Development 135, 1347-1353 (2008) doi:10.1242/dev.009936

Evidence that DIF-1 and hyper-osmotic stress activate a *Dictyostelium* STAT by inhibiting a specific protein tyrosine phosphatase

Tsuyoshi Araki[1,*], Judith Langenick[1,*], Marianne Gamper[2,3], Richard A. Firtel[2] and Jeffrey G. Williams[1,†]

STATc becomes tyrosine phosphorylated and accumulates in the nucleus when *Dictyostelium* cells are exposed to the prestalk cell inducer Differentiation inducing factor 1 (DIF-1), or are subjected to hyper-osmotic stress. We show that the protein tyrosine phosphatase PTP3 interacts directly with STATc and that STATc is refractory to activation in PTP3 overexpressing cells. Conversely, overexpression of a dominant inhibitor of PTP3 leads to constitutive tyrosine phosphorylation and ectopic nuclear localisation of STATc. Treatment of cells with DIF-1 or exposure to hyper-osmotic stress induces a decrease in biochemically assayable PTP3 activity and both agents also induce serine-threonine phosphorylation of PTP3. These observations suggest a novel mode of STAT activation, whereby serine-threonine phosphorylation of a cognate protein tyrosine phosphatase results in the inhibition of its activity, shifting the phosphorylation-dephosphorylation equilibrium in favour of phosphorylation.

KEY WORDS: STAT, *Dictyostelium*, Tyrosine phosphatase, Stress, DIF-1

INTRODUCTION

STAT proteins are important regulators of metazoan gene expression (Bromberg and Darnell, 2000). They are activated when diverse signalling pathways are stimulated, but the various JAK-STAT pathways form the paradigm. In response to the binding of a cytokine to its receptor, an associated tyrosine kinase of the JAK family phosphorylates a STAT at a unique site near its C terminus. This leads, via reciprocal phosphotyrosine:SH2 domain interactions, to dimerisation of the STAT and the STAT dimers accumulate in the nucleus (Reich and Liu, 2006).

Dictyostelium cells use STAT signalling to regulate several aspects of their differentiation (Williams, 2003). Extracellular cAMP signalling activates STATa, which can function as either a repressor or an activator of specific gene expression (Araki et al., 1998; Fukuzawa and Williams, 2000). DIF-1 is a chlorinated hexaphenone that induces differentiation of one of the prestalk cell subtypes, pstO cells (Thompson and Kay, 2000). At the slug stage, STATc is nuclear localised in pstO cells, where it acts to prevent ectopic expression of a marker of pstA cell differentiation (Fukuzawa et al., 2001). Addition of DIF-1 to cells early in development leads to the premature tyrosine phosphorylation, dimerisation and nuclear accumulation of STATc.

Nuclear accumulation of STATc in response to DIF-1 is regulated at the level of nuclear export (Fukuzawa et al., 2003). In uninduced cells, the effect of a nuclear import signal, located near the N terminus of STATc, is negated by a DIF-1-regulated nuclear export signal located near the centre of the protein. The balance between import and export activity seems to be linked to the homo-dimerisation that is triggered by phosphorylation of STATc on tyrosine residue 922. The mechanism by which STATc becomes tyrosine phosphorylated is unknown. There are no apparent *Dictyostelium* homologues of the class of tyrosine kinases that modify metazoan STATs (Goldberg et al., 2006). There are, however, an unusually large number of tyrosine kinase-like enzymes that perhaps subsume their function.

In mammals, STAT1 and STAT3 are activated by specific cytokines but hyper-osmotic stress is also an activator (Gatsios et al., 1998). Similarly, STATc accumulates in the nucleus rapidly when cells are subjected to hyper-osmotic stress (Araki et al., 2003). Tyrosine phosphorylation and nuclear localisation of STATc are maintained for at least 30 minutes. By contrast, the fraction of STATc protein that is tyrosine phosphorylated and nuclear localised after DIF-1 treatment reaches a sharp peak at 3-5 minutes of treatment and STATc is then de-phosphorylated and exits the nucleus.

One likely explanation for the temporal disparity, between the stress and the DIF-1 responses, is a difference in de-phosphorylation kinetics. In metazoa TC45, the nuclear isoform of the T-cell protein tyrosine phosphatase (TC-PTP), serves to de-phosphorylate STAT1 and this causes it to re-localise to the cytoplasm (ten Hoeve et al., 2002). TC-PTP-null cells are also defective in STAT3 de-phosphorylation but TC45 may not be the only phosphatase involved; because the cytosolic form of PTPϵ de-activates STAT3 when overexpressed (Tanuma et al., 2000). STAT5 activation is normal in TC-PTP-null cells and here there is evidence for direct interaction of STAT5 with the non-receptor tyrosine phosphatases SHP2 (Yu et al., 2000) and with PTP1B (Aoki and Matsuda, 2000). Thus, the metazoan STATs, which differ significantly in their mechanisms of nuclear accumulation (Reich and Liu, 2006), are also heterogeneous in their modes and sites of de-activation. The two processes are of course intimately inter-related; a nuclear protein must actively shuttle between nucleus and cytoplasm if a cytosolic tyrosine phosphatase is to serve to de-activate it.

The tyrosine phosphatase that catalyses de-phosphorylation of STATc is unknown. *Dictyostelium* encodes three PTPs, all of which are predicted to be non-transmembrane proteins (Howard et al., 1992; Howard et al., 1994; Gamper et al., 1996). PTP1 and PTP2-null strains show only minor defects in development, but PTP1 and PTP2 overexpressing strains develop aberrantly and contain an altered spectrum of tyrosine phosphorylated proteins. PTP1 is a negative regulator of STATa tyrosine phosphorylation but the failure

[1]University of Dundee, College of Life Sciences, Dow Street, Dundee DD1 5EH, UK. [2]University of California, San Diego, Natural Sciences Building Room 6111, 9500 Gilman Drive, La Jolla, CA 92093-0380, USA. [3]Biomedical Research Foundation (SBF), Lauchefeld 31, CH-9548 Matzingen, Switzerland.

*These authors contributed equally to this work
†Author for correspondence (e-mail: j.g.williams@dundee.ac.uk)

Accepted 18 January 2008

to detect a direct interaction between the two proteins, using a substrate-trapping form of PTP1, suggests that PTP1 acts indirectly, at some point upstream of STATa (Early et al., 2001).

