinase (PKA). A screen for mutants where stalk and spore cells mature in isolation produced three genes which may act as negative regulators of PKA: *rdeC* (encoding the PKA regulatory subunit), *regA* and *rdeA*. The biochemical properties of RegA were studied in detail. One domain is a cAMP phosphodiesterase (K_m ~5 μM); the other is homologous to response regulators (RRs) of two-component signal transduction systems. It can accept phosphate from acetyl phosphate in a reaction typical of RRs, with transfer dependent on Asp212, the predicted phosphoacceptor. RegA phosphodiesterase activity is stimulated up to 8-fold by the phosphodonor phosphoramidate, with stimulation again dependent on Asp212. This indicates that phosphorylation of the RR domain activates the phosphodiesterase domain. Overexpression of the RR domain in wild-type cells phenocopies a *regA* null. We interpret this dominant-negative effect as due to a diversion of the normal flow of phosphates from RegA, thus preventing its activation. Mutation of *rdeA* is known to produce elevated cAMP levels. We propose that cAMP breakdown is controlled by a phosphorelay system which activates RegA, and may include RdeA. Cell maturation should be triggered when this system is inhibited.

Keywords: *Dictyostelium*/phosphorelay/protein kinase A/RegA/response regulator

Introduction

The work described here brings together two well-analysed signal transduction systems in a novel configuration: the cAMP/protein kinase A system and the histidine kinase/two-component system. cAMP is a ubiquitous second messenger which controls the activity of the cAMP-dependent protein kinase (PKA); the system is widespread in eukaryotes, especially in metabolic and neuronal control, and in development (Harwood *et al*., 1992a,b; Jiang and Struhl, 1995; Li *et al*., 1995). The level of intracellular cAMP depends on the relative activities of adenylyl cyclase and cAMP phosphodiesterase, both of which can be regulated through extracellular signals. Two-component systems are the basis of most bacterial sensory pathways, and consist, at their simplest, of a sensory histidine kinase that transfers phosphate from a histidine to an aspartate on a second protein, the response regulator (RR), which controls the effector function (Bourret *et al*., 1991; Parkinson and Kofoid, 1992; Swanson *et al*., 1994). Recently, two-component systems have been discovered in eukaryotes, including a more elaborate phosphorelay system in yeast which controls osmotic responses (Appleby *et al*., 1996; Posas *et al*., 1996). *Dictyostelium* similarly has an osmosensing histidine kinase (Schuster *et al*., 1996), and a further histidine kinase (Wang *et al*., 1996) and an RR (Shaulsky *et al*., 1996) which have important roles in development.

The fruiting body of *Dictyostelium* consists of a cellular stalk, stabilized by a basal disc of stalk cells, supporting a mass of spores. It is formed during culmination by the movement of pre-stalk cells up and into the stalk tube, which extends as more pre-stalk cells are added to the top, so lifting the developing spore mass upwards. These morphogenetic events are coupled to the terminal differentiation of each cell type in its appropriate position: stalk cells at the growing tip of the stalk and round its base, spore cells at the top of the stalk, where they mature in a gradient from the top to the bottom of the spore head (Bonner, 1952; Richardson *et al*., 1994).

Several types of experiment indicate that maturation of both spore and stalk cells is triggered by the activation of PKA. For instance, expression of a dominant inhibitory form of the regulatory subunit of PKA in pre-stalk or pre-spore cells prevents their maturation during normal development (Harwood *et al*., 1992b; Hopper *et al*., 1993b). Conversely, overactivity of the catalytic subunit of PKA causes spore and stalk cells to mature prematurely during development and, in the case of the *rdeC* mutants, which lack a functional PKA regulatory subunit, the final fruiting structure is a mound of differentiated cells (Anjard *et al*., 1992; Simon *et al*., 1992; Hopper *et al*., 1993b; Mann *et al*., 1994). These same genetic activating manipulations also stimulate the maturation of spore cells when amoebae are allowed to develop as submerged monolayers. Finally, wild-type cells can be induced to form both spore and stalk cells by pharmacological treatment with the cAMP analogue, 8-Br-cAMP, which penetrates the cell and activates PKA directly (Kay, 1989; Riley *et al*., 1989; Maeda, 1992; Inouye and Gross, 1993; Kubohara *et al*., 1993; see also Results).

PKA is activated by intracellular cAMP, which is produced largely during development by the adenylyl

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Table I. Stalk and spore cell formation in culture

Section	Strain (No. of assays)	Genotype/comments	% Stalk cells (+DIF-1)	% Spore cells (no DIF-1)
A	NC4 (2)	wild-type	0	0
	DH1 (3)	wild-type	0.4	0
	myc1002 (3)	*rdeC* (REMI)	22	3
	HM332 (3)	*regA* (REMI)	27	4
	HM1015 (4)	*regA* (KO)	42	31
	WTC10-H2 (3)	*rdeA* (KO)	78	8
B	HM338 (2)	*gskA* (REMI)	87	0
	M2–1 (2)	*dokA* his. kinase null	0	0
	NS202 (4)	*dhkA* his. kinase null	0.5	0
C	HTY217 (1)	*rdeC*	42	65
	KIP (1)	control for A7P	0[a]	0
	A7P (1)	PKA-cat overexpressor	10[a]	5
	NC4 (2)	wild type + Br-cAMP	27	68

