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Developmentally and spatially regulated activation of a *Dictyostelium* STAT protein by a serpentine receptor

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Dd-STAT, the protein that in part controls *Dictyostelium* stalk cell differentiation, is a structural and functional homolog of metazoan signal transducers and activators of transcription (STATs). Although present during growth and throughout development, Dd-STAT's tyrosine phosphorylation and nuclear localization are developmentally and spatially regulated. Prior to late aggregation, Dd-STAT is not tyrosine phosphorylated and is not selectively localized in the nucleus. During mound formation, the time at which cell-type specific gene expression initiates, Dd-STAT becomes tyrosine phosphorylated and translocates into the nuclei of all cells. The tyrosine phosphorylation and nuclear localization of Dd-STAT are induced very rapidly by extracellular cAMP through the serpentine cAMP receptor cAR1, with Dd-STAT tyrosine phosphorylation being detectable within 10 s of stimulation. This activation is independent of the only known G_β subunit, suggesting that it may be G-protein independent. Nuclear enrichment of Dd-STAT is selectively maintained within the sub-population of prestalk cells that form the tip, the organizing center of the slug, but is lost in most of the other cells of the slug. This spatial patterning of Dd-STAT nuclear localization is consistent with its known role as a negative regulator of stalk-cell differentiation.

Keywords: *Dictyostelium*/receptor signaling/STAT/tyrosine phosphorylation

Introduction

In *Dictyostelium*, formation of a multicellular structure occurs by the chemotactic aggregation of up to 10^5 cells (Firtel, 1995; Ginsburg *et al*., 1995; Van Haastert, 1995; Chen *et al*., 1996). This process is mediated by cAMP, which functions as a ligand for a family of serpentine, G-protein-coupled cell-surface receptors (cARs). Cyclic AMP binding to cAR1, the major receptor expressed during early development, activates three main regulatory circuits: signal propagation, involving the activation of adenylyl cyclase and the consequent relaying of the cAMP signal; chemotaxis, which is believed to involve the activation of guanylyl cyclase; and the induction of aggregation-stage-specific gene expression via an as yet uncharacterized signaling pathway. The above pathways are heterotrimeric G-protein dependent and require oscillatory pulses of nanomolar cAMP (Firtel, 1995; Van Haastert, 1995; Wu *et al*., 1995; Chen *et al*., 1996); continuous treatment of aggregation-stage cells with a high concentration of cAMP results in a single activation after which the above pathways become adapted.

At the end of aggregation, as the cells within an aggregation territory form themselves into a mound, levels of extracellular cAMP are thought to rise (Abe and Yanagisawa, 1983; Saxe *et al*., 1996) and it is assumed that this adapts aggregation-stage pathways and activates postaggregative gene expression (Firtel, 1995, 1996). Postaggregative gene expression is again mediated by cAR1, which in part functions by activating the transcription factor GBF (Mehdy and Firtel, 1985; Sun and Devreotes, 1991; Insall *et al*., 1994; Soede *et al*., 1994; Schnitzler *et al*., 1995). In contrast to the cAR1-mediated pathways that function during aggregation, the cAR1-mediated pathways functioning at the mound stage require continuous, micromolar levels of cAMP. Furthermore, analyses of strains in which the gene encoding the only known G_β (Lilly *et al*., 1993; Wu *et al*., 1995) subunit of heterotrimeric G proteins is disrupted suggest that postaggregative gene expression may not require functional heterotrimeric G proteins.

Prestalk and prespore cell differentiation initiates as the mound is beginning to form. Prespore differentiation is induced in 80% of the cells by extracellular cAMP (Schaap and van Driel, 1985) and a chlorinated hexaphenone, differentiation-inducing factor (DIF), induces the remaining 20% of cells to differentiate as prestalk cells (Kay and Jermyn, 1983; Morris *et al*., 1987; Williams *et al*., 1987). The prestalk cells move to the apex of the mound forming a tip (Williams *et al*., 1989; Esch and Firtel, 1991; Early *et al*., 1995) that elongates to yield a cylindrical, standing slug that falls over onto the substratum and migrates away.

The anterior of the slug constitutes the prestalk region and is comprised of three sub-domains (Jermyn *et al*., 1989; Early *et al*., 1993): the prestalk (pst) A cells, located in the anterior one-third of the prestalk region; the pstO cells, which lie immediately posterior to the pstA cells; and the pstAB cells, which form a cone embedded within the pstA domain. Additional prestalk cells, the anterior-like cells (ALCs), are scattered throughout the prespore region. At culmination, the ALCs contribute to three ancillary structures: the upper cup, the lower cup and the

basal disc (Jermyn and Williams, 1991; Jermyn *et al*., 1996). This heterogeneity within the prestalk and stalk cell populations is defined by the expression patterns of *ecmA* and *ecmB*, two genes that are induced by DIF and encode closely related extracellular matrix proteins (Jermyn *et al*., 1987; Williams *et al*., 1987).

At the slug stage, the *ecmA* gene is expressed at high levels in pstA and pstAB cells and at lower levels in pstO cells and ALCs, whereas the *ecmB* gene is expressed at high levels in pstAB cells (Jermyn *et al*., 1989, 1996; Ceccarelli *et al*., 1991; Early *et al*., 1993). This quantitative difference in *ecmA* expression in pstA and pstO cells appears to reflect a difference in the signaling pathways in the two cell types because the promoter of the *ecmA* gene contains two distinct regulatory elements: one that is preferentially utilized in pstA cells and one that is utilized in pstO cells and ALCs (Early *et al*., 1993).

The *ecmA* gene is under positive regulation by DIF, and promoter analysis identified a closely spaced, direct repeat of the sequence TTGA as the endpoint of the DIF signal-transduction pathway (Kawata *et al*., 1996). The *ecmB* gene is negatively regulated prior to culmination by two repressor elements that predominantly restrict its expression to the pstAB cells (Ceccarelli *et al*., 1991; Harwood *et al*., 1993). Deletion of the repressor elements results in ectopic activation of the *ecmB* promoter within the pstA and pstO cells. The *ecmB* repressor elements each comprise two inverted repeats, one containing two TTGA half-sites and the other containing a TTGA half-site and a TTGT half-site (Harwood *et al*., 1993). Dd-STAT was discovered as a developmentally regulated activity that binds to both the *ecmA* activator sequence and the two *ecmB* repressor elements (Kawata *et al*., 1996).

Signal transducers and activators of transcription (STATs) were initially identified as components of cytokine-mediated signaling pathways in mammalian cells but are now known to have a much wider evolutionary distribution and more general functions. In mouse, the proteins encoded by the various STAT genes mediate responses to cytokines and growth factors (reviewed by Schindler and Darnell, 1995; Ihle and Kerr, 1995; Leaman *et al*., 1996; Darnell, 1997; Horvath and Darnell, 1997) and are now also known to play regulatory roles during early development (Duncan *et al*., 1997). A STAT has been identified in *Drosophila* (Hou *et al*., 1996; Yan *et al*., 1996) and *Dictyostelium* (Kawata *et al*., 1997), but there are no SH2 domain-containing proteins in budding yeast.

The general structure of all known STATs is highly conserved. They contain in the following order on the protein: a heptad repeat, a DNA-binding domain, a putative SH3 domain, an SH2 domain and a site of tyrosine phosphorylation. In most mammalian STAT isoforms there is also, at the extreme C-terminus, a transcriptional activation domain. Such a domain is either absent from *Dictyostelium* Dd-STAT or is located elsewhere in the protein, because the site of tyrosine phosphorylation is situated just a few nucleotides from the C-terminus of the protein (Kawata *et al*., 1997).

