The RdeA-RegA System, a Eukaryotic Phospho-relay Controlling cAMP Breakdown*

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The regA and rdeA gene products of Dictyostelium are involved in the regulation of cAMP signaling. The response regulator, RegA, is composed of an N-terminal receiver domain linked to a C-terminal cAMP-phosphodiesterase domain. RdeA may be a phospho-transfer protein that supplies phosphates to RegA. We show genetically that phospho-RegA is the activated form of the enzyme in vivo, in that the predicted site of aspartate phosphorylation is required for full activity. We show biochemically that RdeA and RegA communicate, as evidenced by phospho-transfer between the two proteins in vitro. Phospho-transfer is dependent on the presumed phospho-accepting amino acids, histidine 65 of RdeA and aspartate 212 of RegA, and occurs in both directions. Phosphorylation of RegA by a heterologous phospho-donor protein activates RegA phosphodiesterase activity at least 20-fold. Our results suggest that the histidine phosphotransfer protein, RdeA, and the response regulator, RegA, constitute two essential elements in a eukaryotic His-Asp phospho-relay network that regulates Dictyostelium development and fruiting body maturation.

Eukaryotic His-Asp phospho-relay networks are thought to consist of a signal-regulated histidine protein kinase (HPK) containing an ATP-binding catalytic core, a site of histidine phosphorylation (the H-domain), and a receiver domain containing a site of aspartate phosphorylation (1). Downstream of the HPK is a cognate response regulator protein that also possesses a receiver domain, phosphorylation of which modulates the activity of the response regulator. Communication between the two receiver domains occurs via a histidine phosphotransfer protein (HPT), which acts as a histidine-phosphorylated intermediary allowing phosphate to be shuttled between the conserved aspartate residues of the two receiver domains. In bacteria, the HPT moiety can be fused to the C terminus of a hybrid HPK, as with ArcB or BvgS for example, or be a separate protein, such as LuxU (1, 2). In eukaryotes, the only characterized HPT protein is Ypd1 from Saccharomyces cerevisiae, which like LuxU, is a separate protein (2, 3).

The osmo-sensing pathway of S. cerevisiae is the best understood of the two eukaryotic phospho-relays so far characterized (they are both present in yeast and share components) (3). It consists of the histidine protein kinase Sln1, the histidine phosphotransfer protein Ypd1, and the response regulator Ssk1. The catalytic domain of Sln1 is thought to trans-phosphorylate the H-domain of another Sln1 monomer within an HPK dimer complex. The Sln1 receiver domain acquires the phosphate at a conserved aspartate residue and relays it to the Ypd1 H-domain. Lastly, the receiver domain of Ssk1 dephosphorylates Ypd1, transferring the phosphate onto itself. Genetic analyses have shown that the unphosphorylated form of Ssk1 is the active species (4).

The Ssk1 response regulator controls activity of the HOG mitogen-activated protein kinase pathway in S. cerevisiae, by acting as a direct regulator of the Ssk2 mitogen-activated protein kinase kinase kinase (3, 4). Only two other eukaryotic response regulators have so far been studied: Skn7, a yeast transcription factor (5, 6), and the Dictyostelium protein RegA, a cAMP phosphodiesterase (PDE) (7–9).

The cAMP/protein kinase A (PKA) pathway is central to regulating Dictyostelium development (10–16). On starvation, individual cells begin to secrete pulses of cAMP, which acts as a ligand for a family of G-protein-coupled receptors. Receptor activation leads to the production of second messengers including cGMP and cAMP (17). A large proportion of the cAMP is probably exported from the cell to continue intercellular signaling; levels of external cAMP are regulated by the secreted phosphodiesterase, PdsA (18).

cAMP also acts inside the cell to control the activity of PKA (19). Recently an intracellular cAMP PDE has been discovered (8, 9). This enzyme, RegA, serves a critical function in controlling the rate of Dictyostelium development, because a regA null mutant develops rapidly and produces spores prematurely, similar to the rdeC mutants (which lack functional PKA R-subunit) (7–9). A third class of mutant, rdeA, also shares this phenotype (9, 20–22). The RegA phosphodiesterase probably acts to restrain the activity of PKA during development, because dominant negative mutations of PKA are epistatic to a regA mutation (8, 9).

