

# Glycogen synthase kinase-3 enhances nuclear export of a *Dictyostelium* STAT protein

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**Extracellular cAMP stimulates the rapid tyrosine phosphorylation and nuclear translocation of the *Dictyostelium* STAT protein Dd-STATA. Here we show that it also induces serine phosphorylation by GskA, a homologue of glycogen synthase kinase-3 (GSK-3). Tyrosine phosphorylation occurs within 10 s of stimulation, whereas serine phosphorylation takes 5 min, matching the kinetics observed for the cAMP regulation of GskA. Phosphorylation by GskA enhances nuclear export of Dd-STATA. The phosphorylated region, however, is not itself a nuclear export signal and we identify a region elsewhere in the protein that mediates nuclear export. These results suggest a biphasic regulation of Dd-STATA, in which extracellular cAMP initially directs nuclear import and then, via GskA, promotes its subsequent export. It also raises the possibility of an analogous regulation of STAT nuclear export in higher eukaryotes.**

**Keywords:** *Dictyostelium discoideum*/GSK-3/nuclear export/serine phosphorylation/STAT transcription factors

## Introduction

The STAT proteins were discovered as transducers of the cellular response to cytokines (reviewed by Ihle and Kerr, 1995; Schindler and Darnell, 1995). They contain a DNA-binding domain, an SH2 domain, a tyrosine phosphorylation site and most contain a transcriptional activation domain at their C-terminus. When a cytokine binds to its receptor, an associated Janus kinase (JAK) is activated and phosphorylates STAT on a tyrosine residue. This leads to dimerization through binding of the phosphotyrosine on one STAT molecule to the SH2 domain of the other. STAT signalling pathways are also found in other organisms. The *Drosophila* genes *hopscotch* and *marelle* encode JAK and STAT homologues, respectively (Binari and Perrimon, 1994). They are required for blood cell development and control segmentation, imaginal disc cell proliferation and

polarity in the developing eye (Harrison *et al.*, 1995; Strutt and Strutt, 1999). A STAT protein homologue has been cloned from the non-metazoan organism *Dictyostelium* (Kawata *et al.*, 1997). This protein, Dd-STATA, is homologous to metazoan STAT proteins, possessing an SH2 domain, a tyrosine phosphorylation site and a DNA-binding domain. Dd-STATA does not, however, contain a transcriptional activation domain at its C-terminus and it is known to act as a repressor of transcription (Mohanty *et al.*, 1999).

All growing and developing *Dictyostelium* cells appear to contain a small amount of Dd-STATA in their nuclei, the only apparent exception being mature spore cells (Araki *et al.*, 1998). However, during development some cells come to exhibit a strong nuclear enrichment. This is most apparent between 6 and 10 h development, when *Dictyostelium* cells undergo the transition from unicellular amoebae to a multicellular aggregate (the mound). Immediately after formation of the mound >95% of cells show strong nuclear staining with anti-Dd-STATA antibodies. Consistent with these observations, tyrosine phosphorylation of Dd-STATA increases over the same time scale and is rapidly stimulated by extracellular cAMP: the central regulatory signal for aggregation.

After 12 h development a tip appears on top of the mound and this coordinates later development. The tip extends upwards and the mound reshapes into a tall finger-like column of cells, which under some environmental conditions can topple over to form a freely motile slug. There is a dramatic fall in the number of cells showing nuclear enrichment of Dd-STATA during tip formation, so that in the slug only ~15% have strong Dd-STATA nuclear localization and these are present in the slug tip. The ultimate product of *Dictyostelium* development is the fruiting body, which consists of a spore head, a stalk and a basal disc. Fruiting body formation (culmination) is blocked in mutants that lack Dd-STATA protein (Mohanty *et al.*, 1999). *Dd-STATA* null mutants also exhibit abnormal expression of the *ecmB* gene. In the wild type, strong expression of *ecmB* is seen at culmination in cells as they enter the stalk tube (Williams *et al.*, 1989). In a *Dd-STATA* mutant, *ecmB* is prematurely and ectopically expressed in pre-stalk cells before they enter the stalk tube (Mohanty *et al.*, 1999).

The *Dictyostelium* homologue of glycogen synthase kinase-3 (GSK-3), GskA, is also required for the correct pattern of gene expression during development. GSK-3 is a highly conserved protein kinase found in all eukaryotic groups and regulates a wide range of cellular processes, ranging from intermediate metabolism and gene expression to cytoskeletal integrity (Plyte *et al.*, 1992). Genetic studies suggest that GSK-3 family members play a crucial role during development. The *Drosophila* homologue of GSK-3, Shaggy (encoded by the gene *sgg*), is required for

establishing segment polarity, cell specification and correct patterning of the zygotic neuroectoderm (Ruel *et al.*, 1993; Siegfried *et al.*, 1994; Cadigan and Nusse, 1997). GskA is required to establish the correct pattern of cell types in the *Dictyostelium* mound (Harwood *et al.*, 1995) and its activity increases at this stage of development. GskA activity is stimulated by extracellular cAMP, through a process that is mediated by the cAMP receptor carC and the tyrosine kinase ZAK-1 (Kim *et al.*, 1999; Plyte *et al.*, 1999).

