

A STAT-regulated, stress-induced signalling pathway in *Dictyostelium*

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Summary

The *Dictyostelium* stalk cell inducer differentiation-inducing factor (DIF) directs tyrosine phosphorylation and nuclear accumulation of the STAT (signal transducer and activator of transcription) protein Dd-STATc. We show that hyperosmotic stress, heat shock and oxidative stress also activate Dd-STATc. Hyperosmotic stress is known to elevate intracellular cGMP and cAMP levels, and the membrane-permeant analogue 8-bromo-cGMP rapidly activates Dd-STATc, whereas 8-bromo-cAMP is a much less effective inducer. Surprisingly, however, Dd-STATc remains stress activatable in null mutants for components of the known cGMP-mediated and cAMP-mediated stress-response pathways and in a double mutant affecting both

pathways. Also, Dd-STATc null cells are not abnormally sensitive to hyperosmotic stress. Microarray analysis identified two genes, *gapA* and *rtoA*, that are induced by hyperosmotic stress. Osmotic stress induction of *gapA* and *rtoA* is entirely dependent on Dd-STATc. Neither gene is inducible by DIF but both are rapidly inducible with 8-bromo-cGMP. Again, 8-bromo-cAMP is a much less potent inducer than 8-bromo-cGMP. These data show that Dd-STATc functions as a transcriptional activator in a stress-response pathway and the pharmacological evidence, at least, is consistent with cGMP acting as a second messenger.

Key words: *Dictyostelium*, DIF, STAT, Stress response

Introduction

As would be expected of a soil-dwelling single-celled organism, *Dictyostelium* possesses effective response mechanisms to cope with environmental insult – for example, osmotic shock, heat shock or oxidative stress. This has been best characterised for hyperosmotic shock, where cells rapidly respond to high osmolarity by reorganising their cytoskeleton. The cytoskeleton is, in part, restructured as a result of the phosphorylation of three threonine residues in the tail region of the myosin II heavy chain that regulate its state of assembly (Kuwayama et al., 1996). Hyperosmotic stress and several other stress factors, such as ATP depletion, exposure to heavy metals, and heat shock, also induce changes in the tyrosine phosphorylation level of actin and in actin cytoskeletal organisation (Aizawa et al., 1999; Gamper et al., 1999; Howard et al., 1993; Jungbluth et al., 1995). Genetic evidence shows that these cytoskeletal rearrangements are essential for resistance to high osmolarity; cells expressing mutant forms of myosin II, wherein the three threonine residues in the tail are substituted by alanine, are sensitive to hyperosmotic stress (Kuwayama et al., 1996), as are double mutants in two F-actin cross-linking proteins (Rivero et al., 1996). In addition to these important cytoskeletal rearrangements, p130, a protein of unknown function, becomes tyrosine phosphorylated after osmotic shock and the protein tyrosine phosphatase PTP3

becomes serine/threonine phosphorylated, changes its intracellular location and binds to p130 (Gamper et al., 1996; Gamper et al., 1999). Two-dimensional gel electrophoresis also revealed changes in the subcellular distribution of several other proteins after osmotic shock but there were no apparent changes in the expression level of any individual protein (Zischka et al., 1999).

When *Dictyostelium* cells are subjected to hyperosmotic shock, guanylyl cyclase activity increases and cGMP levels within the cell rise (Kuwayama et al., 1996; Oyama, 1996; Roelofs and Van Haastert, 2002). Analysis of KI-8, a chemically induced mutant that is defective in cGMP accumulation, suggested that osmotically induced elevation of intracellular cGMP leads to phosphorylation of the myosin II heavy chain (Kuwayama et al., 1996). This conclusion was supported by the fact that 8-bromo-cGMP induces myosin phosphorylation and prevents osmotic lethality in KI-8 cells. The gene encoding, sGC, a soluble guanylyl cyclase that is activated by osmotic stress, has been isolated, but the relationship between sGC activity, myosin phosphorylation and the response to osmotic stress was not reported (Roelofs et al., 2001; Roelofs and Van Haastert, 2002).

Intracellular cAMP levels also rise in response to hyperosmotic shock and this is mediated by the DokA protein (Ott et al., 2000; Schuster et al., 1996). A null mutant for DokA

(a *dokA*⁻ strain) displays reduced viability when exposed to high osmolarity, and artificial elevation of the intracellular cAMP concentration, by exposure to 8-bromo-cAMP, is osmoprotective in *dokA*⁻ cells (Ott et al., 2000). *DokA* is a hybrid histidine kinase. On exposure to osmotic stress, *DokA* is activated by serine phosphorylation and it functions as a negative regulator of the two-component system that controls intracellular cAMP concentration (Chang et al., 1998; Thomason et al., 1998; Thomason et al., 1999; Wang et al., 1996). *DokA* is believed to regulate this system, the *RdeA:RegA* system, by acting as a phosphatase for *RdeA* (Ott et al., 2000).

The link between the above three response systems is unclear. In a *dokA*⁻ strain cGMP accumulates after hyperosmotic stress and the cells show a normal shrinkage reaction (Schuster et al., 1996; Ott et al., 2000). This suggests that cGMP functions either upstream of *DokA* or in a parallel pathway. Also, PTP3 is phosphorylated in response to osmotic shock in *dokA*⁻ cells (Gamper et al., 1999), suggesting that *DokA* does not lie in an upstream part of the PTP3 modification pathway.

In fission yeast, mammalian cells and plant cells, a variety of cellular stresses activate mitogen-activated protein (MAP) kinase cascades that function by regulating the activity of specific transcription factors (Toone and Jones, 1998; Waskiewicz and Cooper, 1995). There is, however, no evidence for MAP kinase involvement in the hyperosmotic stress response of *Dictyostelium*. In addition to activating MAP kinase cascades, animal cells activate specific Janus kinase-signal transducer and activator of transcription (JAK-STAT) signalling pathways when subjected to osmotic or oxidative stress (Bode et al., 1999; Carballo et al., 1999; Gatsios et al., 1998). The mechanisms are not known in detail but their activation by osmotic shock is thought to be triggered by the cell shrinkage associated with hyperosmosis and is again thought to involve a MAP kinase cascade (Gatsios et al., 1998; Bode et al., 1999). STAT proteins contain three highly conserved domains: a DNA binding site, an SH2 domain and a site of tyrosine phosphorylation (reviewed by Bromberg and Chen, 2001; Chatterjee-Kishore et al., 2000; Horvath, 2000). On tyrosine phosphorylation, most often by a member of the JAK family, STAT monomers dimerise, via mutual SH2 domain:phosphotyrosine interactions, and accumulate in the nucleus.

