Protein Tyrosine Phosphatase PTP1 Negatively Regulates *Dictyostelium* STATa and Is Required for Proper Cell-Type Proportioning

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The protein tyrosine phosphatase PTP1, which mediates reversible phosphorylation on tyrosine, has been shown to play an important regulatory role during *Dictyostelium* development. Mutants lacking PTP1 develop more rapidly than normal, while strains that overexpress PTP1 display aberrant morphology. However, the signalling pathways involved have not been characterised. In reexamining these strains, we have found that there is an inverse correlation between levels of PTP1 activity, the extent of tyrosine phosphorylation on *Dictyostelium* STATa after treatment with cAMP, and the proportion of the slug population exhibiting STATa nuclear enrichment *in vivo*. This suggests that PTP1 acts to attenuate the tyrosine phosphorylation of STATa and downstream STATa-mediated pathways. Consistent with this, we show that when PTP1 is overexpressed, there is increased expression of a prestalk cell marker at the slug posterior, a phenocopy of STATa null slugs. In *ptp1* null strains, STATa tyrosine phosphorylation and nuclear enrichment in the slug anterior is increased. There is also a change in the prestalk to prespore cell ratio. Synergy experiments suggest that this is due to a cell-autonomous defect in forming the subset of prespore cells that are located in the anterior prespore region.   © 2001 Academic Press

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INTRODUCTION

When food sources are exhausted, *Dictyostelium* single-celled amoebae aggregate in response to extracellular cAMP pulses to form an organised multicellular structure. Cells within the aggregate differentiate to produce prespore and prestalk cells, the precursors of spores and stalk cells, which sort to give a defined spatial pattern in the migratory slug (reviewed in Aubry and Firtel, 1999). When conditions are suitable for culmination, terminal differentiation is initiated resulting in a fruiting body consisting of a spore-containing sorus supported by a rigid, slender stalk.

The changes in signaling that accompany the unicellular to multicellular transition and the onset of cellular differentiation include significant changes in the level of phosphorylation of p-Tyr-containing proteins (Schweiger *et al.*, 1990; Howard *et al.*, 1992). These changes are regulated by at least eight protein tyrosine kinases (PTKs) and three protein tyrosine phosphatases (PTPs) (Tan and Spudich, 1990; Howard *et al.*, 1992, 1994; Ramalingam *et al.*, 1993; Adler *et al.*, 1996; Gamper *et al.*, 1996; Nuckolls *et al.*, 1996; Kim *et al.*, 1999; R. Firtel and J. G. Williams, unpublished data). None of the PTKs is closely related to those of higher cells. Spl1 is a dual specificity kinase (Nuckolls *et al.*, 1996), while DPYK3, DPYK4, and ZAK1 each have two candidate tyrosine kinase domains but do not otherwise resemble Janus kinases (JAKs) (Adler *et al.*, 1996; Kim *et al.*, 1999). *ptp1*, *ptp2*, and *ptp3* encode devel-

opmentally regulated, nonreceptor PTPs (Howard *et al.*, 1992, 1994; Gamper *et al.*, 1996). The analysis of mutant strains either lacking or overexpressing *ptp1*, *ptp2*, or *ptp3* suggests that they regulate distinct cellular pathways (Howard *et al.*, 1992, 1994; Gamper *et al.*, 1996). PTP1, unlike PTP2 and PTP3, regulates the level of actin tyrosine phosphorylation (Howard *et al.*, 1993), while PTP3 functions as part of the response to osmotic shock (Gamper *et al.*, 1999).

Dictyostelium, like metazoans, utilises STAT transcription factors to control aspects of multicellular development and cell-type differentiation (Kawata *et al.*, 1997). STATs are SH2 domain-containing transcription factors that were initially identified as targets of cytokine signaling in mammalian cells (reviewed in Ihle and Kerr, 1995; Schindler and Darnell, 1995; Leaman *et al.*, 1996; Darnell, 1997; Horvath and Darnell, 1997). STATs are activated in response to ligand binding to a cell-surface receptor by phosphorylation of a conserved tyrosine residue, which rapidly leads to STAT homo- or heterodimerization and translocation to the nucleus. In mammalian cells, both receptor tyrosine kinases and Janus kinases (JAKs) phosphorylate STATs. The *Dictyostelium* STAT protein, STATa, is activated in response to extracellular cAMP signaling functioning through serpentine, cell-surface receptors via a G-protein-independent pathway (Kawata *et al.*, 1997; Araki *et al.*, 1998). There is evidence for similar serpentine receptor, G-protein-independent pathways of STAT activation in mammals (reviewed in Williams, 1999).