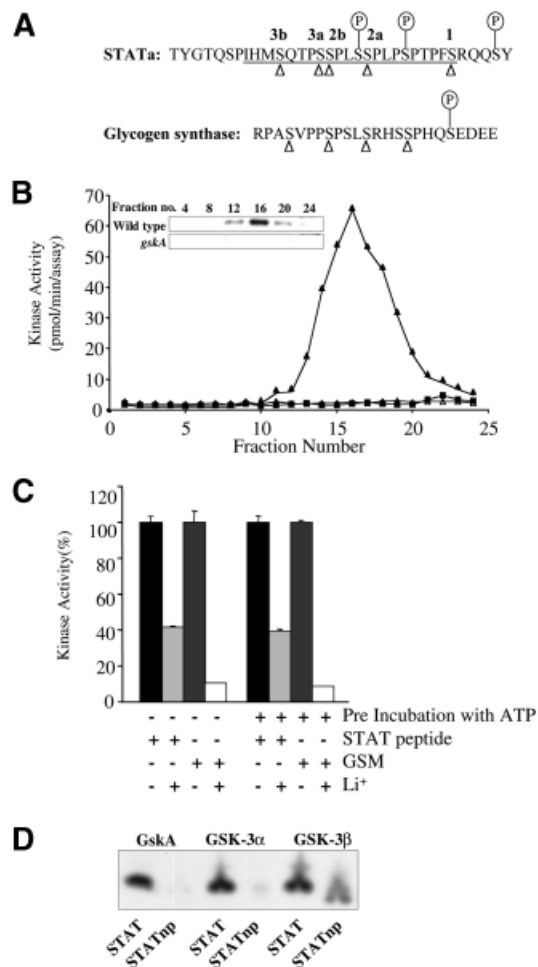
Among the targets of mammalian GSK-3 are a number of transcription factors and GSK-3 phosphorylation has different effects on each of them. Phosphorylation of c-jun interferes with its binding to DNA and hence represses c-jun-directed transcription (Boyle *et al.*, 1991). Conversely, GSK-3 phosphorylation of CREB activates gene transcription (Fiol *et al.*, 1994). GSK-3 phosphorylation of the lymphoid cell transcription factor NF-ATc causes enhanced nuclear exit (Beals *et al.*, 1997). An enhanced rate of nuclear export is also observed when GSK-3 phosphorylates cyclin D1 (Diehl *et al.*, 1998). STAT proteins are also targets of serine/threonine protein kinases (Wen and Darnell, 1997). Phosphorylation by MAP kinase regulates nuclear translocation and transcriptional activity of STAT1a and STAT3 (Stephens *et al.*, 1998). The STAT5a and 5b proteins also contain phosphoserine, but this phosphorylation is independent of MAP kinase activity and the identity of the cognate kinase is not yet known. In this paper we identify a GskA phosphorylation site in Dd-STATa and show that this modification enhances export of Dd-STATa from the nucleus. This demonstrates both a novel GSK-3 interaction and a new mechanism of STAT regulation.

## Results

### Dd-STATa contains potential GSK-3 phosphorylation sites

The sequence of Dd-STATa predicts a cluster of GSK-3 sites within a 34 amino acid sequence (amino acids 112–146) in the N-terminal portion of the protein (Figure 1A). A poly(glutamine)-rich region and a poly(asparagine)-rich region delimit this amino acid sequence at its N- and C-terminus, respectively. We refer to this sequence by the abbreviation GPR (GSK-3 phosphorylation region). Most GSK-3 phosphorylation sites are rich in proline and have the consensus S-P-X-X-S(PO<sub>4</sub>), where the phosphate is transferred to a serine three residues upstream of an existing phosphoserine. The phosphoserine can be formed by a 'priming' kinase, such as when protein kinase A modifies CREB or NF-ATc, or by sequential phosphorylation of overlapping GSK-3 sites, as seen in glycogen synthase (Figure 1A). The GPR of Dd-STATa contains five predicted GSK-3 phosphorylation sites: a single site at residue 140 (site 1) and an upstream cluster of four overlapping sites (sites 2a, 2b, 3a and 3b). This cluster is arranged such that GSK-3 phosphorylation of site 2a generates the priming site for site 2b and phosphorylation of the adjacent site 3a generates the priming site for 3b (Figure 1A).

To examine whether the GPR is a substrate for GSK-3 phosphorylation, a peptide encompassing sites 2a–3b, termed the STAT peptide, was synthesized (underlined in



**Fig. 1.** *In vitro* evidence for a GSK-3 phosphorylation site within Dd-STATa. (A) A comparison of the cluster of putative GSK-3 phosphorylation sites from Dd-STATa (residues 112–146) to that characterized for bovine glycogen synthase. Open triangles mark serine residues that are potentially phosphorylated by GSK-3 (numbered 1–3). 'Priming' phosphoserines, which are required to generate the GSK-3 site, are indicated by P. The sequence of a synthetic peptide, STAT peptide, which contains GSK-3 sites 2a–3b and phosphoserine residues at the putative 'priming' sites is underlined. (B) *Dictyostelium* extracts from wild-type cells (filled symbols) or *gskA* mutant cells (open symbols) were separated on a P11 phosphocellulose column. Each fraction was tested for its phosphorylation activity on the STAT peptide (triangles) or a peptide containing no phosphoserine residues (STATnp, squares). Activity correlated with the presence of GskA protein as detected by western analysis (see insert). No activity was seen using a STATnp peptide or using a *gskA* mutant cell extract. (C) Purified GskA [fraction 16 in (B)] was treated with 50 mM LiCl. Lithium inhibited phosphorylation of peptide substrates derived from glycogen synthase (GSM peptide) and the STAT peptide. The same GskA preparation was pre-incubated with ATP for 30 min. Substrate and 50 mM LiCl were added and phosphorylation assayed as before. (D) GskA, GSK-3 $\alpha$  and GSK-3 $\beta$  were used to phosphorylate STAT and STATnp peptide substrates, and visualized by Tricine-SDS gel electrophoresis.

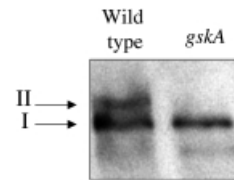
Figure 1A). The predicted priming phosphates were incorporated during peptide synthesis. Extracts from growing *Dictyostelium* cells were fractionated on phosphocellulose and tested against both GSM, a peptide based upon glycogen synthase that has been demonstrated to be a good GSK-3 substrate (Ryves *et al.*, 1998), and the STAT peptide. The phosphorylation activities for GSM (data not

shown) and the STAT peptide co-purified (Figure 1B). This peak of activity exactly matches the peak of GSK-3 protein concentration as judged by western analysis (Figure 1B, insert). No phosphorylation was seen with either an extract from a *gskA* mutant strain that lacks all GskA activity or a STAT peptide that lacks the priming phosphates (Figure 1B). The fraction with peak activity was treated with lithium chloride, which is an inhibitor of GSK-3 kinase activity. Lithium inhibited phosphorylation of STAT peptide, although perhaps not to the same degree as GSM (Figure 1C).