Dictyostelium uses STATs but no JAKs have been reported, and the only known roles for the STATs are developmental. The Dd-STATa protein becomes tyrosine phosphorylated and accumulates in the nucleus when extracellular cAMP binds to its cell-surface serpentine receptor (Araki et al., 1998; Kawata et al., 1997). DIF (differentiation-inducing factor) is a chlorinated hexaphenone, produced by the developing cells, which was originally identified by its ability to induce stalk cell differentiation in a monolayer assay system (Kay and Jermyn, 1983; Morris et al., 1987; Town et al., 1976). DIF induces the differentiation of *pstO* cells (Thompson and Kay, 2000), a band of prestalk cells lying in the rear part of the prestalk region, immediately behind the *pstA* cells (Early et al., 1993). Dd-STATc is activated by DIF and accumulates in the nuclei of *pstO* cells (Fukuzawa et al., 2001). The Dd-STATc null mutant has an accelerated rate of early development and a defect in the regulation of entry into terminal development (Fukuzawa

et al., 2001) Dd-STATc functions as a transcriptional repressor that prevents ectopic *pstA*-specific gene expression in the *pstO* cells (Fukuzawa et al., 2001).

Our previous study showed that there is a low level of tyrosine phosphorylation of Dd-STATc in cells newly set up for development (Fukuzawa et al., 2001). Tyrosine phosphorylation was quickly lost as the cells entered development but reappeared several hours later. We suggested that its presence in newly developing cells might be a stress response induced by the manipulations necessary to transfer the cells out of growth medium. To determine whether Dd-STATc is stress inducible we have characterised its response to hyperosmotic shock, because this is the best-characterised *Dictyostelium* stress-response pathway. We show that Dd-STATc is indeed stress activated and that it mediates specific stress-induced gene transcription.

Materials and Methods

Cell culture, transformation and development

The Ax2 axenic derivative of NC4 (a gift of G. Gerisch) was used in all experiments and was cultured at 22°C in HL5 medium (Watts and Ashworth, 1970). Transformation was by electroporation and strains were selected at 10 µg/ml Blasticidin S. For the stress assays, growing cells were washed twice in KK2 phosphate buffer (16.5 mM KH₂PO₄, 3.8 mM K₂HPO₄, pH 6.2) and resuspended at 2×10⁷ cells/ml in KK2. They were shaken in suspension at 200 rpm and at 22°C for 4 hours before being subjected to various stress conditions. In most cases this was the addition of sorbitol to a final concentration of 200 mM.

Immunohistochemical staining, western and northern transfer

Immunohistochemical detection was performed as described previously (Araki et al., 1998). For Dd-STATc, the 7H3 monoclonal antibody (Fukuzawa et al., 2001) was used and for Dd-STATa monoclonal antibody D4 was used (Araki et al., 1998). For western analysis, 2×10⁷ cells were lysed in 100 µl of SDS sample buffer and proteins were separated by SDS-PAGE and then transferred onto a Hybond C 'extra' membrane (Amersham Bio-science, Amersham, UK). The membrane was blocked with 5% skimmed milk in TBS containing 0.5% Tween-20 for 30 minutes and then reacted overnight with primary antibody, CP22 – a monoclonal antibody that recognises tyr922 of Dd-STATc only when it is in its phosphorylated form (Fukuzawa et al., 2001). The membranes were incubated with secondary antibodies followed by a reagent that allows chemiluminescent detection (Pierce and Warriner, Chester, UK). For checking the amount of Dd-STATc, membranes were stripped and reprobed with 7H3 antibody. Northern analysis was performed as described by Fukuzawa and Williams (Fukuzawa and Williams, 1997), except that total RNA at 15 µg per lane was used.

Microarray analysis

The PCR products from 334 cDNA clones (see supplementary data at jcs.biologists.org/supplemental), chosen from a set of *Dictyostelium*-expressed sequence tags (Morio et al., 1998) (<http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html>), were used as probes in the microarray. The cDNA clones were amplified in a first PCR reaction using primers flanking the vector sequences. The resultant PCR products were used as templates for a second PCR reaction, using nested primers, to generate probes. The first and second PCR reactions were run for 35 and 40 cycles, respectively, with a 2 minute extension time using 'TITANIUM' Taq DNA polymerase (Clontech, BD Bio-science, Oxford, UK). These DNA probes were printed on CMT-GAPS2 coated slides (Corning Ltd, UK)

using a MicroGrid-II microarray robot (BioRobotics, Cambridge, UK). Fluorescently labelled cDNA targets were prepared using the methods described at http://cmgm.stanford.edu/pbrown/protocols/4_yeast_RNA.html. They were produced from 50–60 µg of total RNA with the use of oligo-dT primers, Superscript II DNA polymerase (Life Technologies, Invitrogen Ltd, Paisley, UK) and Cy3- or Cy5-conjugated dCTP (Amersham Pharmacia, Amersham, UK). Unincorporated dyes were removed using QIAquick spin columns (QIAGEN Ltd, W. Sussex, UK) and the targets were purified with microcon-30 filters (Millipore Ltd, Watford, UK) before resuspending in 20 µl of hybridization solution (5×SSC, 0.1% SDS, 50% formamide). Slide processing and hybridisation were performed according to the instructions at <http://www.corning.com/lifesciences>. The denatured targets were deposited on the microarrays after the addition of 37.5 µg of yeast tRNA (GICBCO BRL, Invitrogen Ltd, Paisley, UK) and 50 µg of oligo-dA (Amersham Pharmacia) and covered with coverslips. Hybridisation was carried out overnight in sealed chambers (Corning) at 42°C. The slides were then washed three times, each for 5 minutes, with 0.1×SSC, 0.1% SDS warmed in a water bath at 50°C and rinsed in 0.1×SSC twice before drying. The microarrays were scanned in an Arrayworx scanner (Applied Precision, Issaquah, USA). The images were analysed visually and probes showing a clear enrichment for one or other dye were subjected to secondary analysis by northern transfer.

