Evidence that the RdeA protein is a component of a multistep phosphorelay modulating rate of development in *Dictyostelium*

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We have isolated an insertional mutant of *Dictyostelium discoideum* that aggregated rapidly and formed spores and stalk cells within 14 h of development instead of the normal 24 h. We have shown by parasexual genetics that the insertion is in the *rdeA* locus and have cloned the gene. It encodes a predicted 28 kDa protein (RdeA) that is enriched in charged residues and is very hydrophilic. Constructs with the DNA for the c-Myc epitope or for the green fluorescent protein indicate that RdeA is not compartmentalized. RdeA displays homology around a histidine residue at amino acid 65 with members of the H2 module family of phosphotransferases that participate in multistep phosphoryl relays. Replacement of this histidine rendered the protein inactive. The mutant is complemented by transformation with the *Ypd1* gene of *Saccharomyces cerevisiae*, itself an H2 module protein. We propose that RdeA is part of a multistep phosphorelay system that modulates the rate of development.

Keywords: cAMP-dependent protein kinase (PKA)/H2 module protein/multistep phosphorelay/rapidly developing *rdeA* mutant/two-component system

Introduction

Two-component signaling systems are signal transduction mechanisms that are common in bacteria and have also been reported in several eukaryotic organisms (Parkinson and Kofoed, 1992; Alex and Simon, 1994). The prototypical system consists of two components, a sensor histidine kinase and a response regulator. In response to an appropriate environmental cue, the sensor kinase of such a system autophosphorylates on a histidine residue in its 'transmitter domain' and the phosphate is then transferred to an aspartate residue on the 'receiver domain' of the response regulator, causing a change in activity of the latter. Both transmitter and receiver generally are conserved among members of the class.

More elaborate signalling systems have been discovered in which a phosphoryl group is transferred through four successive steps, the so-called H1–D1–H2–D2 phosphorelay (Appleby *et al.*, 1996; Wurgler-Murphy and Saito, 1997). Signal transduction in such systems begins, as before, with phosphorylation of the transmitter domain on a sensor kinase and ends with phosphorylation of the

receiver domain of a response regulator; between the two are interposed the phosphorylation of an additional aspartate receiver domain followed by phosphotransfer to a histidine residue on a third module (the H2 module). The four steps of such phosphorelays can involve anywhere from two to four separate proteins.

The majority of H2 modules that have been characterized display weak similarity around the centre of the phosphorylated histidine residue (see Figure 1B) and show no homology to the bulk of the H1 sensor kinase modules, exceptions being the 'unorthodox' sensor kinases such as CheA and FtzE (Ishige *et al.*, 1994; Zhou and Dahlquist, 1997). We will refer to these similar H2 modules together with the unorthodox H1 modules as the H2 module family. At present, the only eukaryotic phosphorelay system in which all four modules have been identified is the *Saccharomyces cerevisiae* osmosensor relay Sln1–Ypd1–Ssk1 (Posas *et al.*, 1996) in which Ypd1 bears the H2 module.

Two genes that code for homologues of histidine kinases have been identified in *Dictyostelium discoideum*. Both are hybrid histidine kinases, bearing a receiver as well as a transmitter domain (Schuster *et al.*, 1996; Wang *et al.*, 1996). In addition, a novel gene, *regA*, recently has been described whose N-terminal region is homologous to response regulators and whose C-terminus encodes a cAMP phosphodiesterase (Shaulsky *et al.*, 1996; Loomis *et al.*, 1997). *regA* mutants resemble previously isolated *rdeA* and *rdeC* mutants (the initial *rdeB* mutant is lost) in showing accelerated development (Kessin, 1977; Abe and Yanagisawa, 1983). Mutants at all three loci produce aberrant fruiting bodies, particularly at high population density, with the spores often in a mass at the base of the structure, and are sporogenous, i.e. able to form spores when incubated as submerged monolayers in buffer containing cAMP (Kay, 1989). There is evidence that the rapid development and sporogeny of all three classes of mutants depends ultimately on increased levels of cAMP and/or of cAMP-dependent protein kinase (PKA). For example, wild-type cells can be induced to form spores in submerged monolayers if exposed to the membrane-permeable cAMP analogue, 8-bromo-cAMP (Kay, 1989), and *rdeC* mutants lack a functional PKA regulatory subunit (Simon *et al.*, 1992). Furthermore, as mentioned, *regA*[−] cells lack a cAMP-specific phosphodiesterase while *rdeA*[−] cells have been shown to possess elevated intracellular cAMP levels during vegetative growth (Coukell and Chan, 1980; Abe and Yanagisawa, 1983), and possibly also during development (Abe and Yanagisawa, 1983).

