A bZIP/bRLZ transcription factor required for DIF signaling in *Dictyostelium*

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Summary

The intermingled differentiation and sorting out of *Dictyostelium* prestalk-O and prespore cells requires the diffusible signaling molecule DIF-1, and provides an example of a spatial information-independent patterning mechanism. To further understand this patterning process, we used genetic selection to isolate mutants in the DIF-1 response pathway. The disrupted gene in one such mutant, *dimA*$^-$, encodes a bZIP/bRLZ transcription factor, which is required for every DIF-1 response investigated. Furthermore, the *dimA*$^-$ mutant shows strikingly similar developmental defects to the *dmtA*$^-$ mutant, which is specifically defective in DIF-1 synthesis. However, key differences exist: (1) the *dmtA*$^-$ mutant responds to DIF-1 but does not produce DIF-1; (2) the *dimA*$^-$ mutant produces DIF-1 but does not respond to DIF-1; and (3) the *dimA*$^-$ mutant exhibits cell autonomous defects in cell type differentiation. These results suggest that *dimA* encodes the key transcriptional regulator required to integrate DIF-1 signaling and subsequent patterning in *Dictyostelium*.

Key words: *Dictyostelium*, DIF-1, Pattern formation, Prestalk O cells, *dimA*, *dmtA*

Introduction

A key question in developmental biology concerns the mechanisms by which body pattern is established. Although positional information conveyed by morphogen gradients is a widely accepted way of forming pattern (Tabata, 2001; Wolpert, 1989), an alternative method is conceivable. This method is based on the intermingled differentiation of cells with different fates, followed by their sorting into discrete pattern elements. It has been proposed that *Dictyostelium* prestalk and prespore cells behave in this way (Esch and Firtel, 1991; Leach et al., 1973; Loomis, 1993; Williams et al., 1989). The biology of *Dictyostelium* development is unlike metazoan development. It is based on the aggregation of separate cells to form a multi-cellular organism (Kessin, 2001). However, it is possible that pattern formation by a combination of scattered differentiation and sorting out is a common mechanism. In particular, the cellular properties required to form scattered or spaced patterns (Headon and Overbeek, 1999), and for sorting out different cell types (Xu et al., 1999), are widespread. Furthermore there are indications from chick development that these two cellular properties may be brought together in a pattern-forming process, as both the primitive streak and the limb bud apical ectodermal ridge appear to form by the recruitment of a scattered subset of migratory cells (Altabef et al., 1997; Stern and Canning, 1990).

The developing *Dictyostelium* slug has a clear anteroposterior pattern, with the prestalk and prespore cell types arranged into tissues along this axis. Prestalk cells occupy the anterior quarter and are of two major types: the prestalk-A (pstA) cells, which are at the very front, and the prestalk-O (pstO) cells just behind them (Early et al., 1993; Jermyn et al., 1989). The posterior three-quarters of the slug comprise the prespore zone, and there is some evidence for the subdivision of that region as well (Haberstroh and Firtel, 1990; Kibler et al., 2003). The question of how pattern arises is fundamental to our understanding of *Dictyostelium* development.

The chemical nature and cell culture actions of DIF-1 provide a candidate molecule for the control of *Dictyostelium* patterning. DIF-1 is a chlorinated alkyl phenone produced by developing *Dictyostelium* cells. DIF-1 can drive amoebae to differentiate as vacuolized stalk cells (Morris et al., 1987). It also induces the expression of prestalk markers, represses prespore markers and prevents cells in culture from differentiating as spores. Consequently, DIF-1 has been considered to be a central regulator of the stalk/spore decision (Early et al., 1995; Early and Williams, 1988; Fosnaugh and Loomis, 1991; Kay and Jermyn, 1983).

However, a mutant specifically defective in DIF biosynthesis (*dmtA*) has been generated, which develops relatively normally until the slug stage of development. At that stage, it makes long, thin structures compared with wild type, which later develop spores and a stalk of sorts (Thompson and Kay, 2000b). The only characterized defect in cell type differentiation is a failure to express a subset of pstO markers (Maeda et al., 2003; Thompson and Kay, 2000b). However, several prestalk markers (including a pstA marker) and

prespore markers are expressed normally. This suggests that DIF-1 is only required for the differentiation of a subset of prestalk cells, the pstO cells, but not for the differentiation of pstA cells or prespore cells.

These observations, together with earlier work, suggest that patterning arises by a mechanism whereby the choice between (at least) the pstO and prespore fates is driven by a process akin to lateral inhibition (Clay et al., 1995; Kay et al., 1999; Leach et al., 1973; Loomis, 1993). It is proposed that as cells enter the mound, they all experience similar concentrations of DIF-1. Initial intrinsic differences between the cells distinguish between responding and non-responding populations. Such differences have been noted, and include cell cycle position and growth history (Leach et al., 1973), both of which bias cell fate choice and affect DIF-1 sensitivity (Thompson and Kay, 2000a). As some of the earliest responses to DIF-1 include the downregulation of DIF biosynthesis and upregulation of DIF breakdown (Insall et al., 1992), two populations of cells quickly emerge: DIF-1 responding (prestalk) and DIF-1 producing (prespore). Consistent with this idea, prestalk cells ultimately exhibit the highest levels of DIF-1 breakdown and prespore cells the highest levels of DIF-1 biosynthesis (Kay et al., 1993; Kay and Thompson, 2001). Finally, once distinct populations of cells arise, subsequent tissue patterning may occur by sorting out as a result of differential adhesion and/or cell motility (Clow et al., 2000; Early et al., 1995; Matsukuma and Durston, 1979; Siu et al., 1983; Tasaka and Takeuchi, 1979; Traynor et al., 1992).

In order to further understand this patterning process and its control, it is important to identify the molecular components of the DIF-1 response pathway and to determine how each component may be influenced by other signals, such as those determining intrinsic biases. Only a few components of the DIF-1 signaling pathway have been identified, although several different mechanisms of signal transduction have been proposed. These include a steroid hormone type receptor (Insall and Kay, 1990), signaling through intracellular calcium (Schaap et al., 1996), intracellular pH (Gross et al., 1983) and the control of nuclear export (Fukuzawa et al., 2003).

Studies to identify DIF-responsive transcription factors have also had some success. A minimal DIF-response element has been described that is both necessary and sufficient for DIF-induced gene expression in cell culture (Kawata et al., 1996). Furthermore, several activities have been identified in cell extracts that bind to this element in vitro and, ultimately, led to the identification of the *Dictyostelium* STAT family of transcription factors (Fukuzawa et al., 2001; Kawata et al., 1997). Of these, STATc is tyrosine phosphorylated and translocates to the nucleus of pstO cells in response to DIF-1, where it represses the activity of a pstA marker (Fukuzawa et al., 2001). However, STATc does not seem to play a role in the activation of DIF-1 target genes. Expression of the pstO marker *ecmO/lacZ* is unaffected in the STATc null mutant, and the mutant shows little morphological similarity to the DIF-non-producing *dmtA*$^-$ mutant.

We have taken a forward genetic approach to identify mutants in key signaling molecules required to transduce the DIF signal. One such mutant, *dimA*$^-$, shows no response to DIF-1 in all conditions tested and exhibits morphological phenotypes indistinguishable from those of the *dmtA*$^-$ mutant. However, key differences lie in the cell autonomous nature of the phenotype and the finding that *dimA*$^-$ produces normal levels of DIF-1. As the *dimA*$^-$ gene encodes a transcription factor of the bZIP or bRLZ classes, we propose that *dimA* encodes a key transcriptional regulator required to integrate DIF-1 signaling.