Results

Dd-STATc is activated by hyperosmotic shock but Dd-STATc is not essential for stress resistance

Stress responsiveness of Dd-STATc was analysed under the regime previously used to study its DIF inducibility (Fukuzawa et al., 2001). Cells were removed from axenic growth medium and shaken for 4 hours in a low ionic strength buffer. When sorbitol was added, to a final concentration of 200 mM, Dd-STATc was specifically tyrosine phosphorylated and this occurred as rapidly as when cells were exposed to DIF (Fig. 1A). Thereafter, when cells were treated with DIF, Dd-STATc became rapidly dephosphorylated. However, within the 15 minute period over which we routinely assay induction, there was no dephosphorylation after sorbitol treatment.

After hyperosmotic shock, Dd-STATc translocated to the nucleus with initial kinetics of accumulation that are as rapid as with DIF; the peak of nuclear accumulation was reached approximately 2 minutes after DIF addition (Fig. 1B). Again, as would be expected from the respective activation kinetics (Fig. 1A), Dd-STATc did not detectably exit from the nucleus

when cells were treated with sorbitol but it did rapidly exit the nucleus in cells treated with DIF (Fig. 1B).

The biological relevance of the osmotic-stress-inducible activation of Dd-STATc was assessed by measuring the relative sorbitol sensitivities of Dd-STATc null cells and control cells (Fukuzawa et al., 2001). The strains were either left untreated or exposed to 200 mM sorbitol for 2 hours (Schuster et al., 1996). Samples were then plated onto bacterial lawns to determine the number of viable cells. The parental strain displayed an apparently higher level of resistance than the null strain: parental=84±17%, versus null=59±21% (mean±s.d. from eight experiments, with each viability determination assayed in triplicate). However, there is a very large amount of intrinsic biological variability in this assay and, as can be seen, the difference was not statistically significant. At the level of discrimination of this assay, which we consider to be a difference in viability equal to or greater than twofold, the results would seem to indicate that the Dd-STATc null mutant is not abnormally sensitive to hyperosmotic stress.

Specificity of the response: Dd-STATa is not activated by osmotic stress but other cellular stresses activate Dd-STATc

To determine whether the osmotic stress response of Dd-STATc reflects the existence of a generalised STAT activation pathway, we determined whether osmotic stress also activates Dd-STATa. Cells were treated with various inducers and the nuclear translocation of Dd-STATa was analysed, using Dd-STATc as a positive control (Fig. 2). As expected, nuclear translocation of Dd-STATa was induced by extracellular cAMP but not by DIF. Nuclear translocation of Dd-STATa was also not induced when cells were treated with sorbitol (Fig. 2). The osmotic stress response is therefore specific to the DIF-inducible STAT, Dd-STATc.

We next determined whether stress conditions other than hyperosmotic shock lead to activation of Dd-STATc. We analysed the tyrosine phosphorylation of Dd-STATc protein isolated from cells exposed to a 33°C heat shock or to the uncoupling reagent di-nitro phenol (DNP). Dd-STATc became specifically tyrosine phosphorylated after both treatments but with different efficiencies, relative to a DIF-treated control (Fig. 3A,B). In the case of heat shock there was a slight (2 to 3 minutes) delay relative to DIF (cf. Fig. 1A), but this was

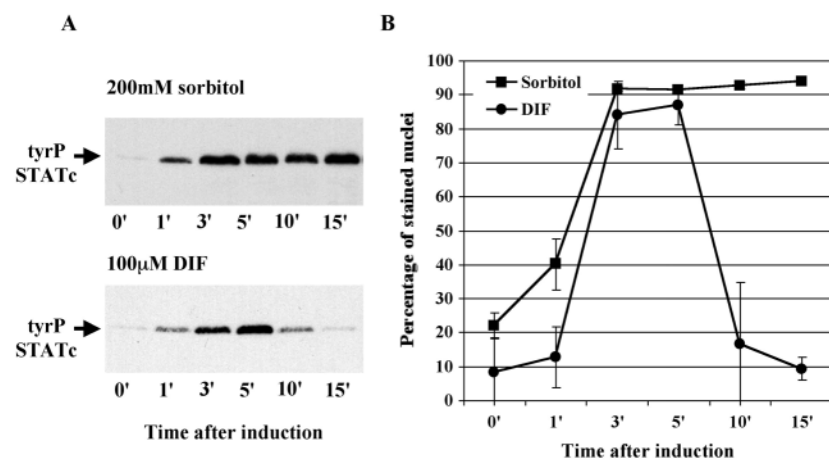


Fig. 1. Activation and nuclear accumulation of Dd-STATc by osmotic stress. Ax2 cells were allowed to develop for 4 hours in shaken suspension, were harvested and then divided into two. One portion was incubated in KK2 containing 200 mM sorbitol and the other portion was incubated in KK2 containing 100 nM DIF. At the indicated times, one aliquot was harvested and the specific tyrosine phosphorylation level of Dd-STATc was determined by western transfer (A). At the same time, a separate aliquot was analysed immunohistochemically to determine the proportion of cells showing nuclear enrichment of Dd-STATc (B). The results are shown as the mean±s.d. but the error bars at 5, 10 and 15 minutes for the sorbitol treated sample are so small as to be obscured by the filled boxes.

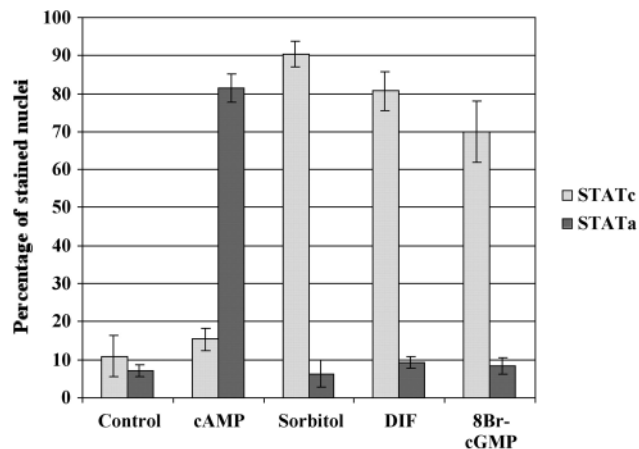


Fig. 2. Dd-STATa shows neither osmotic stress or 8-bromo-cGMP-induced nuclear translocation. Ax2 cells were allowed to develop for 4 hours in shaken suspension and divided into five. One portion was incubated in KK2 alone, and the other portions were incubated in KK2 with the indicated additions (5 mM cAMP, 200 mM sorbitol, 100 nM DIF or 20 mM 8-bromo cGMP). After 3 minutes of incubation, two aliquots were removed. In one aliquot cells were analysed immunohistochemically to determine the proportion of cells showing nuclear enrichment of Dd-STATa (dark grey boxes), and in the other aliquot the proportion of cells showing nuclear enrichment of Dd-STATc was measured (light grey boxes). The results are shown as the mean \pm s.d.