rdeA mutants have elevated cAMP levels, as do regA mutants, consistent with RegA phosphodiesterase activity being lowered in rdeA mutants (20, 23). A biochemical connection between RdeA, RegA, and the adenyllyl cyclase ACB has recently been suggested on the basis of genetic studies and the use of a PDE inhibitor (23). The rdeA gene encodes a 254-amino acid protein with little overall sequence similarity to any other protein. However, RdeA does show homology to histidine phosphotransfer proteins in a 20-amino acid region in its N-terminal half. This region includes a predicted site of histidine phosphorylation, His65, a residue essential for RdeA function in vivo (21). The S. cerevisiae YPD1 gene, which encodes a known HPT protein, complements a Dictyostelium rdeA mutant (21).

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† The abbreviations used are: HPK, histidine protein kinase; PKA, cAMP-dependent protein kinase; PDE, phosphodiesterase; WT, wild type; GST, glutathione-S-transferase; HPT, histidine phosphotransfer protein; PAGE, polyacrylamide gel electrophoresis.

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The RdeA-RegA System, a Eukaryotic Phospho-relay Controlling cAMP Breakdown*

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The N-terminal receiver domain of RegA has been shown to control its PDE activity in vitro: phosphorylation of aspartate 212 in the receiver domain activates the PDE domain. It is not known, however, whether RegA activity is controlled by the receiver domain in the cell, and if so, in what manner. We present results showing that RegA phosphorylated on its receiver domain in vivo is in an activated state. In light of the suggestion that RdeA and RegA function on the same pathway to control cAMP levels, we investigated whether RdeA is an upstream activator of RegA. We present phospho-transfer evidence that this is indeed the case. Furthermore, we show that RegA can be activated by phospho-transfer from an H-domain protein in vitro.

EXPERIMENTAL PROCEDURES

Cell Methods—Cells were grown and developed at 22 °C (24). Expression plasmids were introduced into cells by electroporation using a Bio-Rad Gene Pulser (0.9 kV, 3 microfarad). Transformants were selected at 20 μg/ml G418 and stably maintained at 5 μg/ml G418 in axenic culture.

Cells were developed on K2K-agar (20 mM K2HPO4, 2 mM MgSO4, 1.8% Oxoid L28 agar) at a density of 1.6 × 107 cells/ml. For spore counts, developing structures were disrupted by syringing in KK2 buffer plus 0.3% cemulsol. Detergent-resistant spores were scored by microscopy and plated out for viability after washing.

Molecular Biology—Restriction enzymes and T4 DNA ligase were from New England Biolabs. A rescue plasmid carrying the regA gene positioned in-frame downstream of its own promoter has been described (9). The D212N mutant regA cDNA was used to replace the wild type cDNA in this construct (using BamHI-XhoI) to give plasmid pPT17. These constructs were used to "rescue" the regA null strain HM1015, giving strains HM2040 (with wild type cDNA) and HM2049 (with D212N cDNA), respectively.

The receiver domain of RegA (the region covering amino acids 126–316 was used) was amplified from either the wild type or D212N mutant cDNA by polymerase chain reaction, using oligonucleotides containing 5′ primer XhoI and 3′ primer EcoRI. This DNA was cloned into an actin15 expression vector possessing an in-frame McyC tag at the 3′ end of the insert to give plasmids pPT46 (WT) and pPT43 (D212N). The McyC tag encoded three copies of the amino acid sequence: EQKLISEEDLG. The plasmids were transformed into Ax2 cells to give strains HM2046 (with WT receiver) and HM2047 (with D212N receiver), respectively.

The rdeA cDNA was amplified by polymerase chain reaction and cloned into the bacterial expression vector pGEX-5X1 (Amersham Pharmacia Biotech) at the BamHI and XhoI sites (giving plasmid pPTT5). GST fusion proteins were prepared from isopropyl-1-thio-galactopyranoside-induced Escherichia coli BL21 by standard methods. The purity of the final proteins was about 75%, estimated by Coomassie staining.

The rdeA cDNA was mutagenized to create H63Q and H65Q mutants using the Quikchange™ method (Stratagene). The mutagenized rdeA inserts were transferred to pGEX-5X1 for bacterial expression (plasmids pPT39 (H63Q) and pPT40 (H65Q)). All constructs were confirmed free of errors by DNA sequencing (ABI377).