Materials and methods

Cell culture and *Dictyostelium* strains

Dictyostelium strains AX4, *dmtA*$^-$ (HM1030) and *dimA*$^-$ (CT15) were maintained in HL5 medium or on SM agar plates in association with *Klebsiella aerogenes* (Sussman, 1987). *lacZ* transformants were generated as described (Pang et al., 1999). The *dimA*$^-$ strain was recapitulated after plasmid rescue and the recapitulated strain used in all subsequent experiments. The GenBank Accession Number for the nucleic acid sequence of the coding region of *dimA* is AY428796.

REMI mutagenesis and DIF-resistant mutant selection

REMI mutagenesis was performed as described (Kuspa and Loomis, 1992), except that pools of ~5000 mutants were grown in shaken suspension directly after transformation. Mutant cells were harvested and resuspended at 1×10^5 cells/ml in stalk salts [10 mM MES (pH 6.2), 1 mM $CaCl_2$, 2 mM NaCl, 10 mM KCl, 0.5 mg/ml streptomycin sulphate, 30 μg/ml tetracycline]. 3.75×10^6 cells were plated on tissue culture dishes at a density of 1.6×10^4 cells/cm^2, and supplemented with 10 mM 8-Br-cAMP (Sigma) and 100 nM DIF-1. After 48 hours, detergent was added to a final concentration of 0.1% NP40 and 10 mM EDTA, to eliminate unsporulated cells.

Development and whole-mount *lacZ* staining

Cells were developed at a density of 6.4×10^5 cells/cm^2 on KK2 (16.1 mM KH_2PO_4, 3.7 mM K_2HPO_4) plates containing 1.5% purified agar (Oxoid) with or without 100 nM DIF-1. *lacZ* staining was performed as described (Dingermann et al., 1989).

Monolayer assays and *lacZ* marker quantitation in culture

All stalk and spore cell monolayer assays were performed as described (Thompson and Kay, 2000a). Induction of marker gene expression in dissociated cells was performed as described (Berks and Kay, 1990), except that 200 μM $CaCl_2$ was added to the buffer. For induction of *lacZ* markers in monolayers (I. Sarafimidis, personal communication), mid-log phase cells were harvested, washed and resuspended at 1×10^5 cells/ml in spore medium [20 mM KCl, 20 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM MES (pH 6.2), 100 μg/ml streptomycin sulphate], containing 2 mM cAMP and 50 μM cerulenin, with or without 100 nM DIF-1. 50 μl aliquots were added to each well of a flat-bottomed 96-well tissue culture dish and incubated for 24 hours at 22°C. Cells were lyzed in 50 μl lysis buffer [200 mM HEPES (pH 8.0), 2 mM $MgSO_4$, 4% TritonX-100] containing 2 mM CPRG (Roche). β-galactosidase enzyme activity was monitored by measuring the color change at 575 nm.

Measurement of DIF levels

DIF levels were measured by development on agar containing $^{36}Cl^-$, followed by extraction of DIF with chloroform/methanol, TLC separation and detection on a phosphorimager (Kay, 1998).

Nucleic acid techniques

For northern blots (Berks and Kay, 1990), RNA integrity and loading were monitored by Methylene Blue staining of ribsosmal RNAs (large subunit shown in figures).

Electrophoretic mobility shift assays (EMSA)

For *dimA* expression in *E. coli* strain BL21, the region predicted to encode the DNA-binding and dimerization domains (amino acids 545-

676) was cloned and expressed as a GST-fusion protein. Coomassie-stained gels were used to ensure similar amounts of soluble protein from *dimA*-expressing and control extracts were assayed. Oligonucleotides corresponding to sequences proximal to the transcriptional start site of the *ecmO/lacZ* reporter gene (Oligo1, TTTTTATTTTTTTTTTTTTATTTAAACAGTTACACCCCACAATTTTG; Oligo2, GATCCAAAATTGTGGGGTGTAACTGTTTAAATAAAAAAAAAAAAAATA) were annealed, labeled, and EMSA performed as described (Uv et al., 1994), except that either 0.5 U/ml polydA/dT or 0.5 U/ml polydI/dC (Roche) was included as a non-specific competitor. For competition assays with mutant oligonucleotides (mOligo1, TTTTTATTTTTTTTTTTTTATTTAAACAGTTA**A**AC**A**C**A**ACAATTTTG; mOligo2, GATCCAAAATTG-T**T**GT**G**GT**T**TAACTGTTTAAATAAAAAAAAAAAAAATA; where bold letters indicate mutations) were annealed and used.

Results

A genetic selection for DIF-1 signaling mutants

We developed a selection based on the 8-Br-cAMP monolayer assay (Kay, 1989) to enrich for DIF-insensitive mutants (dims). Wild-type cells incubated at low density under buffered salts in the presence of 8-Br-cAMP differentiate into viable, detergent-resistant spores. However, when 100 nM DIF-1 is included in the medium, DIF-1-responsive cells remain amoeboid (detergent sensitive), or differentiate as vacuolized stalk cells and die, resulting in an ~1500-fold decrease in the number of viable spores after detergent treatment (Fig. 1A). As mutants with a compromised DIF-1 response would be predicted to differentiate as detergent-resistant spores, we used this selection method to isolate DIF-resistant mutants from three independent libraries of 10,000 mutants created by REMI mutagenesis (Kuspa and Loomis, 1992) in two genetic backgrounds, *pkaR*$^-$ (Wang and Kuspa, 1997) and AX4. From this selection, *dimA*$^-$ was chosen for further characterization.

First, we tested DIF-1 responsiveness in 8-Br-cAMP monolayers, and found that *dimA*$^-$ exhibits little if any response when measured either in terms of stalk

