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The *Dictyostelium* prestalk cell inducer DIF regulates nuclear accumulation of a STAT protein by controlling its rate of export from the nucleus

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SUMMARY

Dd-STATc becomes tyrosine phosphorylated, dimerises and accumulates in the nuclei of *Dictyostelium* cells exposed to DIF, the chlorinated hexaphenone that directs prestalk cell differentiation. By performing cytoplasmic photobleaching of living cells, we show that DIF inhibits the nuclear export of Dd-STATc. Within Dd-STATc there is a 50 amino acid region containing several consensus CRM1 (exportin 1)-dependent nuclear export signals (NESs). Deletion of this region causes Dd-STATc to accumulate in the nucleus constitutively and, when coupled to GFP, the same region directs nuclear export. We show that the N-terminal-proximal 46 amino acids are necessary for nuclear accumulation of Dd-STATc and sufficient to direct constitutive nuclear accumulation when fused to GFP. Combining the photobleaching and molecular analyses, we suggest that DIF-induced dimerisation of Dd-STATc functionally masks the NES-containing region and that this leads to nett nuclear accumulation, directed by the N-terminal-proximal import signals. These results show that the regulated nuclear accumulation of a STAT protein can be controlled at the level of nuclear export and they also provide a better understanding of the mechanism whereby DIF directs cell type divergence.

Key words: STAT proteins, DIF, *Dictyostelium discoideum*, prestalk differentiation

INTRODUCTION

When subjected to starvation *Dictyostelium* cells aggregate together and differentiate as either prestalk or prespore cells. Extracellular cAMP signalling induces prespore differentiation and the cells produce a cAMP antagonist, DIF, that diverts 20% of the uncommitted cells into the prestalk differentiation pathway (reviewed by Kay et al., 1999; Early, 1999). DIF is a chlorinated hexaphenone that induces stalk cell differentiation in a monolayer assay system and that is required, during normal development, for the differentiation of one class of prestalk cells (Town et al., 1976; Morris et al., 1987; Thompson and Kay, 2000). In the monolayer assay system DIF rapidly induces transcription of *ecmA*, a prestalk-specific gene that encodes an extracellular matrix protein (Williams et al., 1987). The mechanism of action of DIF is not known but a *Dictyostelium* STAT protein, Dd-STATc, is tyrosine phosphorylated and translocates to the nucleus when cells are exposed to DIF (Fukuzawa et al., 2001).

STAT proteins were discovered as the transcriptional regulators that mediate interferon action but are now known to have many other cellular and developmental functions (Bromberg, 2000; Bromberg and Darnell, 2000; Horvath, 2000; Levy, 1999; Luo and Dearolf, 2001; Watson, 2001; Zeidler et al., 2000). Despite their importance the mechanisms that regulate STAT nuclear accumulation are relatively poorly understood. STATs are activated by tyrosine phosphorylation and dimerise via reciprocal SH2 domain:phosphotyrosine interactions (Shuai et al., 1993). Generally, dimerisation seems a necessary and sufficient trigger to induce nuclear accumulation and or biological activity (Bromberg et al., 1999; Milocco et al., 1999). However, these sometimes seem to occur without an apparent need for dimerisation (Johnson et al., 1999; Kumar et al., 1997). Initially, we reported that mutant forms of Dd-STATc, wherein the site of tyrosine phosphorylation and/or the SH2 domain were destroyed, remain DIF inducible (Fukuzawa et al., 2001). However, we subsequently discovered that, when gene repair by homologous recombination is prevented, a mutation in the tyrosine phosphorylation site is non-inducible (M. F. and J. G. W., unpublished data) (Fukuzawa et al., 2001). Hence Dd-STATc conforms to the general pattern of metazoan STAT behaviour, dimerisation triggers nuclear accumulation.

Here we analyse the Dd-STATc protein to delineate the mechanisms that direct its nuclear accumulation. We show that DIF regulates the nuclear accumulation of Dd-STATc by controlling its rate of export from the nucleus and we map nuclear import and export signals within the protein.

MATERIALS AND METHODS

Cell culture, transformation and development

The Ax2, axenic strain was used in all experiments and cells were

cultured at 22°C in HL5 medium (Watts and Ashworth, 1970). Transformation, was performed by electroporation and strains were selected at 10 μg/ml for Blasticidin S or 20 μg/ml for G418. Except where indicated, Dd-STATc constructs were based on GFP:STATc (Fukuzawa et al., 2001). In GFP:STATc the semi-constitutive Actin15 promoter drives expression of a fusion protein containing GFP at its N terminus, linked to the entire Dd-STATc protein. For DIF assays, cells were washed twice in KK2 phosphate buffer (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH 6.2) and resuspended at 10^7 cells/ml in KK2 for 4 hours at 23°C to render them competent to respond to DIF, which was added to a final concentration of 100 nM and for 5 minutes. Nuclear localisation of GFP fusion proteins was assayed using a confocal fluorescence microscope (Leica DMRBE model TCS-SP2).