The third phosphatase, PTP3, is divergent in several otherwise highly conserved amino acid residues and the bacterially produced enzyme has a very low intrinsic phosphatase activity (Gamper et al., 1996). Analysis of a tagged version of the protein suggests it to be partly cytosolic and partly nuclear (Gamper et al., 1999). In the Ax3-derived strain JH10 there are two copies of the PTP3 gene. It was possible to disrupt one copy, but the second copy was refractory to disruption (Gamper et al., 1996). Antisense inhibition also proved ineffective. Although PTP3 seems to be essential for cell viability, overexpression studies have yielded some insights into its developmental functions. The PTP3 overexpression strain grows slowly and forms large aggregation streams; in addition, many structures arrest development at the mound stage and there are changes in the tyrosine phosphorylation level of several proteins (Gamper et al., 1996; Gamper et al., 1999). In parental cells, hyper-osmotic stress, which is generated by the addition of glucose containing growth medium, induces the tyrosine phosphorylation of a 130 kDa protein. This response is greatly attenuated in the PTP3 overexpressing strain and PTP3 itself becomes serine-threonine phosphorylated under these stress-inducing conditions.

In order to identify a PTP that downregulates STATc after DIF-1 induction, we analysed mutants for the three *Dictyostelium* tyrosine phosphatases. We show that PTP3 directly interacts with and dephosphorylates STATc, and that STATc is the 130 kDa stress-responsive tyrosine phosphorylated protein identified by Gamper et al. (Gamper et al., 1996). We also present evidence that PTP3 has a further, more significant role: as a mediator of the activation of STATc by DIF-1 and osmotic stress.

MATERIALS AND METHODS

Cell culture, transformation and development

Dictyostelium strain Ax2 (Gerisch isolate) was grown axenically and transformed by electroporation (Watts and Ashworth, 1970; Pang et al., 1999). Transformants were selected at 20 μg/ml G418. Clones were isolated by growth on a lawn of bacteria or by plating on 96-well plates. Exponentially growing cells were harvested and washed twice in phosphate buffer (KK2). For suspension, development cells were resuspended in KK2 at a concentration of 1×10^7 cells/ml and were shaken for 4 hours at 200 rpm. For late developmental stages cells were plated on 1.5% non-nutrient agar plates.

Immunohistochemistry

Cells in suspension culture and slugs on agar plates were fixed in absolute methanol for 10 minutes on ice. After rehydration with phosphate-buffered saline (PBS), cells and slugs were incubated with anti-STATc antibody (7H3), followed by incubation with secondary antibody (Alexa488-conjugated goat anti-mouse antibody, Molecular Probes).

Western transfer

Proteins were separated on pre-cast 4-12% polyacrylamide gels (Invitrogen) and electro-transferred to nitrocellulose membranes. Membranes were blocked with 5% skimmed milk in TBS-Tween (Tris-buffered saline, 0.05% Tween20) for 30 minutes and incubated with primary antibody overnight at 4°C. Signals were detected using an HRP-conjugated goat anti-mouse antibody (Bio-Rad) with a chemi-luminescent detection system (Pierce). The 7H3 STATc antibody and anti-GSK3 antibody (clone 4G-1E, Upstate) were used as control.

Substrate-trapping chromatography

Substrate trapping chromatography was performed as described previously using GST:PTP3ΔCS (Gamper et al., 1999). The samples were analysed by western transfer using anti-phosphotyrosine antibodies, a mixture of 4G10 (Upstate) and P-Tyr-100 (Cell Signaling Technology), and the CP22 STATc phospho-specific antibody.

Co-immunoprecipitation of PTP3 and STATc

Parental Ax2 cells and cells expressing myc-tagged PTP3ΔC649S were starved for 4 hours in suspension culture at a cell density of 1×10^7 cells/ml in KK2. After treatment with DIF-1 or sorbitol, 4 ml of cell suspension (4×10^7 cells) was harvested and lysed in 1 ml TT-lysis buffer [50 mM TrisHCl (pH 8.0), 150 mM NaCl, 1.0% TritonX-100, 50 mM NaF, 2 mM EDTA (pH 7.2), 2 mM Na-pyrophosphate, 2 mM benzamidine, 1 μg/ml pepstatin, 1 mM PMSF and Complete EDTA-free proteinase inhibitor cocktail (Roche)] for 10 minutes on ice. After centrifugation at 20,000 ***g*** for 10 minutes, the supernatant was collected and incubated with 9E10 anti-myc antibody (Roche) for 1 hour at 4°C, followed by another 2 hours incubation with Protein G-agarose (Roche). Agarose beads were washed four times in TT-lysis buffer, and finally boiled in 1×SDS-sample buffer for 10 minutes. The samples were analysed by western transfer using the 7H3 STATc antibody and the CP22 STATc phospho-specific antibody.

PTP3 immunopurification and enzyme assay

PTP3 activity was measured with para-nitrophenylphosphate (pNPP) as a substrate and as described by Montalibet et al. (Montalibet et al., 2005). Cells expressing myc:PTP3 were shaken in suspension for 4 hours then treated with the various stimuli for 5 and 15 minutes. Cell suspension (3 ml; 3×10^7 cells) was harvested and cells were lysed in 1 ml NP40 lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1.0% Nonidet-P40 (NP-40), 2 mM EDTA, 50 mM NaF, 2 mM Na-pyrophosphate, 1 mM PMSF, 2 mM benzamidine, 1 μg/ml pepstatin, 0.4 mM TLCK and EDTA-free proteinase inhibitor cocktail (Roche)] on ice for 10 minutes. After centrifugation at 20,000 ***g*** for 10 minutes, the supernatant was pre-absorbed with Protein G-agarose for 30 minutes at 4°C with gentle rocking. After pre-absorption, anti-myc antibody (9E10) was added to samples and left for 30 minutes at 4°C. Then samples were incubated with Protein G-agarose for another 1 hour at 4°C. Beads were washed four times in NP40 lysis buffer and then once in HEPES phosphatase buffer [20 mM HEPES (pH 6.3), 150 mM NaCl, 2 mM EDTA, 5 mM DTT, 2% Glycerol and 0.01% TritonX-100]. The phosphatase activity on pNPP and amount of immunoprecipitated PTP3 protein was measured in each immunoprecipitated PTP3 sample. The phosphatase reaction was carried out with 15 mM pNPP (Sigma) in HEPES phosphatase buffer at 30°C. After 30 minutes, the reaction was terminated by addition of NaOH and the activity was measured as the OD at 405 nm. The amount of immunoprecipitated PTP3 was quantified from western blotting with anti-myc antibody using NIH image software. The PTP3 activity was normalized against PTP3 protein amount. The data are presented as the activity relative to control samples.