The pathway leading to STAT activation is best understood for cytokines (Ihle and Kerr, 1995; Schindler and Darnell, 1995; Leaman *et al.*, 1996; Darnell, 1997; Horvath and Darnell, 1997). These interact with single transmembrane domain receptors that lack an intrinsic kinase activity but are instead associated with members of the Janus family of tyrosine kinases (Jaks). Ligand binding and receptor dimerization activate the Jaks, which then tyrosine phosphorylate the receptors. STATs bind to the tyrosine-phosphorylated receptors via their SH2 domains and are themselves tyrosine phosphorylated by the Jaks. The STATs then form dimers by the reciprocal binding of the pTyr on one STAT molecule with the SH2 domain of its dimerizing partner. In some as yet undefined way, this triggers transport to the nucleus, where the STATs function as sequence-specific DNA-binding proteins.

Insofar as it is understood, Dd-STAT seems to function in a manner similar to the metazoan STATs. Dd-STAT, when purified by DNA-affinity chromatography on the *ecmA* activator sequence, is tyrosine phosphorylated (Kawata *et al*., 1997). Also, its *in vitro* DNA-binding activity can be inhibited by a tyrosine-phosphorylated peptide that has the sequence of the C-terminal region, in which sequence comparisons with metazoan STATs suggest tyrosine phosphorylation to occur (Kawata *et al*., 1997). By analogy to a similar study of a mammalian STAT (Shuai *et al*., 1994), this suggests that Dd-STAT functions as a dimer that is formed by reciprocal SH2–pTyr interactions.

Here we show that extracellular cAMP is the agent that induces tyrosine phosphorylation and nuclear translocation of Dd-STAT. We also demonstrate that there are major developmental changes in the cellular sub-populations in which Dd-STAT is nuclear localized and these are discussed in the context of our very recent work showing that Dd-STAT is the repressor that prevents premature *ecmB* expression and stalk cell differentiation (S.Mohanty, K.A.Jermyn, A.Early, T.Kawata, L.Aubry, A.Ceccarelli, J.G.Williams and R.A.Firtel, in preparation).

Results

Tyrosine phosphorylation of Dd-STAT is regulated developmentally

Dd-STAT was analyzed using a monoclonal antibody (D4) and two affinity-purified polyclonal antibodies (SC7 and SC9). All three antibodies were raised against the 15 amino acids at the extreme C-terminus of Dd-STAT, the site at which tyrosine phosphorylation has been proposed to occur (Kawata *et al*., 1997), and the peptide was synthesized with a phosphate group on the only tyrosine residue located within this region. The antibodies were characterized using a Dd-STAT fusion protein produced in *Escherichia coli* cells that were either expressing (Figure 1, +pY) or not expressing (Figure 1, –pY) the mammalian *lck* tyrosine kinase. Western blotting of the purified fusion protein and analysis using an anti-pTyr antibody show that Dd-STAT is tyrosine phosphorylated in *lck*-expressing cells (Figure 1).

The SC7 polyclonal antisera was purified (see Materials and methods; Lewis *et al*., 1996) by cycles of affinity chromatography, using the tyrosine-phosphorylated and tyrosine-non-phosphorylated C-terminal peptides of Dd-STAT, so as to be highly specific for the tyrosine-phosphorylated form of Dd-STAT (Figure 1, SC7-pTyr). The second polyclonal antibody, SC9, was affinity purified using the glutathione *S*-transferase (GST) fusion protein Dd-STAT–GST, containing the SH2 domain and tyrosine

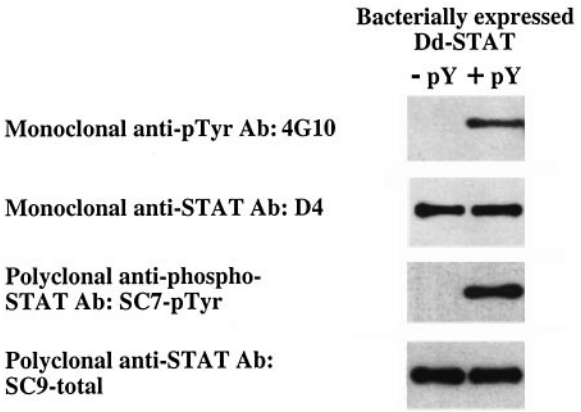


Fig. 1. Comparison of the specificities of the anti-Dd-STAT antibodies. Dd-STAT fusion protein was produced in *E.coli* expressing (+pY) or not expressing (–pY) the mammalian *lck* tyrosine kinase and was analyzed by Western blotting. All three antibodies (monoclonal antibody D4 and polyclonal antibodies SC7-pTyr and SC9-total) were raised against the 15 amino acids at the extreme C-terminus of Dd-STAT. The polyclonal antibodies were purified by affinity chromatography to be specific for the tyrosine-phosphorylated form of Dd-STAT (SC7-pTyr) or to be phosphorylation-state independent (SC9-total). The D4 monoclonal antibody was used without purification. The fact that SC7-pTyr recognizes the Dd-STAT fusion protein only when it is tyrosine-phosphorylated confirms the previous identification of the site of tyrosine phosphorylation (Kawata *et al*., 1997).

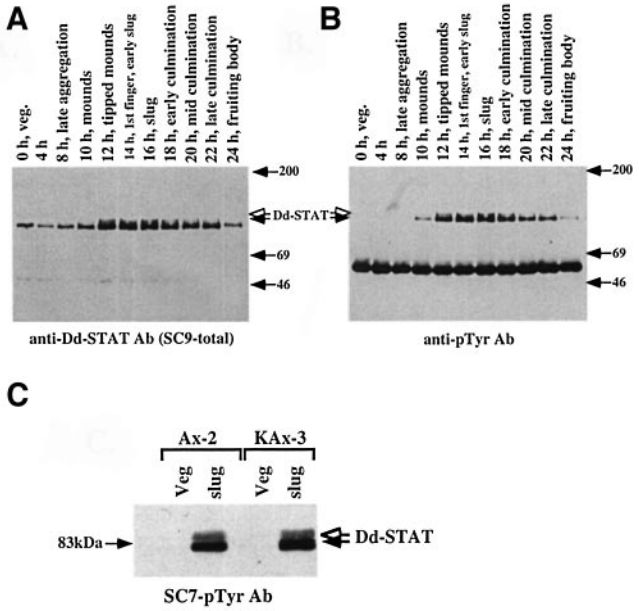


Fig. 2. Developmental changes in the tyrosine phosphorylation of Dd-STAT. (A and B) Using the D4 antibody, Dd-STAT was immunoprecipitated from KAx-3 cells harvested at the indicated development times. The precipitates were fractionated by SDS–PAGE, Western blotted and probed with SC9-total antibody (**A**) or anti-pTyr antibody py72 (**B**). We previously suggested that Dd-STAT might be very susceptible to proteolysis during purification and the absence of lower molecular weight bands (bands C, D and E in Kawata *et al.*, 1997) when cells are extracted directly into SDS and analyzed by Western blotting supports this idea (data not shown). Both bands detected in wild-type cells (indicated with black and white arrows) are entirely absent from cells in which the Dd-STAT gene is inactivated by insertional mutagenesis (data not shown). This holds true when any of the three Dd-STAT antibody preparations described in Figure 1 are used, confirming that all three antibodies are specific for Dd-STAT. This is an important point because very recent work has revelealed there to be two additional *Dictyostelium* STATs (M.Fukuzawa, T.Kawata and J.G.Williams, unpublished data). The fact that the antibodies do not recognize these additional STATs can be explained by the limited sequence homology in their extreme C-terminal regions. (**C**) Growing cells and slug-stage cells of the two parental axenic strains used in this study, KAx-3 and Ax-2, were lysed in SDS buffer and subjected to Western blotting using the SC7-pTyr antibody.

phosphorylation site of Dd-STAT. This antibody recognizes both the tyrosine-phosphorylated and non-tyrosine-phosphorylated forms of Dd-STAT (Figure 1, SC9-total). The monoclonal antibody, D4, also recognizes both the non-tyrosine-phosphorylated and tyrosine-phosphorylated forms of Dd-STAT (Figure 1).