Photobleaching asssay of nuclear export

Dictyostelium cells transformed with GFP:STATc were rendered competent to respond to DIF as described above and then allowed to spread on a Petri dish (Petriperm hydrophilic, In Vitro Systems and Services, GmbH). Even in an uninduced cell there is always a level of nuclear fluorescence that is slightly higher than in the cytoplasm and this fluorescence difference was used to locate the position of the nucleus using a confocal microscope (Leica DMRBE model TCS-SP2). The nucleus was then masked to protect it from irradiation. The cytoplasm was irradiated for 5 seconds at a wavelength of 478 nm and this usually proved to be a level of photobleaching sufficient to reduce the GFP fluorescence by at least 90%. Cells with 10% or less residual cytoplasmic GFP fluorescence were then incubated further and the fluorescence signal within the nuclei was determined at different time points.

RESULTS

DIF inhibits export of Dd-STATc from the nucleus

In order to determine whether DIF-induced nuclear accumulation of Dd-STATc is regulated at the level of nuclear import or export, we used photobleaching to study the kinetics of its nuclear export (Fig. 1A). Cells expressing GFP:STATc, a fusion protein containing the Dd-STATc protein linked via its N terminus to GFP, were identified using a confocal microscope. The cytoplasm was then subjected to photobleaching, to destroy the fluorescence of the cytosolic fusion protein, and cells were further incubated in the absence or the presence of DIF. This protocol allows the fate of the pre-existent nuclear pool of Dd-STATc protein to be determined in individual, living cells.

When the majority of isolated cells within a population (the behaviour is different for cells in clumps and for a minority of the single cells, see legend to Fig. 1A) are photobleached and incubated in the absence of added DIF, 50% of the GFP:STATc protein exits from the nucleus with a $t_{1/2}$ of about 1.5 minutes (Fig. 1B). In contrast, in the presence of DIF, the majority (approx. 65%) of the GFP:STATc protein remains in the nucleus over the entire 5 minute incubation period. The inhibitory effect of DIF on Dd-STATc nuclear efflux is not due to non-specific inhibition of all nuclear export, because the nuclear efflux of Dd-STATa, a *Dictyostelium* STAT that is regulated by cAMP signalling (Araki et al., 1998; Kawata et al., 1997), is not inhibited by DIF (Fig. 1C).

The above results show that DIF inhibits the nuclear export of Dd-STATc but DIF could act to control both the export rate and the import rate of Dd-STATc. In principle, photobleaching could be used to distinguish these possibilities. However,