Lambda phosphatase treatment and 2D gel electrophoresis

Beads bearing immunoprecipitated PTP3 were washed twice in lambda phosphatase buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EGTA, 2 mM DTT, 0.2% NP-40 and 2 mM $MgCl_2$). The samples were incubated with or without 100 U of lambda phosphatase (NEB) for 30 minutes at 30°C. PTP3 proteins were eluted from the beads with 2D sample buffer (Destreak rehydration solution, GE Healthcare). The eluates were applied to first dimension strips (Immobiline drystrip 8 cm pH 6-10, GE Healthcare). After electrophoresis and equilibration for 15 minutes, the strips were run in a second dimension on a 4-12% gradient gel (Invitrogen).

RESULTS

Overexpression of PTP3 inhibits nuclear accumulation of STATc at the slug stage

In order to determine whether PTP1 or PTP2 regulate STATc their null and overexpression mutants were analysed. Parental and mutant cells were developed to the slug stage and immunostained for STATc. There was no consistent difference in staining pattern between the parental, null and overexpression strains (data not shown). In the case of PTP3, where no null mutant is available, we attempted to generate a null using a new disruption construct in an Ax2 background. Out of 171 clones analysed by PCR, there were no disruptants, presumably reflecting the fact that the gene is essential for growth. Hence, it was only possible to analyse a PTP3 overexpressor.

In this construct, myc:PTP3, the coding region of PTP3, bearing a myc tag at its N terminus, is under the control of a semi-constitutive actin promoter. Interestingly, the developmental phenotype in cells transformed with myc:PTP (PTP3OE cells) resembles that observed for null mutants in DIF-1 biosynthesis and signalling (Thompson and Kay, 2000; Austin et al., 2006; Huang et al., 2006; Zhukovskaya et al., 2006); the slugs are long and frequently fragment along their length (Fig. 1A). When such slugs are analysed immunohistochemically, STATc nuclear accumulation is greatly reduced relative to the parental strain (Fig. 1B). Western transfer analysis using an antibody (CP22) directed against the site of modification at tyr922 of STATc confirms there to be only negligible amounts of activated STATc in the PTP3 overexpressing strain (Fig. 1C). The slug splitting phenotype is unlikely to be due to an effect of PTP3 on STATc, because the STATc null strain does not show such a phenotype (Fukuzawa et al., 2001); it presumably reflects an effect of PTP3 overexpression on some other target.

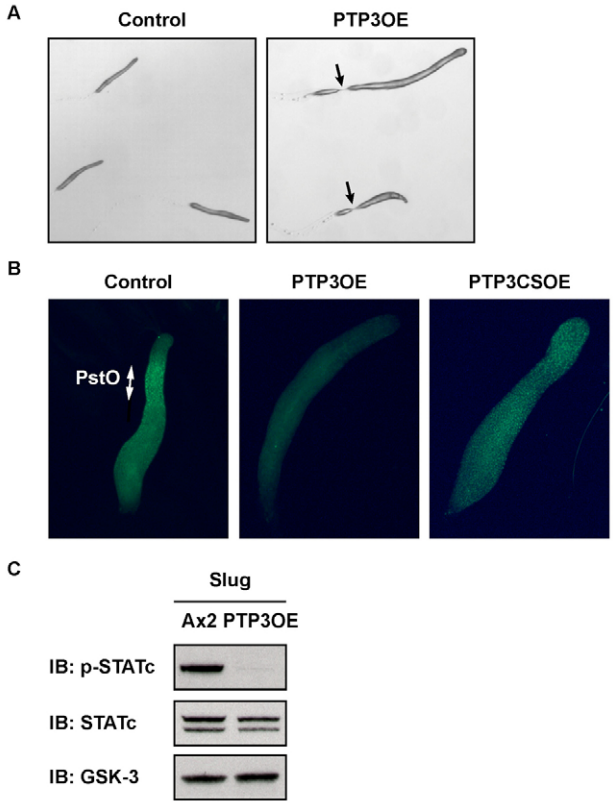

Fig. 1. Analysis of STATc in slugs of a strain overexpressing PTP3 protein tyrosine phosphatase or its dominant-negative form. (**A**) PTP3 and slug morphology. The control slugs (left) are transformed with a myc-GFP overexpression construct, while those on the right overexpress PTP3. The arrows indicate points at which the slugs seem to have split apart. (**B**) PTP3 and STATc staining slugs derived from a non-transformed control and from cells transformed with myc:PTP3 or myc:PTP3CS, the dominant-negative form, were immunostained using a STATc antibody. In the control, STATc is nuclear enriched in the pstO region (double-headed arrow). In the slugs transformed with the PTP3 overexpressor, there is almost no detectable staining; in cells transformed with the dominant-negative PTP3, mutant PTP3 is nuclear enriched in all parts of the slug. (**C**) PTP3 and STATc activation level. Total cell lysate was prepared from slugs of parental Ax2 cells and PTP3OE cells. The samples were analysed using a tyrosine phosphorylation specific STATc antibody (CP22), total STATc antibody (7H3) and, as a loading control, a GSK-3 antibody.

In PTP3 overexpressing cells neither DIF-1 nor stress activate STATc

The above result suggests that PTP3 might be a developmental regulator of STATc. We therefore determined whether activation of STATc by DIF-1 occurs normally in PTP3OE cells. DIF-1 activation was analysed in cells starved in suspension for 4 hours. Tyrosine phosphorylation of STATc was detected by western transfer using a phospho-STATc antibody (CP22). There is no detectable DIF-1 inducible tyrosine phosphorylation of STATc in the PTP3OE strain (Fig. 2A). In parental cells, STATc is predominantly cytosolic but it accumulates in the nucleus within a few minutes of DIF-1 addition (Fig. 2A). In the PTP3OE strain, there is no nuclear translocation of STATc in response to DIF-1. When parental cells are subjected to hyper-osmotic stress, by exposure to 200 mM sorbitol, STATc becomes tyrosine phosphorylated and accumulates in the nucleus (Fig. 2B). In PTP3OE cells, osmotic stress does not induce tyrosine phosphorylation or nuclear accumulation of STATc.