Cells were plated at 4×10^5/ml in either stalk medium + 100 nM DIF-1 or spore medium, both of which contained 10 μM cAMP-S (a non-hydrolysable analogue of cAMP). The *dhkA* and *dokA* histidine kinase null strains behave similarly to wild-type cells, making ~50% stalks and 50% spores with 15 mM Br-cAMP.
[a]These two stalk cell assays were performed in spore medium because more fragile cell lines respond better under these conditions.

cyclase, ACA. In early development, the production of intracellular cAMP is stimulated by extracellular cAMP, through the cell surface family of G-protein-linked, cAMP receptors (cARs). Stimulation of these receptors, in turn, initiates a complex signal transduction pathway resulting in the activation of ACA (Firtel, 1996; Parent and Devreotes, 1996).

Thus, it might seem plausible that extracellular cAMP, acting alone through this known pathway, would be sufficient to trigger terminal differentiation during normal development. However, there are two major problems with such a scheme, both deriving from work on cells differentiating in monolayer culture. First, extracellular cAMP barely induces terminal differentiation of spore and stalk cells in culture, even though it adequately promotes pre-spore and pre-stalk cell differentiation (Kay, 1982; Berks and Kay, 1988). Second, extracellular cAMP actually inhibits the final stages of stalk cell formation and must be removed from the monolayer in order for wild-type NC4 stalk cells to mature (Berks and Kay, 1988). The inhibitory pathway seems to involve the protein kinase GSK3, because null mutants in *gskA* make stalk cells freely, even in the presence of extracellular cAMP (Harwood *et al*., 1995). These problems of PKA activation would be resolved if there was another pathway for regulating intracellular cAMP levels in *Dictyostelium,* apart from that acting through adenylyl cyclase. We present evidence for such a pathway, which appears to function by controlling cAMP breakdown through the cAMP phosphodiesterase RegA.

Results

Screen for stalk and spore cell maturation mutants

We screened for mutants which are able to form mature spore and stalk cells when developed in tissue culture dishes under a simple salts medium. A non-hydrolysable cAMP analogue served to promote differentiation, and the stalk-specific inducer DIF-1 was provided, as appropriate, to enable stalk cell differentiation to occur (Table I). Wild-type cells do not aggregate in these conditions (due to the presence of the cAMP analogue) but remain amoeboid, and neither spore nor stalk cells form efficiently (NC4 cells form <1 in 10 000 viable spores; $<2\%$ stalk cells).

From a collection of >300 mutants created by restriction enzyme-mediated integration (REMI) insertional mutagenesis (Kuspa and Loomis, 1992), we identified strains myc1002 and HM332 which form both stalk and spore cells in the monolayer test conditions (Table I, section A). Subsequently, chemical (Abe and Yanagisawa, 1983) and disruption (Chang *et al*., accompanying paper) mutants of *rdeA* were tested, due to their phenotypic similarity to HM332, and were also found to form stalk and spore cells in monolayer culture.

The myc1002 insertion disrupted *rdeC*, the gene encoding the regulatory subunit of PKA (Simon *et al*., 1992), whereas *rdeA* mutants are known to have elevated levels of intracellular cAMP (Abe and Yanagisawa, 1983). Since mutations of both of these genes should result in an activated PKA, it seemed likely that the gene disrupted in strain HM332 might also encode a negative regulator of PKA activity. The HM332 insertion was identified as disrupting *regA*, a gene previously obtained as a suppressor of a sporulation-defective mutant (Shaulsky *et al*., 1996). To confirm the results with HM332, *regA* was disrupted by homologous recombination in Ax2 cells. The resulting strain, HM1015, formed stalk and spore cells even more efficiently than did HM332, perhaps due the greater health of its parental strain (Table I, section A). The *regA* null phenotype of HM1015 was corrected to wild-type when *regA* was expressed from its own promoter in this strain (not shown).

The three mutant classes, *rdeC*, *rdeA* and *regA* (see below), are thus all implicated in the regulation of PKA activity. In contrast to these three disruptants, mutations in other relevant genes did not promote stalk and spore maturation: *gskA* null cells yielded only stalk cells, as expected (Harwood *et al*., 1995), whereas mutants of the histidine kinases *dokA* (Schuster *et al*., 1996) and *dhkA* (Wang *et al*., 1996) behaved as wild-type cells (Table I, section B). These results suggest that neither

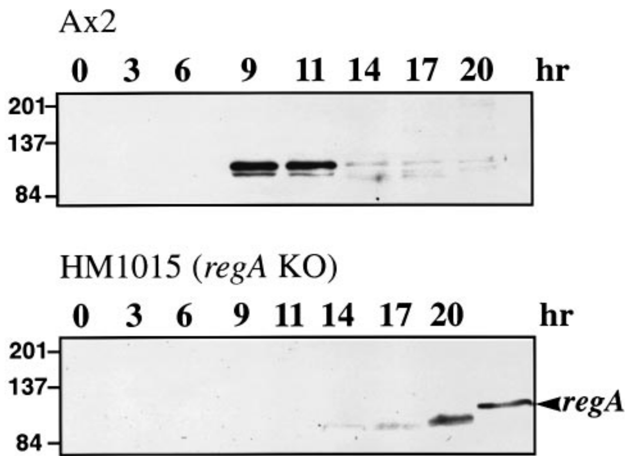


Fig. 1. Developmental expression of RegA protein. Cells of the wild-type Ax2 and the *regA* null, HM1015, were developed on agar, lysates prepared and RegA protein detected by Western blotting, as described in Materials and methods. The position of molecular weight standards (kDa) is shown.