Previous analysis by Northern transfer showed the Dd-STAT mRNA to be strongly regulated in its accumulation, with only a very low level of mRNA in growing cells and cells at developmental stages prior to tip formation (Kawata *et al*., 1997). However, analysis using the SC9-total antibody shows the Dd-STAT protein to be present during growth and throughout development with only a moderate increase in concentration at the tight mound stage (Figure 2A). This difference between mRNA and protein levels could result from either a relative destabilization of the protein or a relative stabilization of the mRNA, but we have no information to distinguish between these two possibilities. When extracted from growing cells and cells during early development, the Dd-STAT protein migrates as a single band. Later on during development, at the mound stage, a second, more slowly migrating band appears. These two closely migrating bands have apparent molecular weights in the range of ~80 kDa, i.e. very similar to the two largest bands observed in purified preparations of Dd-STAT (bands A and B in Kawata *et al*., 1997; Figure 2A).

When tyrosine phosphorylation of the Dd-STAT protein is analyzed by immunoprecipitation with D4 followed by Western blotting with a commercial anti-phosphotyrosine antibody (Figure 2B) or by Western blotting using SC7-pTyr (data not shown), tyrosine phosphorylation is first detected at the tight mound stage and the amount of the tyrosine-phosphorylated protein increases over the following 2–4 h. During this time the second, more slowly migrating band, which is also seen with the SC9-total antibody (Figure 2A), is detected. The second band is poorly resolved using this analysis method, so we confirmed its existence using the SC7-pTyr antibody in Western blotting. The experiment in Figure 2C compares vegetative cells and slug cells of KAx-3, the strain used in Figure 2A and B, with Ax-2, another axenic strain. It shows that the second band is found in both axenic strains and is present when cells are directly lysed in SDS buffer. We do not yet know the significance of this band, but the results presented below show that it is not essential for the initial nuclear localization of Dd-STAT.

The developmental increase in Dd-STAT tyrosine phosphorylation correlates with Dd-STAT nuclear localization

In order to examine the correlation between tyrosine phosphorylation of Dd-STAT and its subcellular localization, the fraction of cells exhibiting nuclear enrichment of Dd-STAT was determined at various stages of development (Figure 3A and B). During early development and aggreg-

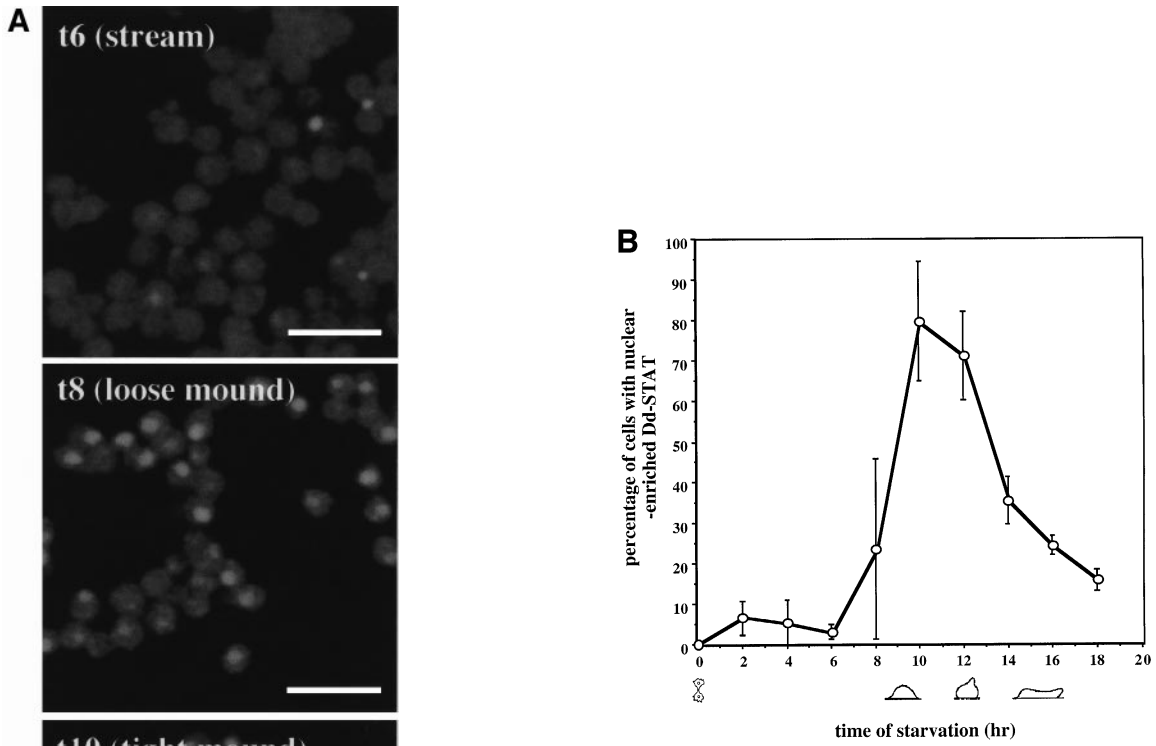


Fig. 3. Change in the subcellular localization of Dd-STAT during development on a substratum. (**A**) Ax-2 cells developing on filters were dissociated at the indicated stages, fixed and stained with monoclonal antibody D4. Scale bar, 20 μm. (**B**) Ax-2 cells were developed on filters and harvested every 2 h during development. The percentage of cells with nuclear enriched Dd-STAT was determined as described in (A). Values are the mean ± SD from at least four separate experiments. The high level of variability observed at 8 h is the result of normal, experiment-to-experiment developmental timing differences. At this time, Dd-STAT is undergoing a rapid change in tyrosine phosphorylation and nuclear enrichment, so experiment-to-experiment differences in the precise rate of development have a major effect on the fraction of cells showing nuclear enrichment.

ation, <5% of cells show detectable Dd-STAT nuclear enrichment. The fraction then increases rapidly, so that by the tight mound stage >95% of cells show nuclear enrichment. This translocation corresponds well with the developmental timing of the tyrosine phosphorylation (Figure 2B) and the appearance of the Dd-STAT DNA-binding activity as determined by gel retardation (Kawata *et al*., 1996).