Conditions that decrease PTP3 activity cause increased STATc tyrosine phosphorylation

Substrate-trapping forms of PTPs contain point mutations that prevent the de-phosphorylation reaction, thereby stabilising interaction with their substrates (Flint et al., 1997). Such constructs are routinely used as dominant inhibitors of PTP activity in vivo. myc:PTP3CS is a myc tagged version of PTP3 and also bears a serine substitution of the catalytically essential cysteine residue (C649) (Gamper et al., 1996). The related mutant myc:PTP3ΔCS bears a deletion of an N terminus-proximal region of PTP3. This deletion, of 244 amino acids, removes several polyN tracts, and results in an increased level of expression of the protein (data not shown).

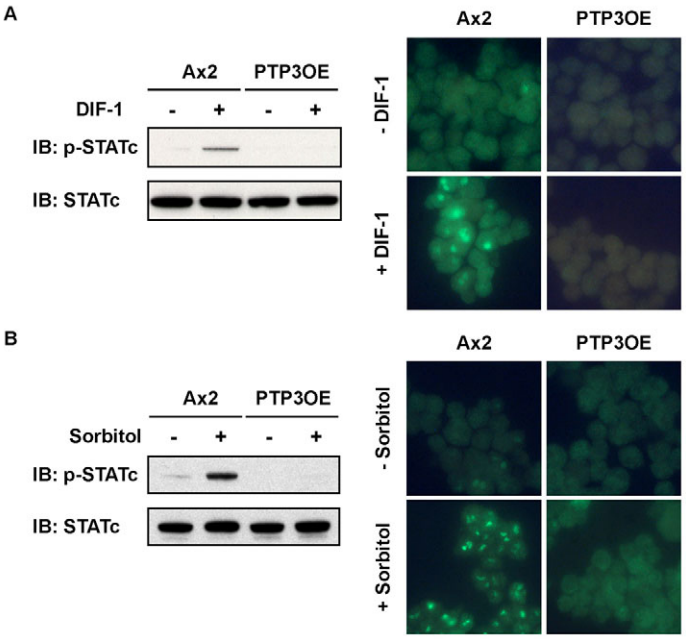

Fig. 2. Analysis of STATc activation by DIF-1 and osmotic stress in a PTP3 overexpressing strain. Parental Ax2 cells and PTP3OE cells were starved for 4 hours and then left untreated, exposed to 100 nM DIF-1 (**A**) for 5 minutes or exposed to 200 mM sorbitol (**B**) for 5 minutes. One aliquot of cells was lysed, subjected to western transfer and the blot was analysed with an antibody specific to the tyrosine phosphorylated form of STATc. As a loading control, a parallel blot was probed with total STATc antibody. The second aliquot of cells was fixed and immunostained for STATc.

When either of these two mutant constructs is expressed in *Dictyostelium*, under the control of the semi-constitutive actin 15 promoter, STATc shows an increased basal and induced level of tyrosine phosphorylation (Fig. 3A; data not shown for myc:PTP3CS). Consistent with its higher level of expression, myc:PTP3ΔCS exerts a stronger stimulatory effect on the level of STATc tyrosine phosphorylation than the full-length PTP3CS protein. Ectopic activation of STATc is also manifest at the slug stage when cells express the substrate-trapping form of PTP3. In parental slugs, STATc is nuclear enriched in pstO cells, while in cells expressing myc:PTP3CS (PTP3CSOE cells) STATc is nuclear enriched in cells throughout the slug (Fig. 1B).

PTP-3 interacts directly with STATc

In order to determine whether PTP3 exerts its effects on STATc by a direct interaction, the C to S substrate-trapping variant of PTP3 was employed in affinity purification. GST:PTP3ΔCS, which contains the same deletion of N terminal sequences and cysteine to serine substitution as myc:PTP3ΔCS but is fused to GST rather than to myc. Parental and STATc-null cells were treated with DIF-1 or sorbitol, cell extracts were subjected to affinity chromatography and replicate eluate samples were analysed by western transfer. As reported previously (Gamper et al., 1996), an osmotic stress-induced protein of 130 kDa (Fig. 4A) and a 60 kDa protein (data not shown) are detected when a phosphotyrosine antibody is used to probe the blot. The 130 kDa protein is also tyrosine-phosphorylated when cells are exposed to DIF-1 but less strongly than by sorbitol (Fig. 4A). The band of 130 kDa is absent in the chromatographic eluates from STATc null extracts (Fig. 4A).

The above data suggest that the 130 kDa protein is STATc and this was confirmed using a phospho-specific STATc antibody to probe the western transfer. Two proteins are detected, a major species running at the position of STATc and a faster running minor species (Fig. 4A). Comparison with the STATc-null eluates indicates that the upper band is STATc, while the lower band is cross-reacting GST-PTP3 adventitiously eluted from the affinity column.

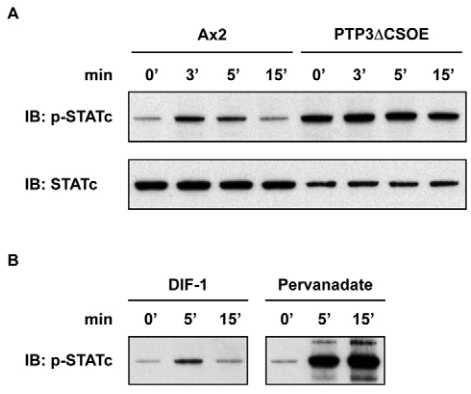

Fig. 3. Analysis of the effects of PTP inhibition on STATc tyrosine phosphorylation. (**A**) Induction assay using dominant-negative forms of PTP3 cells transformed with myc:PTP3ΔCS were starved in suspension for 4 hours and then exposed to DIF-1 at 100 nM. The activation of STATc was analysed by western transfer using an antibody specific to the tyrosine phosphorylated form of STATc. As a loading control, a parallel blot was probed with a non phospho-specific STATc antibody. (**B**) Pervanadate-mediated inhibition of PTP3 Ax-2 cells. Cells were starved in suspension for 4 hours, then exposed to DIF-1 at 100 nM or pervanadate at 2 mM (1 mM H_2O_2 and 2 mM $NaVO_4$) for the indicated times and analysed as in A.