probably caused by the lag period, during which the cells equilibrated at the higher temperature. The extent of the activation at peak level was somewhat greater in the heat-shock-induced cells than in DIF-induced cells (Fig. 3A). DNP was also a slower activator than hyperosmotic stress (Fig. 3B). However, this reflected an intrinsically weaker response because the level of activation never reached that observed with heat shock or osmotic shock. Nonetheless, the results show that Dd-STATc is activated by a generalised stress-response pathway.

Nuclear translocation of Dd-STATc in response to sorbitol requires its specific tyrosine phosphorylation

Although we initially suggested that tyrosine phosphorylation of Dd-STATc is not necessary for its regulated nuclear accumulation (Fukuzawa et al., 2001), subsequent work showed that a mutant form of Dd-STATc, in which the site of tyrosine modification is altered to a phenylalanine residue (a Y to F mutant), does not accumulate in the nucleus after DIF treatment (Fukuzawa et al., 2001). To determine whether this is also true for stress induction, a single copy of the Dd-STATc gene, and of its Y to F mutant form, were transformed into a Dd-STATc null strain. In both cases GFP was fused at the N-terminus of the Dd-STATc protein to yield GFP:STATc and GFP:STATc-YF. When the two strains were exposed to sorbitol, GFP:STATc cells showed translocation of the GFP fusion protein to the nucleus, while the fusion protein remained cytosolic in GFP:STATc-YF cells (Fig. 4). Thus, the key event in stress-induced nuclear translocation of Dd-STATc is, just as in the case of DIF induction, the activating tyrosine phosphorylation.

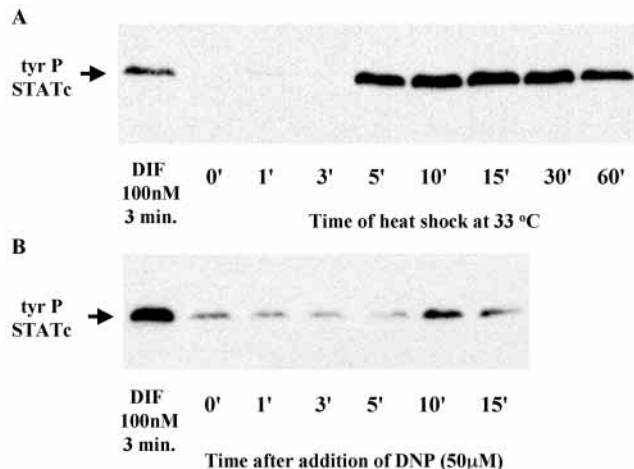
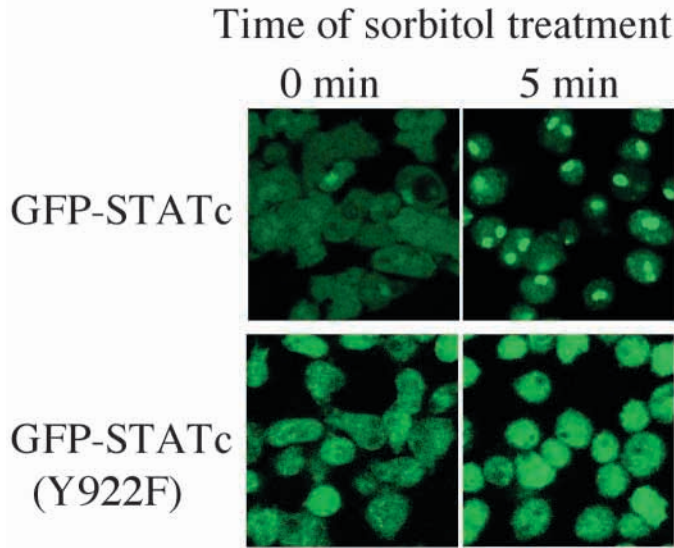


Fig. 3. Activation of Dd-STATc by heat shock and ATP depletion. (A) Ax2 cells were allowed to develop for four hours in shaken suspension at 21°C and then transferred to a heating block at 33°C. At the indicated times thereafter, aliquots were harvested and the specific tyrosine phosphorylation level of Dd-STATc was determined by western transfer. As a positive control for the induction, a portion of the cells was maintained at 21°C, DIF was added to a final concentration of 100 nM and an aliquot was analysed in parallel with the heat shock samples. The results are therefore quantitatively comparable and heat shock appears to be a more effective activator than DIF. (B) Ax2 cells were allowed to develop for 4 hours in shaken suspension and di-nitro phenol (DNP) was added to a final concentration of 50 μM. At the indicated times thereafter, aliquots were harvested and the specific tyrosine phosphorylation level of Dd-STATc was determined by western transfer. DIF was added to a final concentration of 100 nM and an aliquot was analysed in parallel with the oxidative shock samples. The results are therefore quantitatively comparable and oxidative shock is a much less effective inducer than DIF. (N.B. This experiment and the experiment described above, in Fig. 3A, are not directly comparable because they were performed at different times.)

8-Bromo cGMP induces rapid tyrosine phosphorylation and nuclear translocation of Dd-STATc

Because of the evidence that intracellular cAMP and cGMP both act as second messengers in the cellular responses to hyperosmotic shock, we determined the effects of their respective membrane-permeant analogues. Exposure of cells to concentrations of 8-bromo cGMP of >1 mM for just 3 minutes (Fig. 5A) induced tyrosine phosphorylation of Dd-STATc and, at the highest concentration studied (20 mM), activation and nuclear accumulation occurred almost as rapidly as with DIF or sorbitol (cf. Fig. 5B,C with Fig. 1A,B). There was no apparent nuclear efflux of Dd-STATc when cells were treated 8-bromo cGMP, that is, the efflux kinetics mirror that of sorbitol rather than that of DIF. Again, activation was selective for Dd-STATc, because Dd-STATa was unresponsive to 8-bromo cGMP (Fig. 2).