We describe here the isolation by insertional mutagenesis (restriction enzyme-mediated integration; REMI) (Kuspa and Loomis, 1992) of a rapidly developing mutant belonging to the *rdeA* complementation group. This has permitted us to clone the *rdeA* gene by rescue from the

A

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1  TTTTCTTTTCTTCTCCTAATAATTGTCATCAAAAATAAATTTTAAATTCATTTTAAAT
121  AAAAAAATAAAAAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAA
121  AAAAAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAA
181  ATGATCTCGGATTACGAAAGGCAACCACTAAATAAATTTGATCAAGATATATATTT
1  M I S D Y E G P T P T K K F D Q D I L F
241  GATTACTCTGAGGTGAAAGGAATTTACTTTTGAACCTTTAGATTCATATTTTTCATCA
21  D Y S E G E K E F T F E L L D S Y I S S
301  GTAGAAGCAATTTACCAGATTATTAAGATTCATTTGAAGCAAAAGATTTAAGGGTGCA
41  V E E H L P E L L N S F E A K D L K G A
361  GACTTCTATTCATGATATTAAGGGTTCATCAAGTTATATTTGGTTCGAGCTGTGAGA
61  V L H S H D I K G S S S Y I G C E A V R
421  TATGTTAGTGGTAAAGATAGAGCATATTTGAAAAATGATGAATTAGAAAAAGCAGAAATCA
81  Y V S G K I E A Y C K N D E L E K A E S
481  TTTTATCCAGAAATAAAGAAAGATTTGAAGAGTATTTAAATTTTAAAGTATTTTAAAG
101  F Y P E L K K E V E V F K I L S D F K
541  AAAAATTGGGATAAAAAATCATGGTGAAGGTTAGTGTGATGATGGTGGTGTGATAATGAA
121  K N W D K N H G E G G S D D G G D D N E
601  AGTGAACCACTGAAACCAATAATATGATGTTAGTGTGTAATAATAATGATAGTAGT
141  S E P T E N N N N D G S S V N N N D S S
661  AGCTGTGGTGGTGAAGACATAGAAAAATAAACAACAGATAAAATACTGGAAGAAAT
161  S C G G G K D I E N K N T D K N T G K N
721  CTAAACGGAAGATCTAATCACCAGTACCCTTACAACAACATTAATAACAGTAACTATT
181  L N G R S K S P V P L Q T T L K P V T I
781  GAAACACCGAAATCTGCTCTGATAAAATGCAACCGAACTCCAATCTCTTAGCTAAT
201  E T P K T A S D K I A T E T P T S L A N
841  AATACCAATAGTAGCAGCAACATAACAGTAAAAATGAAATGTTTAAATAGTAAACAA
221  N T N S S S N N N S K N E N G L N S K Q
901  CCTCAAACTCTTCAAAATTCACCAACAAAAATTCACCAAAAAATAAAGCAAAAAA
241  P Q T S S N S P T K I Q T K
961  AAAAA

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B

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Dd RDEA  LLNSFEAKDLKGAVLHSHDIKSSSYIGCEAVRYVSGKI (48-86)
Sc YPD1  QRQLDGEKNLTLDNLGHFLKSSSALGLQRIAWVCERI (47-85)
Ec ArcB  LESNLTADQDKKGIIEEGHKKIAGAGSVGLRHLQQLGQQI (700-738)
Ec BarA  VEEQLVGENPEGLVDLTHKLHSGCGYSGVPRMKNLCQLI (844-882)
Bp BvgS  LQKLHQQADWPKVSDMAHRLAGGARVVDKAMIDTVLAL (1155-1193)
[Consensus] D h hH+h+G A Gh

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Fig. 1. (A) cDNA sequence and predicted amino acid sequence of the *rdeA* gene. (B) Sequence comparisons around the histidine (starred) that is thought to be phosphorylated in RdeA and in several H2 modules (Ypd1, ArcB, BarA, BvgS; Posas *et al.*, 1996). h, hydrophobic residue; +, positively charged residue.

mutant. We report that it codes for a small protein that displays the weak similarity around a histidine residue characteristic of members of the H2 module family of phosphotransferases. Moreover, the rapid development phenotype of the mutant is complemented by the *Ypd1* gene of yeast, itself an H2 module protein. In view of the similarities between the *rdeA*⁻ and *regA*⁻ phenotypes and the fact that RegA resembles a typical D2-type response regulator, we propose that the *rdeA* gene product is an H2-type phosphotransferase in a multistep phosphorelay controlling the activity of the RegA phosphodiesterase.