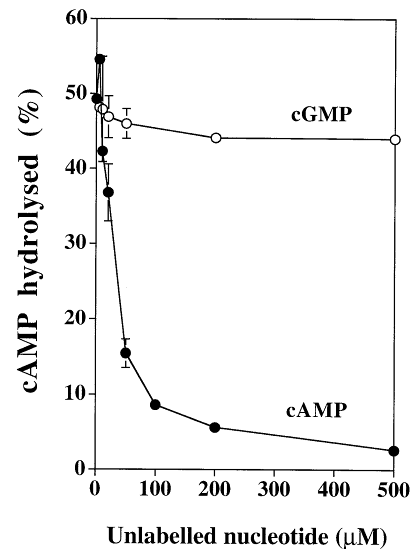


Fig. 2. RegA is a cAMP phosphodiesterase. RegA was immunoprecipitated from wild-type (Ax2) cells at 8 h of development. Phosphodiesterase assays were performed using 0.4 μM [^{3}H]cAMP as substrate, plus the indicated concentrations of unlabelled cAMP or cGMP. The data shown are representative of three experiments, and are the mean of duplicate assays ± range (where no error bars are shown, these are contained within the symbols). Similar results were obtained with bacterially expressed GST–PDE and GST–RegA (data not shown).

of these kinases are negative regulators of the maturation pathway.

Additional evidence that PKA activation is sufficient to induce stalk cell maturation

Not all previous work has shown that activation of PKA is sufficient to induce stalk cell maturation. Thus, *rdeC* mutants did not form stalk cells in monolayers (Kay, 1989); however, in these experiments, exogenous DIF-1 was not supplied and, when it is, stalk cells do form (Table I, section C, strain HTY217). Also, strains overexpressing the PKA catalytic subunit in pre-stalk cells form only a very low number of mature stalk cells in normal development (Hopper *et al*., 1993a; Mann and Firtel, 1993). In contrast, we found that strain A7P, where the catalytic subunit is driven by an actin15 promoter (Anjard *et al*, 1992), did form stalk cells in monolayer culture with DIF-1, though not with great efficiency (we have observed that strains expressing the neomycin resistance gene, such as A7P, are often more fragile in monolayer culture than other strains). Furthermore, wild-type NC4 cells were induced to form stalk cells if Br-cAMP was added to activate PKA, consistent with earlier work (Inouye and Gross, 1993; Kubohara *et al*., 1993). Thus, sufficient activation of PKA can trigger stalk, as well as spore, cell differentiation. We next focused our attention on the *regA* gene product.

RegA protein is present throughout development

The *regA* transcript, which is present in pre-stalk and pre-spore cells, is expressed at a low level in vegetative cells, and is induced rapidly during aggregation, remaining present throughout development (Shaulsky *et al*., 1996). Western blots (Figure 1) show that expression of RegA protein is developmentally regulated. It is not observable before the end of aggregation, peaks at the mound stage (9–11 h) and remains at a lower level thereafter. Although RegA is not evident on Western blots before ~9 h, the activity can be detected by biochemical assay. Using phosphodiesterase assays of immunoprecipitated material (see below), RegA activity is detectable at early stages of development and even in immunoprecipitates from growing Ax2 cells (data not shown). Western blots (Figure 1) and phosphodiesterase assays (see below) show that RegA protein is absent from strain HM1015; the band appearing at a late time in development is not RegA, but could be a related phosphodiesterase or a GST which reacts with the antiserum.

One domain of RegA is a cAMP phosphodiesterase

RegA is homologous to mammalian cyclic nucleotide phosphodiesterases (Shaulsky *et al*., 1996), but not to the *Dictyostelium* PdsA phosphodiesterase (Lacombe *et al*., 1986). We used a biochemical assay to confirm that RegA is a cAMP phosphodiesterase (PDE). Phosphodiesterase activity was evident in immunoprecipitates from developing cells (Figure 2), made using a polyclonal antiserum (R1/2F) against RegA. The R1/2F antiserum selectively precipitated RegA: negligible activity was precipitated from the *regA* null strain HM1015 (<1% that of Ax2 cells, the same level of activity isolated from Ax2 cells using pre-immune serum), whereas the level of activity precipitated from UK7, a strain disrupted in the *pdsA* gene, was very similar to that obtained from Ax2 cells (data not shown). Similar phosphodiesterase activity was seen by expressing intact RegA or its PDE domain in bacteria. The K_m of RegA for cAMP is ~5 μM (5.0 μM for the endogenous enzyme, 6.7 μM for the GST–PDE fusion). The enzyme is specific for cAMP, because cGMP does not compete with cAMP (Figure 2). Like the mammalian PDEs, RegA is sensitive to the general PDE inhibitor 3-isobutyl-1-methylxanthine (50% inhibitory concentration ~250 μM at 1 μM cAMP). The PDE4 inhibitors rolipram or RO20-1724 did not inhibit. RegA is not regulated by Ca^{2+}/calmodulin *in vitro,* and cGMP, at