In contrast to the robust induction of Dd-STATc observed after 3 minutes of induction with 8-bromo cGMP, 8-bromo cAMP barely induced activation of Dd-STATc at this time (Fig. 5A). However, when 20 mM 8-bromo-cAMP was added, in a kinetics experiment, tyrosine phosphorylation and nuclear translocation were detected at the later time points, although the peak levels were lower and were reached more slowly than with 8-bromo cGMP (Fig. 5B,C). Thus, the induction kinetics



for Dd-STATc are more consistent with a second messenger role for cGMP than for cAMP.

Analysis of mutants in the known cAMP-mediated and cGMP-mediated intracellular response pathways

We next studied mutants in the cAMP- and cGMP-mediated stress-response pathways using western transfer (Fig. 6). After probing the filters with CP22, the monoclonal antibody that recognises tyr922 of Dd-STATc only when it is in its phosphorylated form, we re-probed the filter with 7H3, a general Dd-STATc antibody, to check equivalence of loading. Although equal amounts of total protein were loaded onto the gel, the amounts of total Dd-STATc protein varied between

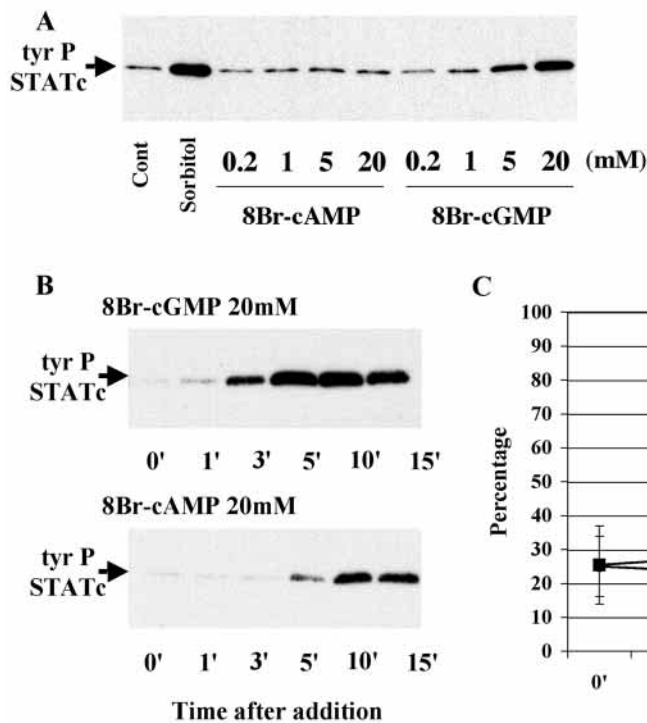


Fig. 4. Osmotic shock activation of Dd-STATc and of a Y to F mutant of Dd-STATc. GFP-STATc contains the entire Dd-STATc protein with GFP fused at its N-terminus, whereas in GFP-STATc(Y922F) the site of tyrosine phosphorylation at residue 922 is replaced with a phenylalanine residue (Fukuzawa et al., 2001). The two constructs were cloned into a vector containing a blasticidin cassette and transformed into Dd-STATc null cells generated using a hygromycin resistance cassette. The Dd-STATc gene displays a very high frequency of homologous recombination. Therefore, to prevent gene conversion/repair of the remnants of the endogenous Dd-STATc gene, the transformation was performed using REMI (restriction enzyme mediated integration); a technique that disfavors homologous recombination (Kuspa and Loomis, 1992). Western transfer using 7H3, the N-terminal-specific monoclonal antibody, showed that the GFP:STATc and GFP:STATc-YF cells express their respective fusion proteins at similar levels (data not shown). The GFP:STATc construct causes reversion of the Dd-STATc null phenotype; the transformant shows normal plaque morphology when plated on a bacterial lawn and loses its 'slugger' phenotype. By contrast, GFP:STATc-YF transformants remain as defective as their Dd-STATc null parent (data not shown). GFP:STATc and GFP:STATc-YF were subjected to shaken development as in Fig. 2 and then induced with 100 mM sorbitol for the indicated time.

different mutants. However, there was very little variation between time-point samples taken from the same strain. Below, we therefore score the results in a plus/minus fashion and disregard any quantitative differences in the absolute induced level of Dd-STATc.

We first analysed a strain that lacks DokA, the histidine kinase that forms part of the cAMP-mediated stress-response pathway. The dokA- strain showed Dd-STATc tyrosine phosphorylation in response to sorbitol treatment (Fig. 6). DokA is believed to function by regulating protein kinase A (PKA) activity, so we also analysed a strain that is defective in PKA-mediated responses to changes in intracellular cAMP concentration. A15:Rm cells are transformed with a dominant-negative form of the R subunit of PKA that blocks C subunit function (Harwood et al., 1992). The response of Dd-STATc to sorbitol stress was found to be normal in A15:Rm cells (Fig. 6). As a further check for any involvement of PKA, we analysed a null mutant for the catalytic subunit of PKA (Mann et al., 1992), and this also displayed sorbitol-inducible activation of Dd-STATc (Fig. 6).

Fig. 5. Activation and nuclear translocation of Dd-STATc in response to membrane-permeant cyclic nucleotide analogues. (A) The tyrosine phosphorylation of Dd-STATc was determined in untreated cells (cont), cells exposed to 200 mM sorbitol (sorbitol) and in cells exposed to the indicated concentrations of membrane-permeant cyclic nucleotide analogue. Induction was for 3 minutes and the samples were analysed as described in Fig. 2. (B) The activation and (C) the nuclear translocation of Dd-STATc in response to 20 mM 8-bromo-cAMP and 8-bromo-cGMP were determined as described in Fig. 2. The results in panel C are shown as the mean±s.d.