Nuclear enrichment of Dd-STAT becomes spatially restricted during multicellular development

The percentage of cells with nuclear enrichment of Dd-STAT falls significantly as the mound transforms into a slug (Figure 3B). In order to determine whether enrichment is retained within a specific subset of cells, indirect immunofluorescence was performed on whole mount structures. The fraction of cells showing nuclear enrichment of Dd-STAT remains high in the upper, prestalk region of tipped aggregates but becomes progressively reduced in the basal, prespore region (data not shown). In migrating slugs, the difference between the prestalk and prespore region is even more pronounced and cells with nuclear enriched Dd-STAT are mainly confined to the tip of the slug (Figure 4A and B).

The slug tip is composed of pstA cells (Fukuzawa *et al*., 1997) but, interestingly, not all of the pstA cells show enrichment of Dd-STAT. The image in Figure 4B was produced by the amalgamation of a set of optical sections made at different depths within the slug (i.e. it is a 'projected image' derived from a 'Z series') and gives the impression that nuclear enrichment is relatively uniform throughout the pstA region. However, analyses of the individual optical sections and computer-generated transverse optical sections (Figure 4C) show that the fraction of cells at the extreme tip with Dd-STAT nuclear enrichment is very high whereas further back enrichment is largely confined to a cortical layer of cells at the base.

Tyrosine phosphorylation, nuclear translocation and DNA-binding activity of Dd-STAT are all activated by extracellular cAMP

In initial experiments [performed before we understood that Dd-STAT is the repressor of ecmB transcription and not the activator of *ecmA* gene expression (Mohanty *et al*., in preparation),] we studied the effect of DIF on nuclear translocation of Dd-STAT and observed no effect (data not shown). Extracellular cAMP is known to regulate a number of signaling pathways at about the time of mound formation (Firtel, 1995), so we investigated whether cAMP

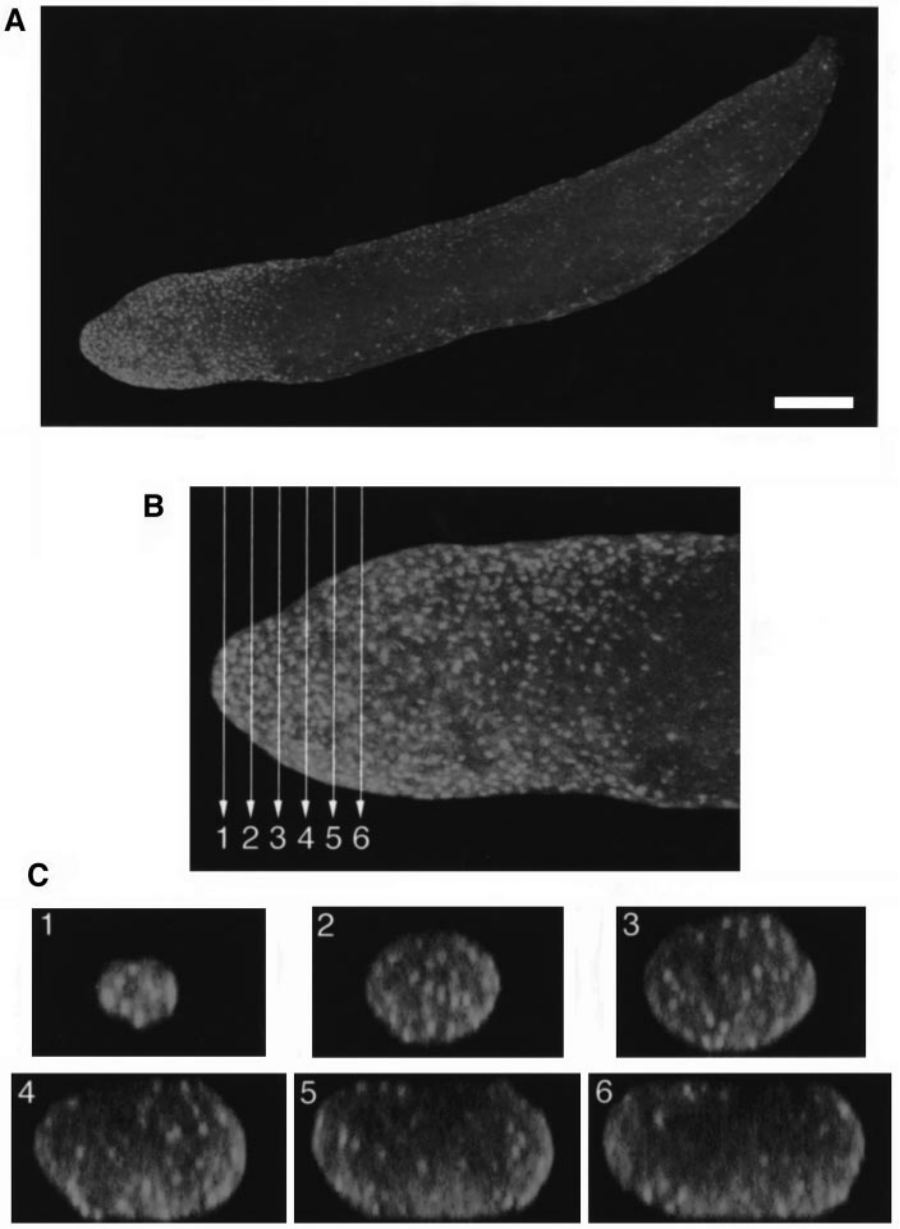


Fig. 4. Whole-mount staining of developing structures obtained using the Dd-STAT antibody D4. Ax-2 cells were developed on a water agar plate until the desired stage of development and the resultant structures were fixed and stained with the D4 antibody. They were then mounted and viewed by confocal microscopy. (**A**) The projected image of a slug derived from a Z series. (**B**) The projected image of the slug in (A) derived from a Z series. We believe the staining that we observe in the rear part of the slug (in the prespore region) is real. It is not observed (data not shown) when the primary antibody is omitted and it is also not observed in slugs derived from Dd-STAT null cells. These two facts are also true for the fluorescence that we observe over the cytoplasm of cells during later development (Figure 3A). This presumably indicates that Dd-STAT is present in the cytoplasm of all cells at the slug stage but only becomes enriched in the nuclei of cells in the pstA regions. The scale bar in (A) represents 50 μm. (**C**) The tip of the slug analyzed in (B) viewed at as a projected image at high power in the upper subpanel and cross-sections in the lower six subpanels. The numbered arrows on the projected image indicate the positions at which the optical cross-sections were taken. These sections indicate that many pstA cells do not show nuclear enrichment of Dd-STAT. We know that this is not a technical problem with variable penetration of the antibodies, because we performed a double-labeling experiment using cells transformed with a pstA cell-specific marker (data not shown). This revealed a uniform distribution of those pstA cells that were detectable (not all pstA cells are detected in such an experiment because even in cloned transformants there is highly variable expression of the marker), whereas the Dd-STAT nuclear enrichment was localized to one surface of the slug tip. We are uncertain of the distribution of Dd-STAT at later stages of development because we do not know whether these fixation conditions allow passage of antibodies through the stalk tube.

induces tyrosine phosphorylation and nuclear translocation of Dd-STAT.