Table II. Cell maturation in *regA* nulls is dependent on PKA

Strain	Genotype/comments	% Stalk cells (+DIF-1)	% Spore cells (no DIF-1)
HM2014 (3)	*regA*, ecmA-Rc (control)	3.4	
HM2013 (3)	*regA*, ecmA-Rm	0.4	
HM2012 (3)	*regA*, D19-Rc (control)		9.4
HM2011 (3)	*regA*, D19-Rm		0.03

The dominant-negative (Rm) and control (Rc) form of the PKA R-subunit was expressed in *regA* null cells under the control of pre-stalk (ecmA)- and pre-spore (D19)-specific promoters. The overall efficiency of differentiation in these cells (expressing the G418 resistance marker) was lower than in untransformed strains and they were more fragile in the monolayer conditions, suggesting that their physiology is perturbed by the selectable marker, as noted before (Schulkes *et al*., 1995). However, this does not interfere with interpretation of the results.

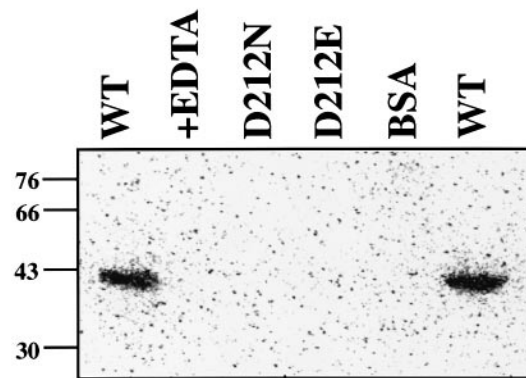


Fig. 3. Phosphorylation of the RegA RR domain by acetyl phosphate. WT, wild-type RR domain; D212N, Asp212Asn mutant RR domain; D212E, Asp212Glu mutant RR domain; +EDTA, 20 mM EDTA present in the reaction (with the wild-type RR domain). Numbers at the side indicate the size of molecular weight standards (kDa).

concentrations up to 20 mM, shows no allosteric effects on RegA activity.

Terminal differentiation in regA mutants is still dependent on PKA

RegA phosphodiesterase activity could control terminal differentiation by regulating PKA, or through some other route involving cAMP. To test whether terminal differentiation in *regA* mutants still requires PKA, differentiation was followed in *regA* null cells expressing the Rm dominant inhibitor of PKA, a form of the PKA R-subunit which cannot bind cAMP or dissociate from the catalytic subunit (Harwood *et al*., 1992b). Expression of Rm in pre-stalk (strain HM2013) or pre-spore (HM2011) cells reduced stalk and spore differentiation compared with control cells expressing the inactive inhibitor, Rc (Table II). Therefore, in *regA* mutants, stalk and spore maturation requires PKA activity.

The second domain of RegA is a response regulator

The second domain of RegA has homology to RRs of two-component systems. RRs receive the phosphate controlling their activity from a histidine of an upstream histidine kinase. This phosphorylation occurs on a conserved aspartate residue (Volz, 1993) corresponding to Asp212 of RegA. Low molecular weight phospho-compounds are also effective phosphate donors, since the RR domain itself catalyses the phosphotransfer (Lukat *et al*., 1992). Accordingly, the RegA RR domain has phosphotransferase activity: a purified GST fusion protein containing the RegA RR domain became phosphorylated when incubated *in vitro* with acetyl-[^{32}P]phosphate (Figure 3). Mutant proteins lacking Asp212 were not labelled by acetyl phosphate, nor was bovine serum albumin (BSA) (Figure 3). Like other RRs, phosphotransferase activity of the wild-type RegA RR domain is Mg^{2+} dependent (Lukat *et al*., 1992; McCleary and Stock, 1994), and the label can be completely removed from the protein by brief heat treatment (100°C, 2 min).

A second property of RRs, phosphdonor phosphatase activity (Lukat *et al*., 1992), can be detected in the RegA RR domain using phosphoramidate as substrate, but the rate of this RR-dependent phosphoramidate hydrolysis was very low. Using ^{31}P NMR analysis, spontaneous hydrolysis of 18 mM phosphoramidate occurred at a rate of ~0.5 μmol/ml/h over 16 h at 25°C (see also Lukat *et al*., 1992). The RegA RR domain (at a concentration of ~10 μM) increased this rate by only 10% (not shown). No enzymatic breakdown of acetyl phosphate could be detected over its high rate of spontaneous hydrolysis. These results suggest that the RegA RR domain has low intrinsic phosphatase activity, resembling Spo0F, whose phosphorylated form is quite stable (Zapf *et al*., 1996), rather than CheY, whose phosphorylated form is very unstable due to its high intrinsic phosphatase activity (Lukat *et al*., 1992).