The pattern of STATa nuclear enrichment during the developmental phase is complex. It is detectable in the nuclei of less than 10% of cells during growth and early development, but is enriched in a rapidly increasing proportion of nuclei during aggregation (Araki *et al.*, 1998). At the tight mound stage, STATa is present in the nuclei of all cells but then is selectively lost from nuclei of all cells except those in the tip of the slug. This pattern of STATa nuclear enrichment is consistent with STATa's genetically defined role as a repressor that holds tip cells in a prestalk state prior to the onset of terminal differentiation (Mohanty *et al.*, 1999). The tip cells in which nuclear STATa is retained are a subset of the pstA cell population (Araki *et al.*, 1998). The anterior, prestalk region of the slug can be divided into three domains, based on the expression pattern of the marker genes *ecmA* and *ecmB*: the prestalk (pst) A region at the front and the pstO region at the rear, in which distinct regions of the *ecmA* promoter are active (Early *et al.*, 1993, 1995); and a core of pstAB cells in the centre of the pstA region, where the stalk-specific *ecmB* gene is also expressed (Jermyn and Williams, 1991). It is these pstAB cells that mark the origin of the stalk tube.

Repressor elements that bind STATa *in vitro* have been defined within the *ecmB* promoter (Kawata *et al.*, 1996). In wild-type slugs, a *lacZ* reporter construct driven by the *ecmB* promoter is expressed only in the pstAB cells (Jermyn and Williams, 1991). However, when the STATa-binding, prestalk repressor element in the *ecmB* promoter is deleted,

reporter expression is seen throughout the pstA domain. A similar expanded pattern of expression is observed with a reporter carrying the wild-type *ecmB* promoter expressed in STATa null cells, supporting the model that STATa is an essential component of the *ecmB* prestalk repressor (Mohanty *et al.*, 1999). STATa may also be required for gene regulation at other stages of development. Paradoxically, although STATa regulates commitment to stalk cell differentiation, the STATa null mutant is not able to complete the culmination process, and it has therefore been postulated that there is a second, uncharacterised step in stalk cell formation that requires STATa (Mohanty *et al.*, 1999). STATa has recently been shown to act as a transcriptional activator of the *cudA* gene, expression of which is essential for correct terminal differentiation (Fukuzawa and Williams, 2000).

In mammalian cells, the mechanisms responsible for deactivation of STATs or JAKs have not been clearly defined, but dephosphorylation, proteolytic degradation, and negative regulatory factors, such as suppressor of cytokine signalling (SOCS) and protein inhibitor of activated STAT (PIAS) proteins, may each have a role (reviewed in Starr and Hilton, 1999). There is evidence from inhibitor studies that an as yet unidentified protein tyrosine phosphatase acts in the nucleus to dephosphorylate STAT1 directly (Haspel and Darnell, 1999). The PTPs most clearly demonstrated to be involved in STAT regulation, SHP1 and SHP2, are nonreceptor, SH2 domain-containing PTPs that act predominantly by regulating JAK activity in the cytosol (reviewed in Tonks and Neel, 1996). In this manuscript, we examine the role of PTP1 in the regulation of *Dictyostelium* STATa function during development. We find that the level of PTP1 affects the degree of STATa activation at the slug stage, and we demonstrate a role for PTP1 in negatively regulating STATa tyrosine phosphorylation. In addition, we show that PTP1 regulates the proportioning of prestalk and prespore cells within the slug, via a pathway that may not involve STATa.