In Vitro Radiolabeling of Proteins—Radiochemicals were from Amersham Pharmacia Biotech. GST fusion proteins were used at 10 μM equivalent. Labeling with acetyl-[32P]phosphate was as before (9); reactions were performed for 30 min. Proteins were separated on SDS-polyacrylamide gel electrophoresis (PAGE) (performed at 4 °C) and then transferred to Immobilon P membrane (Millipore) by electroblotting (at 4 °C). Membrane was exposed to a PhosphorImager screen and then stained with Coomassie Blue to localize proteins.

Phosphorylation of RdeA or CheA H-domain by CheA catalytic domain was done at pH 8.0 in 50 mM Tris-Cl, 50 mM KCl, 5 mM MgCl2, 10% glycerol, containing 10 μM ATP (for RdeA) or 0.2 mM ATP (for CheA H-domain) and γ-[32P]ATP, for 2 h at 25 °C, in a volume of 50 μl. Free ATP was removed by spin column gel filtration using Sepharose G50. For phospho-transfer reactions, all proteins were used at 10 μM.

Activation of RegA by Phospho-H-domain—Preparation of the catalytic and H-domains of CheA has been described (25). H-domain (1–200 μm) was phosphorylated by CheA catalytic domain as above but without radiolabeled ATP. RegA (WT or D212N mutant) and cAMP substrate was added to the prephosphorylated H-domain, and phosphodiesterase activity was determined in a standard assay (9). The amount of phospho-H present at the start of the PDE assay was assessed by performing parallel phosphorylation reactions containing [γ-32P]ATP and measuring the incorporation of radiolabel into H-domain (correction was made for purity of the labeled ATP). For PDE activity measurements, initial rates were used.

For determination of specific activities of RegA proteins, PDE assays were performed with 200 μM cAMP as substrate (Km = 5 μM approximately). PDE assays were performed as described (9); briefly, reactions were performed in 50 mM Tris-Cl, pH 8.0, 50 mM KCl, 5 mM MgCl2, 10% glycerol, at 25 °C for 30 min. Protein amounts were assessed by SDS-PAGE followed by gel densitometry, against a bovine serum albumin standard curve.

Western Blotting—Polyclonal anti-RegA antiserum (R1/2F) was the primary antibody, used at 1:5,000 final dilution. The secondary antibody was goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad), used at 1:12,500 dilution. Detection was by enhanced chemiluminescence (Amersham Pharmacia Biotech). Bands were quantitated by densitometry (Molecular Dynamics).

RESULTS

Requirement of Asp212 for RegA Activation in Vivo—RegA phosphodiesterase activity is stimulated by phosphorylation in vitro. A mutant version of RegA in which the predicted site of phosphorylation in the receiver domain, Asp212, has been replaced by asparagine is neither phosphorylated nor activated (9). To determine whether RegA activity in living cells is similarly regulated through aspartate phosphorylation, we compared the effects of expressing wild type and D212N mutant RegA in the regA null strain HM1015. Transformed cell lines were grown in axenic culture and plated on non-nutrient agar to initiate development. The rate of development of these various strains was followed by the appearance of detergent-resistant spores. In the wild type, these appear at 21–24 h, but in the regA null mutant this is brought forward to about 14 h.

When wild type regA is expressed in a regA null background under the control of its own promoter (Prom::WT), it restores normal timing of development (Fig. 1A). In contrast, expression of the D212N mutant version of RegA (Prom::DN) only slightly delays spore production (by 1–2 h) relative to the regA null mutant (Fig. 1A). Essentially similar results are obtained when RegA expression is driven from the strong actin15 promoter (A15::DN and A15::WT, respectively). For A15::DN, spores are produced at about 20–22 h of development, whereas in A15::WT spore production does not occur until 42–48 h of development (results not shown).

In these experiments, the expression levels of the wild type and mutant forms of RegA are an important factor. Fig. 1B shows that when expressed from the RegA promoter, D212N and WT RegA have similar, though not precisely the same, expression levels, as monitored by Western blotting and densitometry. RegA expression appears to be biphasic in these strains, declining from high, steady levels to a lower level prior to culmination and then increasing again as maturation continues (Fig. 1B). In the Prom::D212N strain, the phase of this expression is shifted forward by about 5 h relative to the Prom::WT strain, possibly as a consequence of the rapid development of the Prom::D212N strain. The ability of WT RegA to delay development more strongly than D212N RegA cannot, therefore, be due simply to expression differences between the two versions of the enzyme.