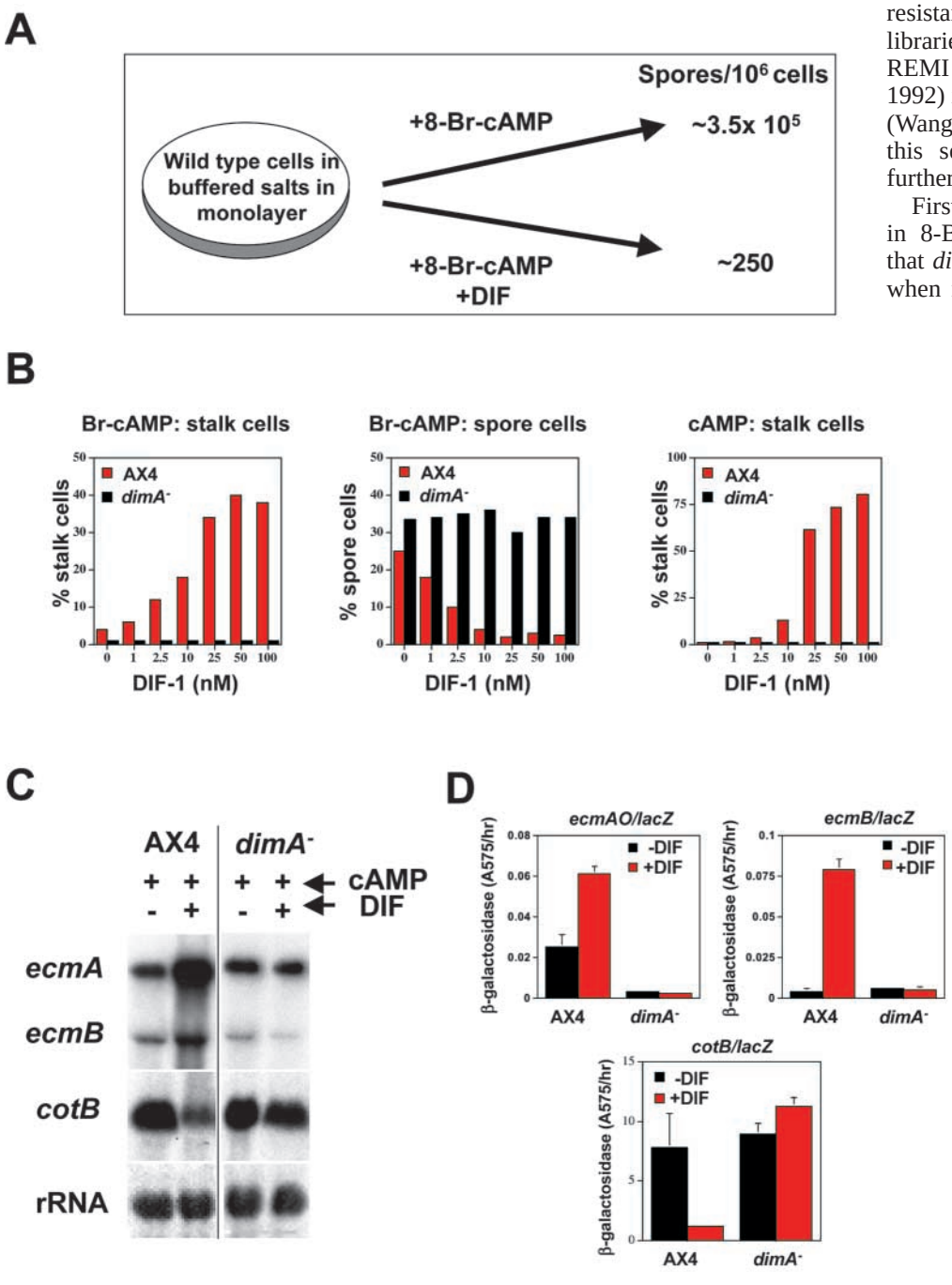

Fig. 1. Isolation of a mutant defective in DIF responses. (A) The 8-Br-cAMP monolayer assay as an enrichment for DIF-1 non-responsive mutants. (B) The *dimA*$^-$ mutant shows no DIF-1 response in monolayer assays; compare with wild type (AX4). DIF-1 responses were measured in 8-Br-cAMP or cAMP removal monolayer assays. Results shown are the mean of three experiments. (C) The *dimA*$^-$ mutant shows no DIF-1 response in a dissociated cell assay. Wild-type AX4 or *dimA*$^-$ mutant cells were harvested at the mound stage of development and disaggregated. Cells were shaken for 4 hours in buffer containing 1 mM cAMP, with or without 100 nM DIF-1. Total RNA was extracted and, following northern transfer, probed with the *ecmA* and *ecmB* (prestalk), and *cotB* (prespore), markers. Methylene Blue staining of ribosomal RNA (rRNA) is shown as a loading control. (D) Prestalk (*ecmAO/lacZ* and *ecmB/lacZ*) and prespore (*cotB/lacZ*) reporter constructs are DIF-1 non-responsive in the *dimA*$^-$ mutant in monolayer assays. Results are averages and standard deviations of two biological replicates, where each assay was performed in triplicate.

cell induction or spore cell repression (Fig. 1B). Even at doses of 100 nM DIF-1, *dimA*⁻ produces essentially no stalk cells, whereas 40% of the parental wild-type cells differentiated as stalk cells. However, *dimA*⁻ is not merely compromised in terminal differentiation as it produces comparable numbers of spores to the wild type in the absence of DIF-1. Furthermore, when spore cell repression is used as a measure of DIF-1 response, *dimA*⁻ spore cell numbers remain unchanged at doses up to 100 nM DIF-1, whereas sporulation of the wild type is greatly reduced.

Second, we examined DIF-1 responsiveness using another monolayer test in which cells are initially brought to competence to respond to DIF-1 by treatment with cAMP, before removing the cAMP and incubating in the presence of DIF-1. Under these conditions, wild-type cells differentiate as stalk cells but not spores (Fig. 1B). Again, *dimA*⁻ showed no DIF-1 response (Fig. 1B), nor did it respond to the chemically related stalk cell inducers DIF-2 and DIF-3 (Morris et al., 1988) (data not shown).

Finally, to test whether any of the observed defects in DIF response were specific to terminal differentiation, we employed two independent tests in which changes in gene expression were monitored in response to DIF-1. First, we used a shaken suspension assay (Berks and Kay, 1990). Cells were developed to the mound stage, dissociated, and shaken in suspension with cAMP and DIF-1. We observed that the prestalk markers *ecmA* and *ecmB* were induced in wild-type cells by DIF-1 treatment, and the prespore marker *cotB* was repressed (Fig. 1C). Under the same conditions, *dimA*⁻ cells showed little or no response to DIF-1 (Fig. 1C). Second, we used a variation on the monolayer assay in which cells are prevented from undergoing terminal differentiation because of the continued presence of high levels of cAMP. Under these conditions, any effects of endogenous DIF-1 are minimal because its biosynthesis is inhibited by the addition of cerulenin (Kay, 1998). The level of the DIF response was determined by quantification of β-galactosidase activity from strains carrying cell-type-specific reporter constructs. Quantification revealed that the prestalk reporter constructs *ecmAO/lacZ* and *ecmB/lacZ* were efficiently induced by DIF-1 in wild-type cells but not in *dimA*⁻ cells (Fig. 1D). Furthermore, the prespore marker construct *cotB/lacZ* was strongly repressed by DIF-1 treatment in wild-type cells, but