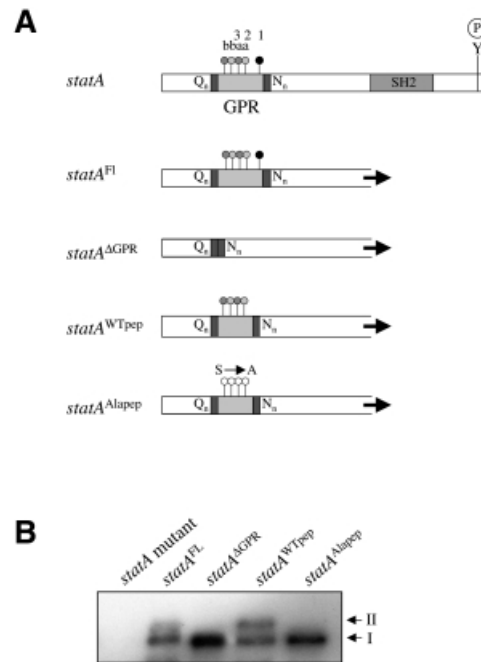
As our GskA preparation was not purified to homogeneity, it is possible that a contaminating kinase is activated by GskA and then is responsible for phosphorylation of the STAT peptide. Two experiments suggest that this is not the case. First, we pre-incubated our GskA enzyme preparation with ATP to allow GskA to activate the potential kinase contaminant. STAT peptide substrate and lithium chloride were then added and the reaction was incubated further. Activation of a kinase contaminant would allow STAT peptide phosphorylation in the presence of lithium. This was not observed (Figure 1C). Secondly, we phosphorylated the STAT peptide with GSK-3 kinases from different sources (Figure 1D). We found that the STAT peptide is a good substrate for both GSK-3 $\alpha$ , prepared from skeletal muscle, and recombinant GSK-3 $\beta$ , prepared from *Escherichia coli*. These observations strongly argue that GSK-3 directly phosphorylates the STAT peptide. Interestingly, we found that GSK-3 $\beta$  was able to phosphorylate an unprimed STAT peptide substrate (STATnp) weakly, suggesting that the STAT GPR may not always require a priming phosphate, as observed for other substrates such as axin,  $\beta$ -catenin and tau (Thomas *et al.*, 1999). Using our purified GSK-3 kinase, we calculate a  $K_m$  of 25  $\mu$ M for the STAT peptide (data not shown). This value is significantly better than the  $K_m$  measured for other GSK-3 substrates, including GSM, GS and eIF2B (Welsh *et al.*, 1997; Ryves *et al.*, 1998), further indicating that the GPR of Dd-STATa is a good *in vitro* GSK-3 substrate.

### GSK-3 targets Dd-STATa *in vivo*

Two forms of tyrosine-phosphorylated Dd-STATa with different electrophoretic mobilities are detected by western analysis (Araki *et al.*, 1998). The faster migrating tyrosine-phosphorylated species appears after 10 s stimulation with extracellular cAMP and its level reaches a plateau at 1 min. Its appearance correlates with the nuclear localization of Dd-STATa and with specific DNA-binding activity. The lower mobility tyrosine-phosphorylated species first appears 5 min after extracellular cAMP stimulation and levels reach a maximum at 15 min, a profile that matches the activation of GskA by cAMP (Plyte *et al.*, 1999). To investigate the potential link between GskA and Dd-STATa, we examined the Dd-STATa protein in a *gskA* mutant that lacks all GskA protein and activity. The lower mobility form of Dd-STATa was absent in the *gskA* mutant, suggesting that this form arises through GskA phosphorylation (Figure 2). The higher mobility form could still be detected using antiserum specific for the tyrosine-phosphorylated Dd-STATa, indicating that GskA activity is not required for tyrosine phosphorylation.

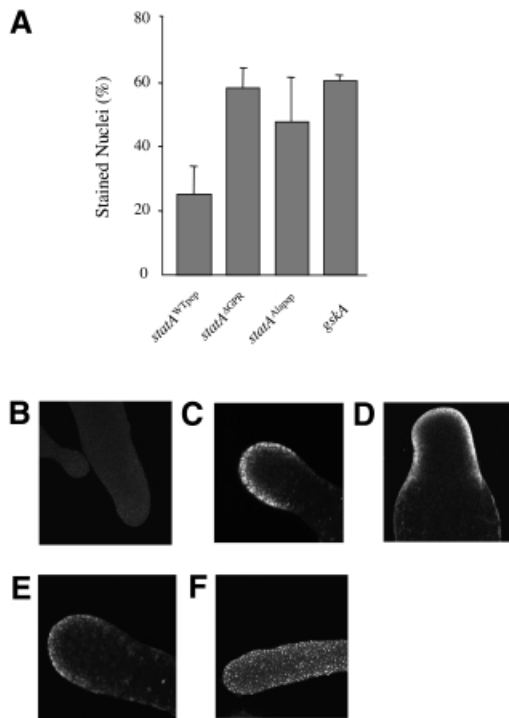


**Fig. 2.** Western analysis of Dd-STATa in wild-type and *gskA* mutant cells. Extracts of cells at 8 h development were investigated by western analysis using affinity-purified SC7-pTyr antiserum, which is specific for tyrosine-phosphorylated Dd-STATa (Araki *et al.*, 1998). The high mobility form of Dd-STATa (I) is present in both strains, but the slow mobility form (II) is not present in a *gskA* mutant. Proteins with higher mobilities than band I result from proteolysis of Dd-STATa.



**Fig. 3.** *In vivo* analysis of the GskA phosphorylation sites of Dd-STATa. (A) The putative GskA phosphorylation site was deleted from the Dd-STATa protein to make Dd-STATa $\Delta$ GPR. A sequence that corresponds to the STAT peptide was inserted into Dd-STATa $\Delta$ GPR to make Dd-STATa $^{WTpep}$ . A peptide sequence with alanine substitutions for those serine residues predicted to be phosphorylated by GskA was also inserted into Dd-STATa to make Dd-STATa $^{ALapep}$ . (B) Western analysis indicates that Dd-STATa proteins which lack the GPR or its GskA sites do not possess the lower mobility form of the Dd-STAT protein (II).

We next deleted the GPR to confirm that this region is required to generate the lower mobility form of Dd-STATa. Both the full-length Dd-STATa (Dd-STATa $^{FL}$ ) and deleted (Dd-STATa $\Delta$ GPR) proteins were expressed from the semi-constitutive *actin 15* promoter in a mutant lacking Dd-STATa (Figure 3A). The lower mobility form was absent in the Dd-STATa $\Delta$ GPR transformed cells (Figure 3B). A sequence corresponding to the STAT peptide was reintroduced into Dd-STATa $\Delta$ GPR. This gene, Dd-STATa $^{WTpep}$ , contains four of the five GSK-3 phosphorylation sites present in the GPR and its introduction restores the lower mobility form (Figure 3B). A mutated sequence was made where alanine residues replace all of the serine residues predicted to be phosphorylated by GskA. This was introduced into Dd-STATa $\Delta$ GPR to make Dd-STATa $^{ALapep}$ . This mutant