The level of RegA expressed in these two strains is approximately 5-fold higher than that of endogenous RegA present in wild type Ax2 cells. When RegA expression is driven from the strong actin15 promoter, however, RegA levels reach about 25-fold those present in wild type cells (data not shown), i.e., a further increase of 5-fold. Thus, the finding that the A15::DN strain has very similar timing of spore production to the Prom::WT strain supports further the hypothesis that RegA D212N is less active than RegA WT in vivo. On Western blots, RegA appears as a doublet consisting of a strong upper band
Promoter::D212N regA

Promoter::WT regA

WT

DN

FIG. 1. Rescue of regA null mutant by expression of wild type or D212N regA. A, the regA null strain (HM1015, n) was transformed with a plasmid encoding wild type or D212N RegA cDNA downstream of the RegA promoter, creating strains HM2040 (Prom::WT, □) and HM2049 (Prom::D212N, □), respectively. The ability of the expressed RegA to inhibit the rapid development of the regA null was determined by monitoring the time course of spore production throughout development. Results are expressed as spores formed (as the percentage of input cells) against time of development and are representative of four similar experiments. B, quantitation of RegA protein in regA rescue strains. Soluble cell extracts (20 μg) were prepared from HM2040 and HM2049 throughout development and probed with RegA antisera to monitor levels of expressed RegA protein (indicated by the arrowheads). Extracts from HM2040 are labeled as WT, and extracts from HM2049 are labeled as DN.

and a more minor lower band, in roughly constant proportions. Neither band occurs in a regA null strain (9). This pattern may represent a modification such as proteolytic processing or phosphorylation. Because both bands are also present in the D212N RegA-expressing strains (e.g., Fig. 1B), this cannot be due to Asp212 phosphorylation.

The finding that D212N RegA causes some rescue of the regA null phenotype indicates that RegA PDE activity is not entirely dependent on Asp212 phosphorylation. To confirm this finding in vitro, the specific activities of WT and D212N RegA were determined. Both forms of the enzyme have PDE activity in vitro, and the specific activity of each (in the absence of phosphorylation) is approximately 0.08 μmol/min/mg protein (at 25 °C).

**Dominant Negative Action of Free Receiver Domain Requires Asp212**—When RegA receiver domain is overexpressed in wild type Ax2 cells, it acts as a dominant negative (9). To determine whether the Asp212 predicted site of phosphorylation is required for this effect, Myc-tagged versions of the receiver domain were expressed in Ax2 cells under control of the actin15 promoter. Expression of the receiver domains was confirmed by Western blotting for the C-terminal Myc epitope (not shown). The wild type receiver domain (strain HM2046) behaved as a dominant negative as expected; development in this strain was rapid and spore production precocious (results not shown), similar to previous findings (9). Expression of the D212N receiver domain (strain HM2047) had no effect on the development of Ax2 (not shown), showing that Asp212 is essential for the dominant negative action.

**Reverse Phospho-transfer from RegA to RdeA**—The above results indicate that RegA functions as a response regulator whose activity in the cell is controlled by phosphorylation. The immediate upstream phosphate donor for the RegA receiver domain is likely to be a Hpt protein (1). Genetic evidence suggests that the RdeA protein may serve this function (see the Introduction).

To investigate whether there is communication between RdeA and RegA, in vitro phospho-transfer between the two proteins was investigated. For this, the ability of some receiver domains to specifically autophosphorylate using the artificial phospho-donor acetyl phosphate was exploited (26). Bacterially expressed (GST-fused) RegA receiver domain was incubated with acetyl [32P]phosphate, resulting in phosphorylation (Fig. 2, lane 1). Phosphorylation required Asp212, because the D212N mutant protein was not phosphorylated (lane 2; as reported previously (9)), nor were three versions of (GST-) RdeA (wild type, H63Q control mutant, and H65Q mutant, lanes 3–5). Addition of wild type or H63Q RdeA to acetyl [32P]phosphate, together with wild type receiver domain, resulted in the appearance of [32P] label on RdeA (lanes 6–7). RdeA phosphorylation was dependent on residue His65 of RdeA, because a H65Q mutant version did not become labeled under these conditions (lane 8). Labeling of wild type RdeA required the presence of wild type receiver domain, because the D212N version did not support phosphorylation of RdeA from acetyl phosphate (lane 9).

