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Cell type specificity of a diffusible inducer is determined by a GATA family transcription factor

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One poorly understood mechanism of developmental patterning involves the intermingled differentiation of different cell types that then sort out to generate pattern. Examples of this are known in nematodes and vertebrates, and in *Dictyostelium* it is the major mechanism. However, a general problem with this mechanism is the possibility that different inducers are required for each cell type that arises independently of positional information. Consistent with this idea, in *Dictyostelium* the signalling molecule DIF acts as a position-independent signal and was thought only to regulate the differentiation of a single cell type (pstO). The results presented here challenge this idea. In a novel genetic selection to isolate genes required for DIF signal transduction, we found a mutant (*dimC*$^{-}$) that is a hypomorphic allele of a GATA family transcription factor (*gtaC*). *gtaC* expression is directly regulated by DIF, and GtaC rapidly translocates to the nucleus in response to DIF. *gtaC*$^{-}$ null cells showed some hallmark DIF signalling defects. Surprisingly, other aspects of the mutant were distinct from those of other DIF signalling mutants, suggesting that *gtaC* regulates a subset of DIF responses. For example, pstO cell differentiation appeared normal. However, we found that pstB cells were mislocalised and the pstB-derived basal disc was much reduced or missing. These defects are due to a failure to respond to DIF as they are phenocopied in other DIF signalling mutants. These findings therefore identify a novel small-molecule-activated GATA factor that is required to regulate the cell type-specific effects of DIF. They also reveal that a non-positional signal can regulate the differentiation of multiple cell types through differential interpretation in receiving cells.

KEY WORDS: DIF, Dictyostelium, GATA, Pattern formation, Sorting out

INTRODUCTION

Multicellular development requires the specialization and correct spatial organization of different cell types. A limited number of patterning mechanisms are thought to regulate this process. For example, unequal partitioning of cytoplasmic determinants between daughter cells by asymmetric cell division can direct their development along different lines (Betschinger and Knoblich, 2004), or short-range signalling can specify cells at a local level and when reiterated produces highly ordered structures (Freeman, 1997; Meinhardt and Gierer, 2000; Simpson, 1990). In addition, some regions of the embryo produce diffusible morphogens that generate 'positional information' because of their local concentration to drive long-range patterning (Wolpert, 1996).

Each of these mechanisms is relatively well characterized at the molecular level. However, a less well understood mechanism has also been shown to operate. This depends on the specification of intermingled cell types in a position information-independent fashion, followed by their sorting out to generate pattern. Such a mechanism has the advantages that it is essentially scale invariant and can result in symmetry breaking without pre-existing organizers. However, it is difficult to envisage how a position-independent sorting mechanism could generate complex patterns. By contrast, in morphogen-based positional signaling, a single graded signal that cells respond to with serial thresholds can readily generate several fates. Despite this, there is now good evidence that patterning by sorting out operates in diverse developmental systems. For example, this model has been used to explain epiblast and primitive endoderm lineage formation during mouse development (Yamanaka et al., 2006). Direct observations of intermingled differentiation have also been made during the development of the chick and mouse limb bud (Altabef et al., 1997; Guo et al., 2003). Furthermore, sorting out plays a key role in *C. elegans* pattern formation, an organism previously thought to exhibit a highly determinate mode of development (Bischoff and Schnabel, 2006; Schnabel et al., 2006). By far the best-studied example of this patterning mechanism, however, is seen during the developmental cycle of the social amoeba *D. discoideum*. Therefore, *Dictyostelium* provides a tractable model system with which to study the molecular regulation of this patterning mechanism.

When *Dictyostelium* amoebae starve, several thousand cells aggregate and begin a program of multicellular development. Different prestalk and prespore cell types have been defined by their position in the migratory slug and fruiting body, as well as the genes they express (Early et al., 1993; Jermyn et al., 1989; Maeda et al., 2003; Maruo et al., 2004). From front to back of the slug, these are the pstA, pstO and prespore cells. In addition, a core of pstAB cells is found near the slug tip and pstB cells are largely clustered around the prestalk-prespore boundary. These cell types go on to form the stalk and spore cells of the terminally differentiated fruiting body, as well as ancillary supporting structures such as the upper cup, lower cup and basal disc (Williams, 2006).

Although the prestalk and prespore cell types adopt a positional arrangement in the migratory slug, at earlier stages no such pattern is present (Thompson et al., 2004b; Williams et al., 1989). Instead, prestalk and prespore cells are scattered throughout the mound produced by aggregation. Then, as the mound elongates into a standing slug, they segregate into distinct zones (Esch and Firtel, 1991; Ozaki et al., 1993; Williams et al., 1989). This change is due to sorting because the different cell types remain intermingled when the cells are prevented from moving within the aggregate (Thompson et al., 2004b). Furthermore, when cell type-specific GFP

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markers are used to follow different cell types in real time during pattern formation, individual prestalk cells move directly towards the forming prestalk mass (Clow et al., 2000; Nicol et al., 1999; Takeuchi et al., 1988). This suggests they sort by differential chemotaxis (Early et al., 1995; Matsukuma and Durston, 1979; Siegert and Weijer, 1995; Traynor et al., 1992). However, prestalk and prespore cells are also differentially adhesive (Lam et al., 1981) and cell-adhesion mutants often have sorting defects (Dynes et al., 1994; Wong et al., 2002) making it likely that differential adhesion assists in sorting.

In order to understand how this patterning mechanism operates, great efforts have been made to identify the signalling molecules that regulate it. One such molecule is the chlorinated alkyl phenone DIF-1 (hereafter referred to as DIF). DIF was identified as a molecule produced by developing *Dictyostelium* cells that can induce amoebae to differentiate as stalk cells in monolayer cell culture assays (Morris et al., 1987). DIF treatment also results in the repression of spore cell differentiation, and in the induction and repression of prestalk and prespore markers, respectively (Early and Williams, 1988; Kay and Jermyn, 1983). Furthermore, when cells are biased towards the prestalk cell fate by manipulation of growth conditions or by cell cycle position, these cells become more sensitive to DIF (Thompson and Kay, 2000a). These findings have led to the proposal that heterogeneity in DIF responsiveness may underlie initial cell type divergence. Consequently, much recent effort has focused on identifying molecules required for DIF signal transduction.

Several mutants in DIF synthesis and responses have been identified. *dmtA*$^{-}$ and *stlB*$^{-}$ are mutants in genes that encode a methyltransferase and polyketide synthase required for DIF-1 biosynthesis (Austin et al., 2006; Thompson and Kay, 2000b). In addition, bZIP (DimA and DimB), myb (MybE) and STAT (STATc) family transcription factors have been identified that regulate DIF responsive gene expression (Fukuzawa et al., 2001; Fukuzawa et al., 2006; Huang et al., 2006; Thompson et al., 2004a; Zhukovskaya et al., 2006). Studies of the common phenotypes of these mutants have led to a clearer understanding of the role of DIF during normal development. For example, mutant slugs are often long and thin and break apart, while fruiting body morphogenesis is aberrant. Importantly, the phenotypes of the *dmtA*$^{-}$ and *stlB*$^{-}$ DIF biosynthesis mutants are rescued by addition of exogenous DIF (Austin et al., 2006; Thompson and Kay, 2000b). Rescue is effective over almost a 1000-fold concentration range, suggesting that concentration gradients of DIF are unimportant. In addition, DIF has only been shown to affect pstO cell differentiation, while the expression of other markers is normal (Fukuzawa et al., 2001; Fukuzawa et al., 2006; Huang et al., 2006; Thompson et al., 2004a).