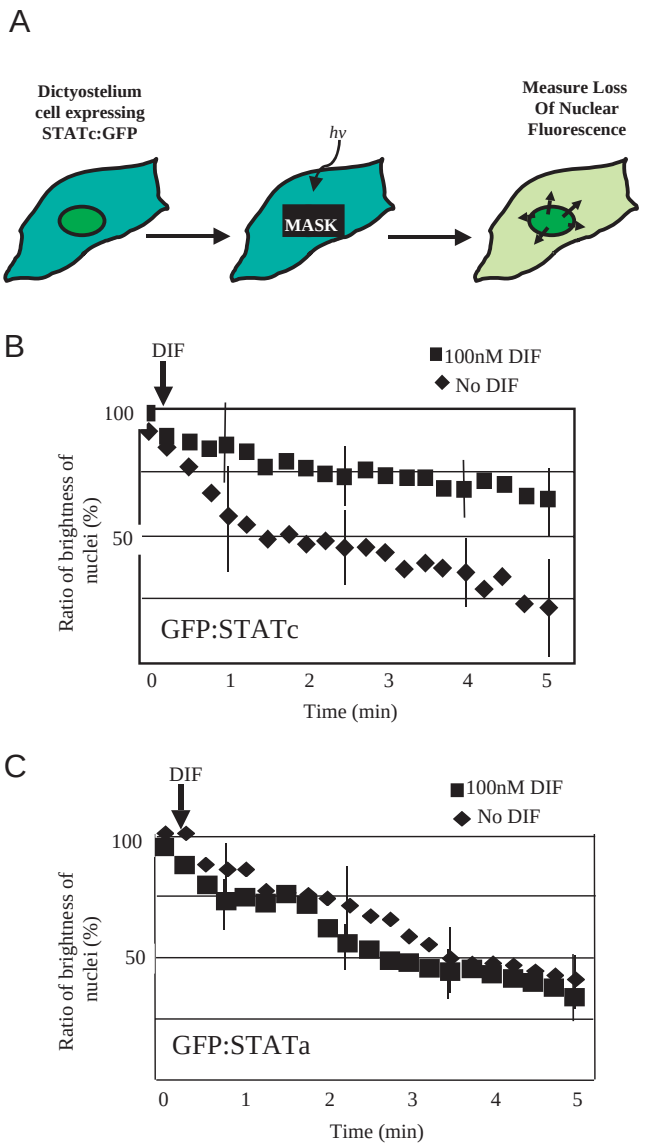

Fig. 1. Photobleaching analysis of the efflux of GFP:STATc from the nucleus. (A) Principle of the photobleaching method. Isolated cells transformed with GFP:STATc, a blasticidin-based, 'single copy' vector (Fukuzawa et al., 2001) in which there was a low to moderate fluorescence signal in the nucleus, were photobleached to reduce only the cytoplasmic fluorescence (the nucleus was masked) and then incubated in either the presence or the absence of 100 nM DIF for 5 minutes. Many of the cells in clumps, and a sub-set (approx. 10%) of the spatially separated cells, show a very high intrinsic level of Dd-STATc nuclear fluorescence. There is a DIF-independent, stress-induced mechanism that directs nuclear accumulation of Dd-STATc (T. Araki, M. Tsujioka, T. A. and J. G. W., unpublished data) and this may account for these 'high background' cells. In such cells, nuclear fluorescence is retained irrespective of the presence or absence of DIF and they were not therefore included in the analysis. (B,C) Analysis of nuclear efflux rates of (B) GFP:STATc and (C) GFP:STATa in the presence and absence of DIF. Cells transformed with GFP:STATc or GFP:STATa were photobleached as described in A. The fluorescence signal from the cytoplasm and nucleus was monitored over 5 minutes. In order to maintain constant conditions, the 'contrast-stretch' function of the microscope was turned off. In each experiment a total of at least 30 cells was analysed and the graph shows the ratio (nuclear signal – background)/(cytoplasmic signal – background) × 100 (%) ± s.d.

technical limitations, imposed by the very small size of the nucleus, the rapid movement of the cells and the toxic effect of the photobleaching radiation on the nucleus made it impossible to perform the reverse experiment: i.e. to photobleach the nucleus and then determine the rate at which GFP:STATc moves into the nucleus in the absence or presence of DIF. Hence we used a molecular genetic approach to further analyse the process.

Dd-STATc contains a sub-region that directs nuclear export

Because the photobleaching experiments suggested that DIF induces Dd-STATc nuclear accumulation by inhibiting its nuclear export, we searched for nuclear export signals (NESs) within Dd-STATc. The region of Dd-STATc between residues 505 and 554 is partially homologous in sequence to a region of Dd-STATa that directs nuclear export when fused to GFP (Fig. 2A) (Ginger et al., 2000). This entire region of Dd-STATc, which we term the EXP region, is very Leu/Ile-rich and this is the characteristic feature of NESs. There is a region (B) near the C terminus of EXP that fits well to the HTV1-rex/rad24 type (LXXXLXL) of NES and another (A), nearer the N terminus, which is also a reasonable match (Fig. 2A). We showed that the EXP region functions as a nuclear export mediator both by deleting it from the intact protein and by fusing it to GFP.

GFP:STATcΔEXP, a GFP fusion protein containing an internal deletion that removes only the EXP region, is constitutively localised within the nucleus (Fig. 2B). We employed photobleaching to determine whether GFP:STATcΔEXP is constitutively localised within the nucleus because it is defective in export from the nucleus (Fig. 3). Comparison of Fig. 3 with Fig. 2A shows that GFP:STATcΔEXP-expressing cells incubated in the absence of DIF behave very much like GFP:STATc-expressing cells incubated in the presence of DIF, i.e. the majority of the GFP fusion protein remains in the nucleus over the entire 5-minute incubation period. This observation readily explains the constitutive nuclear accumulation of GFP:STATcΔEXP. The fact that the GFP:STATcΔEXP fusion protein is itself unaffected by the addition of DIF (Fig. 3) also shows that the EXP region is essential for DIF-induced inhibition of nuclear export.

The EXP region can also act in isolation as an export mediator. This was shown by fusing EXP to GFP, to generate EXP:GFP. GFP is small enough to diffuse into the nucleus freely but the EXP:GFP fusion protein is enriched in the cytoplasm over the nucleus (Fig. 4), indicating that the EXP region contains one or more functional NESs. The best-characterised nuclear export process is that mediated by CRM1 (exportin-1) and the binding sites for CRM1 are, as stated, loosely conserved, leucine-rich sequences (Fig. 2A). In