Results

Isolation of the *rdeA* mutant WTC10

Mutant WTC10, which showed aberrant fruiting body morphology, was isolated by insertional mutagenesis as described in Materials and methods. Aggregation of WT-C10 began at ~4–6 h after the onset of starvation (compared with 8–10 h for wild-type cells) and led to the formation of a large number of small aggregates with no sign of distinct streams (not shown). There was no slug stage and each aggregate underwent a disordered culmination to form a pyramidal structure with a short malformed stalk at the apex and a large mound-like base made up of spores.

By digesting WTC10 genomic DNA with the restriction enzyme *Bgl*II, the inserted plasmid was rescued together

with 0.6 kb of flanking sequence. To confirm that it was this insertion that caused the observed phenotype, we transformed the rescued plasmid into wild-type cells and recreated the mutant phenotype by homologous recombination (Kuspa and Loomis, 1992). Both the original and recreated mutants were used in subsequent experiments.

Identification of the *rdeA* gene

In order to identify the disrupted gene in strain WTC10, we first sequenced the 0.6 kb flanking regions of the rescued genomic DNA and this was then used to isolate a cDNA clone from a *Dictyostelium* 12–16 h cDNA library (Schnitzler *et al.*, 1994). The cDNA obtained contains one long open reading frame of 762 bp that encodes a predicted 254 amino acid protein with molecular mass of 28 kDa. The full-length cDNA contains 180 bp of 5'-untranslated sequence and 24 bp of 3'-untranslated sequence (Figure 1A). The protein is enriched in charged residues, has a calculated pI of 4.55 and is very hydrophilic (Kyte and Doolittle, 1982).

Since the rapid aggregation and later development of WTC10 resemble previously described *rdeA* mutants (Sonneborn *et al.*, 1963; Abe and Yanagisawa, 1983), we made diploids between strain HH201 (Kessin, 1977), carrying the first *rdeA* mutation to be isolated (originally in strain FR17, Sonneborn *et al.*, 1963), along with a mutation conferring temperature-sensitive growth (*tsg*), and a derivative of strain NP2 (Williams *et al.*, 1974) with a different *tsg* mutation, into which the WTC10 disruption mutation had been introduced by homologous recombination. The diploid derivatives obtained showed the Rde phenotype. Control diploids formed by fusion of the insertional mutant with the *rdeA*⁺ strain X22 were *RdeA*⁺, indicating that *rdeA* is recessive. We conclude that WTC10 has an insertion in the *rdeA* locus. Interestingly, this entire sequence is absent from the genomic DNA of strain FR17 as defined by PCR or by Southern blotting (not shown; see Materials and methods), indicating that FR17 has a deletion of the complete *rdeA* coding region.

RdeA as an H2-module protein

Both the nucleotide and amino acid sequences of *rdeA* were compared with other available sequences using the BLAST and FASTA program (Altschul *et al.*, 1990; Henikoff and Henikoff, 1991). RdeA showed no extensive homology to known proteins. However, it did display the weak similarity around a histidine residue (His65) in a region of some 25 amino acids that is characteristic of members of the H2 module family in bacteria (Ishige *et al.*, 1994; Kehoe and Grossman, 1997). Figure 1B compares the amino acid sequence in this region of RdeA with that of Ypd1, the H2 module of the yeast osmosensor relay (Posas *et al.*, 1996), and of three bacterial H2 motifs (Ishige *et al.*, 1994). Identities are highlighted; there are some notable identities between RdeA and Ypd1 around the putative H2 residue. We also give the weak consensus derived by Ishige *et al.* (1994) from an examination of nine H2 modules. RdeA conforms to this consensus at seven of the 11 residues.

We have transformed *rdeA*⁻ cells with constructs carrying the full-length *rdeA* coding region fused to the actin15 promoter (Knecht *et al.*, 1986; Howard *et al.*, 1988) as

Fig. 2. Complementation of the *rdeA* mutation. (A) *rdeA* mutant WTC10 developed on filters showing the formation of pyramidal mounds of cells. (B) Complementation of the *rdeA* mutant with wild-type *rdeA* cDNA using actin15 as promoter and actin8 as terminator. (C) Complementation using the *pspA* (pre-spore-specific) promoter. (D) Complementation using the *ecmA* (pre-stalk-specific) promoter. All frames were taken after 30 h of development.