A key feature of DIF responses involves the regulation of transcription factor activity. For example, STATc is tyrosine phosphorylated in response to DIF (Fukuzawa et al., 2001), and DimA, DimB and STATc rapidly and transiently localize to the nucleus in response to DIF stimulation (Fukuzawa et al., 2001; Huang et al., 2006; Zhukovskaya et al., 2006). Although regulated nuclear localization is an evolutionarily conserved mechanism of gene regulation, and is well understood for several classes of transcription factor, for others such as bZIPs, it is poorly characterized (Cartwright and Helin, 2000). Furthermore, localization of STATc appears to be regulated by a non-canonical pathway as no JAK is encoded by the *Dictyostelium* genome (Eichinger et al., 2005). Consequently, studies of the effects of DIF on transcription factor subcellular localization may provide insights into novel regulatory mechanisms of transcription factor activation.

Although the DIF signalling pathway and the role of DIF has begun to unravel, our understanding is far from complete. Many other regulators of the DIF signalling pathway await identification. The upstream kinase(s) that regulate STATc activity are unknown (Fukuzawa et al., 2001), as are the regulators of DimA and DimB nuclear localization (Fukuzawa et al., 2001; Huang et al., 2006; Thompson et al., 2004a; Zhukovskaya et al., 2006). Additional transcription factors could be required to coordinate complex transcriptional responses to DIF. It has also not been explained why DIF has more dramatic effects in cell culture than normal development. One possibility is that other roles await discovery (Serafimidis and Kay, 2005; Thompson and Kay, 2000b). Some support for this idea comes from the recent finding that lower cup expression of *mrrA* and *ecmB* is aberrant in the *mybE*$^{-}$ mutant (Tsujioka et al., 2007). Furthermore, DimA is required for spore cell formation in chimera with wild-type cells (Foster et al., 2004). In both cases, it is unknown whether the phenotype is due to a defect in DIF signal transduction or pleiotropic effects of the mutation. It is clear, however, that any such additional role of DIF would raise the issue of how a signalling molecule that acts in a concentration-independent fashion could regulate the differentiation of discrete cell types.

To address these issues, we set out to identify other regulators of the DIF signalling pathway. We describe the characterization of a mutant in a DIF-regulated GATA family transcription factor (GtaC). Surprisingly, GtaC is not required for pstO cell differentiation. However, GtaC is instead required to regulate DIF-dependent pstB and basal disc cell differentiation. These studies therefore demonstrate that cell type-specific action of the non-positional signal DIF is determined by GtaC activity.

MATERIALS AND METHODS

Strains, culture and development

Dictyostelium strains were maintained on SM-agar plates in association with *Klebsiella aerogenes* or cultured at 22°C in HL5 axenic medium (Sussman, 1987). Transformed strains were selected with blasticidin (10 μg/ml) or G418 (20 μg/ml). Cells were developed on KK2 [16.1 mM KH_2PO_4, 3.7 mM K_2HPO_4 (pH 6.5)] plates containing 1.5% purified agar (Oxoid) at a density of 1.3×10^6/cm^2.

REMI mutagenesis and library screening

For REMI mutagenesis (Kuspa and Loomis, 1992) pBSRΔBam plasmid was linearized with *Eco*RI and electroporated into *AX4* cells with 50U *Tsp*509I per transformation. A library of 20,000 REMI mutagenized cells was subjected to cAMP removal assays (Thompson et al., 2004a). After 48 hours, filter sterilized HL5 was added and surviving cells left to recover. Plasmid insertion sites were identified by inverse PCR (Keim et al., 2004).

Knock-out, GFP and labile *lacZ* construct generation

For *gtaC*$^{-}$ knockout construct generation, a 1.7 kb *gtaC* genomic fragment (GtaC primers, 5′-CGC**GTCGAC**CATATCAGTTTACGGTTACATCAA-3′ and 5′-CGC**GAATTC**GAGTTTGGTACTTTTGATAAATCC-3′) and 1.9 kb *gtaC* genomic fragment (GtaC primers, 5′-CGC**ACTAGT**TTTC-TCCTGAAAGTGCAATGAGTG-3′ and 5′-CGC**GCGGCCGC**AAG-ATTCTCTTTCCAAATCCGGAG-3′) were cloned into pRHI119. Bold indicates restriction sites added for cloning. For GFP fusion protein generation, the *gtaC* gene was amplified by PCR (GtaC primers, 5′-ACGC**GGATCC**ATGAATCATCAATATATACCATCTCC-3′ and 5′-TGCG**CTCGAG**TTAATCGCTAATTAATTTTGAAACAC-3′) and cloned into pTX-GFP (Levi et al., 2000). GtaC-GFP-expressing cells were starved in KK2 for 4 hours before induction with 100 nM DIF-1. For generation of a labile *lacZ* construct, upstream *gtaC* promoter sequences (GtaC primers, 5′-AATCG**TCTAGA**TTTATGATCTGTGCTTTGATTGGTT-3′ and 5′-ATTTT**GGATCC**CATTCTTTAAATTCGTTGAGAATAC-3′) were cloned into the *Xba*I and *Bgl*II sites of p63iDQ-gal (Detterbeck et al., 1994). Whole-mount *lacZ* staining was performed as described (Dingermann et al., 1989).

qPCR and measurement of DIF responsiveness

Induction of prestalk and prespore markers and mRNA quantification by real-time PCR was performed as described (Huang et al., 2006). qPCR primers for *gtaC* fragment amplification (GtaC primers, 5′-GTATT-GCTAAATCATTACCACCAC-3′ and 5′-TGGAGTTTCCATAGTACC-ACAG-3′) spanned one intron to distinguish cDNA amplification products from genomic contamination. Monolayer and dissociated cell assays were performed as described (Berks and Kay, 1990; Huang et al., 2006).

RESULTS

Isolation of a distinct class of DIF-insensitive mutants

The 8-Br-cAMP monolayer assay provides a method to select for DIF signalling mutants that remain as viable spores when treated with DIF (e.g. *dimA*$^-$) (Thompson et al., 2004a). However, not all DIF signalling mutants behave in this way. Most notably, *dimB*$^-$ mutant cells undergo non vacuolar cell death in response to DIF in the 8-Br-cAMP monolayer assay (Huang et al., 2006). By contrast, both the *dimA*$^-$ and *dimB*$^-$ mutants behave in an identical fashion in the cAMP removal assay. Both mutants fail to make stalk cells when treated with DIF and instead remain as viable amoebae. Consequently, we optimized this assay in an attempt to identify a wider range of genes required for DIF signal transduction. A library of ~20,000 *Tsp509I* REMI mutants was generated and subjected to two rounds of growth and selection (Fig. 1A). From this, independent alleles of previously identified DIF-insensitive mutants, including *dimA*$^-$, as well as several novel mutants were isolated, thus demonstrating the effectiveness of this approach.