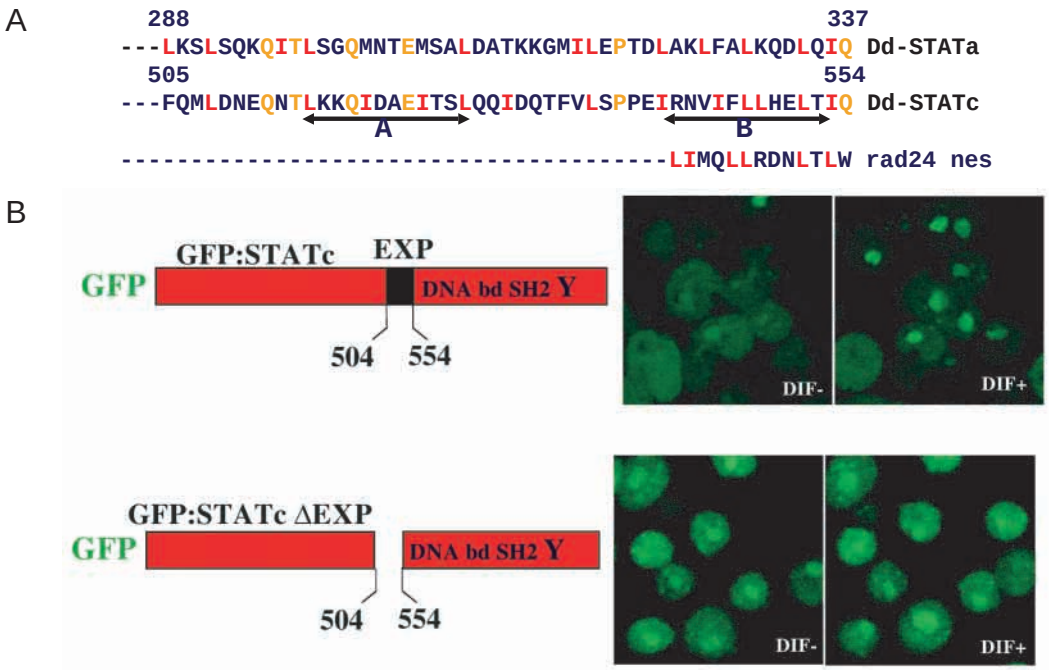

Fig. 2. Identification of NESs within Dd-STATc. (A) Alignment of potential NES sequences. Dd-STATc (middle) contains, near its centre, a leucine/isoleucine rich region (red letters) (the EXP region). A part of Dd-STATa (top) that contains the 50 a.a. region homologous to EXP has been shown to function as an LMB-sensitive NES (see text) and is displayed here for comparison. Identities between Dd-STATa and Dd-STATc are shown in orange. The NES of rad24 is shown (bottom) for comparison, aligned below the leucine rich region near the C terminus of EXP (marked as B). Another candidate NES, i.e. a leucine-rich region, nearer the N terminus is also marked as A . (B) Nuclear accumulation of a Dd-STATc mutant protein lacking EXP. The GFP:STATc construct comprises the entire Dd-STATc protein, with GFP fused at its N terminus. It is a blasticidin-based, 'single copy' vector (Fukuzawa et al., 2001). GFP:STATcΔEXP is an equivalent construct with an internal deletion (residues 505 to 554) that removes just the EXP region. Both these constructs were introduced into cells and stable transformant clones were selected. In order to rule out homologous gene conversion, a frequent event with Dd-STATc, the structure of the two GFP fusion proteins was checked by western blotting and were as expected. Cells transformed with these constructs were developed in shaken suspension for 4 hours and then analysed for nuclear GFP after a further 5 minutes of incubation in either the presence or absence of 100 nM DIF.

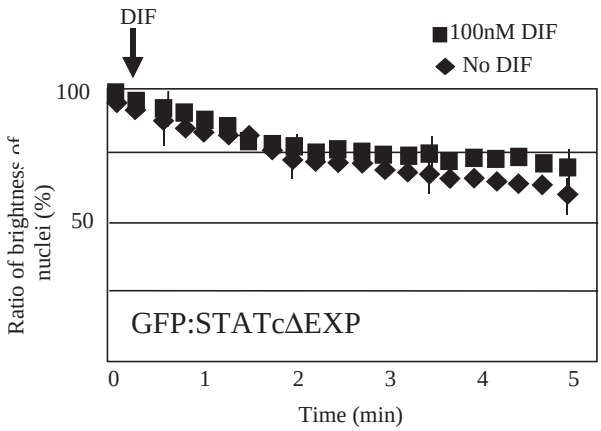

Fig. 3. Analysis of nuclear efflux rates of GFP:STATcΔEXP in the presence and absence of DIF. Cells transformed with GFP:STATcΔEXP (Fig. 2B) were photobleached exactly as described in Fig. 1A,B.

the case of Dd-STATa (Ginger et al., 2000) nuclear export directed by the NES-containing region is sensitive to leptomycin B (LMB), a drug that binds to and inhibits CRM1. When cells expressing the EXP:GFP fusion protein are treated with LMB, nuclear exclusion is lost and the protein accumulates in both the nucleus and the cytoplasm (Fig. 4). Thus the EXP region contains at least one functional, CRM1-dependent NES.