Cells were treated with cAMP after either 2 or 6 h of starvation, i.e. prior to the time when Dd-STAT activation normally occurs. Forty percent of the cells treated with cAMP after 2 h of starvation show nuclear enrichment of Dd-STAT within 1 h compared with ~10% in cells not receiving cAMP (Figure 5A). When treated with cAMP at 6 h after starvation, >95% of the cells show nuclear translocation of Dd-STAT compared with <10% of untreated cells. This same level of response is achieved with cAMP concentrations in the range 50 μM–5 mM

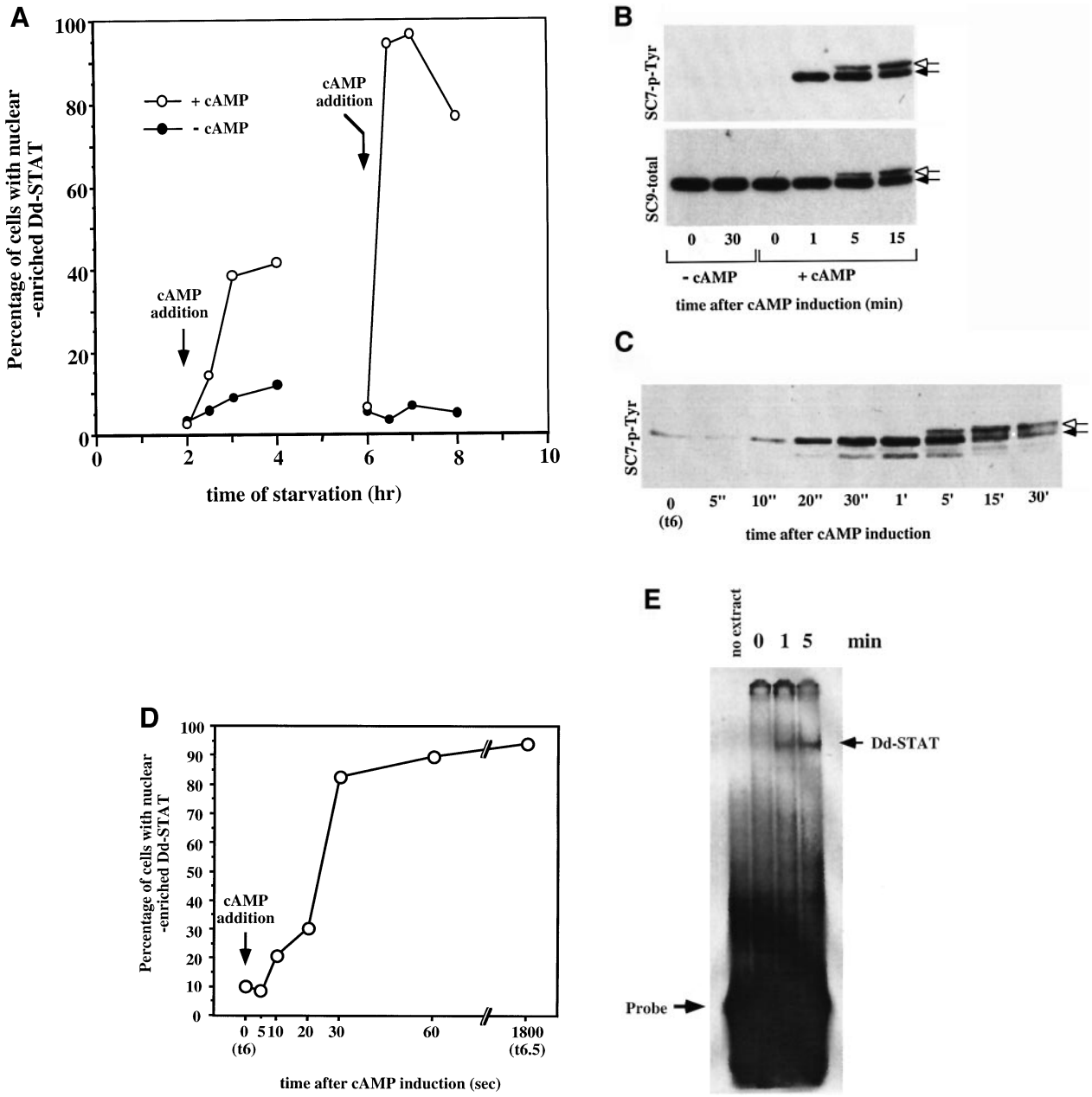


Fig. 5. cAMP induction of nuclear enrichment, tyrosine phosphorylation and DNA binding activity of Dd-STAT. (**A**) Exponentially growing Ax-2 cells were harvested and developed in shaking suspension in KK2 phosphate buffer. After either 2 or 6 h of starvation, cells were placed in fresh flasks and cAMP was added to a concentration of 5 mM. Samples were collected at the times indicated and analyzed by immunohistochemical staining using the D4 monoclonal antibody. (**B**) Ax-2 cells developed for 6 h as described in (A) were treated with 5 mM cAMP. At the indicated times after cAMP addition, cells were lysed in SDS sample buffer. After Western blotting, Dd-STAT was detected with either the SC7-pTyr (upper subpanel) or the SC9-total antibody (lower subpanel). (**C**) Cells developed and induced as in (B) were analyzed at very short times after cAMP stimulation using the SC7-pTyr antibody. (**D**) Aliquots of cells from the experiment described in (C) were analyzed for nuclear enrichment of Dd-STAT using the SC7-pTyr antibody. (**E**) Nuclear extracts were prepared from cells developed and induced as in (B) and a gel retardation assay was performed using the *ecmA* activator as probe (domain I in Kawata *et al.*, 1996).

(data not shown), the range of cAMP concentrations known to activate postaggregative gene expression (Mehdy and Firtel, 1985; Schaap and van Driel, 1985).

Developing *Dictyostelium* cells secrete a cAMP phosphodiesterase that will rapidly degrade small amounts of cAMP but we were able to detect some increase in tyrosine phosphorylation with as little as 1 μM cAMP (data not shown). In order to circumvent the cAMP phosphodiesterase problem we also used cAMPS, a non-hydrolysable cAMP analog, and found concentrations as low as 0.5 μM to be totally effective in directing both tyrosine phosphorylation and nuclear translocation of Dd-STAT (data not shown). However, activation requires continuous cAMP treatment; oscillatory pulses of nanomolar cAMP, of the kind which mediate aggregation-stage pathways, result in minimal Dd-STAT tyrosine phosphorylation and nuclear localization (data not shown). The fact that nuclear translocation is significantly more

rapid in cells starved for 6 h than in cells starved for 2 h, suggests that the cells acquire components of this signaling pathway during the course of early development.

In order to compare the precise kinetics of tyrosine phosphorylation and nuclear translocation, cells were treated with cAMP after 6 h of starvation and the Dd-STAT protein was analyzed rapidly thereafter by Western blotting using the SC7-pTyr and SC9-total antibodies and by immunohistochemistry using the SC7-pTyr antibody (Figure 5B–D). Total Dd-STAT protein levels remain constant over the time courses used in the different experiments and tyrosine phosphorylation is dependent upon the addition of cAMP (Figure 5B).