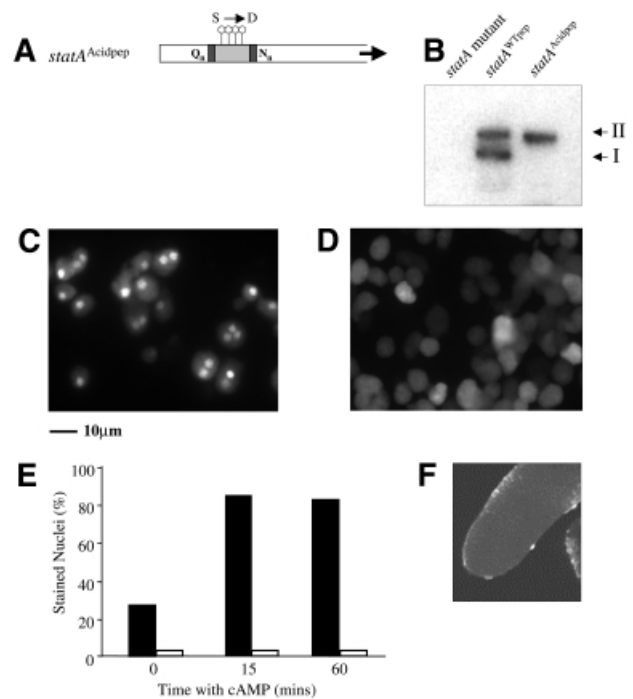


**Fig. 4.** GskA phosphorylation and nuclear localization. (A) *Dictyostelium* cells at 8 h development were fixed and immunostained for Dd-STATA. The number of stained nuclei is expressed as a percentage of the total number. In both a *gskA* mutant and cells expressing Dd-STATA<sup>AGPR</sup> or Dd-STATA<sup>ALapep</sup>, significantly higher numbers of stained nuclei are observed. (B–E) Confocal images of slug structures (formed after 16 h development) using anti-Dd-STATA antiserum: (B) *STATa* mutant; (C) *STATa* mutant cells expressing Dd-STATA<sup>WTpep</sup>; (D) *STATa* mutant cells expressing Dd-STATA<sup>AGPR</sup>; (E) *STATa* mutant cells expressing Dd-STATA<sup>ALapep</sup>. (F) A confocal image of a 16 h structure from a *gskA* mutant using anti-Dd-STATA antiserum; these cells rarely form slug structures, but form an elongated finger-like projection.

Dd-STATA again lacks the lower mobility form (Figure 3B). These results indicate that the cluster of GskA phosphorylation sites identified in our *in vitro* biochemical assay is a likely *in vivo* target of GskA.

#### **GskA phosphorylation of the GPR inhibits Dd-STATA nuclear localization**

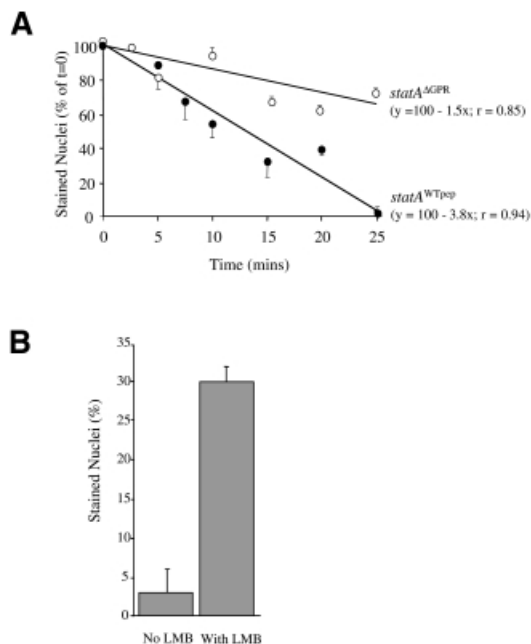
We investigated the function of the GPR and its phosphorylation by GskA. The subcellular distribution of Dd-STATA was examined at various stages of development using an antiserum specific for the tyrosine-phosphorylated form of Dd-STATA. Mutant cells lacking the Dd-STATA gene show no staining, but this is restored by expression of the Dd-STATA<sup>WTpep</sup> gene. As in wild-type cells, the number of stained nuclei increases during early development to peak at 10 h after the beginning of starvation. Expression of the Dd-STATA<sup>AGPR</sup> mutant, which lacks the GPR, again restores cell staining, but this protein has an enhanced rate of nuclear accumulation. This is best seen at 8 h development, when nuclear accumulation of Dd-STATA is undergoing its major increase. At this point, cells expressing Dd-STATA<sup>AGPR</sup> possess approximately twice as many stained nuclei as cells expressing Dd-STATA<sup>WTpep</sup> (Figure 4A). This sug-



**Fig. 5.** Ser→Asp substitution within the GPR. (A) A peptide sequence in which the serine residues of the GSK-3 site are replaced by aspartates was inserted into Dd-STATA<sup>AGPR</sup> to make Dd-STATA<sup>Acidpep</sup>. (B) Western analysis indicates that a Dd-STATA protein with the aspartate substitutions is present as a single band with reduced mobility. (C and D) The distribution of tyrosine-phosphorylated Dd-STATA was examined in disaggregated cells after 8 h development. Dd-STATA<sup>WTpep</sup> cells (C) exhibit strong nuclear staining, whereas Dd-STATA<sup>Acidpep</sup> cells (D) show little distinct nuclear staining, but in many cases show strong cytoplasmic staining. (E) Disaggregated cells after 8 h development were treated with 5 mM cAMP. This increased the number of Dd-STATA<sup>WTpep</sup> cells with nuclear staining but had no effect on Dd-STATA<sup>Acidpep</sup> cells. (F) A confocal image of a slug structure formed by Dd-STATA<sup>Acidpep</sup> cells using anti-Dd-STATA antiserum. Very few nuclei are stained in comparison with Dd-STATA<sup>WTpep</sup> (see Figure 4C).

gests that the GPR acts to limit nuclear accumulation of Dd-STATA. To investigate whether GskA phosphorylation is required, we examined cells expressing the Dd-STATA<sup>ALapep</sup> mutant. The number of strongly stained nuclei is also increased to approximately double that in Dd-STATA<sup>WTpep</sup>-expressing cells (Figure 4A). In addition, we examined the distribution of endogenous Dd-STATA protein in the *gskA* null mutant. Again, an increased number of stained nuclei was observed (Figure 4A).