Phospho-transfer to RdeA also occurred when purified phospho-receiver domain (which had been separated away from acetyl phosphate by gel filtration) was added to RdeA (data not shown). These results show that RdeA can be phosphorylated on His65 by a phospho-transfer mechanism dependent on Asp212 of the RegA receiver domain. In the proposed linear phospho-relay scheme, this represents reverse phospho-relay. Similar in vitro reverse transfer is seen between the Spo0B and Spo0F proteins of the *Bacillus subtilis* sporation phospho-relay (27), and between the ArcA and ArcB proteins of the *E. coli* anoxic redox control phospho-relay (28).
RegA receiver domain was added to \([^{32}\text{P}]\text{RdeA}\), label was transferred to the wild type but not D212N receiver domain (Fig. 3B); in the presence of D212N receiver domain, phospho-RdeA was stable over the time course of the experiment (estimated \(t_{1/2} > 6\) h). Wild type receiver domain rapidly removed the \(^{32}\text{P}\) label from phospho-RdeA, but labeling of the receiver itself was transient. This is likely due in part to the instability of aspartyl-phosphate but also to intrinsic phosphatase activity of the receiver. The half-life of phospho-RdeA in the presence of 10 \(\mu\)M WT receiver domain was approximately 5 min.

**Activation of RegA by Phospho-transfer from an H-domain**—Because phospho-transfer from a cognate H-domain protein to RegA receiver domain is predicted to activate the phosphodiesterase, the ability of phospho-RdeA to activate RegA in vitro was investigated. However, activation could not be demonstrated using CheA catalytic domain to phosphorylate RdeA, because of the inefficiency of this reaction. Nevertheless, the principle could be demonstrated using a heterologous phosphodonor protein. For this, the H-domain (i.e., the P1 domain) of CheA, which is the normal substrate of the CheA catalytic domain, was used as phospho-donor for RegA.

Phospho-H-domain, phosphorylated using CheA catalytic domain and \([\gamma^{32}\text{P}]\text{ATP}\) (as for RdeA above), was rapidly dephosphorylated by RegA receiver domain (\(t_{1/2}\) of phospho-H-domain was \(-3\) min in the presence of 10 \(\mu\)M receiver domain) (results not shown), which concomitantly acquired this phosphate. As with transfer from RdeA to the RegA receiver domain, phospho-transfer from CheA H-domain required Asp\(^{212}\) of the RegA receiver domain (not shown). To investigate the activation of RegA, a coupled assay was employed. A standard phosphodiesterase reaction was supplemented with (unphosphorylated) CheA H-domain and ATP. A PDE assay was initiated by addition of RegA (either wild type or D212N), and the rate of cAMP hydrolysis was monitored under these conditions, prior to the addition of CheA catalytic domain. CheA catalytic domain was then added, and its effect on PDE activity was determined. Fig. 4A shows that CheA catalytic domain stimulated PDE activity only in the reaction containing wild type RegA together with CheA H-domain and ATP. All other reactions, lacking any one of these components or containing D212N RegA instead of wild type RegA, showed no stimulation. Thus, there was no direct effect of CheA catalytic domain on RegA activity, but rather activation was via phospho-transfer from phosphorylated H-domain (Fig. 4B). Furthermore, because D212N RegA showed no response, activation required Asp\(^{212}\).

To analyze the dose-response relationship of RegA activation by phospho-H-domain, a modified version of the coupled assay was used. Fig. 4A shows that there is a lag phase after addition of CheA catalytic domain before RegA is significantly activated. This represents the period during which phospho-H-domain must accumulate to sufficient concentrations to activate RegA. To avoid this lag phase, unphosphorylated CheA H-domain (at various concentrations) was preincubated with CheA catalytic domain and ATP. The amount of phospho-H-domain produced was determined from identical parallel reactions that also contained \([\gamma^{32}\text{P}]\text{ATP}\). RegA was added directly to the above reactions, containing known amounts of phospho-H-domain, and PDE activity was determined. Fig. 5 shows the dose-response curve for RegA activation by phospho-H-domain; activation required a threshold phospho-H-concentration of about 1 \(\mu\)M, and maximal activation occurred at about 50 \(\mu\)M. At saturation,
RegA was activated at least 20-fold by phospho-transfer from CheA H-domain.