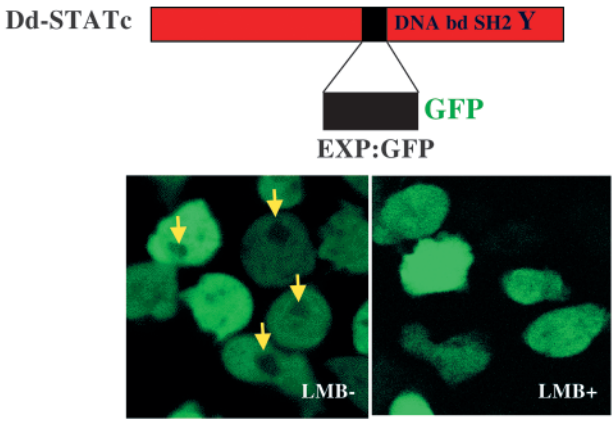

Fig. 4. Demonstration that EXP is an LMB-sensitive NES. The EXP region (residues 505-554) of Dd-STATc was inserted at the N terminus of GFP, to yield EXP:GFP, under transcriptional control of the Actin 15 promoter. This construct contains a G418 resistance cassette and was transformed into *Dictyostelium*. Clones of expressing cells containing multiple copies of EXP:GFP were selected using G418. GFP diffuses freely into the nucleus but in EXP:GFP-transformed cells, here shaken for 3 hours under starvation conditions, the fusion protein is selectively excluded from the nucleus (the positions of the nuclei are indicated by yellow arrows). When, however, cells are incubated for 90 minutes with LMB (20 nM) exclusion is reversed and there is a uniform GFP signal over the cell or, occasionally, even a slight nuclear enrichment [we have previously shown that there is sometimes a low level of nuclear enrichment of GFP itself when it is expressed in *Dictyostelium* (Ginger et al., 2000)].

In the absence of the EXP region the N-terminal-proximal half of Dd-STATc directs constitutive nuclear accumulation

The constitutive accumulation of GFP:STATcΔEXP, the GFP fusion protein containing an internal deletion that removes just the EXP region (Fig. 2B), suggests that there are cryptic nuclear import signals within Dd-STATc. Dd-STATa and Dd-STATc are very differently regulated but they are highly conserved in the proximal regions of their C termini. Hence we first searched for import activity in the N-terminal-proximal half of Dd-STATc. GFP:STATc1-504 encodes a GFP fusion protein containing the region of Dd-STATc extending from the N terminus to a point just upstream of the EXP region. The GFP:STATc 1-504 fusion protein is constitutively enriched in the nucleus (Fig. 5A), showing that there are one or more nuclear import signals in the N-terminal half of Dd-STATc.

In construct GFP:STATc1-554, a 50 residue longer N-terminal fragment fusion wherein the EXP region is retained, the GFP fusion protein is excluded from the nucleus (Fig. 5B). Thus the NESs contained within the EXP region are dominant over the import signals within the N-terminal region. Moreover, the fact that LMB treatment of cells transformed with GFP:STATc1-554 causes nuclear accumulation (Fig. 5B) shows that exclusion from the nucleus depends upon the activity of CRM1.

Essential nuclear import signals are located within the N-terminal 46 amino acids

We next mapped the presumptive NLSs contained within the N-terminal region by performing N to C deletion analysis of the Dd-STATc protein. A construct with five N-terminal amino acids deleted retains DIF-inducible nuclear accumulation but all smaller constructs, including a construct (GFP:STATc47-929) with only 46 amino acids deleted, yield GFP fusion proteins that are equally distributed between the cytoplasm and the nucleus and non-DIF inducible (Fig. 6A). Thus the N-terminal 46 amino acids (we will term this the 'IMP region') are necessary for DIF-inducible nuclear accumulation. [NB We believe that there is at least one other weakly active nuclear import region, located just downstream of EXP, between residues 555 and 607 (M. F. and J. G. W., unpublished data). The presence of this second import domain probably explains why the N-terminally deleted proteins, where IMP is absent, are not excluded from the nucleus, despite the presence of the EXP region.]

When fused to GFP, in IMP:GFP, the IMP region directs constitutive nuclear accumulation (Fig. 6B). Thus, in addition to being necessary for DIF-inducible nuclear accumulation of the Dd-STATc protein, the IMP region can function as an autonomous NLS. Within the IMP region there is a stretch of basic amino acids (^{1}MSNNNP<u>KKR</u>PLD^{12}), that could conceivably form part of a 'classical', importin-α binding NLS. However, when the three basic residues are mutated to alanine, within the context of the whole protein, nuclear accumulation in response to DIF is unaffected (data not shown). This is in accord with previous work on NLSs within STAT1, where importin-α5 is believed to mediate nuclear import but via a totally distinct mechanism from that utilised for classical NLSs (Sekimoto et al., 1997; McBride et al., 2002).