These observations suggest that during early development GskA phosphorylation of Dd-STATA inhibits its nuclear accumulation. This is not, however, the case during the later stages of development. After tip formation, Dd-STATA<sup>AGPR</sup>- and Dd-STATA<sup>ALapep</sup>-expressing cells exhibit the same pattern of nuclear staining as wild-type or Dd-STATA<sup>WTpep</sup>-expressing cells (Figure 4C–E). Enriched nuclear staining is, however, retained in all *gskA* mutant cells during these later developmental stages (Figure 4F). The reason for this difference is unclear, but could be an indirect consequence of the aberrant cell type patterning that occurs in the *gskA* mutant.



**Fig. 6.** GskA regulates nuclear export of Dd-STATa. (A) Cells were developed in shaking culture for 8 h and Dd-STATa nuclear localization induced by treatment with 5 mM cAMP for 15 min. cAMP was removed and timed samples were fixed and immunostained for Dd-STATa. The rate of loss is plotted as stained nuclei, expressed as a percentage of the initial number, against the elapsed time after cAMP removal. Linear regression was used to fit each line; equations and correlation coefficients are shown in parentheses. (B) Dd-STATa<sup>Acidpep</sup>-expressing cells were developed for 8 h and then incubated with cAMP in the presence or absence of the nuclear export inhibitor LMB (20 nM). This treatment leads to nuclear localization, indicating that Dd-STATa<sup>Acidpep</sup> exits the nucleus at a higher rate.

#### Aspartate for serine substitutions within the GPR reduce nuclear localization

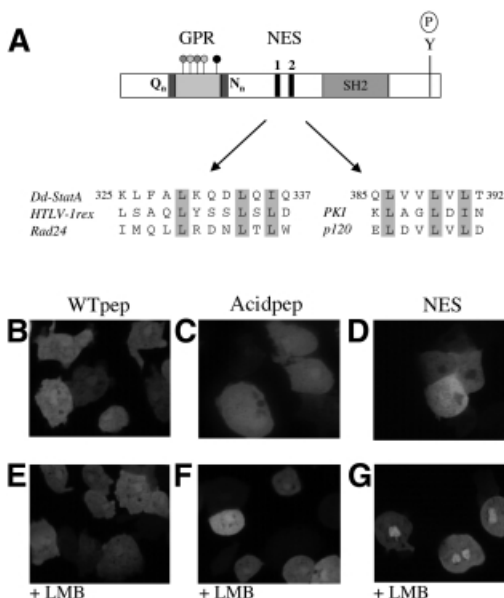
Phosphorylation increases the net negative charge of a protein and in some cases its effects can be mimicked by substitution of the target amino acid with an acidic residue. We made an acidified GPR by replacing the serines of each GskA site with aspartate residues (Figure 5A). This protein was expressed in a *STATa* null mutant and its behaviour examined. As expected for a protein lacking its GskA phosphorylation sites, this mutant, Dd-STATa<sup>Acidpep</sup>, has only a single mobility form (Figure 5B). Dd-STATa<sup>Acidpep</sup> protein, however, is slower migrating than the Dd-STATa<sup>ALApep</sup> mutant and has a mobility that is close to that of the GSK-3 phosphorylated form. The mutant Dd-STATa<sup>Acidpep</sup> protein was detected using antiserum specific for the tyrosine-phosphorylated form of Dd-STATa and the kinetics of this tyrosine phosphorylation appeared to be the same as those of wild-type protein (data not shown). In contrast to cells that express other mutant Dd-STATa proteins, we found very few nuclei in which Dd-STATa<sup>Acidpep</sup> protein had accumulated after 8 h development (Figure 5C and D). To confirm that this is a cell autonomous effect, we incubated cells after 8 h development with 1 mM cAMP. This chronic application of cAMP induces nuclear staining in nearly all Dd-STATa<sup>WTpep</sup> cells. However, even after prolonged cAMP stimulation, we found no accumulation of Dd-STATa<sup>Acidpep</sup> in the nucleus (Figure 5E).

It is important to note that the nucleus is not devoid of Dd-STATa staining, indicating that some Dd-STATa<sup>Acidpep</sup> does enter the nucleus. Interestingly, Dd-STATa<sup>Acidpep</sup> transformed cells exhibit higher levels of cytoplasmic staining than wild-type cells (Figure 5D). As an antiserum specific for tyrosine-phosphorylated Dd-STATa was used in these experiments, this cytoplasmic accumulation represents the tyrosine-phosphorylated form of Dd-STATa. Examination of Dd-STATa<sup>Acidpep</sup> at later developmental stages demonstrates that the failure to accumulate Dd-STATa in the nucleus persists during development (Figure 5F).

#### The GPR sequence regulates nuclear export

The GPR sequence could, in principle, regulate nuclear accumulation of Dd-STATa by either reducing the rate of nuclear import or increasing the rate of nuclear export. Two experiments indicate that the GPR enhances nuclear export. In the first experiment, cells were developed in shaking suspension for 8 h and then stimulated with 1 mM cAMP to induce maximal nuclear enrichment of Dd-STATa. cAMP was then removed and the decrease in nuclear staining monitored by immunocytochemistry. After cAMP removal, Dd-STATa is progressively lost from the nuclei of cells expressing Dd-STATa<sup>WTpep</sup>, with a half-maximal number of cells with stained nuclei reached after ~15 min (Figure 6A). The rate of nuclear exit from cells expressing Dd-STATa<sup>ΔGPR</sup> is 2.5 times lower, with a half-maximal number of cells with stained nuclei reached after ~40 min (Figure 6A). Removal of the GPR therefore reduces the export rate of Dd-STATa.

None of our GPR mutants appears to affect the tyrosine phosphorylation of Dd-STATa and we find no apparent difference between the loss of tyrosine phosphorylation after cAMP removal for Dd-STATa<sup>WTpep</sup> and Dd-STATa<sup>ΔGPR</sup> mutants (data not shown). This suggests that GskA phosphorylation may affect the interaction of Dd-STATa with the nuclear export machinery. To address this question, we further investigated the behaviour of Dd-STATa<sup>Acidpep</sup>, as failure to accumulate in the nucleus could be due to a high rate of nuclear export. Cells were treated with the nuclear export inhibitor leptomycin B (LMB), which in many species binds and inhibits the protein CRM-1/exportin 1 (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997). To date, the use of LMB has not been reported in *Dictyostelium*; however, we confirmed that the *Dictyostelium* homologue of CRM-1/exportin 1 contains the conserved cysteine that is alkylated by LMB (Kudo *et al.*, 1999), thus making it LMB sensitive. Cells expressing Dd-STATa<sup>Acidpep</sup> were developed in shaking culture for 8 h and then induced with 1 mM cAMP for 1 h in the presence or absence of LMB. Exposure to LMB increased the number of cells with nuclear localized Dd-STATa<sup>Acidpep</sup> from 2 to 30% (Figure 6B). This dramatic increase suggests that, in the absence of LMB, Dd-STATa<sup>Acidpep</sup> enters the nucleus but is rapidly exported back to the cytoplasm. It was not possible to obtain a degree of nuclear localization >30%, as concentrations of >20 nM LMB inhibited nuclear import. Taken together, these two sets of experiments suggest very strongly that GskA phosphorylation of GPR enhances nuclear export.