One mutant identified in this selection was termed *dimC*$^-$, as it does not make stalk cells when treated with DIF in a cAMP removal assay (Fig. 1B). This is not due to a defect in terminal differentiation, as *dimC*$^-$ cells efficiently differentiate as viable spore cells in the 8-Br-cAMP assay (Fig. 1C). Importantly, the behaviour of the *dimC*$^-$ mutant in the 8-Br-cAMP assay in response to DIF was different to that of other characterized dims. The addition of DIF resulted in the repression of spore cell formation, yet *dimC*$^-$ cells still did not form stalk cells, but instead remained as amoebae (Fig. 1C). This behaviour is therefore distinct from both the *dimA*$^-$ mutant (that remains as spores) and the *dimB*$^-$ mutant (that undergoes non vacuolar cell death) when treated with DIF.

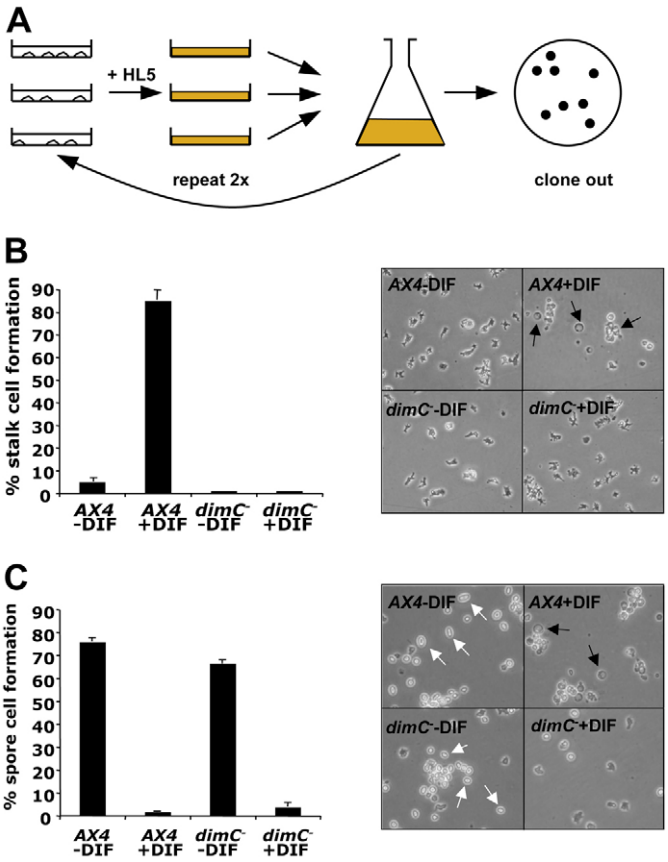

Fig. 1. Identification of a novel DIF resistant mutant. (**A**) Schematic of the selection strategy used to identify the *dimC*$^-$ mutant. REMI mutagenized cells were subjected to the cAMP-removal stalk-cell induction assay before HL5 growth medium was added. Surviving cells were grown up and subjected to a further two rounds of selection before cloning out. (**B**) *dimC*$^-$ cells fail to make stalk cells in the cAMP removal assay. At least three independent assays were performed with comparable results. Data are shown from a representative experiment in which plates were scored in triplicate. Representative pictures are shown on the right. Black arrows highlight vacuolized stalk cells. (**C**) *dimC*$^-$ cells make equivalent numbers of spore cells as wild type in the 8-Br-cAMP assay in the absence of DIF. In the presence of DIF, spore cell formation is repressed in both wild-type and mutant cells. At least three independent assays were performed with comparable results. Data are shown from a representative experiment in which plates were scored in triplicate. White arrows highlight representative spore cells. The pictures on the right show that the mutant cells, in contrast to wild type, still do not make stalk cells when treated with DIF. Black arrows highlight vacuolized stalk cells.

dimC$^-$ is a hypomorphic allele of a DIF regulated GATA transcription factor

The site of insertion of the disruption vector was found to be intergenic, lying downstream of the coding sequence of a predicted ubiquitin ligase and upstream of a predicted GATA transcription factor (*gtaC*) (Fig. 2A). It therefore seemed most likely that this would affect the expression of *gtaC*. This idea was confirmed by quantitative RT-PCR on RNA samples taken at intervals during development. In wild-type cells, expression of *gtaC* rises during the early stages of development and peaks during streaming, with high levels of transcript also present at later developmental stages. However, in the *dimC*$^-$ mutant, *gtaC* transcripts were significantly reduced at all developmental stages, while transcription of the neighbouring gene was unaffected (Fig. 2B,C). The *dimC*$^-$ mutant is therefore a hypomorphic allele of *gtaC*.

GtaC activity is directly regulated by DIF

The behaviour of *dimC*$^-$ mutant cells in monolayer assays is consistent with a role for GtaC in the regulation of a specific subset of DIF responses. However, an alternative explanation is that GtaC is simply required for terminal stalk cell differentiation. Consequently, we sought to further establish a link between direct DIF responses and GtaC by investigating whether GtaC activity is regulated by DIF. When wild-type cells were starved in low density monolayers supplemented with cAMP, quantitative RT-PCR revealed *gtaC* transcription to be significantly induced within 1 hour of DIF stimulation. Furthermore, induction is dependent on the activity of DimA and DimB (Fig. 3A). As DIF-dependent induction of other genes required for DIF signal transduction has not previously been described, this could suggest that *gtaC* is part of a cascade of DIF responses. Two findings argue against this simple interpretation. First, we discovered that *dimA* and *dimB* expression was also induced by DIF-1 (Fig. 3B), suggesting that transcriptional

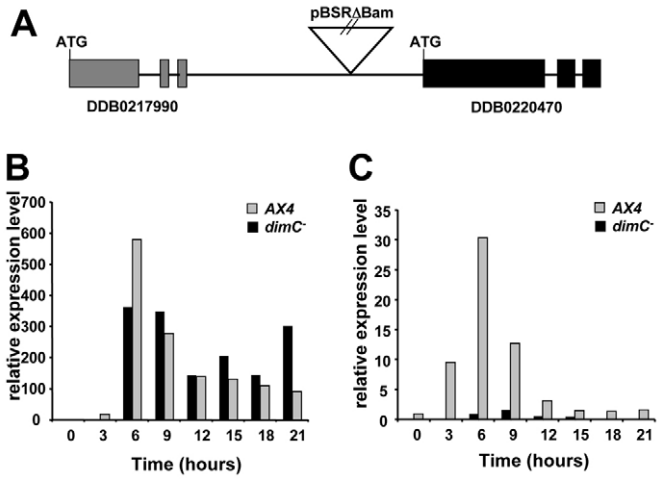

Fig. 2. The disrupted gene in the *dimC*⁻ mutant is a GATA transcription factor. (**A**) Schematic of the site of insertion of the pBSRΔBam disruption vector in the *dimC*⁻ mutant. The numbers refer to *Dictyostelium* database identifiers (www.dictybase.org). DDB0217990 is predicted to encode a ubiquitin ligase and DDB0220470 a GATA family transcription factor. (**B**,**C**) Quantitative PCR analysis of DDB0217990 (B) and DDB0220470 (C) gene expression in developing wild-type (grey bars) and *dimC*⁻ mutant cells (black bars). PCR reactions were performed in triplicate and data averaged. Identical results were obtained with mRNA extracted from two independent developmental time courses.