The substrate-trapping results show that PTP3 and STATc interact in vitro. In order to determine whether they also interact in vivo, immunoprecipitation was performed on cells expressing the myc tagged, substrate-trapping form of PTP3. Cells were treated with DIF-1 or sorbitol and extracts were immunoprecipitated with a myc antibody and analysed by western transfer using a STATc antibody (7H3) that recognizes total STATc, and using CP22, the phospho-specific STATc antibody. The substrate-trapping form of PTP3 co-immunoprecipitates with tyrosine-phosphorylated STATc, confirming an in vivo interaction (Fig. 4B).

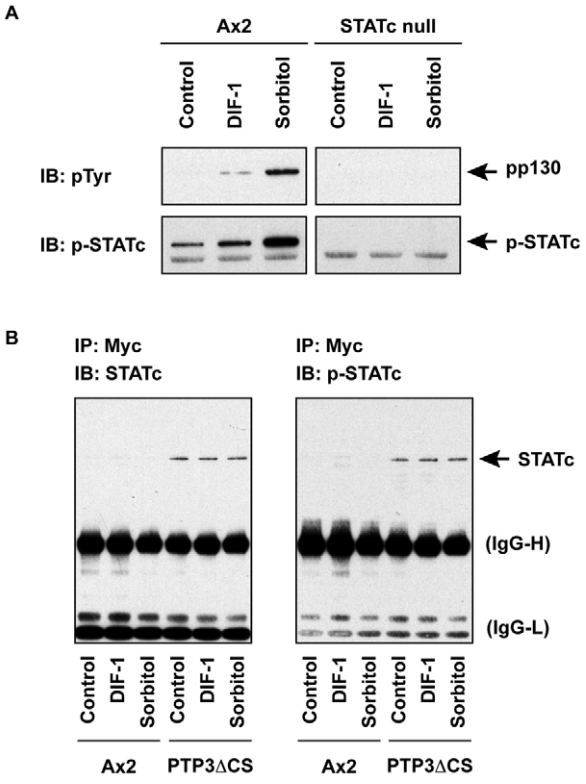

Fig. 4. Interaction of PTP3 with STATc revealed by substrate-trapping. (**A**) In vitro binding of PTP3. STATc parental Ax2 cells and STATc-null cells were starved for 4 hours and then left untreated (control), exposed to DIF-1 (100 nM) or exposed to sorbitol (200 mM). After 5 minutes of treatment, extracts were prepared from the cells and subjected to affinity chromatography using the C to S substrate-trapping form of PTP3. The eluates from the columns were subjected to western transfer using a general phosphotyrosine-specific antibody (upper panel) or a tyrosine phosphorylation-specific STATc antibody (lower panel). The strongest DIF-1 and stress-induced signal detected by the phosphotyrosine-specific antibody is a 130 kDa species that corresponds to a previously described protein (Gamper et al., 1999). This protein co-migrates with the strongest signal detected by the STATc antibody and both species are absent from the STATc null. (**B**) In vivo interaction of PTP3 and STATc parental Ax2 cells or cells expressing the myc tagged C to S substrate-trapping form of PTP3 (myc:PTP3ΔCS, labelled as PTP3ΔCS) were starved for 4 hours and then left untreated (control), exposed to DIF-1 at 100 nM or exposed to sorbitol at 200 mM. After 5 minutes of treatment, extracts were prepared from the cells and immunoprecipitated using a myc antibody. After western transfer, the blot was stained using a general STATc antibody and a tyrosine phosphorylation-specific STATc antibody. We estimate that ~5% of STATc protein in the cell is recovered by the co-IP procedure.

DIF-1 and hyper-osmotic stress treatment inhibit cellular PTP3 activity

In order to determine whether sorbitol or DIF-1 affect the activity of PTP3, we immunoprecipitated the myc tagged form of PTP3 from control and induced cells and determined its enzymatic activity (Fig. 5). This was previously analysed using growth medium as the stressor and, under non-reducing conditions, stress induced a 2.5- to 5-fold reduction in PTP3 activity (Gamper et al., 1999). We find that, under similar conditions, exposure to sorbitol results in a 40% decrease in PTP3 activity at both 5 and 15 minutes of treatment. DIF-1 produces a 20% reduction in PTP3 activity after 5 minutes of treatment but the activity reverts to its initial level by 15 minutes. These changes mirror the activation kinetics of STATc after the two different treatments (bottom of Fig. 5). Pervanadate irreversibly modifies the active site cysteine residue of PTPs. Therefore, as a control, we also analysed immunoprecipitates from pervanadate-treated cells. They display negligible PTP3 activity (Fig. 5). As would be predicted from this observation, pervanadate induces extremely high levels of tyrosine phosphorylation of STATc in the absence of any other added inducer (Fig. 3B).

DIF-1 induces serine-threonine phosphorylation of PTP3

Stress causes serine-threonine phosphorylation of PTP3 (Gamper et al., 1996). To determine whether this also holds true for DIF-1, cells expressing myc:PTP3ΔCS were induced with DIF-1 and analysed by western transfer using a myc antibody. On 2D gels, the uninduced myc:PTP3ΔCS protein migrates as a series of irregular spots (Fig. 6). This suggests some level of pre-existent phosphorylation. On addition of DIF, there is a shift in the spot distribution, with an increase in the proportion of more acidic, putatively phosphorylated, spots. Pre-incubation with lambda phosphatase causes the tagged protein to migrate as a mass at a more alkaline pI. Lambda phosphatase de-phosphorylates serine, threonine and tyrosine residues but PTP3 does not become tyrosine phosphorylated after DIF-1 treatment (data not shown). Hence, by elimination, these observations indicate a change in serine-threonine phosphorylation status. In addition, stress, a known inducer of serine threonine phosphorylation of PTP3, causes a similar pI shift as DIF-1 and lambda phosphatase has the same, counteracting effect (data not shown). These results suggest the existence of multiple serine-threonine phosphorylation states for PTP3, with an elevation in the level of modification after DIF-1 addition.