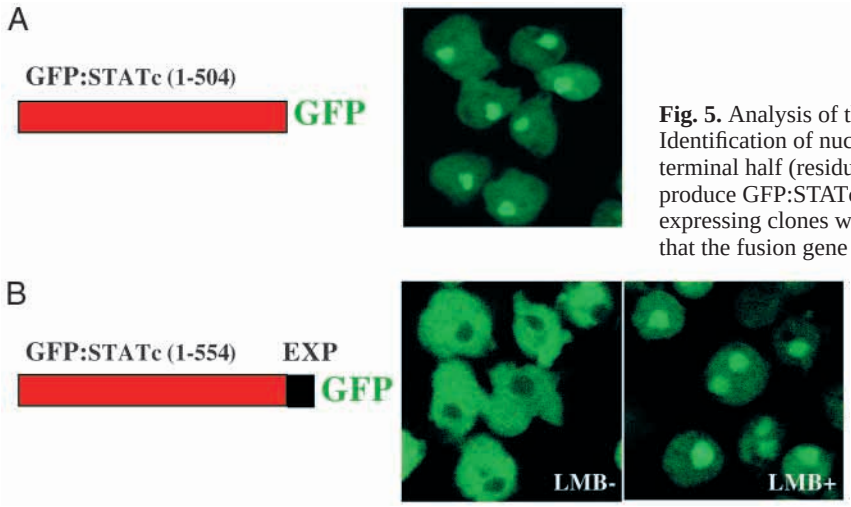

Fig. 5. Analysis of the N-terminal-proximal region of Dd-STATc. Identification of nuclear import signals in Dd-STATc. The approximate N-terminal half (residues 1-504) of Dd-STATc was fused upstream of GFP, to produce GFP:STATc1-504 and introduced into *Dictyostelium* cells. Stably expressing clones were selected and western transfer analysis confirmed that the fusion gene produced a fusion protein of the expected size. The cells were then analysed as in Fig. 4. (B) Analysis of the effect of the EXP region on the N-terminal-proximal import region. Construct GFP:STATc1-554 differs from construct GFP:STATc1-504 only in that it contains the EXP region. GFP:STATc1-554 was transformed into cells and they were analysed as described in Fig. 4. The panel at the right shows cells treated with LMB for 90 minutes, resulting in nuclear accumulation.