We next analysed mutants in the cGMP pathway. The *sgc* gene encodes a guanylyl cyclase activity that is inducible by hyperosmotic stress (Bosgraaf et al., 2002). Activation of Dd-STATc is responsive to hyperosmotic shock in the sGC null mutant (Fig. 6). *Gca* encodes a guanylyl cyclase that is not stress inducible but, in order to rule out absolutely some form of functional redundancy with *sgc*, we analysed the double null, sGC-/GCA-, strain (Bosgraaf et al., 2002). The strain was stress inducible for Dd-STATc activation (Fig. 6). Finally, to determine whether there is functional redundancy between the cGMP and cAMP pathways, we generated and analysed a *dokA*-/sGC- strain. This strain was also stress inducible for Dd-STATc activation (Fig. 6).

Microarray analysis identifies two genes that are osmotic stress induced and Dd-STATc dependent

Although there has been a significant body of work characterising the hyperosmotic stress response in *Dictyostelium* there has, to our knowledge, been no report of

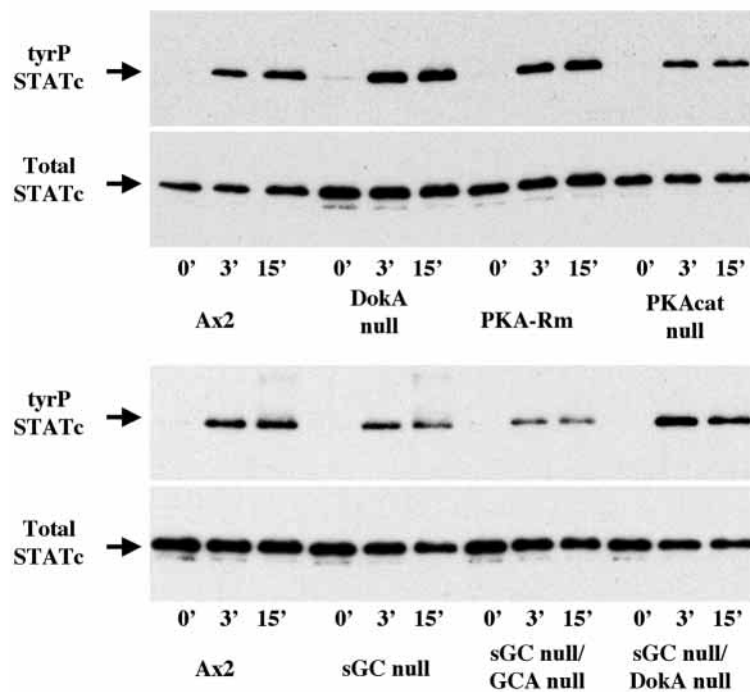


Fig. 6. Osmotic stress-induced activation of Dd-STATc in *Dictyostelium* strains mutant in the cAMP- and cGMP-mediated stress-response pathways. The activation of Dd-STATc in response to sorbitol in the parental (Ax-2) strain and the indicated mutants was determined essentially as described in Fig. 2. However, after probing with CP22 (tyrP-STATc), the filter was re-probed with 7H3 (Total STATc) as described in Materials and Methods. The latter signal provides a convenient loading control. All the strains are published (see text), with the exception of the *sgc*-/*dokA*- strain. This was generated using as a start point the *dokA*- strain (Schuster et al., 1996), which was created using a G418 resistance cassette. An *sgc* blasticidin-based disruption cassette was created using a novel in vitro transposition technique (T. Abe and J. G. Williams, unpublished). PCR analysis shows that the mutated *Dictyostelium* cells contain a blasticidin-resistance cassette in the centre of the *sgc* gene, in the approximate position of the C1/C2 catalytic domains (Roeloffs et al., 2001). Consistent with this, we analysed osmotic stress induced guanylyl cyclase activation in the *sgc*-/*dokA*- strain (Roeloffs et al., 2000) and it is absent (data not shown).

associated gene expression changes. Indeed, proteomic analysis suggested that there may be none (Zischka et al., 1999). Given the results presented above, we were nonetheless encouraged to search for genes that might be regulated when Dd-STATc is activated by hyperosmotic stress. We screened for such genes using a microarray of 334 expressed sequence tags (ESTs) derived from the *Dictyostelium* cDNA sequencing project (Morio et al., 1998). The cDNA library used for this project was prepared from slug stage cells and the ESTs were selected because they have all previously been described in published papers. Information obtained from any positive signals seemed more likely, therefore, to be more functionally interpretable.

The microarrays were hybridised with a mixed probe prepared using RNA isolated from cells that were either untreated or exposed to sorbitol for 15 minutes. We identified two ESTs that reproducibly showed a higher signal with the probe from osmotically stressed cells and that could be confirmed by northern transfer. These derive from the *rtoA* (Brazill et al., 2000) and the *gapA* genes (Adachi et al., 1997). Both genes are rapidly induced by osmotic stress but neither gene was inducible by DIF (Fig. 7).

We next analysed the induction of the two stress-regulated genes in the Dd-STATc null strain. Expression of both genes was noninducible by hyperosmotic stress in the Dd-STATc null strain (Fig. 7). Although osmotic stress inducibility is lost in Dd-STATc disruptants, the *gapA* and *rtoA* genes show their normal patterns of semiconstitutive gene expression in the null strain (data not shown) (Adachi et al., 1997; Wood et al., 1996).

Differential effects of 8-bromo-cGMP and 8-bromo-cAMP on *gapA* and *rtoA* gene expression

Because Dd-STATc is rapidly activated by 8-bromo-cGMP, and because Dd-STATc is required for stress-induced transcription of *gapA* and *rtoA*, we determined whether 8-bromo-cGMP treatment would activate *gapA* and *rtoA* gene expression (Fig. 8). The addition of 8-bromo-cGMP produced a detectable increase in the concentration of both *rtoA* and *gapA* mRNA after 15 minutes of induction. By 30 minutes of induction the expression of *rtoA* was strongly induced, whereas that of *gapA* was less strongly induced. 8-Bromo-cAMP was a much less potent inducer of the expression of both genes than 8-bromo-cGMP (Fig. 8). As expected, neither cyclic nucleotide was effective in inducing *gapA* or *rtoA* gene expression in Dd-STATc null cells but the *sgc* null mutant remained fully stress inducible for expression of both genes (data not shown).