**DISCUSSION**

**The RdeA-RegA Phospho-relay System—**Previous in vitro studies suggested that the phosphorylated form of RegA is the more active species. We have shown that this is also the case in vivo, in that a nonphosphorylatable version of RegA fails to restore wild type development to a regA null Dictyostelium strain and therefore is much less active than wild type RegA in the cell.

A possible connection between RdeA and RegA was suggested on genetic grounds (9, 21). The phospho-transfer experiments presented under “Results” provide direct evidence for biochemical communication between RdeA and RegA. Importantly, all of the phospho-transfer activities between RdeA and RegA require both His65 of RdeA and Asp212 of RegA; both of these are essential residues in vivo.

It seems very likely that RdeA functions upstream of RegA on a phospho-relay pathway to control cAMP levels. The proposed phospho-relay pathway is shown in Fig. 6 (after Refs. 9 and 21). As outlined above, rdeA and regA null mutants share many characteristics, both having rapid development, premature spore maturation, and a common biochemical defect, i.e. elevated cAMP. However, the rdeA null phenotype appears more severe, suggesting that RdeA may supply phosphate to other response regulators. The loss of regulation of such putative response regulators in an rdeA mutant could contribute to the severity of the rdeA null phenotype.

In devising a method for RdeA phosphorylation, we showed that RdeA behaves as an H-domain protein in vitro, in that it acts as a substrate for CheA catalytic domain. This supports previous in vivo evidence showing that an rdeA null mutant can be complemented by the yeast H-domain-encoding gene, YPD1 (21). Interestingly, a different method has recently been used to phosphorylate Ypd1 itself in vitro, that of phospho-transfer from phospho-CheY (29). CheY is a cognate response regulator (i.e. downstream target) of CheA; phosphorylation of Ypd1 by CheA catalytic domain has not been tested, but our results with RdeA suggest that this method would also work. Thus, the known eukaryotic H-domain protein, Ypd1 and the proposed H-domain protein RdeA will directly couple to proteins of the bacterial CheA-CheY system in vitro. It may be possible to use the CheA-CheY network to help study further eukaryotic response regulator and H-domain proteins when they are discovered. Phosphorylated CheA H-domain, for example, may activate other response regulators as it does RegA.

**Control of RegA Activity—**As with all enzymes whose activity is regulated by phosphorylation, RegA activity in vivo must reflect the relative levels of kinase and phosphatase activities directed toward RegA. One factor regulating phospho-RegA levels may be intrinsic aspartate phosphatase activity, as mentioned above, but there are likely other factors too. Bacteria possess specific aspartate phosphatases (e.g. RapA/B and SpoOE in B. subtilis (30) and phosphatase-activating proteins (e.g. CheZ) (31). However, little is known about the dephosphorylation of aspartyl-phosphate in eukaryotes and homologs of the above genes have not been found so far.

Because we have shown that phosphate can be transferred either forward, from RdeA to RegA, or backward, from RegA to RdeA, depending on the relative concentrations of the phosphodonor proteins, reverse phospho-relay could contribute to control of RegA activity. Such a mechanism is thought to occur in the regulation of the ArcB-ArcA system in E. coli (28), under conditions where the receiver domain of the hybrid kinase ArcB exhibits aspartate phosphatase activity. The balance of opposing kinase/phosphatase activities of HPK enzymes represents an important regulatory area in phospho-relay networks.

**Integration of the RdeA-RegA Phospho-relay—**RegA controls many aspects of Dictyostelium development. regA null mutants begin development earlier, aggregate faster, do not undertake developmental arrest at the slug stage, and display precocious terminal differentiation compared with wild type strains. Potentially, RegA activity is controlled at all of these stages.