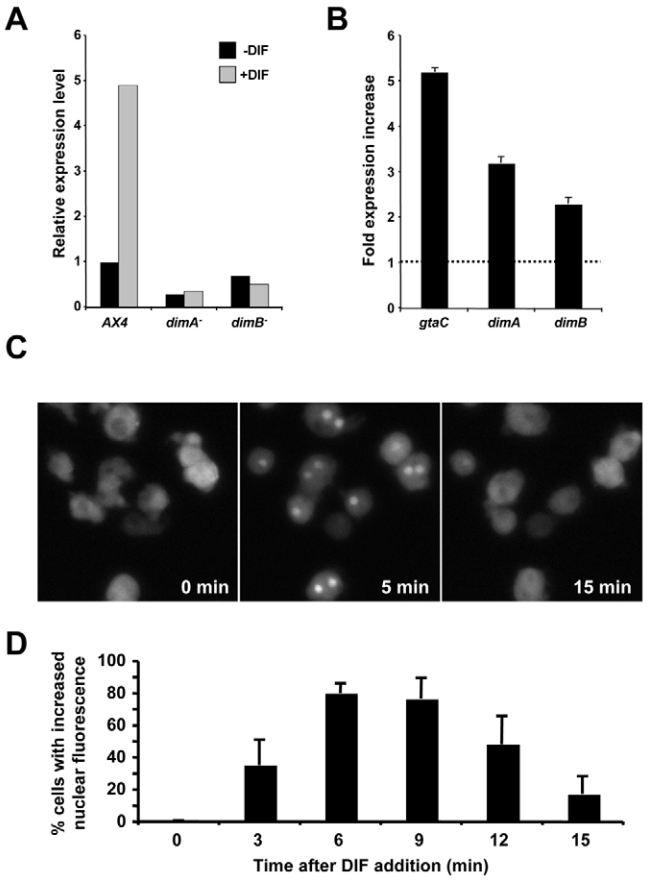

Fig. 3. GtaC activity is regulated by DIF. (**A**) *gtaC* expression is DIF inducible and dependent on DimA and DimB. Expression of *gtaC* was measured by qPCR in wild-type, *dimA*⁻ and *dimB*⁻ cells with and without a 1 hour DIF treatment. Results shown are an average of three independent experiments. (**B**) *dimA* and *dimB* expression is also DIF inducible in wild-type cells. Transcript levels were measured by qPCR with and without a 1 hour DIF treatment. Results shown are an average of three independent experiments. (**C**) GtaC shows DIF-dependent nuclear localization. GtaC-GFP-expressing cells were starved for 4 hours before induction with DIF. Maximal nuclear localization can be seen after 5 minutes. After 15 minutes, nuclear accumulation is no longer visible. (**D**) Quantification of the kinetics of GtaC-GFP nuclear import in response to DIF. Results shown are an average of three independent experiments.

positive feedback may be a general feature of the DIF response. Secondly, we found that the subcellular localization of GtaC activity was also modified by DIF. *dimC*⁻ mutant cells were transformed with a constitutively expressed GtaC-GFP fusion protein. The fusion protein was fully functional, as expression rescued all defects of *dimC*⁻ mutant cells (data not shown). In starving cells and control mock-treated cells, GtaC-GFP was uniformly distributed in the cytoplasm and nucleus. By contrast, most GtaC-GFP rapidly and transiently localized to the nucleus upon stimulation with DIF (Fig. 3C,D). GtaC-GFP was predominantly localized in the nucleus, even after only a 5-minute stimulation. However, after only 15-20 minutes this localization was no longer observed. GtaC activity, like DimA and DimB, is therefore tightly regulated by DIF at the level of subcellular localization and transcript abundance.

A *gtaC*⁻ null mutant exhibits distinct defects in DIF responses

As the *dimC*⁻ mutant is a hypomorphic allele, in order to further characterize the role of GtaC in the regulation of DIF responses, we generated a null allele. The GtaC gene encodes a protein of 587 amino acids. BLAST searches revealed homology in the GATA DNA-binding domain (Fig. 4A), especially to fungi and plant GATA factors, although no significant matches were found in other parts of the protein. In fact, most of the N-terminus consists of homopolymeric tracts encoding asparagine and glutamine. Although such tracts have been demonstrated to encode transcriptional activation domains in other organisms, they are common within *Dictyostelium* proteins. Therefore, in order to ensure that the mutant was a null allele, we deleted most of the coding sequence of the *gtaC* gene and replaced it with a blasticidin resistance cassette (Fig. 4B). The resulting null mutant (*gtaC*⁻) phenocopied the hypomorph in cAMP removal and 8-Br-cAMP monolayer assays (data not shown), supporting the idea that GtaC is distinct from previously identified components of the DIF signalling pathway. These differences are further highlighted by more direct measurements of DIF responsive gene expression. In the first assay, cells were initially brought to competence to respond to DIF by treatment with cAMP, before a 1- or 3-hour DIF treatment. The expression of representative DIF induced prestalk (*ecmA* and *ecmB*) and DIF repressed prespore (*psA* and *cotB*) transcripts in the *gtaC*⁻ null mutant was used to quantify DIF responsiveness. Surprisingly, these DIF responses were unaffected (Fig. 4C). This behaviour is therefore different from that of *dimA*⁻ and *dimB*⁻ cells as both these mutants exhibit specific defects in DIF responses in this assay. In order to determine whether this behaviour was specific to the monolayer assay, we also measured DIF responsive gene expression using an alternative assay in which cells were first developed to the mound stage, before being dissociated and incubated with or without cAMP and DIF. Under these conditions, *dimA*⁻ cells show little or no responses to DIF (Fig. 4D). However, consistent with the findings in the monolayer assay,