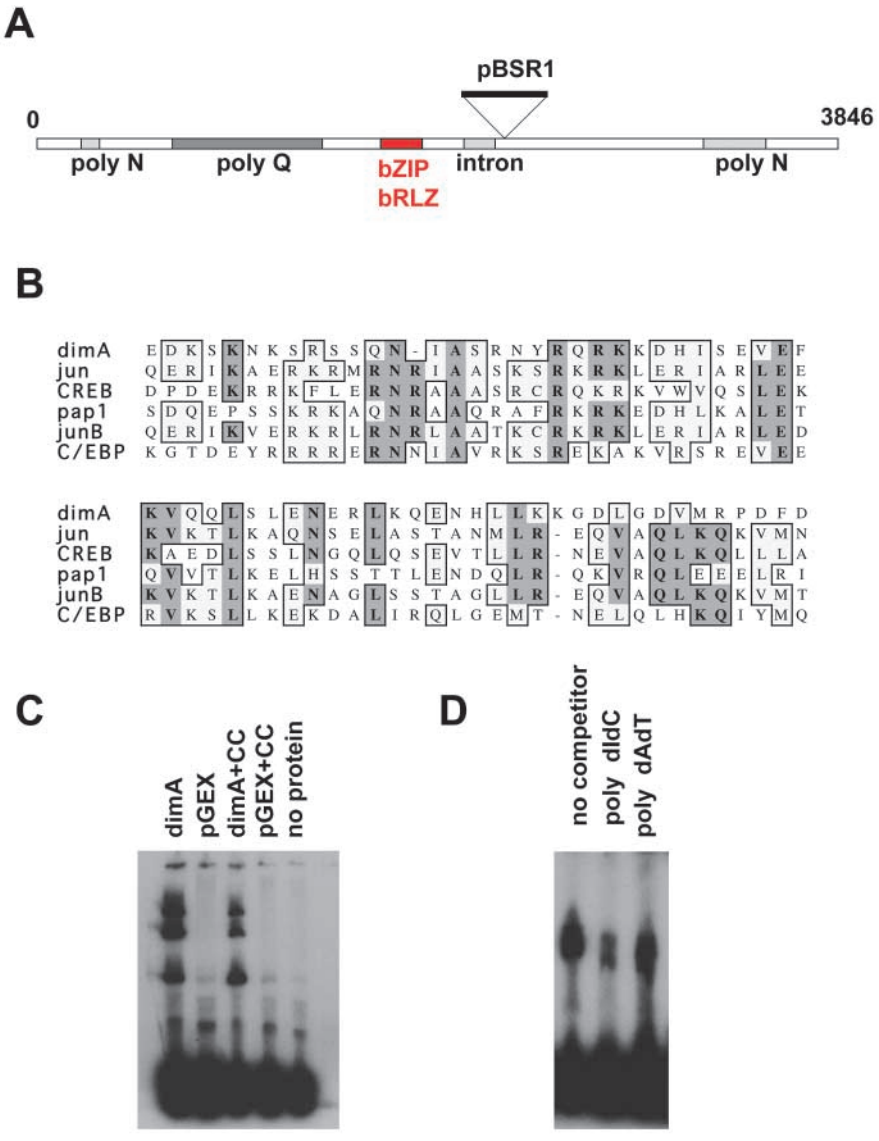

Fig. 2. Structure and function of the *dimA* gene. (A) Site of insertion of the disruption plasmid and structure of the *dimA* gene. The pBSR1 disruption vector was recovered from the *dimA*⁻ mutant by plasmid rescue. The insertion lies in the second exon of a 3846 bp gene, encoding a putative 1242 amino acid protein. The protein contains long stretches of asparagine (N) and glutamine (Q) residues as indicated. The region between amino acids 545-676 shows extensive homology to the DNA-binding and dimerization domains of bZIP and bRLZ transcription factors. (B) Sequence alignment of the putative *dimA* DNA-binding and dimerization domain with examples of bZIP and bRLZ proteins from human, mouse, *Drosophila* and yeast (gi:19745184, gi:10835484, gi:135304, gi:17647933 and gi:135867). (C) DimA binds DNA. Binding of total soluble protein extracts prepared from bacteria expressing the putative DimA DNA-binding/dimerization domain (*dimA*) fused to GST was compared with extract from cells expressing GST alone (pGEX). Equal amounts of total protein were assayed and loaded. A 48 bp fragment from the 3′ half of the minimal *ecmO/lacZ* promoter was used as a probe and poly dAdT was included as a non-specific competitor. The probe is only retarded when mixed with DimA-expressing extract. The amount of binding is reduced by the addition of a 10-fold excess of unlabeled oligonucleotide (CC). (D) The effects of varying non-specific competitor species on DNA binding. Strongest binding is evident in the absence of non-specific competitor. The addition of poly dIdC strongly reduces binding, whereas poly dAdT addition results in a small reduction in binding. Fewer retarded bands are visible (compared with C), as electrophoresis was performed at 4°C to stabilize protein DNA interactions.

was unaffected in *dimA*⁻ cells (Fig. 1D). Therefore, in all conditions tested, *dimA* is required for cellular responses to DIF-1.

dimA encodes a bZIP or bRLZ transcription factor

We identified the disrupted gene by plasmid rescue and found the insertion to lie in the second exon of a gene with an ~3700 bp ORF (Fig. 2A). The predicted protein product of the *dimA* gene shows strong sequence similarity over 66 amino acids to the DNA-binding and dimerization domains of bZIP and bRLZ transcription factors (Fig. 2B), which are required for the activation and repression of gene expression in response to a wide variety of signals (Hurst, 1995; Jakoby et al., 2002).

In order to determine if the sequence similarity reflects functional homology, we tested whether the predicted DNA-binding and dimerization domain of the DimA protein could bind DNA. As the *ecmO/lacZ* reporter gene requires DIF-1 for its normal expression (Thompson and Kay, 2000b), and sequences proximal to the transcriptional start site are necessary for its efficient expression in wild-type cells (Early et al., 1993), we chose these sequences to assay binding. The putative DNA-binding/dimerization domain was expressed as a GST-fusion protein in *E. coli* and was found to strongly bind sequences from this promoter in vitro, when compared with control extracts (Fig. 2C). As the probe contains regions of both high A/T and G/C content, we used competition assays to determine binding specificity. Both poly dAdT and poly dIdC reduced binding when compared with no cold competitor. However, we found that the reduction in binding was significantly greater in the presence of poly dIdC (Fig. 2D), indicating that most of the specific binding is likely to be in regions of higher G/C content. Taken together, these findings suggest that *dimA* encodes a DNA-binding protein with a likely binding preference for G/C rich sequences.

dimA⁻ and *dmtA*⁻ show similar developmental phenotypes

The experiments with cells in culture show that *dimA* is required for each response to DIF-1 that we have investigated. To determine the function of *dimA* in normal development, we investigated the developmental phenotype of the *dimA*⁻ mutant, comparing it as appropriate with the *dmtA*⁻ mutant, which is specifically defective in DIF-1 synthesis.

dimA⁻ cells grow normally in axenic medium, but when starved on buffered agar exhibit clear morphological defects. Aggregation takes place with relatively normal timing, although there is a tendency for the streams to break up (data not shown). However, clear defects are observed at the finger and slug stages of development. The *dimA*⁻ mutant fingers are extremely long and thin, resulting in the formation of similarly defective migratory slugs (Fig. 3D). Furthermore, after a period of migration, *dimA*⁻ slugs tend to break apart (Fig. 3E). Finally, at the time of fruiting body formation, rather than the stalk lifting the sorus from the agar, as in the wild-type, stalks and spores lie on the surface of the agar, resulting in plates with an untidy appearance (Fig. 3F). Time-lapse microscopy reveals that this is due to both the collapse of the comparatively fine stalks and repeated attempts at culmination (data not shown). The overall morphology of the mutant is strikingly similar to that of the 'DIF-less' *dmtA*⁻ mutant (Fig. 3G-I), supporting the notion that *dimA*, like *dmtA*, functions in the DIF signal transduction pathway.

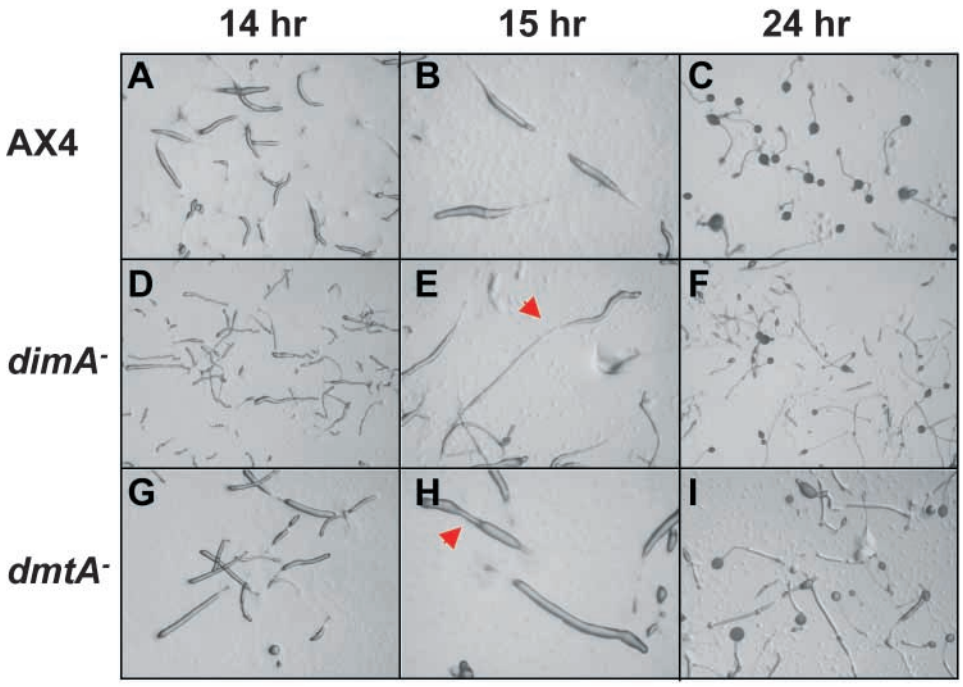

Fig. 3. The developing *dimA*⁻ mutant phenocopies the *dmtA*⁻ mutant. (A,D,G) *dimA*⁻ and *dmtA*⁻ fingers tend to appear long and thin compared with wild type. (B,E,H) Migratory slugs of both mutants are also long and thin, and have a tendency to break apart (arrowheads). (C,F,I) After 24 hours the wild type has produced fruiting bodies, but both mutants produce fewer normal fruiting bodies, although stalks and spores litter the agar.