The kinases that input phosphate to the RegA pathway (or extract phosphate by reverse phospho-relay) are not yet defined. Several genes encoding putative hybrid histidine kinases have been cloned from Dictyostelium (32–35) and further candidates also exist (36). Mutations in genes encoding upstream kinases that activate RegA should resemble the regA null. None of the mutants in the four characterized genes have this phenotype, but this may be due to some degree of functional overlap. However, because individual null mutants in these four genes all have distinct developmental phenotypes, there must be significant genetic specificity among the kinases.

Of the four putative histidine kinases, DhkC seems to be the clearest candidate activator of the RegA pathway, because the dhkC null strain shows aspects of rapid development (35). For DhkA and DhkB, genetic evidence suggests that they may be inhibitors of the RegA pathway (33, 34). One way in which they could do this is by regulating the stability of phospho-RegA, perhaps by a mechanism such as that discussed above. Finally, DokA may also have a role in cAMP signaling during late development, because a dokA null mutant fails to make mature.

**FIG. 5. Dose-response curve of RegA activation by phospho-H-domain.** GST-RegA incubated in a coupled assay with the indicated concentrations of phospho-H-domain was assayed for phosphodiesterase activity. The concentration of phospho-H shown is the calculated initial concentration. Changes in phospho-H concentration during the assay were not determined; however, reactions were linear with time over the whole course of the assay, and all rates of hydrolysis shown are initial rates. Results shown are from one experiment representative of three similar experiments. Errors were typically <5% (error bars not shown).

**FIG. 6. Model of proposed RdeA-RegA phospho-relay pathway.** The model as previously proposed (9, 21) is shown with the newly established biochemical connection between RdeA and RegA indicated as a bi-directional phospho-transfer. For simplicity, phosphorylation of the HPK H-domain by the HPK catalytic domain is not shown. Connections between RdeA and upstream HPKs remain undefined and are shown by dashed arrows. The H-domain protein RdeA is a point of integration in the phospho-relay network, allowing communication between the two receiver domains. It may also communicate with other receiver domains on response regulators that have yet to be discovered.

![Diagram of RdeA-RegA Phospho-relay](image-url)
spores during culmination (32) and thus may have defects in PKA activation.

Multiple histidine kinases in Dictyostelium, with the potential to act as receptors, may control RegA (and thus cAMP) signaling in response to multiple extracellular ligands, many of which may remain to be discovered. Candidate ligands so far include ammonia, the proposed ligand for DhkC (35), the spore germination inhibitor discadenine (a proposed ligand for DhkB (34)), and the spore differentiation factors. Spore differentiation factor-2 has been proposed to signal via DhkA to inhibit the RegA pathway (37). Whichever ligands and receptors do contribute to RegA control, their inputs must be integrated to produce an appropriate output, in terms of PKA activity, to co-ordinate development.