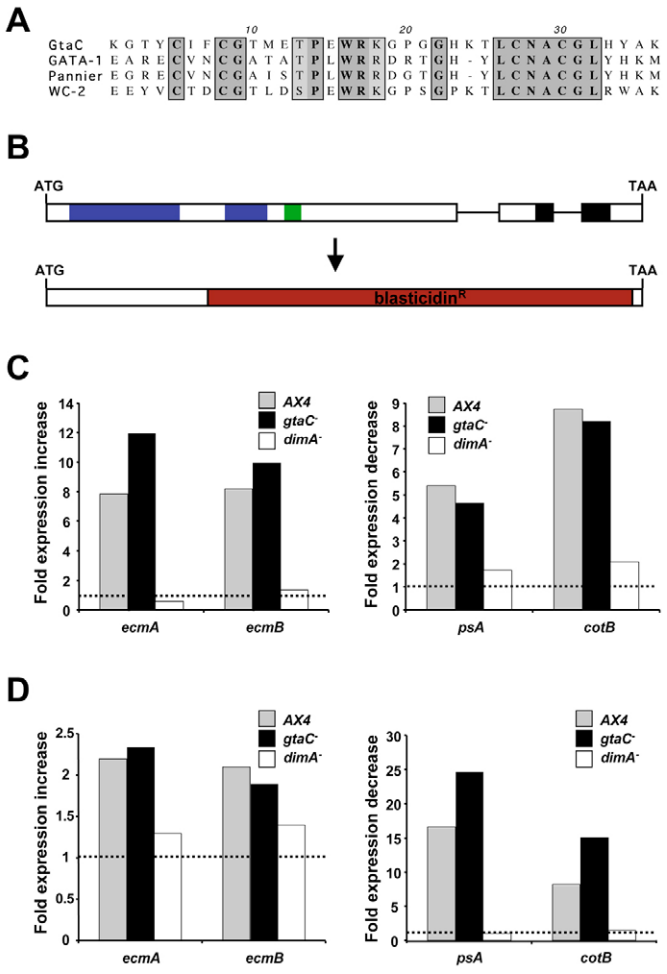

Fig. 4. *gtaC*⁻ null cells exhibit DIF responses that are absent from *dimA*⁻ mutant cells. (**A**) Alignment of the GATA DNA-binding domain of GtaC with the DNA-binding domain of mouse GATA1, *Drosophila* Pannier and *Neurospera* WC-2. (**B**) Schematic of the *gtaC* gene and gene disruption. The region encoding the GATA DNA-binding domain is shaded black, poly asparagine repeats are blue and poly glutamines are green. Approximately three-quarters of the *gtaC*-coding sequence was replaced with a blasticidin cassette, including the predicted GATA DNA-binding domain. (**C**) Measurement of DIF responsive gene expression in monolayer assays. Expression of prestalk markers (*ecmA* and *ecmB*) and prespore markers (*psA* and *cotB*) was measured by qPCR. Cells were treated with cAMP for 9 hours before addition of DIF for 1 (*ecmA* and *ecmB*) or 3 hours (*psA* and *cotB*). *gtaC*⁻ cells exhibit responses comparable with those of wild-type cells, whereas no responses were seen in *dimA*⁻ cells. Results shown are from one experiment. Comparable results were seen in at least three independent experiments. (**D**) DIF responsive gene expression in dissociated cell assays. Mound stage cells were dissociated before shaking in cAMP with or without DIF for 2 hours. Expression of prestalk markers (*ecmA* and *ecmB*) and prespore markers (*psA* and *cotB*) was measured by qPCR. *gtaC*⁻ cells exhibit comparable responses with those of wild-type cells, whereas no responses were seen in *dimA*⁻ cells. Results shown are from one representative experiment. Comparable results were seen in at least three independent experiments.

gtaC⁻ null cells also showed no defect in DIF responsive gene expression in the dissociated cell assay when measured using these well-defined markers (Fig. 4D). Therefore, although GtaC activity is directly regulated by DIF, and *gtaC*⁻ null cells fail to differentiate as stalk cells in response to DIF, some DIF responses are unaffected. Taken together, these data suggest that GtaC is required only to coordinate a subset of DIF responses.

gtaC⁻ mutant cells exhibit similar developmental defects to DIF signalling mutants

Many DIF signalling mutants exhibit hallmark developmental defects (Austin et al., 2006; Fukuzawa et al., 2006; Huang et al., 2006; Thompson et al., 2004a; Thompson and Kay, 2000b; Zhukovskaya et al., 2006). We therefore tested whether *gtaC*⁻ mutant cells exhibit similar developmental phenotypes. *gtaC*⁻ mutant cells aggregated with normal timing, although the streams rapidly broke up, resulting in the formation of large numbers of small mounds that formed small and aberrantly shaped slugs (Fig. 5). Like other DIF signalling mutants, *gtaC*⁻ slugs also often broke apart, resulting in the formation of even smaller slugs that eventually went on to form fruiting bodies that often did not stand upright (Fig. 5). These phenotypes are therefore reminiscent of the defects observed in other DIF signalling mutants and provide further support for the idea that GtaC is required to regulate DIF responses.

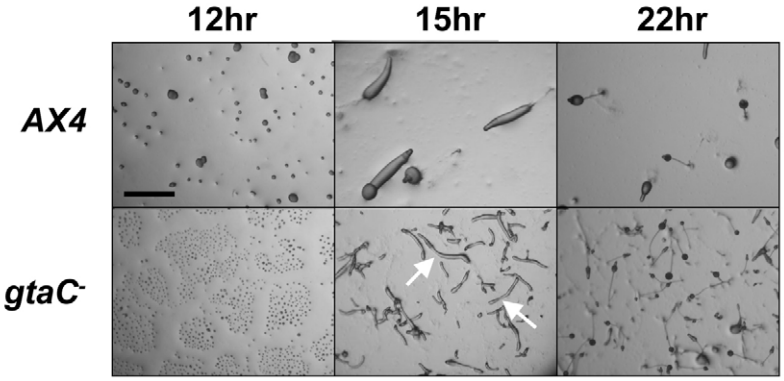

Fig. 5. Morphological defects of developing *gtaC*⁻ mutant cells. Cells were developed on non-nutrient agar for the times shown. Mutant structures are smaller than those of wild type. In addition, mutant slugs were often found to break apart (arrows). At the fruiting body stage, plates appeared messy with stalks often laying on the agar surface. Scale bar: 1 mm.

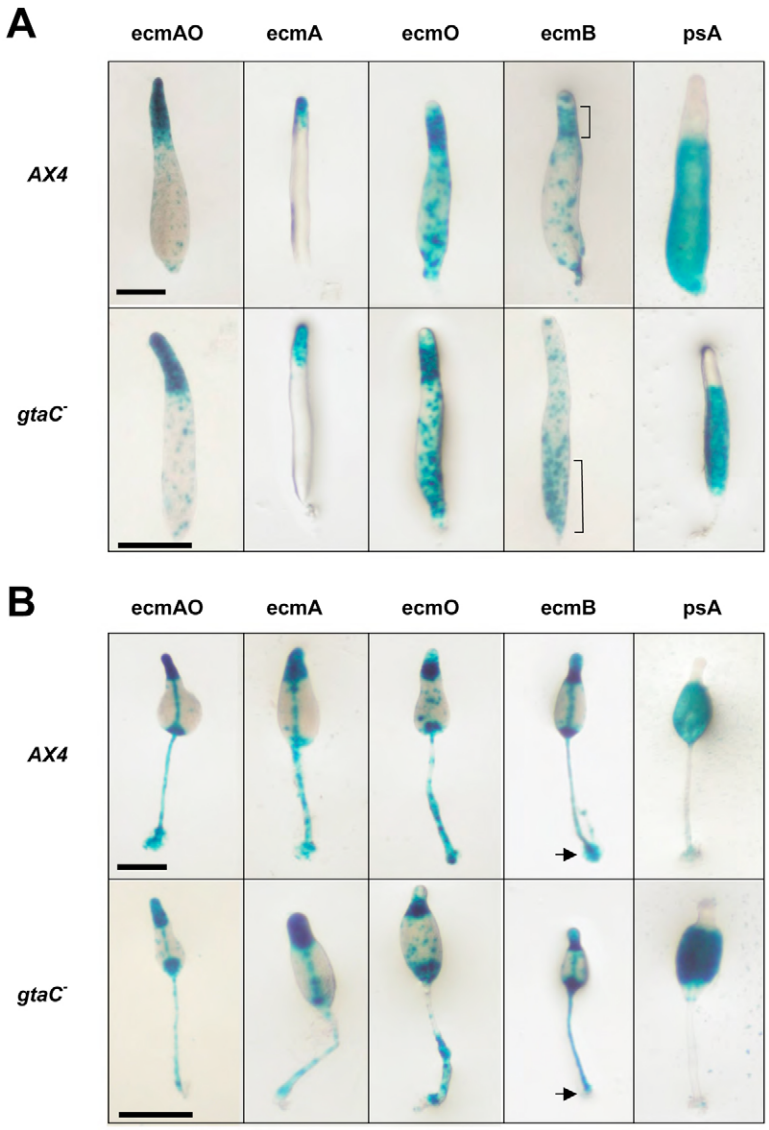

Fig. 6. *gtaC*⁻ null mutant cells exhibit defects in pstB cell and basal disc cell differentiation. Wild-type and *gtaC*⁻ mutant cells were transformed with representative prestalk and prespore reporter genes, and developed and stained for β-galactosidase activity at the slug (**A**) and early culminant (**B**) stages. *ecmB-lacZ* expression was aberrant as many staining cells were found at the rear of slugs and was absent from the basal disc. Brackets highlight the pstB cell population. Arrows indicate basal disc. Expression of all other markers was normal at the slug stage. However, *ecmAO* and *ecmA-lacZ* expression was absent at the expected position of the basal disc in mutant structures. Scale bars: 0.25 mm.