Developmental gene expression in the *dimA*⁻ mutant

The observation that DIF-inducible prestalk markers are expressed in the *dmtA*⁻ mutant resulted in the proposal that DIF-1 is unlikely to be absolutely required for their expression (Thompson and Kay, 2000b). However, because it could be argued that the *dmtA*⁻ mutant may produce low levels of DIF-1, which are sufficient to drive the expression of prestalk genes, it was also important to determine whether a mutant that shows no measurable DIF response showed similar profiles of gene expression. Therefore, we examined the expression profiles of a panel of developmentally regulated genes in the *dimA*⁻ mutant by northern blotting.

First, we found that the timing of the initiation of development appears normal, as indicated by the repression of *cprD* transcripts. Both the wild type and the mutant express *cprD* during growth, and

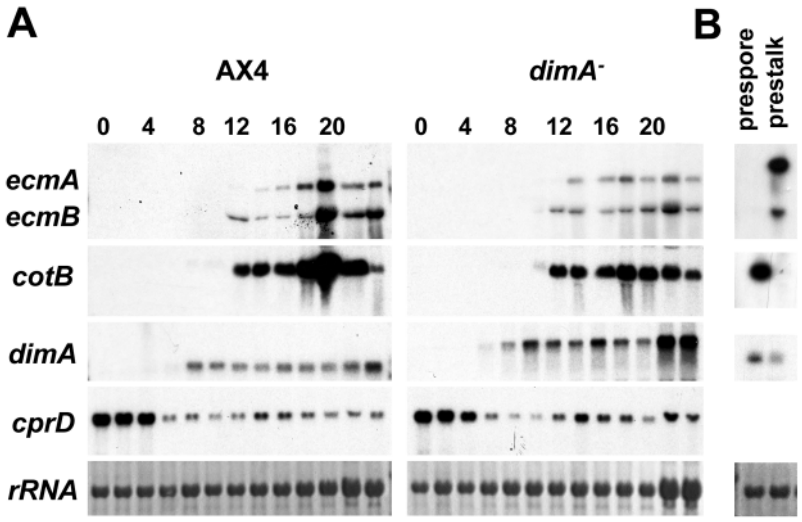

Fig. 4. Gene expression profiles in the *dmtA*⁻ mutant. (A) Developmental time course of gene expression. The *ecmA* and *ecmB* (prestalk), *cotB* (prespore) and *cprD* (growth/differentiation transition) markers are expressed with comparable levels and timing in *dimA*⁻ and wild-type cells, from 0-24 hours. The ~5 kb *dimA* transcript is also developmentally regulated in wild-type cells but is absent in the mutant. However, a larger transcript (>7 kb), due to a readthrough transcription into the blasticidin resistance cassette, is detectable. Methylene Blue staining of ribosomal RNA (rRNA) is shown as a loading control. (B) *dimA* transcripts are expressed in both prespore and prestalk cells at the slug stage of development, although the highest levels are detectable in prespore cells. The purity of the isolated cell populations is indicated by the great enrichment of *ecmA* and *ecmB* in prestalk cells, and *cotB* in prespore cells.

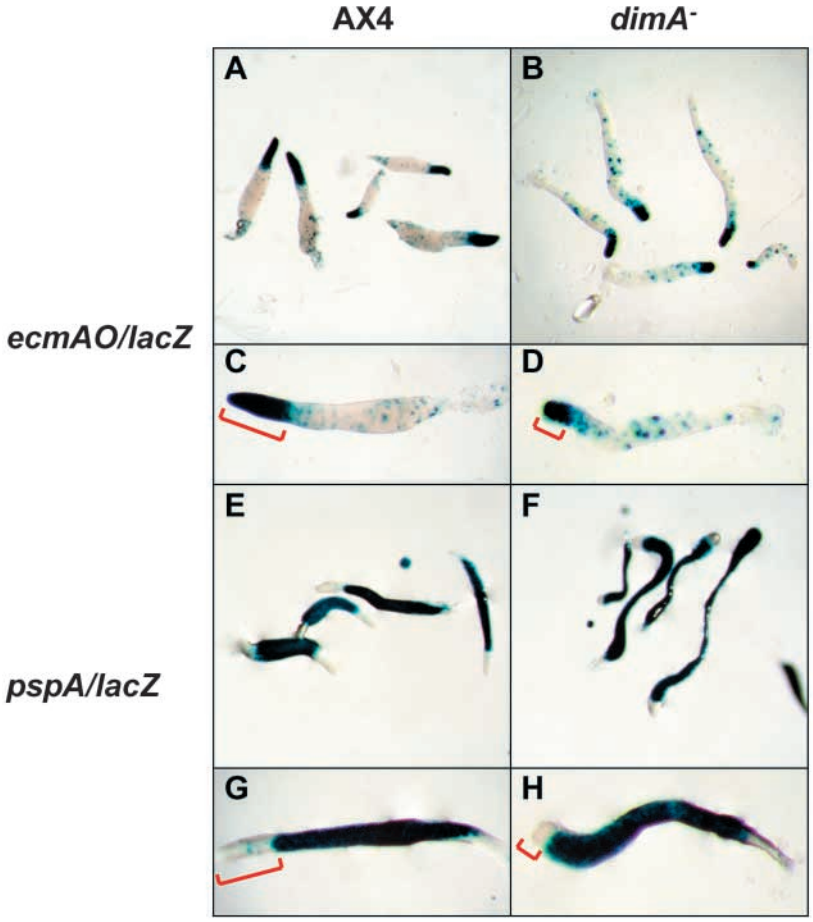

Fig. 5. The *dimA*⁻ mutant exhibits pstO patterning defects. (A-D) Expression pattern of the prestalk specific *ecmAO/lacZ* reporter at the late finger stage of development. Mutant slugs show a greatly reduced zone of expression. (A,B) Several representative samples are shown. (C,D) Higher magnification highlights the shortening of the prestalk zone in the mutant (brackets). (E-H) Expression pattern of the prespore specific *pspA/lacZ* reporter at the late finger stage of development. (E,F) Representative samples show that the marker is expressed throughout the prespore zone of wild-type and mutant slugs. (G,H) Higher magnification highlights the small size of the unstained prestalk zone in mutant structures (brackets).

downregulate its transcripts during the first 6 hours of development (Fig. 4A). Second, we tested the expression of cell-type-specific products, namely the expression of the prestalk markers *ecmA* and *ecmB* (which can be induced by DIF-1) and prespore marker *cotB* (which can be repressed by DIF-1). Expression of these markers in the mutant was unaffected both in terms of timing and levels of expression (Fig. 4A). Therefore, like the *dmtA*⁻ mutant, the DIF non-responsive mutant *dimA*⁻ expresses prestalk and prespore markers.