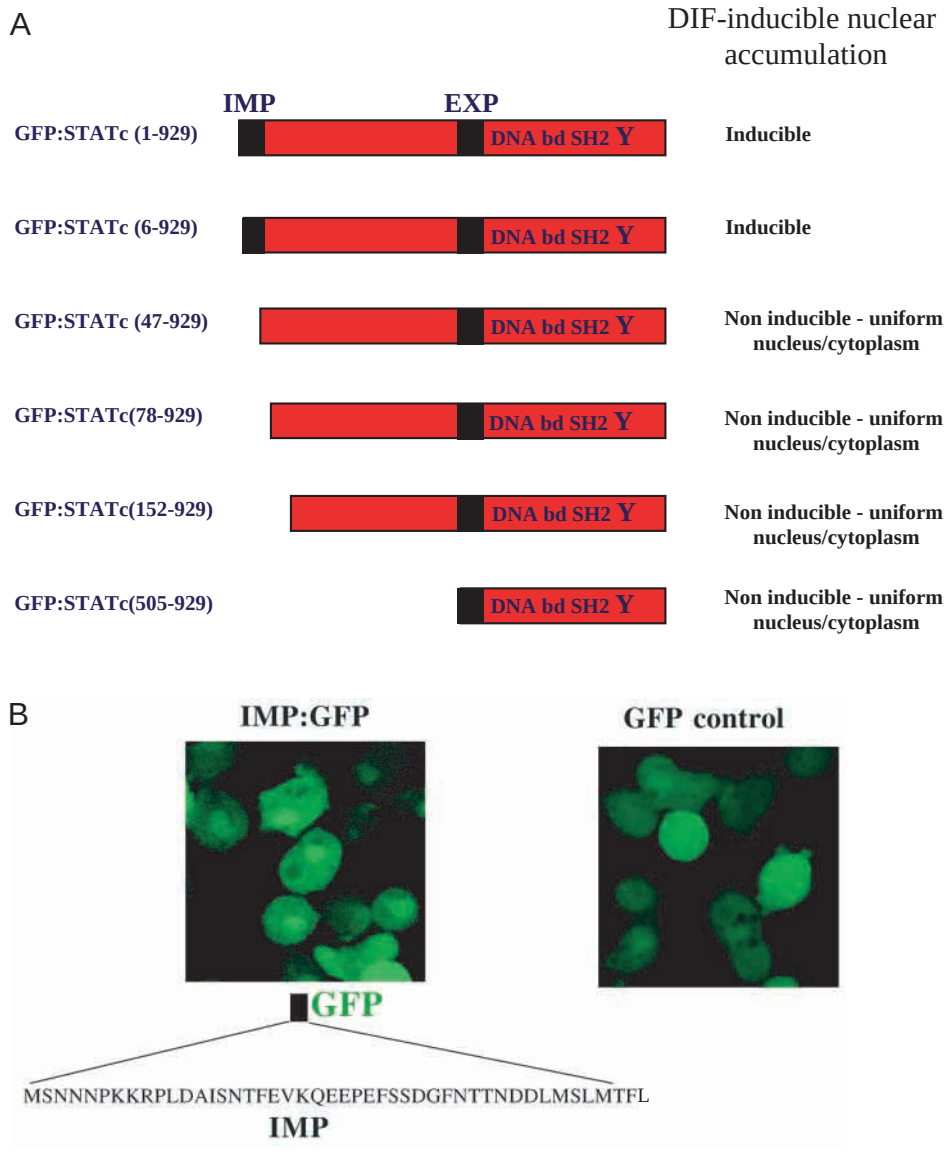

Fig. 6. Mapping nuclear import signals in Dd-STATc. (A) Analysis of an N to C deletion series of Dd-STATc. A set of constructs, bearing the indicated N to C deletions, was constructed by PCR. The indicated regions were amplified and cloned immediately downstream of the Actin 15 promoter:GFP fusion present in GFP:STATc replacing the original Dd-STATc sequences (Fukuzawa et al., 2001). The constructs were transformed into cells and analysed for DIF inducibility as in Fig. 2B. (B) Identification of a nuclear import signal at the N terminus of Dd-STATc. Construct IMP:GFP contains the N-terminal-proximal 46 residues of Dd-STATc fused upstream of GFP. This construct contains a G418 resistance cassette and was transformed into *Dictyostelium*. Clones of expressing cells containing multiple copies of IMP:GFP were selected using G418. GFP accumulation in the nucleus was monitored in growing cells (left) and, in parallel, with an identical construct expressing GFP alone (right).

DISCUSSION

Photobleaching analysis of living cells shows that DIF is an inhibitor of Dd-STATc nuclear efflux

The photobleaching analysis suggests that DIF functions to control the nuclear accumulation of Dd-STATc by repressing its export from the nucleus. The DIF-induced decrease in export rate we measure by photobleaching is not complete but it can, in principle, account for the nuclear accumulation induced by DIF; if the rate of Dd-STATc import into the nucleus is only slightly below its rate of export then even a small change in export rate will have a disproportionately large effect on its nett rate of accumulation.

The notion of Dd-STATc nuclear accumulation as a balance between competing import and export processes accords with the now generally held view of regulated nuclear translocation. One reagent that has been valuable in arriving at this perspective in other systems is LMB, because it can be used to selectively inhibit nuclear export and thus reveal any constitutive import. However, we find that LMB treatment does not induce nuclear accumulation of the intact Dd-STATc protein (M. F. and J. G. W., unpublished data). A similar observation has been made for STAT1 (Begitt et al., 2000). Again, the intact STAT1 protein failed to accumulate in the nucleus of cells not treated with interferon, in the presence of LMB, although molecular dissection showed that it contains an LMB-sensitive NES. In this case, however, LMB did slow the efflux of STAT1 after adaptation to the interferon stimulus. We find no such effect in the case of DIF-treated cells (M. F. and J. G. W., unpublished data).

LMB resistance of the intact Dd-STATc protein may be the result of co-operative interaction between EXP and sequences in the C-terminal half of the protein. Multiple nuclear export signals within a protein can act co-operatively to direct nuclear export by CRM1 (Gaubatz, 2001) and there are additional, weak nuclear export signals in the region of Dd-STATc between residue 716 and the C terminus at residue 929 (M. F. and J. G. W., unpublished data). Perhaps, therefore, co-operating NESs in the intact Dd-STATc protein produce a substrate with a high relative affinity for CRM1, that can function at sub-saturating doses of LMB. This effect would be compounded by the relative non-susceptibility of *Dictyostelium* cells to LMB; at 20 nM the concentration used here for Dd-STATc only 30% of cells transformed with a nuclear excluded mutant of Dd-STATa (Dd-STATaAcidpep) showed nuclear accumulation and higher concentrations of LMB blocked both export and import (Ginger et al., 2000). A similar mechanism has been proposed to explain the LMB insensitivity of a mutant form of the ICP27 protein of Herpes Simplex Virus (Murata et al., 2001).

Deletion of a region containing LMB-sensitive NESs leads to constitutive nuclear accumulation

Although LMB does not affect the intact Dd-STATc protein, we were able to verify the photobleaching result by mutating the Dd-STATc protein. The fact that deletion from Dd-STATc of the LMB-sensitive nuclear export mediators located in the EXP region leads to constitutive nuclear accumulation complements the conclusion from the photobleaching results very well; in cells transformed with this deletion construct the fusion protein accumulates in the nucleus without the need for DIF treatment. This again suggests that the cellular processes directing nuclear import of the intact protein are constitutively active and that the rate of export from the nucleus, partly or wholly mediated by the EXP region, acts to determine the [cytoplasm]/[nucleus] ratio for Dd-STATc. In support of this suggestion we showed, using photobleaching, that deletion of the EXP region drastically reduced its rate of efflux from the nucleus and rendered it DIF insensitive.