pstO cell differentiation is unaffected in *gtaC*⁻ mutant cells but *ecmB* expression is aberrant

In addition to hallmark morphological defects, most DIF signalling mutants exhibit defects in pstO gene expression. This has been described as a reduction in the domain of *ecmAO-lacZ* expression (that marks both pstA and pstO cells), an expansion of the domain of expression of prespore markers, a lack of expression of the pstO-specific marker *ecmO-lacZ*, or an expansion of the domain of *pstA-lacZ* gene expression (Fukuzawa et al., 2001; Fukuzawa et al., 2006; Huang et al., 2006; Thompson et al., 2004a; Thompson and Kay, 2000b; Zhukovskaya et al., 2006). We therefore examined the expression of these markers in developing *gtaC*⁻ mutant cells. Surprisingly, given the morphological similarities shown by the *gtaC*⁻ mutant, each of these markers was expressed normally (Fig. 6). However, as *gtaC*⁻ mutant cells do display obvious developmental defects, we also carefully examined the expression pattern of other markers. These studies revealed defects in the pattern of expression of *ecmB-lacZ*. In wild-type slugs, *ecmB-lacZ* is expressed in a group of cells located near the anterior of the prespore zone (pstB cells), as well as a core of cells near the slug tip (pstAB). Although no defect in pstAB expression of *ecmB-lacZ* could be detected, pstB cell localization was clearly aberrant. *ecmB-lacZ*-expressing cells were not found clustered at the anterior prestalk-prespore boundary, but instead a large number of staining cells were present towards the rear of *gtaC*⁻ mutant slugs (Fig. 6). This defect is independent of the stage at which slugs were stained. Even at the tip mound or standing finger stages, when pstB cells are normally first enriched at the prestalk-prespore boundary of wild-type structures, they are evenly scattered throughout the prespore zone of mutant structures (Fig. 7). Consequently, it appears that the pstB cell defect is due to a failure to sort to, or remain in the correct location. It is thought that the pstB cell population mainly contributes to the lower cup (with some contribution to the upper cup) and outer basal disc of wild-type fruiting bodies (Dormann et al., 1996; Jermyn et al., 1996; Sternfeld and David, 1982). We therefore tested whether these structures were affected in *gtaC*⁻ mutant fruiting bodies. Although expression of lower cup markers, including *ecmB-lacZ* was normal, outer basal disc expression was much reduced or missing (Fig. 6). Together these findings suggest that GtaC is required for the normal differentiation of the pstB cell population of anterior-like cells and basal disc.

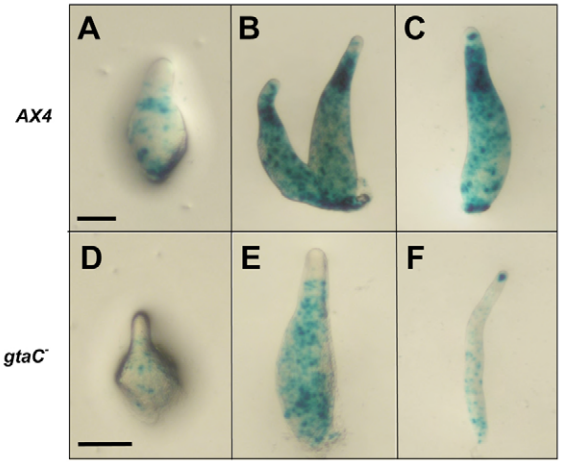

Fig. 7. pstB cells do not sort to the prestalk-prespore border at any developmental stage. Wild type and *gtaC*⁻ mutant *ecmB-lacZ* transformants were stained at the tip mound (**A**,**D**), early finger (**B**,**E**) and standing (**C**,**F**) slug stages. A collection of staining cells is visible towards the prestalk-prespore boundary in wild-type specimens as soon as morphological pattern appears. In mutant specimens, staining cells always appeared to be scattered throughout the prespore zone and became concentrated towards the rear as development progressed. Scale bars: 0.25 mm.

gtaC is expressed in anterior like cells and ancillary fruiting body structures

As GtaC is required for normal pstB and basal disc differentiation, we also tested whether it was expressed in these cells. A reporter line was generated in which upstream *gtaC* promoter sequences were used to drive *lacZ* expression. β-galactosidase staining revealed more staining cells towards the anterior of the prespore zone, although expressing cells were also present throughout the entire rear of the slug (Fig. 8A). Several lines of evidence demonstrate that these stained cells are anterior-like cells. First, when fruiting bodies were stained, highest expression was found in the lower cup and basal disc, with significant levels also found in the upper cup, whereas little if any staining was detectable in the spore head (Fig. 8B,C). A similar pattern of expression was also seen by in situ hybridization (data not shown). Second, when *gtaC-lacZ*-expressing cells were co-transformed with the prespore reporter *psA-nucGUS* and dissociated at the slug stage, β-glucoronidase and β-galactosidase co-expressing cells could not be detected (data not shown). Therefore *gtaC* is expressed predominantly in a population of anterior-like cells that contributes to the upper cup, lower cup and basal disc of fruiting bodies.

DIF signalling is required for pstB cell sorting and basal disc differentiation

To date, DIF signalling has been shown only to be required for pstO cell differentiation. The effects of GtaC on pstB cell differentiation can therefore be explained in two ways: (1) GtaC regulates pstB cell differentiation in a DIF-independent fashion; or (2) DIF signalling is required to regulate pstB cell differentiation as well as pstO cell differentiation. In order to distinguish between these possibilities, we used *ecmB-lacZ* expression to follow pstB cell differentiation and *st-lacZ* (a specific stalk and basal disc marker derived from *ecmB-lacZ*) to follow basal disc differentiation in different DIF signalling mutants. Mutants in both DIF signal production and DIF responses displayed indistinguishable phenotypes from those

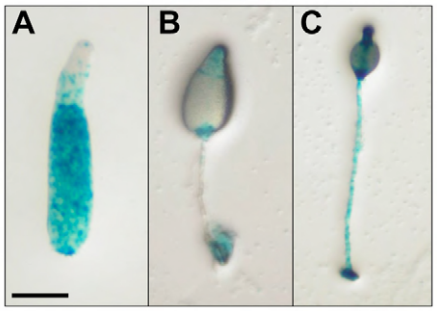

Fig. 8. *gtaC* expression at the slug and fruiting body stages in wild type. A *gtaC* promoter-*lacZ* reporter gene construct is expressed in scattered cells throughout the rear of slugs, but with highest levels of staining towards the prestalk-prespore boundary (**A**). At the early culminant (**B**) and mature fruiting body (**C**) stages, expression is restricted to the upper and lower cup, as well as to the stalk and basal disc. Scale bar: 0.25 mm.

observed in the *gtaC*⁻ mutant. *ecmB-lacZ* expression in pstB cells was mislocalised to cells in the rear of slugs (Fig. 9A). Furthermore, although *st-lacZ* was expressed in the stalk, neither staining cells nor a well-defined basal disc structure could be detected in each DIF signalling mutant examined (Fig. 9B). Lack of basal disc expression is due to a failure of outer basal disc cells to differentiate. Examination of unfixed specimens revealed that although a clear basal disc could be observed in wild-type fruiting bodies, the corresponding structure was absent from unfixed mutant fruiting bodies (Fig. 9C). Together, these data demonstrate that DIF signalling is required to regulate patterning of the pstB cell population that is ultimately required to form the outer basal disc.