well as to the *pspA* (Early and Williams, 1989; Harwood *et al.*, 1992) and *ecmA* (Early *et al.*, 1993, 1995) promoters. All three constructs gave rise to fruiting bodies of essentially wild-type appearance (Figure 2), confirming that the mutant phenotype was due to loss of the function specified by the cDNA we had isolated. As had been anticipated, the *pspA-rdeA* transformants retained the rapid aggregation pattern typical of *rdeA*⁻ cells (as the *pspA* promoter is only active after aggregation), but each of the numerous small aggregates then produced a normal-looking fruiting body (Figure 2C). On the other hand, we were surprised to find that introduction of the *ecmA-rdeA* construct appeared to correct both the aggregation defect and the fruiting body morphology of the *rdeA*⁻ cells: like the *act15-rdeA* transformants, the transformed cells aggregated at the normal time, forming large aggregation territories with well-developed streams, and gave rise to fruiting bodies of wild-type appearance (Figure 2D). We believe, with others (Fukuzawa *et al.*, 1997; Mann *et al.*, 1997), that this result is due to a low level of *ecmA* promoter activity early in development, prior to the time when expression can be detected with *lacZ* reporter constructs.

Fig. 3. Complementation of the *rdeA* mutation by constructs generated from the full-length *rdeA* cDNA. (A) Full-length *rdeA* cDNA with actin15 promoter and actin8 terminator (+ signifies formation of normal wild-type fruiting body structures). (B) Deletions of the *rdeA* cDNA from the 5' and 3' ends showing complementation by all constructs containing codons 1–130. (C) Substitution of the codon for His65 with glutamine abolishes complementation while substitution of His63 does not. (D) The *Ypd1* gene from yeast complements the *rdeA* mutation.

In order to test whether the His65 residue of RdeA is essential for activity, as it should be if it is part of a phosphorelay, we mutated it to glutamine, fused the modified cDNA to the actin15 promoter and transformed *rdeA*⁻ cells. The modified construct was unable to complement the *rdeA* mutant. In a control experiment, when a nearby histidine (at position 63), which is not part of the H2 consensus, was replaced with glutamine, the construct did complement the *rdeA* lesion (Figure 3C).

To define the region of RdeA that is essential for activity, we created a series of deletion constructs and tested for complementation of the *rdeA* mutant. Removal of the first 15 N-terminal amino acids rendered the construct inactive, as did deletion from the C-terminus into the region between amino acids 115 and 130 (Figure 3B). We conclude that RdeA activity depends upon the integrity of a region extending roughly between amino acids 1 and 130. This region includes the H2 consensus motif and is similar in size to that of the *S.cerevisiae* Ypd1 protein (167 amino acids) (Posas *et al.*, 1996), the H2 module of members of the unorthodox sensory kinase family (~150 amino acids) (Ishige *et al.*, 1994) as well as to the N-terminal H2 phosphotransferase domain (amino acids 1–134) of *Escherichia coli* CheA (Swanson *et al.*, 1993).

The suggestive similarities between RdeA and *S.cerevisiae* Ypd1 encouraged us to test whether the latter could complement the *rdeA* defect. The *Ypd1* gene was cloned from *S.cerevisiae* genomic DNA by PCR and fused downstream of the *Dictyostelium* actin15 promoter. This construct fully complemented the *Dictyostelium rdeA* mutation (Figure 3D). This finding strongly supports the view that RdeA, like Ypd1, is an H2 module protein.

Fig. 4. Expression of *rdeA* during development. Northern blot of *rdeA* expression in wild-type cells. RNA extracted at various times of development was probed with a full-length *rdeA* cDNA, and with *IG7*, a gene that is expressed at a constant level during development.

Expression of the *rdeA* gene during development

Since the *rdeA* defect is already expressed early in development in the form of precocious cell aggregation, we would expect *rdeA* to be transcribed in vegetative cells. In order to examine its developmental expression, RNA was extracted from wild-type cells at various times during development and hybridized with the full-length *rdeA* cDNA as a probe. The results show that *rdeA* encodes a single transcript of ~1 kb that is expressed at a significant level during vegetative growth and peaks at 8–16 h of development (Figure 4). Even at its peak, the level of expression is low and detection requires long exposures of the Northern blot. This mRNA was absent from the *rdeA*⁻ REMI mutant (not shown). We have also isolated the *rdeA* promoter region (see Materials and methods) and made a reporter construct in which the β -galactosidase gene is under its control. Examination of β -gal expression in strains harbouring this construct showed that *rdeA* is expressed in both pre-stalk and pre-spore cells (not shown).