Phosphorylation of the RR domain activates the phosphodiesterase domain

Phosphorylation of RRs modulates their activity, e.g. the activities of CheB, NtrC and BvgA are enhanced by phosphorylation (Feng *et al*., 1992; Lukat *et al*., 1992; Sanders *et al*., 1992; Boucher *et al*., 1994). Addition of phosphoramidate, as phosphodonor, to RegA stimulated its PDE activity by up to 8-fold *in vitro*. Activation was dose dependent and saturable (not shown). Half-maximal activation occurred at ~2 mM phosphoramidate and, by extrapolation to saturating phosporamidate concentrations, a maximal stimulation of ~10-fold is expected. Kinetic analysis (Figure 4) showed that the activation of RegA by phosphoramidate is due to an increase in the V_{max} of the phosphodiesterase, with no appreciable change in the K_m for cAMP. Activation was also observed with immunoprecipitated RegA from *Dictyostelium* lystates, though to a lesser extent than with bacterially expressed protein.

Other potential phosphodonors (acetyl phosphate, ATP and phosphoenol pyruvate) had no effect at a concentration of 10 mM, whilst carbamyl phosphate gave a small stimulation (~2-fold; not shown). Acetyl phosphate is probably ineffective due to the low stoichiometry of phosphorylation, as labelling of the RegA RR domain with 10 mM acetyl-[^{32}P]phosphate yielded no more than 4% phosphorylated protein.

Phosphoramidate did not activate the PDE domain expressed alone, nor mutant forms of RegA in which Asp212 of the RR domain was mutated to either asparagine

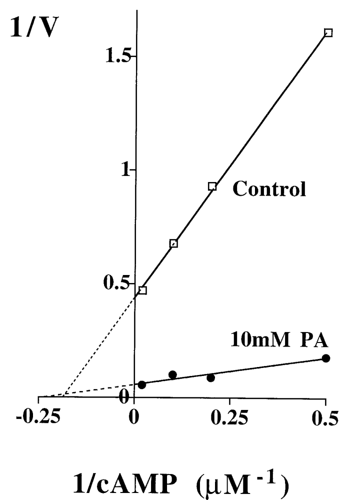


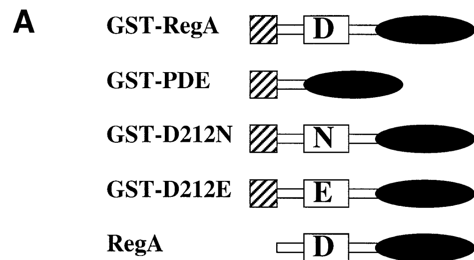
Fig. 4. Phosphoramidate activates RegA by increasing the V_{max}. cAMP phosphodiesterase assays were performed in the absence (Control) or presence (10 mM PA) of 10 mM phosphoramidate, using cAMP concentrations in the range 2–50 μM. The kinetics of the reaction were determined using a Lineweaver–Burk double-reciprocal plot. In the example shown (representative of three experiments), the kinetic parameters are as follows: V_{max} = 2.2 (control) and 17.5 arbitrary units (with phosphoramidate; 8-fold increase); K_m = 5.4 μM (control) and 4.2 μM (with phosphoramidate).

or glutamic acid (Figure 5). This shows that activation is not due to a non-specific effect of phosphoramidate on the PDE domain, but depends on the RR domain and, within it, on the putative phosphoacceptor Asp212.

The flow of phosphate onto RegA determines the activity of the maturation pathway

Preventing the flow of phosphate onto Asp212 of RegA should prevent its activation and, therefore, decrease RegA activity in the cell. To attempt to divert the phosphate flow away from RegA, the RegA RR domain was expressed in wild-type *Dictyostelium* cells using the strong, non-specific actin15 promoter (giving strain HM2045). This caused a similar phenotype to the *regA* null mutant. Aggregation was completed by 6 h in HM2045, compared with 10 h for wild-type Ax2 cells. The terminal structures made by HM2045 were club-shaped (Figure 6), rather similar to *regA* nulls, and caused distinct yellowing of the agar, a common feature of strains which have precocious spore maturation. Indeed, spore production during development of HM2045 was accelerated compared with wild-type cells: HM2045 yielded 50% of its total spores by 14 h of development, whereas Ax2 cells required 19 h to make 50% of its final spore yield, both strains giving very similar spore numbers. Finally, HM2045 formed spores efficiently in monolayers (>10%), unlike the wild-type but similarly to *regA*, *rdeC* and *rdeA* mutant strains. In contrast, expression of the full-length *regA* gene, driven by the actin15 promoter, resulted in slower development than for the wild-type (although fruiting bodies eventually formed).

Rapid development and de-regulated cell maturation in HM2045 indicate that the phosphorelay pathway governing RegA activity is operative during early as well as late development, and thus that RegA is likely to have roles throughout *Dictyostelium* development.