We also tested the expression profile of the *dimA* mRNA. Transcripts were first observed weakly at 2-4 hours after starvation. Expression levels rose to a peak at 8 hours and were sustained throughout the remainder of development. The expression profile of *dimA* therefore closely resembles that of DIF-1 biosynthesis (Thompson and Kay, 2000b). The endogenous *dimA* transcript was absent in the mutant, but a larger transcript was visible, which is due to transcriptional readthrough into the blasticidin resistance cassette. Nevertheless, we believe that this insertion results in a null or severe loss-of-function of the *dimA* gene. In support of this, we have isolated additional *dimA*⁻ alleles with small or large deletions of the *dimA* gene, and each of these shows an identical phenotype to the *dimA*⁻ mutant described here (C.R.L.T. and G.S., unpublished).

Finally, we tested whether the *dimA* gene is expressed in a cell-type-specific manner. mRNA was extracted from separated prestalk and prespore cells at the slug stage of development (Ratner and Borth, 1983), and *dimA* transcripts were detected by northern blot. We found *dimA* mRNA in both prestalk and prespore cells, with the highest levels of expression in prespore cells (Fig. 4B). The developmental timing and broad expression of *dimA* is therefore consistent with a role in DIF-1 signaling.

dimA⁻ exhibits defects in pstO cell differentiation

Although prestalk and prespore transcripts were detected on northern blots, this gives no information about spatial patterns of gene expression. As the *dmtA*⁻ mutant shows non-cell autonomous defects in pstO differentiation, whereas pstA differentiation appears normal (Thompson and Kay, 2000b), we tested whether *dimA* exhibits similar defects. First, as the *ecmAO/lacZ* marker is expressed in both pstA and pstO cells in wild-type slugs, a shortening of its zone of expression would be expected in the mutant as a result of expression in pstA cells but not in pstO cells. Indeed, similar results were described in *dmtA* mutant slugs (Thompson and Kay, 2000b). Consistent with this, we found the *ecmAO/lacZ* staining region in *dimA*⁻ mutant slugs to be approximately 50% shorter than that of wild-type control transformants (Fig. 5A-D).

Second, an expansion of the prespore zone, which is marked by *pspA/lacZ* staining, might be expected to result from the expression of this marker in the part of the slug normally occupied by pstO cells. Although this has not been demonstrated in the *dmtA*⁻ mutant, a decrease in the number of prestalk cells with a concomitant rise in prespore cell number has been described (Thompson and Kay, 2000b). We indeed found the expression domain of *pspA/lacZ* to be markedly increased in size in *dimA*⁻ slugs (Fig. 5F,H). This is especially evident when the small size of the unstained prestalk zone in the mutant (Fig. 5H) is compared with that of wild-type controls (Fig. 5G). Therefore, the specific defect in pstO cell differentiation, similar to that observed in the *dmtA*⁻ mutant, further supports the idea that *dimA* functions to regulate cellular responses to DIF-1.

dimA⁻ produces DIF-1 and *dmtA*⁻ responds to DIF-1

All the above results support a model in which *dimA* is required to transduce the DIF-1 signal. However, an alternate explanation for the similarity between the phenotypes of the *dimA*⁻ and *dmtA*⁻ mutants is that the DIF response is required for DIF production, or vice versa. We therefore sought to determine whether *dimA*⁻ produces DIF-1 and whether the *dmtA*⁻ mutant responds to DIF-1. First, we measured DIF-1 production in *dimA*⁻ cells by developing the mutant on agar containing $^{36}Cl^-$, before extraction of organic compounds and TLC separation. The results, in Fig. 6A, clearly illustrate that *dimA*⁻ produces DIF-1 and its breakdown product DIF-3. Although the levels are slightly lower in the mutant than in the wild type, this is likely to be due to the slight developmental delay exhibited by *dimA*⁻, especially at later stages of development. Second, we tested the response of the *dmtA*⁻ mutant to DIF-1 in 8-Br-cAMP monolayers. The *dmtA*⁻ mutant shows a response indistinguishable from that of wild-type cells, either when measured in terms of stalk cell induction (Fig. 6B) or spore cell repression (Fig. 6C).

Therefore, *dimA* is required to transduce the DIF-1 signal, but not to produce the signal itself. Furthermore, as *dmtA*⁻ cells respond to DIF-1 but do not produce DIF-1, the similarity between the *dimA*⁻ and *dmtA*⁻ mutants probably lies in their respective requirement for DIF-1 signal transduction and production.

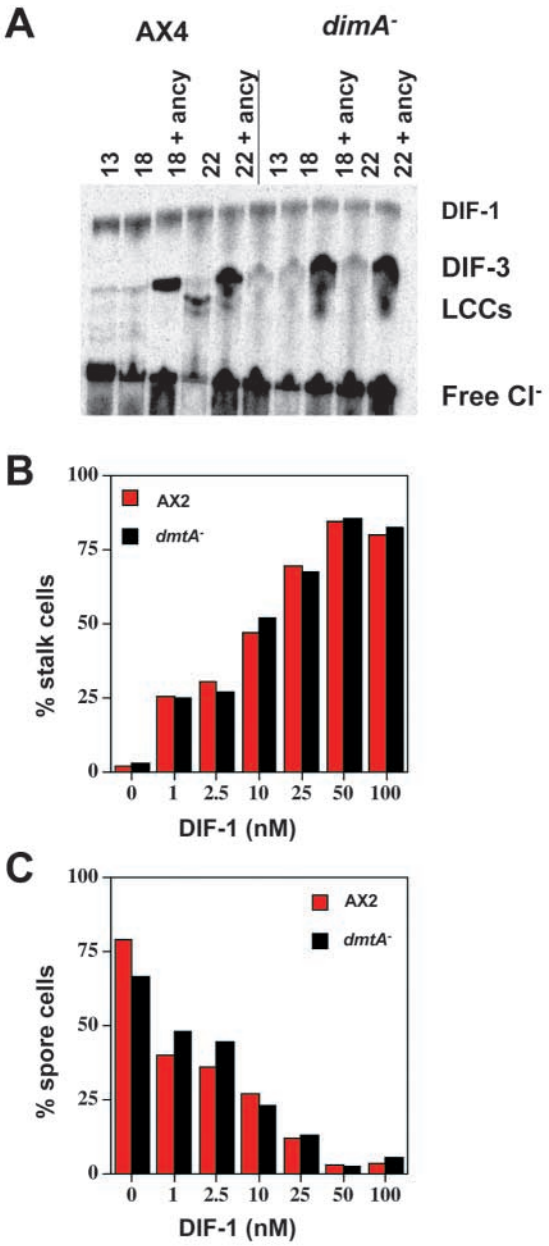

Fig. 6. Measurement of DIF biosynthesis in *dimA*⁻, and DIF response in *dmtA*⁻. (A) *dimA* produces normal levels of cell-associated DIF-1. Cells were developed for the indicated times (hours) on agar containing $^{36}Cl^-$ and labeled compounds extracted with organic solvents, resolved by TLC and detected using a phosphorimager. The inclusion of the P450 inhibitor ancymidol (ancy) in the agar at the time points indicated results in the build-up of the DIF-1 breakdown product DIF-3. LCCs are late chlorinated compounds produced by stalk cells. (B,C) Stalk cell induction and spore cell repression in *dmtA*⁻. The efficiency of stalk cell induction (B) or spore cell repression (C) by DIF-1 was measured in 8-Br-cAMP monolayers. No difference was observed between *dmtA*⁻ and wild type. Results shown are from a representative experiment. The experiment was performed at least three times.

dimA exhibits cell autonomous defects

Although there are great similarities between the developmental phenotypes of the *dimA*⁻ and *dmtA*⁻ mutants, if *dimA*⁻ is defective in DIF-1 responses, then any defects would be predicted to be cell autonomous. We therefore tested this hypothesis.