Cellular location of the RdeA protein

To examine the localization of RdeA protein within the cell, constructs driven by the *actin15* promoter were made with either the complete *rdeA* gene with an attached c-Myc epitope (EQKLISEEDL), or amino acids 1–130 of RdeA fused in-frame with the highly sensitive C3 version of the green fluorescent protein (GFP) (Cramer *et al.*, 1996) (Figure 5A). No compartmentalization was observed. This is consistent with the idea that RdeA is a cytosolic protein, relaying a phosphoryl group to a cytosolic RegA cAMP phosphodiesterase. The result for the GFP construct is shown in Figure 5B.

Effects of the *rdeA* mutation on developmental gene expression

In order to gain some insight into the role of RdeA in early development, we compared the expression of a number of early genes in *rdeA*⁻ and wild-type cells. The time of appearance of transcripts of the early genes *car1* (a cell surface cAMP receptor), *aca* (developmental adenylyl cyclase) and *pde* (extracellular phosphodiesterase) was advanced in the mutant and their expression level was enhanced (Figure 6A). An interesting finding is that both *pdi* (the extracellular phosphodiesterase inhibitor) and *disc1* (coding for discoidin 1 protein) were already

Fig. 5. Cellular location of the RdeA protein. (A) Constructs of the *rdeA* cDNA with: (a) a c-Myc tag (EQKLISEEDL) under the control of the *actin15* promoter and *actin8* terminator; (b) the green fluorescent protein (GFP-C3) fused in-frame with codons 1–130 of the *rdeA* cDNA, under the control of the *actin15* promoter and *actin8* terminator. (B) Fluorescence image of the GFP-C3 construct.

expressed at a low level during growth of the mutant (zero time sample) but not of the wild-type. Since transcription of both of these genes is strictly PKA dependent (Wu *et al.*, 1995; Endl *et al.*, 1996), this observation is consistent with reports that loss of RdeA function leads to abnormally elevated levels of cAMP during vegetative growth (Coukell and Chan, 1980; Abe and Yanagisawa, 1983) since this would be expected to lead to activation of PKA catalytic activity.

Figure 6B presents results for the expression of later genes in *rdeA*⁻ and wild-type cells. Expression of each of the genes examined was advanced in the mutant (as also reported for some pre-spore cell-specific genes in FR17 by Saxe and Firtel, 1986). However, the pattern of expression of the PKA catalytic and regulatory subunits was only slightly affected in the *rdeA* mutant. Two other as yet unexplained features of these results are that expression of the pre-stalk marker, *ecmA*, was markedly (and consistently) reduced in the *rdeA* mutant and that expression of the pre-spore marker, *pspA*, and spore maturation marker, *spiA*, was markedly biphasic.

We have also generated *rdeA*⁻ strains that carry the *lacZ* gene under the control of the *pspA*, *ecmA* and *ecmB* promoters. The expression of *pspA-lacZ* and *ecmA-lacZ* appeared normal, indicating that cell type differentiation is not disrupted in the mutant. Cells expressing *lacZ* under the control of the *ecmB* promoter appeared to be more numerous early in development than normal and, surprisingly, ~5–10% of the spores formed in this strain stained

Fig. 6. Gene expression during *rdeA*⁻ development. Northern blots were prepared from RNA extracted at intervals during the development of wild-type and *rdeA*⁻ cells. (A) Blots were visualized with probes for aggregation-stage genes: *car1* (cAMP receptor 1), *aca* (the developmental adenylyl cyclase), *pde* (cyclic nucleotide phosphodiesterase), *pdi* (phosphodiesterase inhibitor) and *disc1* (discoidin 1); *pde** was exposed for an additional period to visualize the 1.9 kb transcript. (B) Probes for cell type-specific genes: *psA* (D19) (pre-spore cell-specific), *spiA* (late sporulation marker), *ecmA* and *ecmB* (pre-stalk cell-specific), and *PKAcat* (catalytic subunit of PKA) and *PKAreg* (regulatory subunit of PKA); *IG7* is expressed at a constant level during development and is used as a reference.

with X-gal, indicating that some of the cells that formed spores had expressed the *ecmB* gene earlier in development.