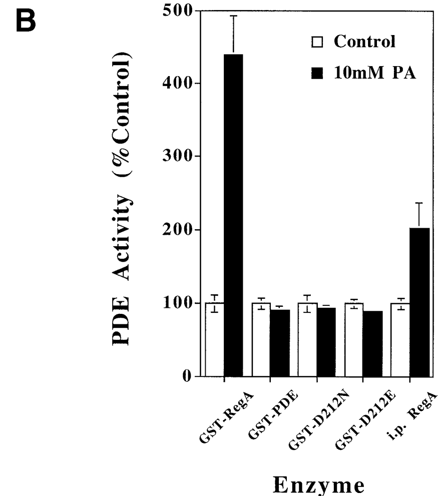


Fig. 5. Activation of RegA cAMP phosphodiesterase activity by phosphoramidate is dependent on the RR domain. (**A**) Outline of proteins used in *in vitro* activation experiments. Hatched squares, GST; white rectangles, RR domain (the wild-type has aspartate, D, at residue 212, mutants have either asparagine, N, or glutamate, E, at this position); black ovals, PDE domain. GST fusions were produced in bacteria; the native RegA protein was immunoprecipitated from *Dictyostelium* cell lysates. (**B**) *In vitro* phosphodiesterase assays were performed without (Control; white bars) or with (10 mM PA; black bars) 10 mM phosphoramidate. Results are expressed as activity measured in the presence of PA relative to that in its absence (basal activity = 100%). Results show the mean values, error bars are the standard error (n = 6).

Discussion

The RegA protein functions at an intersection between the cAMP and two-component signal transduction systems, combining in itself domains characteristic of both. The PDE domain has good homology to mammalian phosphodiesterases (Shaulsky *et al.*, 1996), and we have shown biochemically that it is a cAMP-specific phosphodiesterase (see also Shaulsky *et al.*, 1998). The RR domain has clear homology to other response regulators, and it can accept phosphates from acetyl phosphate, in a reaction typical of RRs, with transfer dependent on the predicted phosphoaccepting residue, Asp212. Our results using phosphoramidate indicate that phosphorylation of the RR domain activates the PDE domain.

In the simplest configuration of a two-component system, phosphates are transferred directly from a histidine kinase to the Asp of the RR (Parkinson and Kofoid, 1992; Swanson *et al.*, 1994). However, in more complex systems such as those controlling initiation of sporulation in *Bacillus subtilis*, and in the yeast osmo-regulatory pathway, phosphates flow through a four-step phosphorelay

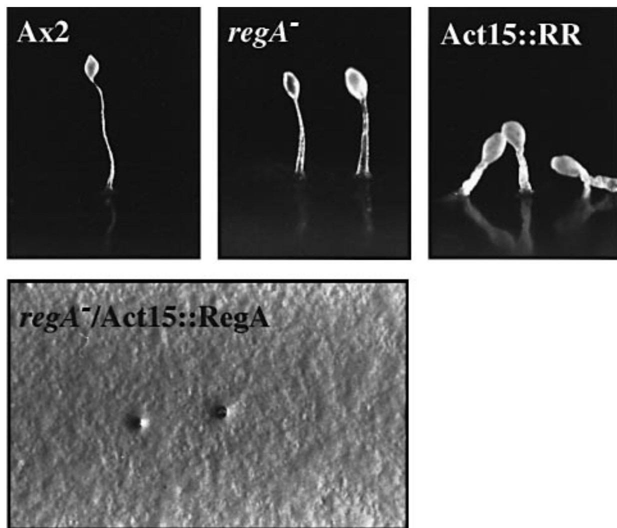


Fig. 6. Developmental morphology of wild-type and mutant strains. Wild-type (Ax2), *regA* null (HM1015), *regA*⁻/actin15::RegA (*regA* null cells transformed with *regA* cDNA driven by the actin15 promoter; HM2042) and Ax2 cells overexpressing the RR domain (actin15::RR; HM2045) were developed on KK2 agar for 26 h. The final morphology of all strains is shown, except for *regA*⁻/actin15::RegA (see text). The *regA* null strain has a greatly thickened lower portion of the stalk, often more severe than in the image shown, such that the structures have a pyramid of mature stalk/spore cells at their base, from which a tapering stalk extends. Likewise, the HM2045 strain can also form these structures.

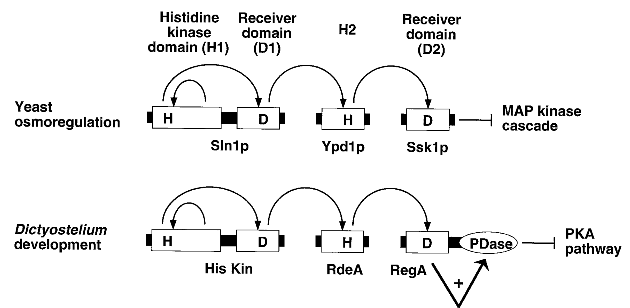


Fig. 7. Proposed phosphorelay pathway controlling RegA activity. The upper scheme is the current model of the yeast osmo-regulatory phosphorelay system (Posas *et al*., 1996), the lower scheme is our proposed phosphorelay pathway for RegA regulation. The flow of phosphate is shown by curved arrows (the first of which is autophosphorylation of the histidine kinase), and occurs by a four-step process. The first component of the pathway in *Dictyostelium* is not known, but is shown as being a hybrid histidine kinase (labelled His Kin); phosphates flow from this to RdeA and then to RegA, where phosphorylation at Asp212 of the RR domain activates the phosphodiesterase domain (this is shown by the crooked arrow). In both schemes, when the phosphorelay pathway is active, the downstream signalling pathways are less active.