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REFERENCES
1. Appleby, J. L., Parkinson, J. S., and Bourret, R. B. (1996) Cell 86, 845–848
2. Freeman, J. A., and Bassler, B. L. (1999) J. Bacteriol. 181, 899–906
3. Posas, F., Wurgler-Murphy, S. M., Maeda, T., Witten, E. A., Thai, T. C., and Saite, H. (1996) Cell 86, 865–875
4. Posas, F., and Saite, H. (1996) EMBO J. 15, 1385–1394
5. Brown, J. L., Bussey, H., and Stewart, R. C. (1994) EMBO J. 13, 5186–5194
6. Li, S., Ault, A., Malone, C. L., Raitt, D., Dean, S., Johnston, L. H., Deschenes, R. J., and Fassler, J. S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 718–722
7. Shaulsky, G., Escalante, R., and Loomis, W. F. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15260–15265
8. Shaulsky, G., Fuller, D., and Loomis, W. F. (1998) Development 125, 691–699
9. Posas, F., Wurgler-Murphy, S. M., and Maeda, T. (1998) EMBO J. 17, 6952–6962
10. Shaulsky, G., Escalante, R., and Loomis, W. F. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15260–15265
11. Mann, S. K. O., and Firtel, R. A. (1993) Development 119, 135–146
12. Mann, S. K. O., Robertson, D. L., Lee, S., Kimmel, A. R., and Firtel, R. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10561–10565
13. Harwood, A. J., Hopper, N. A., Simon, M. N., Bouzid, S., Veron, M., and Williams, J. G. (1992) Dev. Biol. 149, 90–99
14. Harwood, A. J., Hopper, N. A., Simon, M. N., Driscoll, D. M., Veron, M., and Williams, J. G. (1992) Cell 69, 615–624
15. Hopper, N. A., Harwood, A. J., Bouzid, S., Veron, M., and Williams, J. G. (1993) EMBO J. 12, 2459–2466
16. Hopper, N. A., Anjard, C., Reynolds, C. D., and Williams, J. G. (1993) Development 119, 147–154
17. Parent, C. A., and Devreotes, P. N. (1996) Dev. Biol. 179, 200–210
18. Chang, W. T., Thomason, P. A., Gross, J. D., and Newell, P. C. (1998) EMBO J. 17, 2809–2816
19. Firtel, R. A. (1996) Curr. Opin. Genet. Dev. 6, 545–54
20. Abe, K., and Yanagisawa, K. (1983) Dev. Biol. 95, 200–210
21. Chang, W. T., Thomason, P. A., Gross, J. D., and Newell, P. C. (1998) EMBO J. 17, 2809–2816
22. Kessin, R. (1977) Cell 10, 703–708
23. Kim, H.-J., Chang, W.-T., Meima, M., Gross, J. D., and Schaap, P. (1998) J. Biol. Chem. 273, 30859–30862
24. Watts, D. J., and Ashworth, J. M. (1970) Biochem. J. 119, 171–174
25. Levit, M., Liu, Y., Surette, M., and Stock, J. (1996) J. Biol. Chem. 271, 32057–32063
26. Lukat, G. S., McMeen, W. R., Stock, A. M., and Stock, J. B. (1992) Proc. Natl. Acad. Sci. U. S. A. 90, 718–722
27. Burbulys, D., Trach, K. A., and Hoch, J. A. (1991) Cell 64, 545–552
28. Georganides, D., Kwon, O., De Wulf, P., and Lin, E. C. C. (1998) J. Biol. Chem. 273, 32864–32869
29. Janiak-Spens, F., Sparling, J. M., Gurfinkel, M., and West, A. H. (1999) J. Biol. Chem. 273, 411–417
30. Perego, M., and Hoch, J. A. (1996) Trends Genet. 12, 97–101
31. Bourret, R. B., Borkovich, K. A., and Simon, M. I. (1991) Annu. Rev. Biochem. 60, 401–441
32. Bussey, H., and Stewart, R. C. (1994) EMBO J. 13, 5186–5194
33. Li, S., Ault, A., Malone, C. L., Raitt, D., Dean, S., Johnston, L. H., Deschenes, R. J., and Fassler, J. S. (1998) EMBO J. 17, 6952–6962
34. Wang, N., Shaulsky, G., Escalante, R., and Loomis, W. F. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15260–15265
35. Shaulsky, G., Fuller, D., and Loomis, W. F. (1998) Development 125, 691–699
36. Thomason, P. A., Traynor, D., Cavet, G., Chang, W.-T., Harwood, A. J., and Kay, R. R. (1998) EMBO J. 17, 2838–2845
37. Parent, C. A., and Devreotes, P. N. (1996) Dev. Biol. 179, 200–210
38. Posas, F., and Maeda, T. (1998) Dev. Biol. 208, 200–210
39. Shakhar, G., and Hoch, J. A. (1998) J. Biol. Chem. 273, 32864–32869
40. Shaulsky, G., Escalante, R., and Loomis, W. F. (1996) EMBO J. 15, 3880–3889
41. Wang, N., Shaulsky, G., Escalante, R., and Loomis, W. F. (1996) EMBO J. 15, 3880–3889
42. Shaulsky, G., Escalante, R., and Loomis, W. F. (1996) EMBO J. 15, 3880–3889
43. Zinda, M. J., and Singleton, C. K. (1998) Dev. Biol. 196, 171–83
44. Singleton, C. K., Zinda, M. J., Mykytka, B., and Yang, P. (1998) Dev. Biol. 205, 345–357
45. Zinda, M. J., and Singleton, C. K. (1998) Dev. Biol. 196, 171–83
46. Thomason, P., Traynor, D., and Kay, R. (1999) Trends Genet. 15, 15–19
47. Anjard, C., Zeng, C., Loomis, W. F., and Nellen, W. (1998) Dev. Biol. 193, 146–155