The behaviour of an *aca*⁻ *rdeA*⁻ double mutant

As mentioned in the Introduction, both *rdeA* and *rdeC* mutants are rapidly developing and sporogenous, i.e. able to form spores when incubated as submerged monolayers in buffer containing cAMP (Kay, 1989). This is readily accounted for in the case of *rdeC* mutants since they lack a functional PKA regulatory subunit and therefore have constitutive PKA catalytic activity (Simon *et al.*, 1992). On the other hand, there is no reason to believe that PKA activity is directly affected in *rdeA* mutants; rather they have been reported to possess elevated intracellular levels of cAMP (Coukell and Chan, 1980; Abe and Yanagisawa, 1983). If the *rdeA* phenotype depends on elevated intracellular cAMP levels, we could expect that the sporogenous behaviour of *rdeA*⁻ cells (but not of *rdeC*⁻ cells) might depend upon the presence of the developmental adenylyl cyclase enzyme (ACA; Pitt *et al.*, 1992). In order to examine this, we have compared the sporogenous behaviour of *aca*⁻*rdeA*⁻ and *aca*⁻*rdeC*⁻ double knockout strains by disruption of the *aca* gene in the *rdeA*⁻ and

rdeC⁻ strains. As predicted, the *aca*⁻*rdeA*⁻ double mutant formed only a very small number of spores, whereas the REMI-induced *rdeA* mutant itself, like previously studied *rdeA* mutants (Kay, 1989), was sporogenous (Table I). In contrast, the sporogeny of *rdeC*⁻ cells was not abolished (but for unknown reasons actually increased) by elimination of the adenylyl cyclase gene.

Discussion

The *rdeA* mutant generated by insertion has characteristics similar to those seen in previously isolated irradiation-induced *rdeA* strains (Sonnenborn *et al.*, 1963; Kessin, 1977; Abe and Yanagisawa, 1983), such as rapid aggregation without streaming, formation of spores under submerged conditions (sporogeny) and aberrant fruiting body formation. The fact that the *rdeA* phenotype can be generated by gene disruption (WTC10) or by deletion of the entire gene, as shown here for FR17, demonstrates that it is due to a loss of function, as is also indicated by the fact that the *rdeA* mutation is recessive (Kessin, 1977 and above).

We have presented evidence that the *rdeA* locus encodes a novel protein (RdeA) that comprises in its N-terminal

half an H2-type module similar to those in proteins involved in multistep phosphorelays. This evidence is: first, that RdeA displays the weak similarity around a histidine residue (His65) characteristic of members of this family of phosphotransferases; second, that this histidine residue is essential for RdeA function; and, finally, and most convincingly, that Ypd1 of yeast can complement the RdeA defect.

We propose that RdeA acts immediately upstream of RegA in the multistep relay shown in Figure 7. This proposal is based on the similarities in the phenotype of *rdeA⁻* and *regA⁻* cells. Both are sporogenous (Kay, 1989; Loomis *et al.*, 1997; Thomason *et al.*, 1998), and, in both, aggregation begins several hours before it does in wild-type cells and leads to the formation of numerous small aggregates with no sign of streaming (see Results; P.A. Thomason, D.Traynor, G.Cavet, W.-T.Chang, A.J. Harwood and R.R.Kay, personal communication). Furthermore, spore formation—a PKA-dependent process—occurs prematurely in each case, resulting in the formation of disorganized fruiting structures. The behaviour of the mutants may therefore be understood if they each lack an intracellular cAMP phosphodiesterase activity. Support for this interpretation comes from experiments in which

a novel adenylyl cyclase activity that is not normally apparent in wild-type cell lysates was detected in lysates of vegetative-stage *rdeA⁻* and *regA⁻* cells and in lysates of vegetative wild-type cells provided the latter were supplemented with the cAMP phosphodiesterase inhibitor, IBMX (H.J.Kim, W.-T.Chang, J.D.Gross, R.Brandt and P.Schaap, in preparation).

According to the scheme in Figure 7, phosphotransfer from RdeA to RegA results in activation of the RegA cAMP phosphodiesterase. This assumption is required to account for the evidence that inactivation of RdeA, like that of RegA, causes effects consistent with elevation of cAMP levels, and is supported by the findings of Thomason *et al.* (1998). The identity of the H1 and D1 elements upstream of RdeA in the scheme of Figure 7 is unknown. However, it does not seem likely that either of the hybrid kinases DokA (Schuster *et al.*, 1996) or DhkA (Wang *et al.*, 1996) could be upstream components since disruption of *dokA* or of *dhkA* does not generate a rapidly developing phenotype. We note, however, that Loomis and his collaborators (Shaulsky *et al.*, 1996; Loomis *et al.*, 1997; Anjard *et al.*, 1998) have suggested a relay scheme that does involve DhkA. According to their scheme, the RegA response regulator is phosphorylated when the DhkA sensor kinase is activated by a peptide released from pre-stalk cells. Phosphorylation of RegA in turn inhibits its cAMP phosphodiesterase, and the resulting increase in intracellular cAMP and PKA activity switches aggregates from migration to fruiting body formation. If phosphorylation of RegA stimulates its phosphodiesterase activity, as the results of Thomason *et al.* indicate, it seems more likely that DhkA antagonizes the activity of RegA by a process other than phosphorylation. It might, for example, initiate a relay that stimulates a (putative) RegA phosphatase similar to the phosphatases acting on the SpoF response regulator of *Bacillus subtilis* (Perego and Hoch, 1996; Perego, 1997).