(Burbulys *et al*., 1991; Posas *et al*., 1996). In the yeast system, phosphates are relayed from His to Asp on the kinase (Sln1p) and then to a His on an intermediate phosphotransfer protein, Ypd1p, before reaching the RR (Ssk1p).

In the case of *Dictyostelium*, mutants of a second gene, *rdeA*, strongly resemble *regA* mutants phenotypically: fruiting body formation is accelerated and final morphogenesis abherrant (Abe and Yanagisawa, 1983), and both strains are able to form mature stalk and spore cells in our monolayer test. Recent work demonstrates that the *Dictyostelium rdeA* gene in fact encodes a functional homologue of the yeast *Ypd1* gene, as shown most strikingly by the complementation of an *rdeA* mutant by *Ypd1* (Chang *et al*., accompanying paper). *rdeA* mutants are also known to have high cAMP levels (Abe and Yanagisawa, 1983), consistent with reduced RegA activity. It therefore seems likely that RegA is controlled by a phosphorelay system configured in a similar way to the osmo-regulatory pathway of yeast, and that RdeA is the immediate upstream phosphodonor (Figure 7). Thus we propose that stalk and spore cell maturation are both controlled by a common pathway, involving RdeA and RegA, that controls cAMP levels and hence PKA activity.

The cognate upstream histidine kinase(s) and ligand(s) that control RegA activity remain unknown. Since phosphorylation on Asp212 appears to activate the RegA phosphodiesterase, we predict that the flow of phosphates from the upstream kinase should also be activating and, therefore, that kinase-null mutants should resemble *regA* mutants phenotypically. Consistent with this prediction, genetic manipulations expected to interrupt the flow of phosphates to RegA (*rdeA* knock out or RR domain overexpression) cause similar phenotypes to a *regA* null. On these grounds, the suggestion by Loomis and co-workers (Loomis *et al*., 1997; Shaulsky *et al*., 1998) that phosphorylation is inhibitory to RegA and that the upstream kinase is DhkA seem unlikely, especially since *dhkA* null mutants behave as wild-type in monolayers and differ significantly from *regA* in their developmental phenotype: they develop long, fragile stalks and are defective in spore production (Wang *et al*., 1996).

The discovery of the *regA* pathway for controlling cAMP levels also suggests a solution to the problem of why extracellular cAMP alone is insufficient to induce stalk and spore cell maturation in cultured cells, even though it can activate adenylyl cyclase. We propose that only the highest levels of intracellular cAMP (and hence PKA activity) are sufficient to drive spore and stalk maturation and that they are attained only when adenylyl cyclase is active and the RegA phosphodiesterase is inactive (Houslay and Milligan, 1997). Since the activity of these proteins is probably controlled by two different extracellular signals, this condition will only be met where both ligands are present at the appropriate concentrations. In this way, the very precise spatial and temporal control over terminal differentiation can be obtained, which is presumably necessary for morphogenesis of the fruiting body.

Materials and methods

Cell methods

Cells were grown and developed at 22°C (Watts and Ashworth, 1970; Kay, 1987) and REMI mutants isolated in the DH1 background as before (Harwood *et al*., 1995). Monolayer differentiation was as before (Kay, 1987) but used 10 μM cAMP-S (Sigma), and (for stalk assays) 100 nM DIF-1 or 15 mM 8-Br-cAMP, as indicated.

Rc and Rm cell lines were made by introducing the pre-stalk- or pre-spore-specific constructs (Harwood *et al*., 1992a; Hopper *et al*., 1993a) into HM1015 by the standard $CaPO_4$ method (Harwood *et al*., 1992a). Initial transformants were selected at 40–80 μg/ml and maintained at 50 μg/ml G418; stable transformants were maintained at 20 μg/ml G418 (10 μg/ml for 'rescued' strains). HM1015 was made by electroporation of 50 μg of *Hin*dIII–*Sac*II-linearized p*RegA*KO into Ax2 ($1.6{\times}10^7$

cells). Transformants were selected with 20 μg/ml blasticidin S. Null mutants were confirmed by Western and Southern blotting.

Molecular biology

The REMI insert and flanking regions in myc1002 were isolated by plasmid rescue; this failed for HM332 and so inverse PCR was used instead. The PCR product (3′ end of *regA*) was used to probe a λgt11 cDNA library, prepared from cAMP pulse-induced cells (a gift of P.Devreotes). This yielded an 80% full-length cDNA which was used to construct a full-length cDNA in conjunction with a genomic DNA clone covering the missing 5′ end of the gene, by PCR (introducing a *Bss*SI site at nucleotides 487–492; this is a silent mutation).