First, we compared the effects of exogenously added DIF-1 on the development of the *dmtA*$^-$ and *dimA*$^-$ mutants. Consistent with previous reports (Thompson and Kay, 2000b), the developmental defects of the *dmtA*$^-$ mutant are effectively rescued by development on DIF-1 agar (Thompson and Kay, 2000b) (Fig. 7A;g-i). However, despite morphological similarity with *dmtA*$^-$, the *dimA*$^-$ mutant is not rescued by development on agar containing 100 nM DIF-1. Most notably, *dimA*$^-$ slugs remain long, thin and broken (Fig. 7A;d,e), whereas *dmtA*$^-$ slugs are of wild-type appearance (compare with Fig. 7A;g,h; and see Fig. 3). In addition, the culmination defects are rescued in the *dmtA*$^-$ mutant but not in *dimA*$^-$ (Fig. 7A;c,f,i). Furthermore, unlike wild-type cells, which show a developmental delay on DIF agar, *dimA*$^-$ cells appear largely unaffected. Therefore, the morphological defects of the *dimA*$^-$ mutant are unaffected by exogenously added DIF-1. This is consistent with the idea that *dimA* is required to transduce the DIF-1 signal rather than to produce it.

Second, we tested whether the *dimA*$^-$ mutant exhibits cell autonomous defects when developed in chimeras with wild-type cells. *dimA*$^-$ or wild-type cells were labeled with the ubiquitously expressed *actin15/lacZ* marker, mixed with unlabeled cells and cell fate addressed by the position of the labeled cells in the resulting chimeric slugs. These mixing experiments revealed several interesting behaviors. When a small proportion of marked wild-type cells were mixed in with a majority of *dimA*$^-$ cells, the wild-type cells preferentially populate the pstO region and the front of the prespore zone (Fig. 7B;c), as might be expected if the *dimA*$^-$ mutant were unable to form pstO cells. In the reverse experiment, *dimA*$^-$ mutant cells were found scattered throughout the body of largely wild-type chimeric slugs. However, the mutant cells avoided the pstO region and the front part of the prespore zone and congregated preferentially in the rear of the slug (Fig. 7B;d). To test whether these *dimA*$^-$ cells were bone fide prespore cells, rather than misplaced prestalk cells, we used *cotB/lacZ*-marked mutant cells. When *dimA*$^-$ cells carrying the prespore cell specific *cotB/lacZ* reporter gene were developed in chimeras with unlabelled wild-type cells, the marked cells were found at the rear of the prespore zone (Fig. 7C). This finding defines the mutant cells in the posterior region as being prespore cells. These results further highlight the

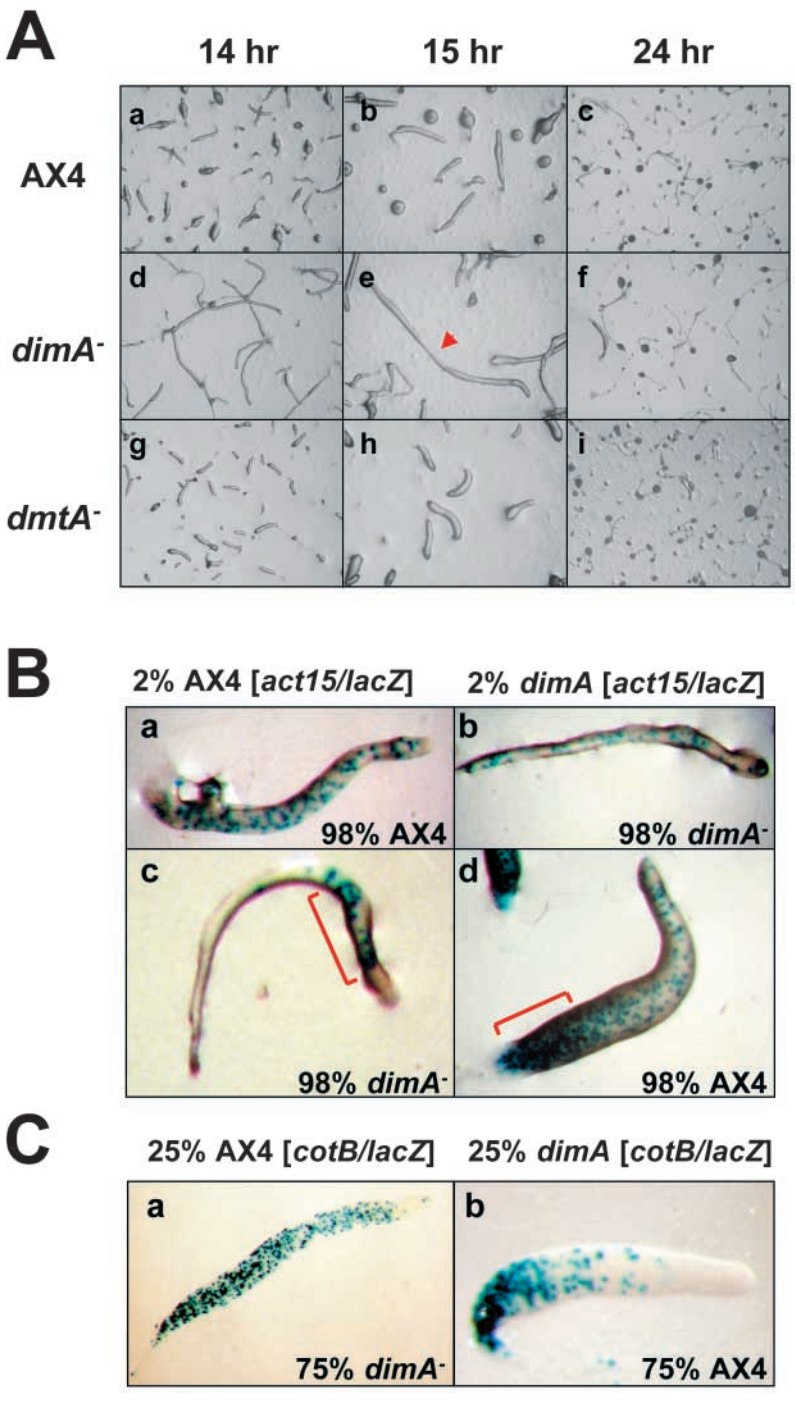

Fig. 7. Cell autonomous defects of the *dimA*$^-$ mutant. (A) Development on DIF-agar. 100 nM DIF-1 slightly slows the development of the wild type as some tip mounds are still visible up to 15 hours (a,b), although all structures ultimately fruit normally (c). *dimA*$^-$ development is unaffected by the addition of exogenous DIF-1, as slugs remain long and thin (d,e) with a tendency to break (arrowhead), and fruiting bodies still lie on the surface of the agar (f). By contrast, 100 nM DIF-1 is sufficient to rescue the phenotype of the *dmtA*$^-$ mutant, as both slugs (g,h) and fruiting bodies (i) appear normal. (B) *dimA*$^-$ cell-autonomous defects in chimeras with wild-type cells. Wild-type or *dimA*$^-$ mutant cells were transformed with the constitutively expressed *actin15*/*lacZ* marker and mixed with unlabeled cells. (a,b) Control samples illustrate that expression of the marker itself does not affect cell fate or position (c) Labeled AX4 cells localize to the pstO and anterior prespore zones in chimeras with unlabeled *dimA*$^-$ mutant cells. (d) Labeled *dimA*$^-$ mutant cells are strongly enriched in the posterior prespore zone in chimeras with unlabeled wild-type cells. (C) Expression of the *cotB/lacZ* prespore marker in chimeric slugs. (a) *cotB*/*lacZ*-expressing AX4 cells are scattered throughout the prespore zone of chimeras with unmarked *dimA*$^-$ cells. (b) *dimA*$^-$ cells that express the *cotB*/*lacZ* prespore marker are predominantly found at the rear of the prespore zone in chimeras with unmarked wild-type cells.

importance of DIF signaling and *dimA* function in the differentiation of pstO cells. They also hint at further complexities in the organization of the slug, and reveal an additional unexpected function for *dimA* (or DIF signaling) in prespore cell differentiation.