DISCUSSION

We have identified a novel GATA class transcription factor (GtaC) that is required to regulate a specific subset of DIF responses in *Dictyostelium*. GtaC activity is directly regulated by DIF, as DIF increases *gtaC* gene transcription and directs rapid nuclear translocation of GtaC protein. A *gtaC*⁻ null mutant does, however, exhibit unique behaviours when compared with other DIF signalling mutants. Most importantly, pstO cell differentiation is unaffected. These differences led us to re-evaluate the role of DIF during *Dictyostelium* development and resulted in the discovery of new roles for DIF in pstB cell and basal disc differentiation. Therefore, although the initial differentiation of different cell types in *Dictyostelium* does not involve positional information, a single diffusible signalling molecule affects the behaviour of multiple cell types. In this case, cell type specificity is dependent on GtaC activity.

pstO and pstB cell differentiation and the role of DIF

There is now good evidence that initial cell type differentiation in *Dictyostelium* does not depend on positional information (Thompson et al., 2004b; Williams et al., 1989). Consistent with this idea, DIF does not appear to act as a classical morphogen at the mound stage, when the different prestalk cells arise. For example, studies have only shown a requirement for DIF in pstO cell differentiation (Fukuzawa et al., 2001; Fukuzawa et al., 2006; Huang et al., 2006; Thompson et al., 2004a). Furthermore, a uniform concentration of DIF, when supplied in the agar, is sufficient to rescue both the pstO gene expression and developmental defects of the *dmtA*⁻ mutant (Thompson and Kay, 2000b). Consequently, it was

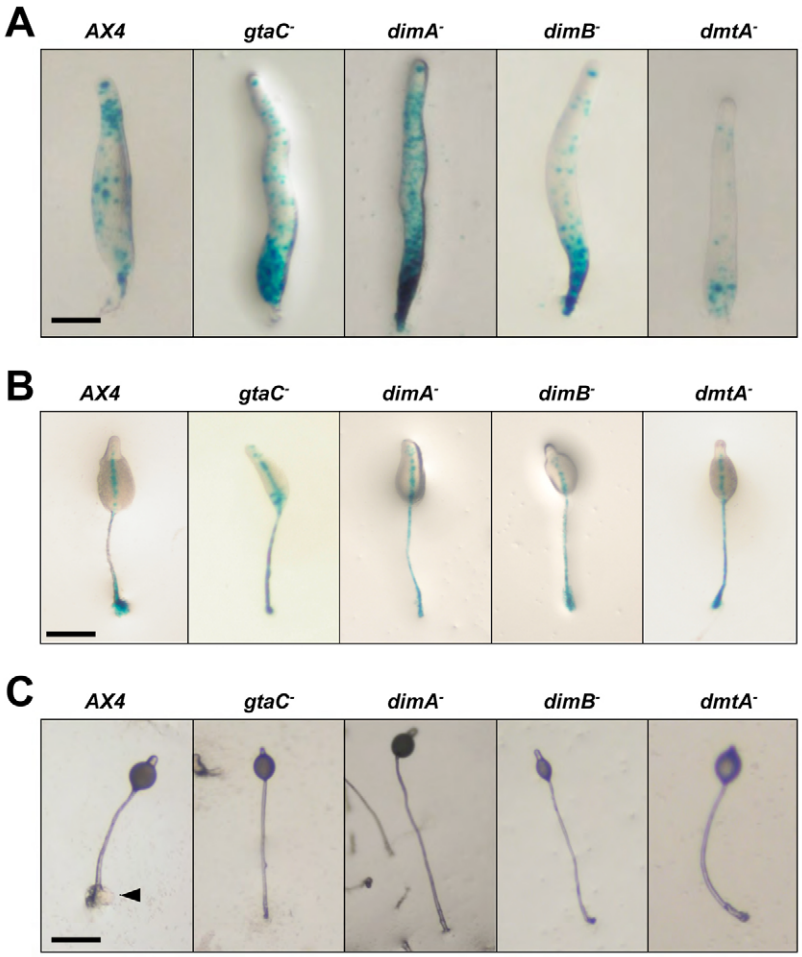

Fig. 9. *gtaC*⁻ mutant defects are phenocopied by DIF signalling mutants. (**A**) Expression of *ecmB-lacZ* at the slug stage. In wild-type structures clusters of high expressing cells are found at the prestalk/prespore boundary (pstB cells). In DIF signalling mutants (*gtaC*⁻, *dimA*⁻, *dimB*⁻ and *dmtA*), these cells are mostly found clustered towards the slug rear. (**B**) Basal disc differentiation is aberrant in DIF signalling mutants. *st-lacZ* expression was used to label the stalk and basal disc. Although staining can be observed in the stalk proper in mutant fruiting bodies, no staining is present in the basal disc. (**C**) The basal disc is absent from DIF signalling mutants. The arrowhead shows the basal disc of a typical wild-type fruiting body. Although a morphological fruiting body with stalk and spore is formed in each DIF signalling mutant, the basal disc structure is much reduced or absent. Scale bars: 0.25 mm.

simple to envisage a scenario where stochastic differences in DIF responsiveness would result in a proportion of cells responding to DIF and differentiating as pstO cells (Thompson and Kay, 2000a).

Like pstO cells, pstB cells also arise scattered in the mound and differentiate in the absence of positional signals (Early et al., 1995; Jermyn and Williams, 1991; Thompson et al., 2004b). Similarly, we have found that the normal differentiation of both pstO and pstB cells is dependent on DIF. One difference between the cell types, however, is that whereas pstO cells fail to differentiate, *ecmB-lacZ*-positive pstB cells can be detected in all DIF signalling mutants tested (Fig. 9). Instead, they fail to reach (or remain at) the prestalk-prespore boundary at the finger/slug stage (Fig. 7). This finding therefore raises the possibility that DIF does not directly affect the behaviour of pstB cells, but is instead required to set up positional information in the slug (e.g. via directing pstO cell differentiation) to which the pstB population responds by sorting out. Although this simple idea is appealing, two findings argue against it. First, GtaC activity is directly regulated by DIF. Second, GtaC is actually expressed in the pstB anterior-like cell population. A key question, therefore, is how does DIF affect both pstB and pstO cell differentiation, if not acting in a concentration-dependent fashion? We propose that GtaC provides part of the explanation. In this model, stochastic differences in GtaC activity define a subpopulation of DIF-responsive cells that differentiate as *ecmB*-positive pstB cells with the correct adhesive and motile properties to enable them to sort to and remain at the prestalk border (Fig. 10). One intriguing possibility, therefore, is that GtaC may directly affect the activity of other DIF response regulators such as DimA, DimB or MybE. Indeed, regulatory protein complexes between GATA, bZIP and myb family transcription factors have previously been described and shown to regulate mammalian hematopoietic and adipoctye differentiation (Takahashi et al., 2000; Tong et al., 2005).