There is a nuclear import signal very near the N terminus that falls under the control of the EXP region

We localised nuclear import signals by showing that the 46 amino acid residue region at the N terminus, the IMP region, is essential for the nuclear accumulation of Dd-STATc. We also showed that the IMP region itself will direct nuclear accumulation when fused to GFP. The nuclear export signals contained within the EXP region are, however, dominant to the IMP region; so that a fragment containing the entire N-terminal half of the protein, including EXP and IMP, is excluded from the nucleus. This result again indicates the existence of an equilibrium between the nuclear import and export of Dd-STATc. Analysis of the LMB sensitivity of this construct also supports the notion discussed above, that co-operative interaction between EXP and C-terminal-proximal export signals leads to the LMB insensitivity of the whole Dd-STATc protein; because this fragment, which contains only the approximate N-terminal half of the Dd-STATc protein, accumulates in the nucleus in the presence of LMB.

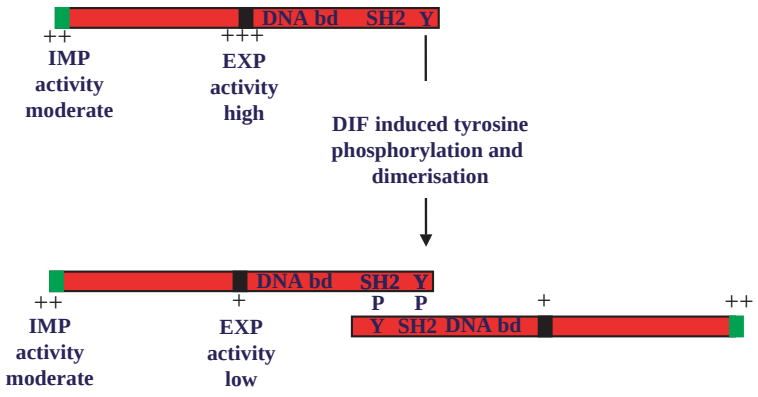

Fig. 7. A model for DIF-induced nuclear accumulation of Dd-STATc. The Dd-STATc protein is proposed to be predominantly cytosolic in the absence of DIF, because the NESs in the EXP region predominate over the NLSs present within the IMP region (illustrated by the number of crosses). The model proposes a conformational change when Dd-STATc monomers dimerise, in reponse to DIF treatment, that masks the activity of EXP. The increased relative activity of the nuclear import signal(s) in the IMP regions then causes Dd-STATc to accumulate within the nucleus.

A model for the DIF-induced nuclear accumulation of Dd-STATc

The C-terminal-proximal half of Dd-STATc is essential for the regulation of nuclear accumulation by DIF. This region contains the DNA binding site, the SH2 domain and the site of tyrosine phosphorylation and we now know that tyrosine phosphorylation is essential for DIF-induced nuclear accumulation of Dd-STATc. We therefore suggest that the DIF-induced dimerisation of Dd-STATc in some way masks EXP from CRM1 (Fig. 7). This allows the nuclear import signals in the IMP region to become dominant over the NESs in the EXP region, and as a result Dd-STATc accumulates in the nucleus.

Regulated export controls the nuclear accumulation of other transcription factors, e.g. p53, YAP1p, NF-AT (reviewed by Fonseca, 2002) but has not, thus far been reported for STAT proteins. While the efflux of STAT1 from the nucleus after cessation of interferon signalling (McBride et al., 2000), and of Dd-STATa upon adaptation to cAMP signalling (Ginger et al., 2000), are both mediated by CRM1 this is, to our knowledge, the first case in which the initial nuclear accumulation of a STAT protein has been shown to be regulated at the level of nuclear export. Indeed, in the best characterised STAT induction system, the activation of STAT1 by interferon γ, regulation appears to be at the level of nuclear import (McBride et al., 2002). Although STAT1 does not seem to contain a classical nuclear localisation signal (NLS), importin α5 binds to STAT1 via sequences near its C terminus (Sekimoto, 1997). Activation, by intereferon treatment, increases the degree of importin α5 binding to STAT1 and this increase correlates quantitatively with the formation of STAT1 dimers (McBride et al., 2002). The mechanism suggested by the STAT1 study is in one respect similar to that suggested here; dimerisation state governs the balance between import and export signals. The significant difference is that STAT1 dimerisation seems to unmask a latent NLS while we suggest that dimerisation of Dd-STATc masks an active NES.

Several different nuclear export signals have been mapped within STAT1 (Begitt et al., 2000; Ginger et al., 2000; McBride et al., 2002; McBride et al., 2000; Melen et al., 2001) but the EXP region of Dd-STATc is not conserved in position with respect to any of these. Also, as stated, regulated nuclear translocation of STAT1 appears to occur by a quite different mechanism than that of Dd-STATc. However, the mammalian STAT protein family has 6 other members that could, in principle, be regulated differently from STAT1. It remains possible therefore that nuclear export-based regulation features in mammalian STAT signalling.

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