**Fig. 7.** The Dd-STATa NES is separated from the GPR. (A) Schematic diagram showing the relative position of the GPR and two putative NESs within Dd-STATa. An alignment is shown for each NES with those established for other proteins. Numbering corresponds to the amino acid position within the Dd-STATa protein. (B–G) Living cells expressing GFP fusions with the GPR, Wtpep (B and E) and Acidpep (C and F) or the region of Dd-STATa that contains both NESs. Both GPR–GFP fusions are present in both the cytoplasm and nucleus, although they are excluded from vesicles and the contractile vacuole. The NES–GFP fusion is excluded from the nucleus (D). Treatment with LMB (20 nM) has no effect on cells expressing GPR–GFP fusions (E and F), but causes nuclear accumulation of the NES–GFP fusion (G).

### GPR is not a nuclear export signal

The GPR sequence does not conform to that of any known nuclear export signal (NES) (Figures 1A and 7A), but to examine whether the GPR may nonetheless possess NES activity we fused it to green fluorescent protein (GFP). GFP is a small protein that is able to enter the nucleus by diffusion and it hence equilibrates throughout the cell: although in our hands a slight nuclear enrichment is seen (data not shown). Fusion of either the wild-type or the aspartate-substituted form of the STAT peptide to GFP had no effect on its nuclear localization (Figure 7B and C). This argues that the GPR sequence alone does not have intrinsic NES activity. We examined the rest of the Dd-STATa sequence for NES sequences. These have a distinctive pattern of leucine residues, although substitutions by isoleucine and valine residues are permitted (van Hengel *et al.*, 1999). Two potential NES sequences were found in the Dd-STATa sequence. The first (at positions 329–336) conforms to the less frequent LXXXLXL pattern seen in the NES sequences of HTLV-1rex and Rad24, but contains an isoleucine substitution for the third leucine (Figure 7A). The second (at positions 386–391) conforms to the more general LXXLXL pattern seen for the majority of NES sequences (Figure 7A). A region of Dd-STATa (positions 288–444) containing both potential NES sequences was fused to GFP. In contrast to STAT peptide–GFP fusions, this protein was excluded from the nucleus (Figure 7D). Blocking nuclear export with LMB caused nuclear accumulation of this GFP fusion protein

(Figure 7E–G), suggesting that this region also contains a weak nuclear import signal. No nuclear accumulation of GFP was observed for the STAT peptide–GFP fusions when the cells were treated with LMB (Figure 7E and F). These results indicate the presence of NES sequences within Dd-STATa that utilize an exportin/CRM-1-dependent nuclear export mechanism but that are distinct from the GPR.

## Discussion

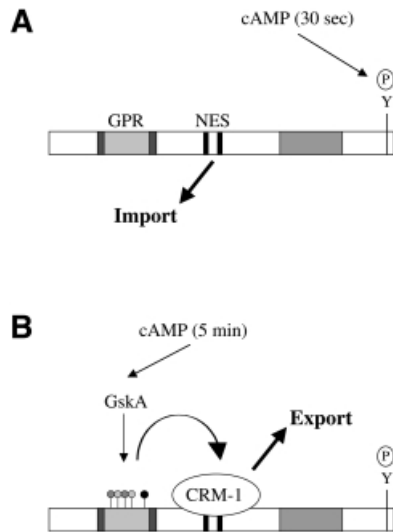
### Dd-STATa is a substrate of GSK-3

We have identified a sequence within Dd-STATa that contains several overlapping GSK-3 phosphorylation sites, which we named the GPR. Biochemical analysis demonstrates that this sequence is an excellent substrate for GskA. These results explain the observations of Araki *et al.* (1998), who discovered a lower mobility form of the Dd-STATa protein. Our analysis indicates that this lower mobility form is generated by GskA phosphorylation, strongly suggesting that Dd-STATa is an *in vivo* substrate for GskA. The incorporation of up to eight phosphate moieties at this position could explain the significant change in mobility observed by SDS–PAGE. This is the first target of GskA to be positively identified in *Dictyostelium* and this is, to our knowledge, the first report of GSK-3 phosphorylation of a STAT protein, increasing the list of nuclear targets known to be phosphorylated by GSK-3.

### Phosphorylation by GskA enhances nuclear export of Dd-STATa

Removal of the GPR reduces the rate of nuclear export and increases the number of cells with nuclear enrichment of Dd-STATa during early development. This effect is not, however, an absolute block as even in its absence Dd-STATa protein does eventually exit the nucleus. This indicates that other mechanisms also act on Dd-STATa and the role of the GPR is to enhance an intrinsic level of nuclear export. Specific mutation of the GskA phosphorylation sites within the GPR has the same effect as its removal. Furthermore, serine to aspartate substitution within the GPR creates a protein that accumulates in the cytoplasm rather than the nucleus, the opposite behaviour to GPR removal. The behaviour of the aspartate substitution mutant can be reversed by inhibition of nuclear export using LMB, indicating that the mutant enters the nucleus but is then rapidly exported. Collectively, these observations argue strongly that GskA phosphorylation is required for the effect of the GPR on nuclear export of Dd-STATa.

GSK-3 phosphorylation has previously been shown to regulate nuclear export of NF-ATc and cyclin D1 (Beals *et al.*, 1997; Diehl *et al.*, 1998), but the mechanism by which GSK-3 regulates nuclear export of these proteins is unclear. Our results suggest a mechanism for Dd-STATa. The GPR sequence does not contain a leucine-rich NES motif and we can find no evidence for it acting as an NES by itself. As fusions between the STAT peptide and GFP are not exported from the nucleus, we can also eliminate the role of an additional protein that possesses a NES and promotes export by binding to the Dd-STATa GPR, as seen when the 14.3.3 protein Rad24p binds to Cdc25p after Chk1 phosphorylation (Lopez-Girona *et al.*, 1999). We



**Fig. 8.** A scheme for GskA regulation of Dd-STATa. (A) Stimulation by extracellular cAMP leads to rapid (within 30 s) tyrosine phosphorylation of Dd-STATa and nuclear import. (B) cAMP stimulation also leads to slower (>5 min) phosphorylation at the GPR by GskA. This causes a conformational change that enhances exportin/CRM-1 binding to the NES and nuclear export.

have identified two potential NES sequences within Dd-STATa, and the GPR could interact, either positively or negatively, with one or both of these. We can, however, exclude the possibility of the GPR ‘masking’ the activity of these NES sequences because GPR removal leads to reduced rather than enhanced nuclear export. The mechanism we favour is for phosphorylation of the GPR by GskA to cause an intramolecular change within Dd-STATa that enhances its intrinsic NES activity (Figure 8).