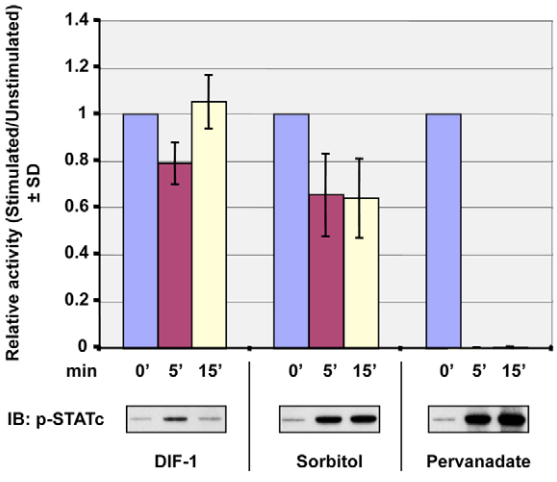

Fig. 5. Osmotic stress and DIF-1 treatment decrease cellular PTP3 activity. Cells at 4 hours of development in suspension and expressing myc:PTP3 were left untreated or exposed to DIF-1 (100 nM), sorbitol (200 mM) or pervanadate (1 mM H_2O_2 and 2 mM Na_3VO_4) for 5 and 15 minutes. They were lysed in a non-ionic detergent and immunoprecipitated using a myc antibody. The precipitates were dissolved and assayed for tyrosine phosphatase activity using the general substrate pNPP. As controls, parental Ax2 cell extracts or cells transformed with myc:PTP3CS were immunoprecipitated with myc antibody. Neither precipitation yielded significant activity (data not shown). For purposes of comparison, the western blot at the bottom shows the level of the STATc phosphorylation in parental Ax2 cells after the treatments described above.

DISCUSSION

PTP3 is highly diverged from metazoan PTPs and it has a relatively low intrinsic tyrosine phosphatase activity (Gamper et al., 1999). Identification of PTP3 as the protein tyrosine phosphatase responsible for de-activation of STATc is important, therefore, because it provides a specific function for this otherwise rather enigmatic enzyme. Our results further suggest that PTP3 is itself subject to direct biochemical regulation, induced by DIF-1, and that it forms part of the signalling mechanism that determines the tyrosine phosphorylation and nuclear localisation status of STATc (Fig. 7). The evidence for this derives from a combination of genetic and biochemical observations.

Overexpression of PTP3 inhibits tyrosine phosphorylation and nuclear accumulation of STATc in response to DIF-1 or osmotic stress. Conversely, inhibition of PTP3, using substrate-trapping mutants of PTP3 as dominant-negative forms, causes constitutive STATc activation; thus, if PTP3 is rendered inactive, DIF-1 and stress become dispensable for activation. The dominant-negative construct also exerts a striking effect on normal development; now rather than being nuclear enriched predominantly in the pstO region, STATc becomes enriched in nuclei throughout the slug. These observations suggest that activation of STATc by DIF-1 and stress is bought about by the inactivation of PTP3 but leave open the possibility of additional regulation at the level of the tyrosine kinase. Because we do not have access to the STATc tyrosine kinase, we cannot test its involvement directly, but the fact that cells

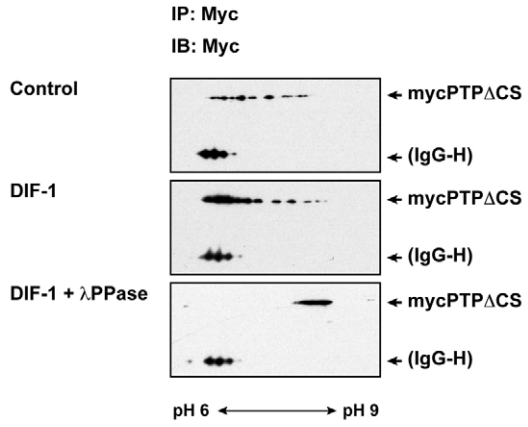

Fig. 6. Electrophoretic mobility shifts of myc-PTP3CS after DIF-1 treatment. Cells transformed with myc:PTP3ΔCS were starved for 4 hours, exposed to DIF-1 at 100 nM for 3 minutes or left untreated, where indicated the samples were treated with lambda phosphatase. All samples were analysed by 2D gel electrophoresis (pH 6-10 for first dimension and 4-12% gradient gel for second dimension). After western transfer, the resultant blots were stained using a myc antibody.

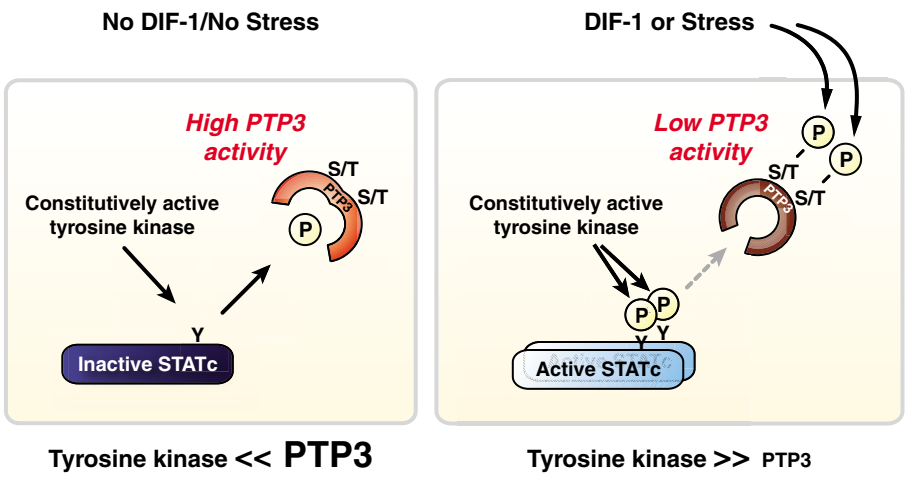

Fig. 7. A model for DIF-1 and stress-induced activation of STATc. The model proposes regulation of STATc at the level of de-phosphorylation. In untreated cells, PTP3 activity is relatively high and STATc is, therefore, minimally tyrosine phosphorylated. Upon stress or DIF-1 treatment, PTP3 is serine-threonine phosphorylated. This reduces PTP3 activity and the level of tyrosine phosphorylation of STATc increases.

transformed with the dominant-negative constructs show little if any activation of STATc by DIF-1 argues against the kinase playing the dominant regulatory role.