The *regA* KO vector was made from a 3.5 kb genomic fragment of the *regA* promoter and the 5′ end of the gene (isolated from a genomic minilibrary of *Eco*RI–*Bcl*I-digested Ax2 DNA in pBluescript KS II). 5′ distal sequences were removed using *Hin*dIII, and the blasticidin S deaminase cassette (from pRHI119, obtained from R.H.Insall) inserted, giving p*RegA*KOD3′. A 1.2 kb genomic *Not*I–*Sac*II PCR product from *regA*, covering the 3′ end of the gene to the stop codon, was inserted 3′ to the *bsr* cassette in p*RegA*KOD3′, giving rise to p*RegA*KO, with a 263 amino acid deletion (229–491).

Full-length *regA* cDNA, the RR domain and the PDE domain were expressed as GST fusion proteins in *Escherichia coli*, using pGEX-2T (Pharmacia). The RR and PDE domain constructs encompassed amino acids 127–335 and 385–793, respectively. Soluble GST fusion proteins were purified using glutathione–agarose resin (Fluka). The RR domain construct expressed from the actin15 promoter in *Dictyostelium* cells encompassed RegA amino acids 1–430. All constructs were confirmed by DNA sequencing (ABI377).

Mutagenesis and rescue plasmids

Asp212 of RegA was mutagenized to Asn or Glu using the QuikChange site-directed mutagenesis kit (Stratagene). Actin15::RegA was made by replacing the gfp cDNA from actin15::gfp with the *regA* cDNA from pKSIIΔ*Sal*I:regA (*Bam*HI–*Xho*I fragment). The *regA* promoter rescue construct was made by digesting the *regA* 3.5 kb genomic fragment with *Bgl*II–*Sal*I and inserting the 1.8 kb promoter fragment into pKSIIΔ*Sal*I: regA digested with *Bam*HI–*Sal*I, to give an expression cassette of the *regA* cDNA driven in-frame by its own promoter. This cassette was removed as a 5.0 kb *Xba*I–*Xho*I fragment and used to replace the actin15::gfp cassette. Both rescue vectors conferred resistance to G418.

Immunochemistry

Polyclonal antiserum (R1/2F) was raised in rabbits to the GST–PDE fusion. RegA was detected by Western blotting of cell lysates in 50 mM Tris pH 7.4, 1 mM EDTA, 0.05% Triton X-100, containing protease inhibitors [1 mM benzamidine, 10 μM L64, 10 μg/ml leupeptin, 1 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM TLCK]. Proteins (40 μg/lane) were resolved by 10% SDS–PAGE, electroblotted onto Immobilon P (Millipore), incubated with R1/2F and then with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma), and bands were visualized by ECL (Amersham). R1/2F detects a band of ~110 kDa on a Western blot.

RegA was immunoprecipitated from cell lysates of 1.1×10^8 cells/ml in IP buffer (50 mM $K_1K_2PO_4$ pH 7.3, 1 mM $MgCl_2$ and 10% glycerol plus protease inhibitors as above), pre-cleared by centrifugation. The equivalent of 16 mg (dry weight) of protein A–Sepharose CL-4B (Pharmacia) per 10^8 cells and 30 μl of R1/2F serum were added. After 1 h at 4°C, beads were pelleted and washed six times in eight bed volumes of IP buffer, then assayed for PDE activity. Protein concentrations were determined using the Bio-Rad dye-binding assay.

Phosphodiesterase assays

Assays were in 50 mM Tris–HCl, 50 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol (DTT), pH 8.0 at 25°C, usually containing 0.33 μM (4×10^5 d.p.m.) [^{3}H]cAMP (Amersham), 1–100 μM total cAMP, final volume 20 μl, at 25°C. Reactions were stopped with trichloroacetic acid (TCA) (to 5%), and nucleotides were resolved by TLC on PEI-cellulose plates with a fluorescent indicator (Sigma), using 1 M ammonium acetate pH 7.5/ethanol (30:75 v/v). Nucleotide spots were excised and radioactivity measured by scintillation counting. Using potassium phosphate buffers, the pH optimum of RegA was 8.0, and 5 mM $MgCl_2$ was optimal. Tris buffer was used instead of phosphate buffer for assays to avoid any potential problems when analysing the effects of phosphodonor compounds. Phosphoramidate was pre-incubated with enzyme for 5 min. Phosphoramidate was the kind gift of Dr Ann Stock.

In vitro ^{32}P labelling

Acetyl-[^{32}P]phosphate was synthesized as described in Kornberg *et al*. (1956), but using only 50 μmol of K_2HPO_4 as substrate. Five μg of fusion protein was used per labelling reaction (10 μl), containing 10 mM $MgCl_2$ (Lukat *et al*., 1992) for 10 min at 25°C. SDS–PAGE and electroblotting were done at 4°C to minimize loss of label from proteins.

Accession number

The DDBJ/EMBL/GenBank accession number for the *regA* locus, including 1.8 kb of promoter sequence, is AJ005398.

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