#### **A complex regulation of Dd-STATa by extracellular cAMP**

The appearance of a lower mobility form of STATa correlates well with the regulation of GskA activity, suggesting that GskA is the major kinase active on this site. GskA activity has previously been shown to be both developmentally regulated and to be induced by extracellular cAMP. The pattern of GskA activation matches that of the appearance of the lower mobility form of Dd-STATa; it increases during early development (6–10 h) and occurs after 5 min of cAMP stimulation.

The two effects of extracellular cAMP stimulation on Dd-STATa suggest a biphasic regulation of nuclear translocation. Extracellular cAMP rapidly stimulates tyrosine phosphorylation of Dd-STATa and promotes its nuclear import. Activation of GskA and phosphorylation of the GPR sequence promote nuclear export with slower kinetics. Under chronic cAMP stimulation, this would be expected to drive a dynamic equilibrium, with Dd-STATa molecules shuttling between the cytoplasm and nucleus. The major developmental activation of GskA is via carC, which accounts for >80% of the GskA activation seen at the mound stage (Plyte *et al.*, 1999). This receptor has a slightly lower affinity than carA (Johnson *et al.*, 1992), the receptor shown to mediate tyrosine phosphorylation of Dd-STATa, and this may lead to differential regulation of

Dd-STATa nuclear import and export with varying external cAMP concentrations. carC is also differentially expressed during multicellular stages of development (Yu and Saxe, 1996), suggesting further possibilities for the regulation of Dd-STATa translocation.

Although our observations clearly demonstrate a role of GskA in the regulation of Dd-STATa, mutations of the GPR alone have very little effect on gene expression or morphogenesis. All of the Dd-STATa GPR mutants described in this report are able to rescue the *Dd-STATa* null mutant to give apparent wild-type patterns of gene expression and development, at least under standard laboratory conditions (data not shown). In part, this may reflect the additional complexity we observe in the regulation of Dd-STATa during later stages of development. We find that elimination of the GskA phosphorylation sites only affects nuclear enrichment of Dd-STATa during early developmental stages, arguing for additional mechanisms that affect nuclear translocation during later development. One such mechanism could be stimulation of nuclear import, an obvious prerequisite for export. As stimulation by extracellular cAMP is required for tyrosine phosphorylation and nuclear import, reduced signalling would negate the effects of loss of GskA phosphorylation. Preliminary evidence suggests that there is indeed a reduced level of extracellular cAMP in the posterior part of the slug and that this is responsible for the lower levels of nuclear Dd-STATa observed in the cells of this region (T.Abe, D.Dorman, K.Weijer and J.G.Williams, unpublished observation).

As no nuclear enrichment of the aspartate-substituted GPR is seen in the slug tip, GskA regulation of Dd-STATa is likely to be present throughout development. This means that the reduced amount of Dd-STATa<sup>Acidpep</sup> in the nucleus at any given time is either sufficient for development or that Dd-STATa is functionally redundant to other STAT proteins. The most significant role for GskA phosphorylation of the GPR could be in early development when GskA activity levels begin to rise during late aggregation. Preliminary observations suggest that under certain conditions our Dd-STATa GPR mutants may affect cell adhesion (data not shown). Finally, these observations indicate that failure to phosphorylate Dd-STATa is not a major cause of the strong phenotype observed for the loss of *gskA* (Harwood *et al.*, 1995).

#### **GSK-3 and STAT signal transduction**

Our results demonstrate an interaction between GSK-3- and STAT-mediated signal transduction in *Dictyostelium* and raise the possibility that similar interactions exist in metazoa. Although no direct interactions have been reported, there are a number of potential situations where they may occur. In *Drosophila* both the Wg and JAK-STAT pathways act in parallel to establish cell polarity in the developing eye (Strutt and Strutt, 1999). Both pathways converge to regulate expression of the *four jointed* gene (Zeidler *et al.*, 1999) and hence could directly interact within the *four jointed* promoter. Components of the Wnt-1 pathway are active during mammalian lymphocyte development. In mammalian lymphocytes the transcription factors LEF-1 and TCF-1 regulate transcription and, consistent with an active Wnt-1 pathway, they form nuclear complexes with  $\beta$ -catenin (Schilham and Clevers,

1998; Prieve and Waterman, 1999; Staal *et al.*, 1999). Furthermore, T-cell activation leads to a decrease in GSK-3 activity (Staal *et al.*, 1999). These events alone, however, are not sufficient to regulate transcription in native peripheral T lymphocytes, suggesting the requirement for additional signal pathways (Prieve and Waterman, 1999). Further support for a role of GSK-3 signalling in lymphocytes is suggested by frequent loss of the GSK-3 inhibitor FRAT-1 in lymphoblastomas (Yost *et al.*, 1998) and by regulation of the lymphoid transcription factor NF-ATc by GSK-3 (Beals *et al.*, 1997; Diehl *et al.*, 1998). GSK-3 therefore is situated at a potentially significant junction point in the signal transduction network of lymphocytes. Extrapolation of our results suggests that STAT transcription factors could be an additional component of this signalling network. In support of this interaction, potential GSK-3 phosphorylation sites are present in the N-termini of mammalian STATs 4, 5B and 6 and in the *Drosophila* STAT homologue Marelle (A.J.Harwood, unpublished observations). There are, however, no reports to date of serine phosphorylation in the N-terminal region of a metazoan STAT molecule.

In summary, we have demonstrated that Dd-STATa is a substrate for GSK-3 in *Dictyostelium* and that its phosphorylation enhances its nuclear export. This form of regulation is consistent with the paradigm established for NF-ATc, whereby GSK-3 phosphorylation regulates the rate of nuclear export. As both tyrosine phosphorylation and GSK-3 activity are determined by extracellular cAMP stimulation, this suggests a complex regulation of Dd-STATa nuclear translocation.