The higher mobility tyrosine-phosphorylated band (marked with a black arrow in Figure 5B), which is either not detectable (Figure 5B) or barely detectable (Figure 5C) in unstimulated cells, shows a detectable increase in its level of tyrosine phosphorylation after just 10 s of stimulation (Figure 5C). Its intensity increases greatly within 30 s of cAMP stimulation, reaches a plateau by 1 min and begins to decline by 30 min. It co-migrates approximately with Dd-STAT from unstimulated cells, although after cAMP stimulation a slight decrease in its mobility is sometimes discernible (Figure 5B). The fact that this band is recognized by both the SC9-total and SC7-pTyr antibodies reveals it to be Dd-STAT that is modified by tyrosine phosphorylation within the predicted tyrosine phosphorylation domain. We will therefore term it Dd-STAT:pTyr. (The higher mobility bands seen just below the Dd-STAT:pTyr band in Figure 5C are, we think, proteolytic degradation products, because they are only sporadically detected; compare Figure 5B and C.) After 5 min of cAMP stimulation, the lower mobility band (marked with a white arrow in Figure 5B) is detected. Because it is recognized by SC7-pTyr and is of lower mobility than the main Dd-STAT:pTyr band, we assume it to be Dd-STAT:pTyr that is additionally modified in some way (see below). We will therefore term it Dd-STAT:pTyr+.

Aliquots of cells taken at the same times as the samples used for the Western blotting experiment described in Figure 5C were stained with D4, the anti-STAT monoclonal antibody, and examined by indirect immunofluorescence. Approximately 80% of the cells exhibit nuclear enrichment of Dd-STAT at 30 s and >95% of the cells have nuclear localized Dd-STAT by 1 min (Figure 5D). The data in Figure 5C show that in cells stimulated for 30 s or 1 min, the more slowly migrating Dd-STAT:pTyr+ band is not detected. However, over this period, Dd-STAT translocates into the nuclei of essentially all cells. Thus, tyrosine phosphorylation correlates with STAT nuclear localization. We assume that Dd-STAT:pTyr+ is formed as the result of additional phosphorylation(s) or other post-translational modifications and, from the kinetics of its appearance, it is possible that this second modification occurs within the nucleus. Consistent with this notion, cellular fractionation experiments (data not shown) indicate that Dd-STAT:pTyr+ is enriched in a partially purified preparation of nuclei.

Tyrosine phosphorylation and nuclear localization of Dd:STAT both correlate very well with the appearance of specific DNA-binding activity. Binding activity to the DNA sequence from the promoter of the *ecmA* gene that was used to identify and purify Dd-STAT (Kawata *et al*., 1997) is not observed in unstimulated cells but is observed at just 1 min after cAMP stimulation (Figure 5E). Similar results are obtained when the RII repressor element from the *ecmB* gene is used as a probe (data not shown).

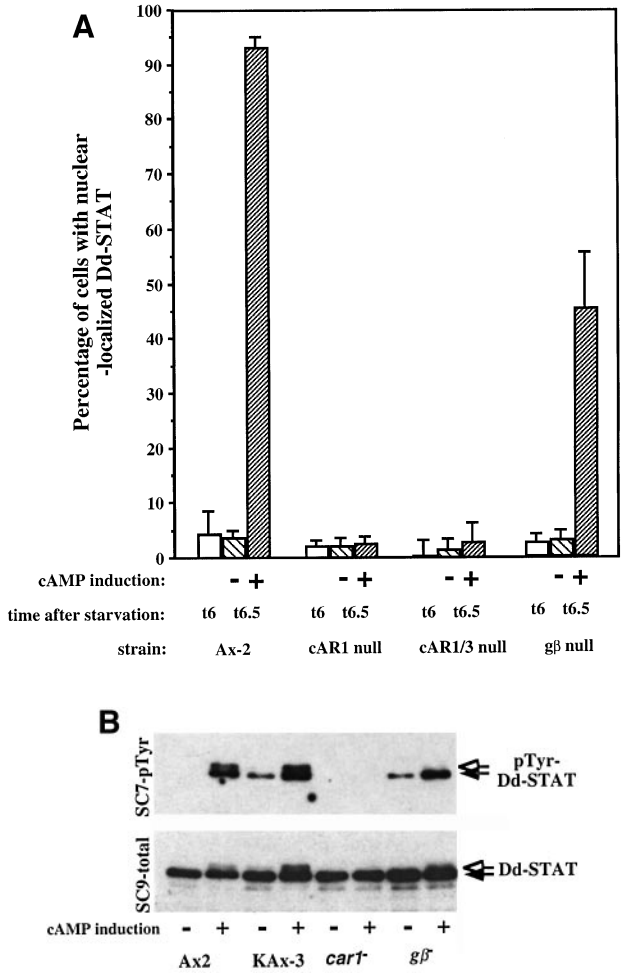
Activation of Dd-STAT is dependent upon cAR1

The primary cAMP receptor that functions during the developmental period when nuclear enrichment of Dd-STAT becomes cAMP inducible is cAR1 (Insall *et al*., 1994; Soede *et al*., 1994; Firtel, 1995; Ginsburg *et al*., 1995; Schnitzler *et al*., 1995); hence we investigated nuclear translocation and tyrosine phosphorylation in a *cAR1* disruptant strain. There is no detectable tyrosine phosphorylation or nuclear translocation in response to extracellular cAMP in the *car1* null strain (Figure 6A and B).

In order to confirm that the failure to activate Dd-STAT is due to the absence of cAR1 and not a cryptic mutation, we also analyzed a *car1* null strain transformed with a construct wherein *cAR1* is expressed under the control of the semi-constitutive *Actin15* promoter. Because the cAR3 receptor is expressed during early development and could complicate the analysis, the strain that was used contained both a disrupted *cAR1* and a disrupted *cAR3* gene (Johnson *et al*., 1993; Insall *et al*., 1994; Soede *et al*., 1994). As expected, the *car1/car3* null strain shows no detectable nuclear translocation of Dd-STAT in response to cAMP treatment (Figure 6C). However, in the *car1/car3* null strain expressing cAR1 from the *Actin15* promoter, the induction of nuclear translocation occurs just as efficiently as in Ax-2 cells or the parent strain KAx-3. The same approach was used to show that cAR2, a receptor that normally functions during later multicellular development (Saxe *et al.*, 1993), can subsume the function of cAR1 (data not shown).

Downstream steps in the signaling pathway

A significant amount of biochemical and molecular data suggest that there is only a single gene encoding a heterotrimeric G protein β subunit (G_β) in *Dictyostelium* (Lilly *et al*., 1993; Wu *et al*., 1995; Chen *et al.*, 1996) and so we investigated Dd-STAT activation in cells in which this gene is disrupted. Since g_β null cells do not accumulate cAMP receptors in response to cAMP pulsing, a g_β null strain constitutively expressing high levels of the cAR1 receptor was used to ensure that receptor levels were not limiting (Wu *et al*., 1995). Cyclic AMP stimulation induces a rapid tyrosine phosphorylation of Dd-STAT, although to a lower level than in the parental strain (Figure 6B). Cyclic AMP also induces nuclear translocation in the g_β null strain, but to only half of the level found for the parental strain (Figure 6A). A similar reduction in level of activation in g_β null cells compared with wild-type cells has been observed for GBF-mediated gene expression, the activation of the MAP kinase ERK2, and calcium influx (Milne *et al*., 1995; Schnitzler *et al*., 1995; Maeda *et al*., 1996). This reduced activation in g_β null cells compared with wild-type cells may be due to a requirement of G_β-mediated pathways for maximal activation of these responses or the effect may be indirect, i.e. g_β null cells may be deficient in one or more general components of signaling pathways. Both the Dd-STAT:



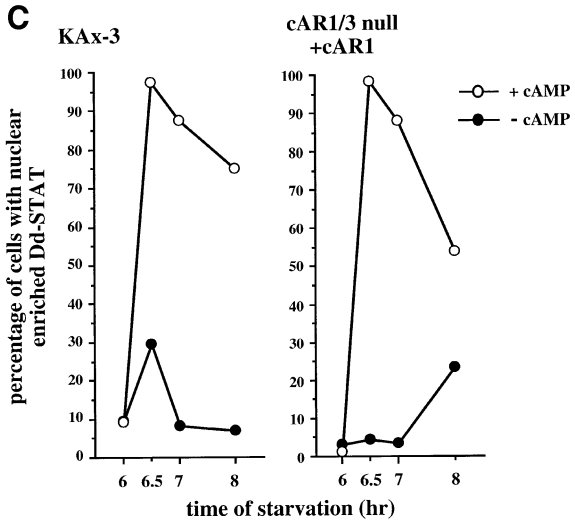


Fig. 6. cAMP-induced nuclear localization and tyrosine phosphorylation of Dd-STAT in signaling mutants. (**A**) Ax-2, *car1* null, *car1/car3* null and g_β null cells were allowed to develop in shaking culture for 6 h and either treated with 5 mM cAMP for 30 min or left untreated. [The parental strain for the two mutants is KAx-3 and Ax-2 is therefore used here only as a control for the induction conditions. However, KAx-3 behaves similar to Ax-2 in such an experiment (Figure 6C).] Samples were taken for staining with the D4 monoclonal antibody before cAMP addition (unfilled bars), after shaking for 30 min in the absence of cAMP (lightly hatched bars), or after 30 min in the presence of cAMP (densely hatched bars). (**B**) Ax-2, KAx-3, *car1* null and g_β null cells were allowed to develop in shaking culture for 6 h and either treated with 5 mM cAMP for 30 min or left untreated. Total cell lysates were prepared in SDS gel sample buffer and the samples were analyzed by Western blotting. The blot was first probed using SC9-total antibody and, after stripping, it was reprobed with SC7-pTyr antibody. (**C**) The cAR1 receptor was expressed in *car1/car3* double disruptant cells under the control of the semi-constitutive, Actin15 promoter and compared with KAx-3 parental cells using the assay procedure described in (A). Samples were analyzed for Dd-STAT nuclear enrichment 30 min, 1 h and 2 h after cAMP was added to the test samples.

pTyr and the Dd-STAT:pTyr+ bands are present in cAMP-stimulated g_β null cells, although the amount of the more slowly migrating species is reduced significantly (Figure 6B) suggesting that the secondary modification may be in some way dependent on a G_β-controlled pathway.

Cell-type differentiation in *Dictyostelium* is known to require the function of both cAMP-dependent protein kinase (PKA) and the MAP kinase ERK2 (Harwood *et al*., 1992; Hopper *et al*., 1993; Reymond *et al*., 1995; Firtel, 1996; Gaskins *et al*., 1996; Zhukovskaya *et al*., 1996; Mann *et al*., 1997). To examine whether activation of Dd-STAT might be dependent on either of these kinases, null strains for both ERK2 and the PKA catalytic subunit were analyzed, but both strains showed tyrosine phosphorylation and nuclear translocation of Dd-STAT (data not shown).

Discussion

We have shown that Dd-STAT is not nuclear enriched in the majority of cells during growth and early development, but that as the mound forms, it becomes nuclear enriched in most or all cells. Nuclear translocation occurs synchronously with tyrosine phosphorylation of Dd-STAT and this is consistent with mammalian STATs, in which tyrosine phosphorylation also correlates closely with nuclear translocation. Subsequently, during slug formation, nuclear enrichment is lost in the majority of the cells but is retained in a proportion of cells in the anterior, pstA domain. This pattern of activation can perhaps be understood when the function of Dd-STAT is considered.

Normally, the *ecmB* gene is not expressed in pstA cells and pstO cells but is expressed in the cone of pstAB cells which lies at the position where stalk formation is initiated at culmination. In contrast, in slugs derived from *Dd-stat* null cells, *ecmB* is expressed throughout the prestalk region (S.Mohanty, K.A.Jermyn, A.Early, T.Kawata, L.Aubry, A.Ceccarelli, J.G.Williams and R.A.Firtel, in preparation). Thus Dd-STAT is the repressor that prevents premature *ecmB* expression in the slug tip. It would therefore be predicted to be bound *in vivo* to the two copies of the inverted TTG repeat that comprise the repressor elements (Harwood *et al*., 1993), in those cells where repression occurs. The presence of nuclear localized Dd-STAT within cells in the pstA domain of the slug is consistent with just such a function, because the pstA cells are the direct precursors of the pstAB cells, and are therefore the cells in which repression of *ecmB* expression needs to be exerted. It is not, however, obvious why all cells have nuclear-localized Dd-STAT at the tight mound stage.

It is also not apparent why only a spatially restricted

subset of the pstA cells show nuclear enrichment. It may reflect the fact that the slug tip is a constantly changing structure in which rapidly altering signaling conditions direct a highly dynamic movement of Dd-STAT in and out of the nucleus. The precise shape of the tip, for example, alters constantly and the tip spends only a proportion of the time in contact with the substratum. Variability also extends to the conus of pstAB cells, which periodically, and with no known stimulus, moves back from the tip and is lost from the slug (Sternfeld, 1992). Again, this could relate to Dd-STAT's function as a repressor of stalk-cell differentiation, because after such a shedding event a new cohort of pstAB cells must presumably be produced by activation of *ecmB* expression in a subset of the pstA cells. The evidence for a highly dynamic movement of Dd-STAT comes from our identification of the signal that induces its nuclear translocation as cAMP.

Several pieces of evidence indicate that extracellular cAMP is the inducer that activates Dd-STAT. First, there is the fact that cAMP added to cells at developmental times before there is a significant level of tyrosine phosphorylation and nuclear enrichment induces both processes to occur. The evidence that cAMP is the proximal inducer derives from the speed of response. There is a detectable increase in Dd-STAT tyrosine phosphorylation by 10 s after cAMP addition and ~80% of the cells exhibit nuclear localized Dd-STAT within 30 s. This compares with the peak of cAMP receptor activation of guanylyl cyclase which occurs 10 s after cAMP addition and of adenylyl cyclase, which occurs 60 s after cAMP addition (Firtel, 1995; Chen *et al*., 1996).

Additional support for the notion that cAMP is the inducer of activation comes from the observation that tyrosine phosphorylation and nuclear translocation do not occur to a detectable level in a *cAR1* null mutant. In null cells expressing an *Actin15–cAR1* fusion gene tyrosine phosphorylation of Dd-STAT can be induced by cAMP very quickly, within just 30 min of the initiation of development (data not shown). Endogenous cAMP levels are very low at that time. Hence this result argues for a direct role of cAR1 and against the notion that some other receptor, dependent upon activated cAR1 for its expression, mediates the activation of Dd-STAT.

Knowing that cAMP is the signal that activates Dd-STAT raises the intriguing possibility that Dd-STAT may be localized in cells in the slug tip because extracellular cAMP signaling is higher in this region. This is an attractive idea because the tip functions as an organizing center for the slug, most probably by acting as the cAMP oscillator (Bretschneider *et al*., 1995; Siegert and Weijer, 1995). However, there is no direct evidence for this in the case of Dd-STAT and there could equally well be factors that repress the Dd-STAT activation pathway in the posterior regions of the slug.