Table I. Spore induction by cAMP in cell monolayers

Strain	Genotype	% spore cells cAMP concentration	
		5 mM	200 μ M
WTC10	<i>rdeA⁻</i>	21	5
WTC101	<i>rdeA⁻aca⁻</i>	2	0
HTY217	<i>rdeC⁻</i>	24	17
WTC001	<i>rdeC⁻aca⁻</i>	42	48

Ellipsoid spore cells were visualized and counted by inverted phase microscopy.

Fig. 7. Top: osmosensor relay in *S.cerevisiae* showing the path of phosphate from the sensor kinase Sln1, via Ypd1 to the response regulator Ssk1. Phosphorylation of Ssk1 leads to inhibition of the MAP kinase cascade. Bottom: the proposed RegA relay in *Dictyostelium*. The RdeA protein plays a similar role to Ypd1: it is phosphorylated on His65 by an unknown (hybrid) sensor kinase; we suggest that the phosphoryl group is then transferred to the D2 aspartate of RegA, an intracellular cAMP phosphodiesterase, and stimulates its activity.

Materials and methods

Growth and maintenance of stocks

Dictyostelium discoideum strains were maintained in association with *Klebsiella aerogenes* on SM-agar plates at 22°C in the dark (Sussman, 1987). The DdPYR5-6 strain DH1 (Ura⁻: lacking uridine monophosphate synthase activity) was grown in axenic medium supplemented with uracil (20 µg/ml). Ura⁺ transformants were selected in FM medium (Gibco-BRL). Neomycin-resistant (Neo^r) transformants were selected in axenic medium supplemented with 10 µg/ml of geneticin (G418) and grown in this medium, and blasticidin-resistant (Bsr^r) transformants were selected and grown in axenic medium with 5 µg/ml of blasticidin S (Sutoh, 1993).

Isolation of the rapidly developing (rde) mutant

REMI allows the isolation of insertional mutants from which the disrupted genes can be rescued and cloned (Kuspa and Loomis, 1992). It was carried out with plasmid pDIV5 (Chang *et al.*, 1996). The pDIV5 was linearized with *Xba*I and electroporated into DH1 cells together with *Xba*I. Transformants were selected for prototrophy in FM medium lacking uracil and plated clonally onto SM-agar plates in association with *K. aerogenes*.

The genomic DNA flanking the integrated vector was isolated from strain WTC10 by digesting the DNA with *Bgl*II, re-ligating and selecting for ampicillin-resistant transformants in *E. coli*. The resulting plasmid pWTC10/*Bgl*II contained 600 bp of flanking genomic DNA. To recreate the mutant phenotype by homologous recombination, the rescued plasmid was linearized with *Bgl*II and electroporated into the parental Ura cells. Ura⁺ transformants were selected in FM medium and screened for transformants with the same phenotype as the original mutant.

Genetic complementation by parasexual cell fusion

The *ura* gene in pWTC10/*Bgl*II was replaced with the blasticidin resistance gene (*bsr*, Sutoh, 1993). The resulting construct was transformed by electroporation into strain NP2 (Williams *et al.*, 1974) which is temperature-sensitive for growth (*tsg*). Selection was for blasticidin resistance and generated a *tsg/rde*⁻ derivative by homologous recombination. Parasexual diploids were then produced by fusion of this derivative with the *tsg/rdeA*⁻ mutant strain, HH201 [a derivative of the original *rdeA* mutant FR17 (Kessin, 1977)], as well as with X22 (*tsg/rdeA*⁺) cells, as described previously (Kessin *et al.*, 1974).

Genetic complementation by transformation

A series of deletion constructs were created by PCR from *rdeA* cDNA with appropriate primers and fused downstream of a *Dictyostelium* actin15 promoter which was generated by digestion of pAct15Gal plasmid with *Bgl*III and *Xho*I to delete the β-galactosidase gene. The constructs were used for complementation of *rdeA*⁻ cells using electroporation and selection by resistance to G418.