Small-molecule activation of a GATA transcription factor

GATA family transcription factors have been described in a wide variety of organisms, including plants, fungi and metazoa. They have been shown to play many roles, including the regulation of cell proliferation and development, and are characterized by the zinc finger $CX_2CX_{17\text{-}20}CX_2C$ DNA-binding domain. Animal GATA factors have two such motifs of the $CX_2CX_{17}CX_2C$ form. A wider variety of motifs are found in plants and fungi, although a single motif of the $CX_2CX_{18}CX_2C$ form predominates (Patient and McGhee, 2002; Reyes et al., 2004). Interestingly, searches of the *Dictyostelium* genome reveal 24 widely diverse zinc finger-containing GATA transcription factors ($CX_2CX_{17\text{-}21}CX_2C$). Fourteen of these, including GtaC, are of the plant/fungal $CX_2CX_{18}CX_2C$ form. To date, however, only the genomes of plants have been found to encode such a large and diverse complement.

The roles of two *Dictyostelium* GATA transcription factors have previously been described. *stkA* is required for terminal spore cell differentiation (Chang et al., 1996), while the role of *comH* is less well defined, having been shown to be required only for development beyond the mound stage and for spore cell formation (Kibler et al., 2003). Importantly, GtaC represents the first *Dictyostelium* GATA factor to be implicated in DIF signal

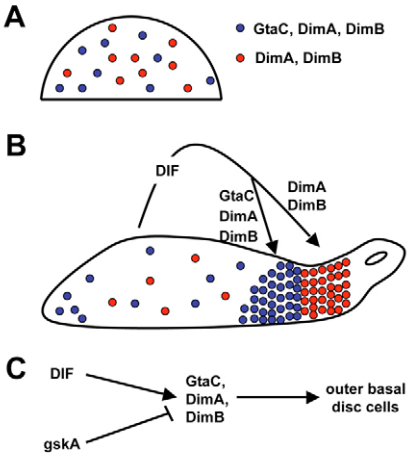

Fig. 10. Model for the role of GtaC in the regulation of DIF responses. (**A**) At the mound stage, stochastic differences due to growth history result in two populations of DIF-hypersensitive cells. Those with highest GtaC activity (blue) will behave as pstB cells while the rest will differentiate as pstO cells. (**B**) At the slug stage, pstB cells (blue) with highest GtaC activity have sorted to the prestalk/prespore boundary, with pstO cells (red) lying just anterior, where they continue to respond to DIF produced by prespore cells. (**C**) In the monolayer assay, DIF induces outer basal disc cell differentiation. DIF activity is dependent on the transcription factors GtaC, DimA and DimB, and is inhibited by the GskA protein kinase.

transduction. A defining feature of this is its rapid and transient relocalization to the nucleus in response to DIF. Regulated transcription factor localization is well characterized for several classes of transcription factors. However, in the case of GATA family members, little is known, even though the regulation of subcellular localization of this class is also a key regulatory feature. This is well illustrated in the case of the founding member of the GATA factor family, GATA1 (Orkin, 1992). GATA1 is strongly expressed during erythroid cell differentiation and is essential for erythroid development (McDevitt et al., 1997; Pevny et al., 1991). Importantly, GATA1 is actually expressed in erythroid cells before the induction of differentiation, but is inactive as it is predominantly localized in the cytoplasm. However, it rapidly relocalises to the nucleus upon the induction of differentiation (Briegel et al., 1996; Gillet et al., 2002). Like GtaC relocalisation upon DIF treatment, GATA1 nuclear localisation is transient (Gillet et al., 2002). Despite the importance of this regulatory step, little is known about the molecular control of GATA1 subcellular localization, or indeed that of any GATA family member. One possible clue comes from the finding that GATA1 becomes hyperphosphorylated upon erythroid cell differentiation (Briegel et al., 1996; Partington and Patient, 1999). Under some conditions, phosphorylation is concomitant with nuclear translocation, although a causal relationship has yet to be demonstrated. Interestingly, DIF stimulation leads to increased levels of tyrosine phosphorylated nuclear STATc (Fukuzawa et al., 2001). DIF treatment also leads to the nuclear translocation of bZIP family members (Huang et al., 2006; Zhukovskaya et al., 2006), but we can find no obvious sequence homology with GtaC (C.R.L.T., unpublished). Consequently, it is possible that DIF-stimulated transcription factor phosphorylation represents a common mechanism. The identification of GtaC therefore provides a route to understand the mode of action of DIF and the regulated nuclear import of GATA family members in other organisms. It will therefore be of great interest to discover whether other *Dictyostelium* GATA transcription factors are also regulated by DIF, or by other signals, at the level of subcellular localisation. Indeed, it is possible that this may represent a widespread mode of regulation because homologues of accessory proteins known to control GATA factor activation in other systems, such as FOG-1 and CBP/p300 (Cantor and Orkin, 2002; Cantor and Orkin, 2005), appear to be absent from the *Dictyostelium* genome.

DIF signalling, pstB cell differentiation and the monolayer paradox

The stalk of the *Dictyostelium* fruiting body consists of the basal disc and stalk proper, and is derived from several different subtypes of prestalk cell (Kessin, 2001; Williams, 2006). For example, the pstA and pstAB cells make up the stalk and inner basal disc, while the pstB population contributes to the outer basal disc, as well as the lower cup. Although DIF functions as a binary switch between the stalk and spore cell fate in cell culture monolayer assays, a role for DIF in stalk cell differentiation during development has not been reported. Most strikingly, stalk cell differentiation does occur in DIF signalling mutants.

The great difference between the action of DIF in cell culture and development has proven difficult to explain. It has been suggested that multiple, redundant stalk-inducing signals may be produced or that the 3D signalling environment in the slug is different from that in cell culture (Huang et al., 2006; Thompson et al., 2004a; Thompson and Kay, 2000b; Zhukovskaya et al., 2006). This study provides a simple alternative explanation. We have shown that GtaC is specifically required to regulate DIF-dependent differentiation of outer basal disc cells. No structure corresponding to the basal disc can be detected in either the *gtaC*$^{-}$ mutant or other previously characterized DIF signalling mutants. Importantly, the basal disc is composed of vacuolised stalk cells. As *gtaC*$^{-}$ cells do not make stalk cells in monolayer culture, it is possible that the stalk cells that arise in monolayer assays are actually basal disc cells. Some support for this idea comes from the *gskA*$^{-}$ mutant, in which the gene encoding the GSK3 serine threonine kinase has been disrupted (Harwood et al., 1995; Schilde et al., 2004). *gskA*$^{-}$ mutant cells exhibit increased stalk cell formation in monolayer assays in response to DIF. Furthermore, during normal development, *gskA*$^{-}$ mutant fruiting bodies have a greatly expanded basal disc. Both defects are therefore the opposite of those of DIF signalling mutants, including *gtaC*$^{-}$. We therefore propose that the stalk cells that differentiate in monolayer assays are basal disc cells (Fig. 10). Although it is currently impossible to test this idea as no specific basal disc markers are available, GtaC target genes probably represent good candidates. Consequently, the *gtaC*$^{-}$ mutant described here will provide a tool for the identification of basal disc specific marker genes. Such studies will provide an exciting opportunity to better understand pattern formation by sorting and the general mechanisms that regulate this recently appreciated and widespread developmental process.

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