The cARs couple to various effectors via heterotrimeric G proteins but, interestingly, Dd-STAT activation occurs in cells with a disruption of the gene encoding the G_β subunit. Three other cAMP-activated processes, GBF-mediated gene expression, the activation of ERK2, and calcium influx all share this same property (Milne *et al*., 1995; Schnitzler *et al*., 1995; Maeda *et al*., 1996). Strong biochemical evidence that there is only a single G_β subunit in *Dictyostelium* (Lilly *et al*., 1993; Wu *et al*., 1995; Chen *et al*., 1996) seems to indicate the existence of a G-protein-independent pathway in *Dictyostelium*. However, because it is impossible to rule out entirely the existence of another, as yet undiscovered G_β protein, it is necessary to reserve final judgment on this point.

The steps further downstream in the activation of Dd-STAT are unknown but a clue to the kind of signaling pathway that may be functioning in *Dictyostelium* comes from two mammalian signaling pathways. Metazoan STATs are usually activated via single span receptors but the angiotensin II AT1 receptor and the skeletal muscle serotonin 5-HT_{2A} receptor, both seven transmembrane domain proteins, are believed to interact directly with Jak proteins to activate STATs (Marrero *et al*., 1995; Ali *et al*., 1997; Guillet-Deniau *et al*., 1997; McWhinney *et al*., 1997). There are tyrosine kinases in *Dictyostelium* that have the gross structure of the metazoan Jaks and, although none of these seems a possible candidate for activation of Dd-STAT (Kawata *et al*., 1997), it is tempting to speculate that a yet to be discovered Jak-like kinase interacts directly with the cAR1 receptor to activate Dd-STAT.

Materials and methods

Growth and development of cells

Strains used in this study were: the parental, axenic strains Ax2 and KAx-3; *car1* null (Sun and Devreotes, 1991); *car1/car3* null (Insall *et al*., 1994); g_β null (Lilly *et al*., 1993); *erk2* null (Gaskins *et al*., 1996); PKA C-subunit null (Mann and Firtel, 1991); and *car1/3* null cells expressing the cAMP receptor under control of the Actin15 (*Act15*) promoter (Insall *et al*., 1994). The cells were grown in HL5 medium (Watts and Ashworth, 1970) at 22°C and transformed strains were grown in HL5 medium supplemented with G418 at a final concentration of 20 μg/ml.

For development, cells in the exponential phase of growth (i.e. at $<2\times10^6$/ml) were washed twice in KK2-phosphate buffer (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH 6.2) and allowed to develop on Millipore filters or in shaking culture. For development on filters, washed cells were resuspended in KK2-phosphate buffer at a concentration of 1×10^8 cells/ml. Part of this suspension (500 μl) was plated uniformly onto a Millipore filter (HABP; diameter 47 mm) and the filters were incubated on KK2-phosphate-buffer-soaked prefilter in Petri dish within a humid box. Every 2 h, or as indicated, the cells of half a filter (2.5×10^7 cells) were harvested for immunohistochemical staining and/or protein analysis. For development in suspension, washed cells were resuspended at a density of 1.0×10^7 and shaken in conical flasks at 150 r.p.m.

Production and purification of antibodies

Three different antibody preparations were used. A mouse monoclonal antibody (clone D4, an IgG) was prepared using an antigen against a tyrosine-phosphorylated peptide corresponding to the C-terminal sequence of Dd-STAT (RTAPVPVGGYEPLNS). Ascites fluid from a single mouse was used in all the experiments described. Two rabbit polyclonal antisera, SC7 and SC9, were prepared against the C-terminal peptide and purified in two different ways. In order to obtain a reagent that could be used to quantitate total levels of Dd-STAT protein, SC9 was purified by binding to a GST fusion protein containing the approximate C-terminal half of Dd-STAT (residues 392–708). In order to obtain a reagent specific for the tyrosine-phosphorylated form of Dd-STAT, SC7 was purified through sequential rounds of affinity chromatography exactly as described by Lewis *et al*. (1997). In brief, the SC7 serum was first bound and eluted from a column bearing the phosphorylated C-terminal peptide and then counter-purified by multiple passages through a column bearing the non-phosphorylated form of the same C-terminal peptide. The final flow through constituted the purified material.

Western blotting and immunoprecipitation

For Western analysis, cells were solubilized in SDS gel sample buffer containing 1.6 μg/ml leupeptin, 40 μg/ml aprotinin, 2.5 mM benzamidine

and 0.25 mM PMSF as proteinase inhibitors. The proteins were analyzed on a 7.5% SDS–polyacrylamide gel and blotted onto Hybond-C extra filters (Amersham, Ltd) which were incubated with one of the following primary antibodies: the D4 monoclonal anti-Dd-STAT antibody, the py72 monoclonal anti-pTyr antibody (Glenney *et al*., 1988), the 4G10 commercially produced anti-pTyr antibody (Upstate Biotechnology, Inc.) or the polyclonal SC7-pTyr and SC9-total anti-Dd-STAT antibodies. Horseradish peroxidase-coupled anti-mouse or anti-rabbit antibodies from Amersham or Bio-Rad were used as secondary antibodies and ECL detection was performed following the protocol recommended by the manufacturers (Amersham, Ltd).

For immunoprecipitation, ~2.0×10^7 cells were lysed in 1 ml of NP-40 buffer (1× PBS, pH 7.4, 50 mM NaF, 1% Nonidet P-40, 2 mM EDTA, pH 7.2, 1 mM Na-pyrophosphate, 1.6 μg/ml leupeptin, 4.0 μg/ml aprotinin) and the lysate was incubated on ice for 5–30 min. After a 10 min centrifugation at 4°C the supernatant was transferred to a new tube. Ascites fluid containing D4 monoclonal anti-Dd-STAT antibody was added at a dilution of 1:400 and the lysate was incubated at 4°C on a slow rocker for 1 h. A quantity (40 μl) of protein A-beads (as a 50% slurry) was added and incubation at 4°C on the slow rocker was continued for another 45 min. The beads were washed four times in 500 μl NP-40 buffer and resuspended in 20–30 μl 2× SDS protein sample buffer. A quantity (10 μl) of this immunoprecipitate was separated by SDS–PAGE and analyzed by Western blotting.

Immunohistochemical staining

For individual staining, cells were dissociated from developing structures by titration through a syringe needle, pelleted and resuspended in 50% methanol. They were then pelleted, resuspended in absolute methanol, and incubated for 10 min on ice. Finally, they were plated on glass slides and allowed to dry.

Whole mounts of multicellular structures were allowed to settle on poly-L-lysine-coated slides and fixed with methanol for 10 min. Before the methanol dried, they were passed through a rehydration series of 75, 50 and 25% methanol (in PBS) with 2 min per step (in order to avoid dehydration and subsequent shrinkage of the structures).

Both kinds of specimen (i.e. dissociated cells and whole mounts of developing structures) were incubated with antibodies D4, SC7-pTyr or SC9-total for >20 h in PBS. After washing in PBS they were then secondarily incubated with pre-absorbed FITC-conjugated goat anti-mouse IgG antibody (Sigma) or FITC-conjugated goat anti-rabbit IgG antibody (Bio-Rad) for >20 h. Finally, the samples were mounted and visualized in a Leica DMRBE confocal microscope (model TCS-NT). The images were processed using NIH-Image version 1.62.

Nuclear fractionation and gel retardation assay

Preparation and fractionation of nuclear extracts and gel retardation assays were performed exactly as described by Kawata *et al*. (1996).

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