When the effect of pre-treatment with DIF-1 or hyper-osmotic stress on biochemically assayable PTP3 activity was determined, DIF-1 induced a transient 20% reduction in activity, while stress induced a stable 40% decrease. The fact that stress is a more effective inhibitor than DIF-1 is in accord with the stronger STATc activation engendered by stress. The additional fact, that inhibition by DIF-1 is transient while inhibition by stress is stable, is also concordant with the respective kinetics of activation of STATc observed after the two different treatments. Although the inhibitions that are observed, even in the case of stress, are incomplete, they fall in the range observed by others; e.g. Garton and Tonks (Garton and Tonks, 1994) observed a 35% decrease in PTP-PEST activity in vivo when they treated cells with TPA, which activates PKC to phosphorylate a serine residue (S39). They are presumably sufficient to tip the phosphorylation-dephosphorylation equilibrium in favour of net phosphorylation. In addition, there may be multiple spatially separated pools of PTP3 [as reported by Yudushkin et al. (Yudushkin et al., 2007) for PTP1B], with a tightly DIF-1 and sorbitol-regulated pool functioning to de-phosphorylate STATc.

We do not know the precise mechanism whereby phosphorylation acts to inhibit phosphatase activity. In principle it could be the direct result of a conformational change induced by phosphorylation. However, the stress-induced reduction in PTP3 activity is not observed when a reducing agent is included in the extraction buffer (Gamper et al., 1999). This suggests that the effect of PTP3 phosphorylation is indirect and that it might involve selective oxidation of the phosphorylated forms by reactive oxygen species (ROS). ROS, such as H_2O_2, are believed to be generated in a localized manner when growth factor or antigen receptors bind their ligands. They direct reversible oxidation of the active site cysteine in PTPs, inhibiting their enzymatic activity (reviewed by Tonks, 2005), and they are known to affect *Dictyostelium* development (Bloomfield and Pears, 2003). This could explain why the apparent inhibition engendered by DIF-1 and stress is observed only under non-reducing conditions. How does phosphorylation favour oxidation? After cells are exposed to stress PTP3 accumulates in endosome-like structures (Gamper et al., 1999). If these contain a relatively high concentration of ROS, this might explain why the phosphorylated PTP3 population becomes selectively inactivated.

Although it was known that medium-induced stress induces an increase in total serine-threonine phosphorylation of PTP3 (Gamper et al., 1999), we have now shown that phosphorylation of PTP3 also increases after DIF-1 treatment. This is, to our knowledge, the first example of DIF-1-induced serine-threonine phosphorylation. Is this likely to be a more general mechanism for ligand-induced STAT activation? The paradigmatic mechanism of STAT activation is via the regulated activation of a tyrosine kinase family member. There is, to our knowledge, no demonstration of ligand-induced STAT activation mediated via an inhibitory effect on a specific PTP. However, PTP activity is negatively regulated, in other signalling contexts, by ROS.

H_2O_2 activates both STAT1 and STAT3 in murine fibroblasts and rat vascular smooth muscle cells (Simon et al., 1998; Madamanchi et al., 2001), and STAT3 is activated by H_2O_2 in human lymphocytes (Carballo et al., 1999). In two of the studies, activation could in principle be explained by concurrent JAK stimulation (Simon et al., 1998; Madamanchi et al., 2001). However, the fact that pervanadate was in itself a potent stimulator of STAT activation led two of the groups involved to posit an additional inhibitory effect of H_2O_2 on an unidentified PTP (Carballo et al., 1999; Madamanchi et al., 2001). Another, more fully documented case of STAT regulation by ROS derives from a study of virus infection. When respiratory syncytial virus (RSV) infects cells, there is a burst of ROS production and STAT1 and STAT3 are activated (Liu et al., 2004). The pathway seems to involve ROS inhibition of PTP activity, but the presumptive PTP target is again unknown.

We thank the Wellcome Trust (Program Grant 053640/Z/98ß) for funding this work and the Boehringer Ingelheim Foundation for supporting J.L. with a PhD studentship. We also acknowledge the initial experiments on STATc dephosphorylation performed by Anne Early.

References

Aoki, N. and Matsuda, T. (2000). A cytosolic protein-tyrosine phosphatase PTP1B specifically dephosphorylates and deactivates prolactin-activated STAT5a and STAT5b. *J. Biol. Chem.* **275**, 39718-39726.

Araki, T., Gamper, M., Early, A., Fukuzawa, M., Abe, T., Kawata, T., Kim, E., Firtel, R. A. and Williams, J. G. (1998). Developmentally and spatially regulated activation of a Dictyostelium STAT protein by a serpentine receptor. *EMBO J.* **17**, 4018-4028.

Araki, T., Tsujioka, M., Abe, T., Fukuzawa, M., Meima, M., Schaap, P., Morio, T., Urushihara, H., Katoh, M., Maeda, M. et al. (2003). A STAT-regulated, stress-induced signalling pathway in Dictyostelium. *J. Cell Sci.* **116**, 2907-2915.

Austin, M. B., Saito, T., Bowman, M. E., Haydock, S., Kato, A., Moore, B. S., Kay, R. R. and Noel, J. P. (2006). Biosynthesis of Dictyostelium discoideum differentiation-inducing factor by a hybrid type I fatty acid-type III polyketide synthase. *Nat. Chem. Biol.* **2**, 494-502.

Bloomfield, G. and Pears, C. (2003). Superoxide signalling required for multicellular development of Dictyostelium. *J. Cell Sci.* **116**, 3387-3397.