Cloning of the *Ypd1* gene

The *Ypd1* gene was cloned from *S. cerevisiae* genomic DNA by PCR with 5'-GAAGATCTATGTCTACTATTCCGTCAGAAATCATCAAT-TGGACCATC-3' and 5'-CCGCTCGAGTTATAGGTTTGTGTTGTAATATTAGATAACTCAATTCTCGCC-3' primers and ligated to *Bgl*III + *Xho*I-digested pAct15Gal. The resulting construct conferred resistance to G418.

Isolation of *rdeA* cDNA

cDNA clones containing the *rdeA* coding region, the 3'-untranslated region and a portion of the 5'-untranslated region were isolated from a λZAP (Stratagene) cDNA library prepared from cells at t₁₂₋₁₆ (Schnitzler *et al.*, 1994), using as a probe a flanking region of DNA from the rescued plasmid and standard protocols (Stratagene).

Identification of the lesion in the *rdeA* mutant, FR17

Primers corresponding to the first 28 and last 33 bases of the coding sequence were used for amplification by PCR using DNA from wild-type DH1 cells and the *rdeA* mutant HH201 (FR17).

For Southern blots, DH1 and HH201 genomic DNAs were digested with *Eco*RI, *Csp*45I, *Spe*I and *Xba*I, and probed with the full-length *rdeA* cDNA.

Northern blot analysis

Total cellular RNA was prepared using catrimox-14 (Iowa Biotech Corp., IA) according to Dahle and Macfarlane (1993) and analysed on Northern blots as described (Chang *et al.*, 1996).

Isolation of the *rdeA* promoter

The *ura* gene in the rescued plasmid pWTC10/*Bgl*II was replaced with a *bsr* gene containing a multiple cloning site. This construct was introduced into wild-type cells by transformation selecting for blasticidin resistance, and *rdeA*⁻ transformants resulting from homologous recombination were purified. The promoter region was then isolated by digesting genomic DNA isolated from this strain with *Csp*45I. The β-gal reporter construct was made by digesting the *Csp*45I fragment with *Eco*RI and ligating the resulting fragment into *Eco*RI-digested pDdGal16.

Aggregation under submerged conditions

Cells were harvested from axenic medium at 3–8×10⁶ cells/ml, washed three times in aggregation buffer (5 mM MES, 5 mM MgCl₂, 10 mM KCl, pH 6.2), and 4 ml of cell suspension were plated in 5 cm tissue culture dishes (Sterilin) to a final density of 1×10⁵ cells/cm². Plates were incubated at 22°C in a humid box.

Spore induction in cell monolayers

Spore induction was performed as described by Kay (1987). Cells were grown in axenic medium, harvested at 3–8×10⁶ cells/ml, and washed three times with spore medium. The cells were then gently resuspended at 5×10⁵ cells/ml in spore medium, and 2 ml of cell suspension was plated in 5 cm tissue culture dishes (Sterilin) with or without 5 mM or 200 µM cAMP. Spore formation was examined by phase contrast microscopy after 48 h of incubation.

β-galactosidase staining

In situ detection of β-galactosidase activity was carried out as described by Dingermann *et al.* (1989), with the following modifications: after development for the appropriate times, structures were fixed with 1% glutaraldehyde in Z-buffer, permeabilized with 0.1% NP-40 in Z-buffer, washed in Z-buffer and incubated with staining solution for 24 h at 37°C.

Mutagenesis of the His codons in the *rdeA* cDNA

The His63 or His65 residues in RdeA protein were changed to Gln by PCR overlap extension using four primers (Horton *et al.*, 1993). The primers for His63 were 5'-GGGTGCAGTACTTCAATCTCATGATAT-TAAAGTTTCATCAAGTTATATTGGTTGCG-3' and 5'-CGCAACC-AATATAACTTGATGAACCTTTAATATCATGAGATTGAAGTACT-GCACCC-3', and for His65 were 5'-GGGTGCAGTACTTCAATCTC-AAGATATTAAAGGTTTCATCAAGTTATATTGGTTGCG-3' and 5'-CGCAACCAATATAACTTGATGAACCTTTAATATCTTGAGAAT-GAAGTACTGCACCC-3'. The primers for the complete *rdeA* coding region were 5'-CGCGGATCCATGATCTCGGATTACGAAGGACCA-3' and 5'-CCGCTCGAGTTATTTTGTGTTGAATTTTGTGTTGGAAT-TTTGAAGA-3'.

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