Discussion

Osmotic stress is as effective an activator of Dd-STATc as DIF

Although the best-characterised roles of metazoan JAK-STAT signalling pathways are in mediating cytokine responses and in regulating development, oxidative and hyperosmotic stresses also lead to JAK-STAT activation (Carballo et al., 1999; Gatsios et al., 1998). Similarly, the *Dictyostelium* STAT protein, Dd-STATc, was originally characterised as a DIF-dependent,

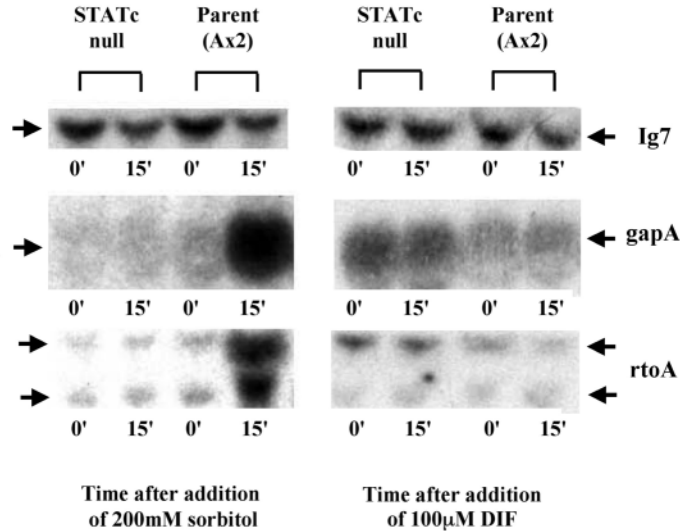
Fig. 7. Expression of the *gapA* and *rtoA* genes in parental and Dd-STATc null cells. Ax2 cells and a Dd-STATc null cells were subjected to development and induced with either sorbitol or DIF, as described in the legend to Fig. 1. After 15 minutes of induction total cellular RNA was isolated and analysed by northern transfer. The blot was hybridised sequentially with the indicated probes. Ig7 is a constitutively expressed RNA that serves as a loading control. The *gapA* mRNA migrates as a broad band at the expected size of 3.2 kb (Adachi et al., 1997). The *rtoA* transcript is reported to be 1.6 kb and we also observed a band of 1.6 kb when cells were growing or developing on a substratum (data not shown). However, when cells were developing in suspension we again observed a band of 1.6 kb, but there is an additional, higher molecular weight species of 2 kb. Because the two RNAs show parallel concentration changes we believe that the longer species is an RNA processing variant of the shorter species. There is a 430 nt intron in the *rtoA* gene and it has a very unorthodox (GG) splice donor site (Wood et al., 1996). The size difference between the longer and shorter transcripts (c. 2 kb minus c. 1.6 kb=0.4 kb) is therefore consistent with a splicing defect in the cells placed in suspension. The result shown is for a Dd-STATc null strain generated using a construct containing a blasticidin cassette. We also analysed strains generated using a hygromycin disruption cassette and, in this case, we compared homologous integrants (i.e. Dd-STATc disruptants) and non-homologous, random integrants from the same transformation (data not shown). This strategy corrects for any unsuspected genetic divergence between the parental strain and the null strain. Again, expression of *gapA* and *rtoA* in the Dd-STATc disruptants was unresponsive to osmotic stress (data not shown).

developmental regulator, but we have now shown that a variety of stress conditions also induce its tyrosine phosphorylation and nuclear translocation. Nuclear translocation after hyperosmotic shock occurs with the same initial, rapid kinetics as with DIF. Also, both inductive events depend on tyrosine phosphorylation. However, Dd-STATc persists in the nucleus for a longer period of time after osmotic shock. This difference between DIF and sorbitol may reflect an adaption process that is specific for DIF signalling or it could simply reflect the fact that sorbitol is a more stable molecule: there is a specific enzymatic activity that inactivates DIF (Nayler et al., 1992) and we have not measured DIF stability under our stress-induction conditions.

cGMP is the most likely candidate for second messenger in the stress pathway

We were able to mimic the effect of sorbitol using 8-bromo cGMP, but 8-bromo-cAMP is a much less effective inducer. Cyclic AMP and cGMP binding proteins often show a degree of cross-reactivity (reviewed by Francis and Corbin, 1999). Hence, we suggest that cGMP is the primary second messenger but that it interacts with an intracellular cGMP binding protein that also binds cAMP at low relative affinity. We took advantage of the fact that there are mutants in the cAMP-mediated stress-response pathways, to further investigate the weak 8-bromo cAMP response. The *doka*⁻ strain lacks the histidine kinase that is an element in the intracellular cAMP mediated pathway and it is hypersensitive to osmotic shock. We also studied two differently generated strains that are defective in PKA-mediated signalling. Both forms of mutant show a normal sorbitol-induced nuclear translocation response for Dd-STATc.

We were not able to perform a similarly definitive genetic analysis for the cGMP-mediated osmotic stress pathway because there are no molecularly defined mutants that block the stress pathway. However, we have analysed a null strain for sGC, the only known osmotically inducible guanylyl cyclase,



and found that Dd-STATc is stress activated. We have also analysed a double null mutant with the other known guanylyl cyclase GCA, and we have ruled out possible redundancy between the cGMP and cAMP pathways by analysing a *Doka*/sGC double mutant.

Because of the above results, we favour the notion of a cGMP-regulated signalling pathway that utilises a guanylyl cyclase that is yet undiscovered; the genome project is not yet complete and biochemical analysis may also have failed to reveal the proposed enzyme. The conclusion that cGMP is the second messenger is supported by our analyses of specific gene activation because, as we go on to discuss, the two stress-inducible genes we identify are much better induced by 8-bromo-cGMP than by 8-bromo-cAMP.