## Materials and methods

### Growth and development of cells

Wild-type (DH1) and mutant (*Dd-STATa* and *gskA*) strains were grown in HL5 medium at 22°C (Watts and Ashworth, 1970). Cells were transformed by electroporation (Howard *et al.*, 1988) and grown in HL5 medium supplemented with 20 µg/ml G418. For development, cells growing at  $2-3 \times 10^6$ /ml were washed twice in  $\text{KK}_2$  (16.5 mM  $\text{KH}_2\text{PO}_4$ , 3.8 mM  $\text{K}_2\text{HPO}_4$  pH 6.2) and either plated onto  $\text{KK}_2$  agar ( $\text{KK}_2$ , 2% agar, 1 M  $\text{MgSO}_4$ ) or resuspended in  $\text{KK}_2$  ( $1 \times 10^7$  cells/ml) and shaken at 150 r.p.m. and 22°C for 8 h.

### DNA constructs

PCR was carried out on the Dd-STATa cDNA using the primers 5'-CCGCTCGAGCGGTTAACTATTTAAAGGTTTCATAACCAC-3' and 5'-GCATATGGTAACAACAACAATAGTAATAACTTCATCA-3'. This reaction incorporated a *NdeI* restriction enzyme fragment at nucleotide 941 and a *XhoI* site at nucleotide 2634, immediately 3' of the termination codon. This fragment was subcloned into *NdeI* and *XhoI* double-digested pAct15-STATa (Mohanty *et al.*, 1999) to create a deletion mutant of Dd-STATa (Dd-STATa<sup>ΔGPR</sup>) that lacks amino acids 112–146 (the GPR). The deletion site contained a *NdeI* restriction enzyme into which a double-stranded oligonucleotide could be subcloned. Oligonucleotides encoding wild-type and mutant versions of the STAT peptide were inserted to give constructs that express Dd-STATa<sup>WTpep</sup>, Dd-STATa<sup>ΔLpep</sup> or Dd-STATa<sup>Acidpep</sup> from the *actin15* promoter.

GFP was also expressed from the *actin15* promoter. Constructs for expression of GFP fusion proteins were constructed by an in-frame insertion into an *EcoRI* restriction site 5' of the *gfp* gene. WTpep-GFP and Acidpep-GFP fusions encoded the sequences HMSQTSSP-LSSPLSPPTPF and HMDQTPDDPLDDPLDPDPTPF, respectively. The Dd-STATa-NES fusion encoded protein sequence from position 288 to 444.

### Measuring GSK-3 activity

Cell extracts were prepared from  $1 \times 10^9$  *Dictyostelium* cells by sonication/lysis ( $3 \times 5$  s bursts on ice with a Vibratome probe sonicator at setting 4) in homogenization buffer [25 mM Tris-HCl pH 7.5, 250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 100 µg/ml leupeptin, 0.1 U/ml aprotinin, 10 mM dithiothreitol, 1 µM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 100 µM sodium vanadate, 10 mM benzamide] and centrifugation at 100 000 g (30 min). The supernatant was loaded onto a base/acid-treated phosphocellulose P11 column (10 ml volume) equilibrated in buffer A (25 mM Tris-HCl pH 8.0, 40°C) connected to an FPLC apparatus (Pharmacia). After washing with buffer A, a linear salt gradient was initiated with buffer A + 500 mM NaCl and 1 ml fractions collected. The GSK-3 activity assay was performed on each fraction as described in Ryves *et al.* (1998) using synthetic peptide substrates (Zinsser Analytical Ltd). Assays were conducted in duplicate and means  $\pm$  SEM of separate experiments are shown. Peptides were separated by Tricine-SDS gel electrophoresis as described by Schagger and von Jagow (1987). GSK-3 $\alpha$  and GSK-3 $\beta$  were supplied from Upstate Biotechnology and New England Biolabs, respectively.

### Western analysis

Cells were lysed at  $1 \times 10^7$  cells/ml in NP-40 buffer (phosphate-buffered saline pH 7.4, 50 mM sodium fluoride, 1% NP-40, 2 mM EDTA pH 7.2, 1 mM sodium pyrophosphate, 1.6 µg/ml leupeptin, 4 µg/ml aprotinin, 10 mM benzamide, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride) and cleared by centrifugation. Supernatant equivalent to  $5 \times 10^5$  cells was separated by 7.5% SDS-PAGE and transferred to nitrocellulose (Hybond-C Extra; Amersham Pharmacia Biotech) using standard procedures. Membranes were blocked overnight at 4°C in 10% milk in  $1 \times$  TBS-T (0.02 M Tris, 0.137 M NaCl, 0.05% Triton X-100 pH 7.6), washed in  $1 \times$  TBS-T and incubated at 4°C overnight with a 1:500 dilution of affinity-purified SC7-pTyr antiserum, which is specific for tyrosine-phosphorylated Dd-STATa (Araki *et al.*, 1998). Membranes were washed in  $1 \times$  TBS-T and incubated with horseradish peroxidase-coupled anti-rabbit IgG (1:1000) for 1 h at room temperature. Antibody binding was visualized by chemiluminescence (Supersignal; Pierce & Warriner Ltd). Total STATa was detected using the monoclonal antibody D4 (Araki *et al.*, 1998) and GskA by a monoclonal antibody raised against the Shaggy protein (Harwood *et al.*, 1995).

### Immunohistochemical staining

Specimens were fixed in cold methanol, then rehydrated for 5 min in 70% methanol/ $\text{KK}_2$ , followed by 30 min in  $\text{KK}_2$  containing 10 mg/ml bovine serum albumin. Specimens were incubated overnight at 4°C with a 1:200 dilution in  $\text{KK}_2$  of affinity-purified SC7-pTyr antiserum, followed by 1:500 anti-rabbit IgG-Texas red for 4 h at room temperature. Specimens were dehydrated using 70% ethanol followed by 100% ethanol and then mounted in Mowiol. Fluorescence microscopy was carried out using either a Nikon Optiphot II confocal microscope with Bio-Rad Lasersharp software or a Zeiss Axioskop fluorescence microscope with an attached Sony camera.

### Nuclear export assay

Cells were developed for 8 h in shaking culture and then treated with 1 mM cAMP for 15 min. Cells were allowed to settle onto microscope cells, washed twice in  $\text{KK}_2$  to remove the cAMP and fixed in cold methanol at timed intervals. Samples were stained using D4, an anti-Dd-STATa monoclonal antibody, and scored for nuclear staining. The numbers of cells with stained nuclei were normalized so that the cell count at 2.5 min was taken as 100% and linear regression lines fitted using the program StatView 5.0.1 (SAS Institute Inc., Cary, NC).

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