Discussion

A transcription factor required for DIF signaling

This work describes the identification and molecular characterization of a gene (*dimA*) required for the cellular response to DIF-1. First, we find that the mutant shows none of the known responses to DIF in monolayer assays, shaken suspension of dissociated cells, or when developed on agar containing DIF-1. Second, the mutant shows strikingly similar morphological and patterning defects to the DIF biosynthesis mutant *dmtA*$^-$. Third, despite similarities with *dmtA*$^-$, the *dimA*$^-$ mutant produces normal levels of DIF-1. Finally, the gene disrupted in the *dimA*$^-$ mutant shows sequence similarity to the bZIP/bRLZ classes of transcription factors. In addition, a bacterially expressed DimA protein encoding the predicted DNA-binding/dimerization domain binds DNA in vitro. Taken together, these results strongly support the idea that *dimA* is required to integrate the cellular response to DIF signaling through the control of DIF target gene expression.

How is *dimA* activity regulated?

DIF-1 induces prestalk markers and represses prespore markers both in vivo and in cell culture (Berks and Kay, 1990; Thompson and Kay, 2000b; Williams et al., 1987). However, there are no reports of common elements required to mediate the transcriptional effects of DIF in prestalk and prespore promoters. It was therefore unknown whether both target gene activation and repression were mediated by the same transcription factors. The results described in this paper suggest that *dimA* is a common factor in both pathways, as it is required for both the activation and repression of DIF-responsive gene expression. This raises the question of how the activity of DimA might be controlled in order to function as both an activator and repressor. One possibility arises from the sequence similarity of DimA to bZIP/bRLZ transcription factors. bZIP/bRLZ proteins bind DNA as obligate dimers. Their ability to form heterodimers and the choice of partners is important in the regulation of their activity (Lee, 1992). As DimA represents the first functionally characterized bZIP/bRLZ transcription factor in *Dictyostelium*, it is unknown whether it is also able to form heterodimers. However, searches of the public databases reveal that a number of related proteins are likely to be encoded by the *Dictyostelium* genome (C.R.L.T. and G.S., unpublished).

bZIP/bRLZ transcription factors have been described in a wide variety of organisms (Chinenov and Kerppola, 2001; Hurst, 1995; Jakoby et al., 2002). The largest number of putative bZIP/bRLZ proteins has been identified in plants (Jakoby et al., 2002); however, to date, the signaling pathways regulating most bZIP transcription factors in plants are largely uncharacterized. Similarly, little is known about the genes required for DIF signaling beyond *dmtA* (signal production) and *dimA* (transcription factor). However, a number of factors that influence DIF signaling, or correlate with cell fate choice, have already been described. These include intracellular calcium levels, intracellular pH, growth history and cell cycle position (Azhar et al., 2001; Gomer and Firtel, 1987; Gross et al., 1983; Leach et al., 1973; Schaap et al., 1996; Thompson and Kay, 2000a; Weijer et al., 1984). It will therefore be of interest to determine whether these factors affect *dimA* directly, both to further our understanding of DIF signaling and to shed light on the regulation of bZIP/bRLZ activity in other organisms. Our selection strategy provides a means to identify other genes required for DIF signal transduction and should enable us to identify such factors.

Does DIF play a role in prespore cells?

DIF-1 has been widely viewed as a prestalk inducer, but most DIF-1 biosynthesis takes place in prespore cells (Kay and Thompson, 2001). This scheme requires that prespore cells become somewhat DIF-1 insensitive. However, prespore cells do not lose their ability to respond to DIF-1 altogether. For example, dissociated prespore cells downregulate prespore markers when treated with DIF-1 (Berks and Kay, 1990). Furthermore, low levels of DIF-1 have been reported to stimulate the expression of prespore markers in cell culture (Oohata, 1995). The results presented here provide evidence that *dimA* is the link between the prespore and prestalk responses to DIF-1. First, *dimA* is required for the repression of the prespore gene *cotB*, in addition to being required for the activation of prestalk markers. Second, *dimA* transcripts are expressed in both prestalk and prespore cells at the slug stage of development, and prespore cells express the highest levels of *dimA*. Finally, the *dimA* mutant exhibits cell autonomous defects in both prestalk and anterior prespore differentiation. Taken together, these results suggest that *dimA* is present in and required for normal prespore cell differentiation.

If *dimA* is indeed dedicated to the regulation of DIF-1 signaling, as might be inferred from the phenotypic similarities between the *dimA*$^-$ and *dmtA*$^-$ mutants, then our results suggest a novel role for DIF signaling in prespore cell differentiation.

DIF-1 signaling and pstO cell function

Studies of the patterns of marker gene expression in the *dmtA*$^-$ mutant suggest that DIF-1 signaling is required for the normal differentiation of pstO cells, but not for the differentiation of pstA cells (Thompson and Kay, 2000b). As we found the classical markers of these cell types to be poorly expressed in the AX4 parental strain of the *dimA* mutant, we were unable to test this directly (C.R.L.T. and G.S., unpublished). Nevertheless, the patterns of expression of more robustly expressed prestalk and prespore markers reveal the only detectable cell-type defects in *dimA*$^-$ to be consistent with defects in pstO cell differentiation, whereas pstA differentiation is unaffected. These observations strengthen the idea that DIF-1 is only required for the differentiation of pstO cells.

Little is known about the role of pstO cells during normal development. However, these studies further highlight the possibility that the defects in pstO cell differentiation in the *dmtA*$^-$ and *dimA*$^-$ mutants can explain the major morphological defects visible at the slug stage of development. For example, as mutant slugs tend to break apart, it might be proposed that pstO cells play a role in maintaining slug integrity. In order to understand the role of pstO cells during normal development, it will be important to identify the compliment of genes

expressed specifically in this cell type. As it seems likely that a number of these genes will be directly regulated by *dimA*, this mutant provides another valuable tool for the study of pstO differentiation and function.

DNA binding and *dimA* target genes

Although bacterially expressed DimA protein binds a fragment from the *ecmO* promoter, it is unclear whether this binding is functionally relevant in the context of the minimal region required for pstO gene expression, as in subsequent mutational studies we were unable to pinpoint the exact residues bound (see Materials and methods; C.R.L.T and G.S., unpublished). However, we do not believe the binding to be non-specific, as it can be detected in the presence of excess non-specific DNA. It is more likely to reflect that at present we do not know (1) whether the *ecmO* promoter is a direct DimA target gene, (2) whether DimA normally binds DNA as a homodimer, or, consequently, (3) the optimal DimA-binding site. Therefore, in order to understand DimA DNA binding and its regulation, it will first be important to identify true DimA target genes, and to define the DimA- or DIF-response elements in these.

dimA and pattern formation

It has been proposed that pstO cells initially differentiate scattered amongst prespore cells in response to DIF-1 (Early et al., 1995; Thompson and Kay, 2000b), and then subsequently sort out as a result of differential adhesion or chemotaxis (Clow et al., 2000; Early et al., 1995; Matsukuma and Durston, 1979; Siu et al., 1983; Tasaka and Takeuchi, 1979; Traynor et al., 1992). The identification and study of the *dimA*$^{-}$ mutant, together with studies on the *dmtA*$^{-}$ mutant, provide important tools to dissect the regulation of this developmental mechanism in *Dictyostelium*. For example, an understanding of how the various inputs might generate stochastic differences in *dimA* activation could explain why a subset of cells adopt the pstO rather than the prespore cell fate. As this developmental mechanism is likely to be used in other organisms, these studies will provide insights into conserved features of its mechanism and regulation.

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