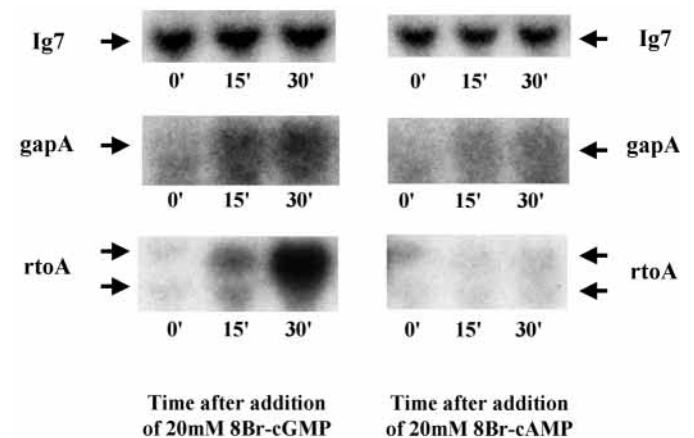
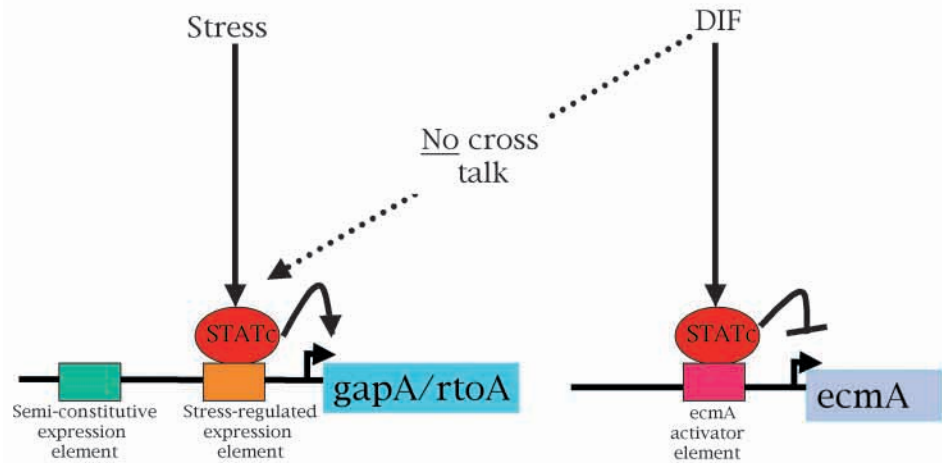


Fig. 8. Expression of the *gapA* and *rtoA* genes in response to membrane permeant cyclic nucleotide analogues. Ax2 cells were subjected to development and induced with 8-bromo AMP or 8-bromo GMP as described in the legend to Fig. 5. After 15 and 30 minutes of induction, total cellular RNA was isolated and analysed by northern transfer as in Fig. 7.

Fig. 9. A schematic representation of the regulatory roles fulfilled by Dd-STATc. This is a representation of the promoters of the two classes of gene known to be regulated by Dd-STATc. The promoter of the *ecmA* gene is under the negative control of Dd-STATc; the element(s) that normally direct *ecmA* expression only in *pstA* cells are repressed by Dd-STATc; such that in a Dd-STATc null strain they become ectopically active in the *pstO* cells (Fukuzawa et al., 2001). Activation of Dd-STATc in *pstO* cells is under the direct control of DIF. As we have shown, *gapA* and *rtoA* are regulated at two levels. They display semiconstitutive activity during growth and development and they are super-inducible, above this level, by hyperosmotic stress. We assume that different promoter elements are utilised for these different activities. We also know that *gapA* and *rtoA* are not induced by DIF, that is, the DIF and the stress signalling pathways do not display 'cross talk', and two possible explanations for this are presented in the text.



The *rtoA* and *gapA* genes are stress inducible

We identified genes that are regulated by hyperosmotic stress using microarrays bearing the PCR products derived from 334 characterised *Dictyostelium* cDNAs. We identified two ESTs, *gapA* and *rtoA*, that are strongly inducible by hyperosmotic stress and by 8-bromo cGMP. Thus, as in other organisms, hyperosmotic stress induces gene expression changes in *Dictyostelium*. The previous failure of two-dimensional gel analysis to reveal such changes (Zischka et al., 1999) presumably reflects the greater sensitivity, and reliability of sample comparison, afforded by the microarray technique.

The principal aim of our study was to determine whether there are stress-induced gene transcripts that are regulated by Dd-STATc. It was not intended as a comprehensive microarray analysis, an endeavour that can only be properly undertaken when the genome sequence is complete. However, the identity of even this very limited sample of regulated transcripts is of interest. *rtoA* encodes a protein that affects cell type choice and it is thought to bring about this effect by acting on the cell cycle (Wood et al., 1996). RtoA acts to promote vesicle fusion and in *rtoA* null strains cytosolic pH regulation during the cell cycle is perturbed (Brazill et al., 2000). GAPA is a RasGAP-related protein that is needed for normal cytokinesis (Adachi et al., 1997; Lee et al., 1997; Sakurai et al., 2001). GAPA is homologous in sequence to mammalian IQGAPs and these are effectors for Rac and CDC42 – Rho family members that regulate the actin cytoskeleton (Epp and Chant, 1997; Kuroda et al., 1996; Osman and Cerione, 1998).

The known cellular functions of GAPA and RtoA are perhaps consistent with a role in re-structuring the cell after hyperosmotic shock but they are not likely to act in isolation; we assayed only a small number of ESTs and there seems certain to be additional Dd-STATc-regulated genes. GAPA, RtoA and these putative co-induced proteins presumably act in concert to help confer hyperosmotic stress resistance. Also, because Dd-STATc null cells are not hyper-sensitive to the lethal effects of sorbitol treatment, we believe that there may be functional redundancy with other osmoprotective, stress-activated pathways.

The stress-response mechanism can be uncoupled from developmental and DIF-inducible gene expression

In the absence of stress, expression of the *gapA* and *rtoA* genes in the Dd-STATc null strain is normal. Hence, gene transcription during growth and development presumably uses different promoter elements from those used for stress-induced gene expression (Fig. 9). Furthermore, even though Dd-STATc transiently accumulates in the nucleus in response to DIF, the *gapA* and *rtoA* genes are completely non-DIF-inducible (Fig. 9). Possibly, *gapA* and *rtoA* activation require the relatively prolonged nuclear persistence of Dd-STATc that is observed after osmotic shock but not after DIF treatment. Alternatively, hyperosmotic stress may bring about additional changes, beyond those brought about by DIF, that result in specific gene activation. There may be, for example, transcriptional co-activators for Dd-STATc that are recruited to the promoters after osmotic shock but that are not activated by DIF signalling. Distinguishing these possibilities will require detailed promoter analyses of the *gapA* and *rtoA* genes.

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