Bromberg, J. and Darnell, J. E., Jr (2000). The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* **19**, 2468-2473.
Carballo, M., Conde, M., El Bekay, R., Martin-Nieto, J., Camacho, M. J., Monteseirin, J., Conde, J., Bedoya, F. J. and Sobrino, F. (1999). Oxidative stress triggers STAT3 tyrosine phosphorylation and nuclear translocation in human lymphocytes. *J. Biol. Chem.* **274**, 17580-17586.
Early, A., Gamper, M., Moniakis, J., Kim, E., Hunter, T., Williams, J. G. and Firtel, R. A. (2001). Protein tyrosine phosphatase PTP1 negatively regulates Dictyostelium STATa and is required for proper cell-type proportioning. *Dev. Biol.* **232**, 233-245.
Flint, A. J., Tiganis, T., Barford, D. and Tonks, N. K. (1997). Development of 'substrate-trapping' mutants to identify physiological substrates of protein tyrosine phosphatases. *Proc. Natl. Acad. Sci. USA* **94**, 1680-1685.
Fukuzawa, M. and Williams, J. G. (2000). Analysis of the promoter of the cudA gene reveals novel mechanisms of Dictyostelium cell type differentiation. *Development* **127**, 2705-2713.
Fukuzawa, M., Araki, T., Adrian, I. and Williams, J. G. (2001). Tyrosine phosphorylation-independent nuclear translocation of a dictyostelium STAT in response to DIF signaling. *Mol. Cell* **7**, 779-788.
Fukuzawa, M., Abe, T. and Williams, J. G. (2003). The Dictyostelium prestalk cell inducer DIF regulates nuclear accumulation of a STAT protein by controlling its rate of export from the nucleus. *Development* **130**, 797-804.
Gamper, M., Howard, P. K., Hunter, T. and Firtel, R. A. (1996). Multiple roles of the novel protein tyrosine phosphatase PTP3 during Dictyostelium growth and development. *Mol. Cell. Biol.* **16**, 2431-2444.
Gamper, M., Kim, E., Howard, P. K., Ma, H., Hunter, T. and Firtel, R. A. (1999). Regulation of Dictyostelium protein-tyrosine phosphatase-3 (PTP3) through osmotic shock and stress stimulation and identification of pp130 as a PTP3 substrate. *J. Biol. Chem.* **274**, 12129-12138.
Garton, A. J. and Tonks, N. K. (1994). PTP-PEST: a protein tyrosine phosphatase regulated by serine phosphorylation. *EMBO J.* **13**, 3763-3771.
Gatsios, P., Terstegen, L., Schliess, F., Haussinger, D., Kerr, I. M., Heinrich, P. C. and Graeve, L. (1998). Activation of the Janus kinase/signal transducer and activator of transcription pathway by osmotic shock. *J. Biol. Chem.* **273**, 22962-22968.
Goldberg, J. M., Manning, G., Liu, A., Fey, P., Pilcher, K. E., Xu, Y. and Smith, J. L. (2006). The dictyostelium kinome-analysis of the protein kinases from a simple model organism. *PLoS Genet.* **2**, e38.
Howard, P. K., Sefton, B. M. and Firtel, R. A. (1992). Analysis of a spatially regulated phosphotyrosine phosphatase identifies tyrosine phosphorylation as a key regulatory pathway in Dictyostelium. *Cell* **71**, 637-647.
Howard, P. K., Gamper, M., Hunter, T. and Firtel, R. A. (1994). Regulation by protein-tyrosine phosphatase PTP2 is distinct from that by PTP1 during Dictyostelium growth and development. *Mol. Cell. Biol.* **14**, 5154-5164.
Huang, E., Blagg, S. L., Keller, T., Katoh, M., Shaulsky, G. and Thompson, C. R. (2006). bZIP transcription factor interactions regulate DIF responses in Dictyostelium. *Development* **133**, 449-458.
Liu, T., Castro, S., Brasier, A. R., Jamaluddin, M., Garofalo, R. P. and Casola, A. (2004). Reactive oxygen species mediate virus-induced STAT activation: role of tyrosine phosphatases. *J. Biol. Chem.* **279**, 2461-2469.
Madamanchi, N. R., Li, S., Patterson, C. and Runge, M. S. (2001). Reactive oxygen species regulate heat-shock protein 70 via the JAK/STAT pathway. *Arterioscler. Thromb. Vasc. Biol.* **21**, 321-326.
Montalibet, J., Skorey, K. I. and Kennedy, B. P. (2005). Protein tyrosine phosphatase: enzymatic assays. *Methods* **35**, 2-8.
Pang, K. M., Lynes, M. A. and Knecht, D. A. (1999). Variables controlling the expression level of exogenous genes in Dictyostelium. *Plasmid* **41**, 187-197.
Reich, N. C. and Liu, L. (2006). Tracking STAT nuclear traffic. *Nat. Rev. Immunol.* **6**, 602-612.
Simon, A. R., Rai, U., Fanburg, B. L. and Cochran, B. H. (1998). Activation of the JAK-STAT pathway by reactive oxygen species. *Am. J. Physiol.* **275**, C1640-C1652.
Tanuma, N., Nakamura, K., Shima, H. and Kikuchi, K. (2000). Protein-tyrosine phosphatase PTPepsilon C inhibits Jak-STAT signaling and differentiation induced by interleukin-6 and leukemia inhibitory factor in M1 leukemia cells. *J. Biol. Chem.* **275**, 28216-28221.
ten Hoeve, J., de Jesus Ibarra-Sanchez, M., Fu, Y., Zhu, W., Tremblay, M., David, M. and Shuai, K. (2002). Identification of a nuclear Stat1 protein tyrosine phosphatase. *Mol. Cell. Biol.* **22**, 5662-5668.
Thompson, C. R. and Kay, R. R. (2000). The role of DIF-1 signaling in Dictyostelium development. *Mol. Cell* **6**, 1509-1514.
Tonks, N. K. (2005). Redox redux: revisiting PTPs and the control of cell signaling. *Cell* **121**, 667-670.
Watts, D. J. and Ashworth, J. M. (1970). Growth of myxameobae of the cellular slime mould Dictyostelium discoideum in axenic culture. *Biochem. J.* **119**, 171-174.
Williams, J. G. (2003). The STAT proteins of *Dictyostelium*. In *Signal Transducers and Activators of Transcription (STATs): Activation and Biology* (ed P. B. Sehgal, D. E. Levy and T. Hirano), pp. 105-121. Boston: Kluwer Academic Publishers.
Yu, C. L., Jin, Y. J. and Burakoff, S. J. (2000). Cytosolic tyrosine dephosphorylation of STAT5. Potential role of SHP-2 in STAT5 regulation. *J. Biol. Chem.* **275**, 599-604.
Yudushkin, I. A., Schleifenbaum, A., Kinkhabwala, A., Neel, B. G., Schultz, C. and Bastiaens, P. I. (2007). Live-cell imaging of enzyme-substrate interaction reveals spatial regulation of PTP1B. *Science* **315**, 115-119.
Zhukovskaya, N. V., Fukuzawa, M., Yamada, Y., Araki, T. and Williams, J. G. (2006). The Dictyostelium bZIP transcription factor DimB regulates prestalk-specific gene expression. *Development* **133**, 439-448.