MATERIALS AND METHODS

Dictyostelium Cell Culture

The cell strains used were as follows: the axenic strains KAx-3 and JH10, a thymidine auxotroph derived from KAx-3 (Mann and Firtel 1991; Hadwiger and Firtel, 1992); the *ptp1* null (Howard *et al.*, 1992); PTP1^{OE}, a KAx-3-based strain that overexpresses the *ptp1* cDNA (Howard *et al.*, 1992); PTP1^{C310S}, a KAx-3-based strain expressing a mutated version of *ptp1* with a cysteine to serine substitution at position 310; and the STATa null (Mohanty *et al.*, 1999). *ptp1* null and PTP1^{OE} cell lines were made that additionally contain the following cell-type-specific markers (by cotransformation in the case of the cells lines overexpressing PTP1): *ecmAO/ lacZ*, *ecmB/lacZ*, *ecmA/lacZ* (marking the pstA cells), *ecmO/lacZ* (marking the pstO cells), *SP60/lacZ* (marking prespore cells), and *pspA/lacZ* (also marking prespore cells) (Jermyn and Williams, 1991; Early *et al.*, 1993, 1995; Haberstroh and Firtel, 1990; Detterbeck *et al.*, 1994). The *ecmB/lacZ*-marked PTP1^{OE} was additionally

created in an AX-background. *ptp1* null cells were also transformed with *pAct15/lacZ* (Harwood and Drury, 1990). β-galactosidase staining of these cell lines was performed as described previously (Early *et al.*, 1993). All cells were grown in HL5 medium (Watts and Ashworth, 1970) at 22°C. The medium was supplemented with 200 μg/ml thymidine in the case of JH10, and with 20 μg/ml G418 for the *lacZ*-marked *ptp1* null and for PTP1[OE]. PTP1[OE] was additionally selected at 80 μg/ml G418.

Immunohistochemical Staining

To obtain whole mounts, agar blocks with attached slugs were submerged in ice-cold methanol for 10 min, and the slugs were then transferred singly to poly-L-lysine coated slides. The primary antibody used was either the anti-STATa mouse monoclonal antibody D4, which recognises phosphorylated and nonphosphorylated STATa (Araki *et al.*, 1998), or the affinity-purified rabbit polyclonal antiserum SC9P. SC9P, which is specific for the phosphorylated form of STATa, was derived from SC9 total serum following the procedure of Lewis *et al.* (1996). The slides were secondarily incubated with preabsorbed FITC-conjugated goat anti-mouse IgG antibody (Sigma) or Texas red-conjugated goat anti-rabbit IgG antibody (Molecular Probes). The samples were mounted and visualised with a Leica DMRBE confocal microscope (with TCS-NT laser head) and the images were processed using NIH-Image version 1.62.

For antibody staining of individual slug cells, the slugs were dissociated by trituration through successively smaller syringe needles, ending with 25G needles. The cells were allowed to settle on poly-L-lysine-coated multiwell slides for 10 min, which were then dipped in ice-cold methanol for a further 10 min. The slides were incubated with the anti-PspA mouse monoclonal MUD-1 (Krefft *et al.*, 1983), followed by a FITC-conjugated goat anti-mouse IgG antibody (Sigma) preabsorbed against *Dictyostelium* cells. MUD-1 positive cells were detected using the 100× objective of an inverted microscope (Leica model DMRBE).

Western Blotting and Immunoprecipitation

Immunoprecipitation was carried out using ascites fluid produced from a single mouse using the anti-Dd-STAT monoclonal D4 (Araki *et al.*, 1998). The monoclonal anti-pTyr antibody PY72 (Glenney *et al.*, 1988) and the anti-Dd-STAT polyclonal SC7 (Araki *et al.*, 1998) were used for subsequent Western blotting. Cells to be analysed by immunoprecipitation were developed in shaking culture in KK2 phosphate buffer and pulsed with 30 nM cAMP every 6 min for 5 h, before the addition of 5 mM cAMP. For each time point, 2×10^7 cells were lysed on ice in 1 ml of NP-40 buffer [1 × PBS (pH 7.4), 1% Nonidet P-40, 10 mM NaF, 2 mM EDTA (pH 7.2), 1 mM sodium PPi, 0.8 μg leupeptin/ml, and 4 μg aprotinin/ml] for 10 min. After a 10-min centrifugation, the supernatant was incubated with 2.5 μl D4 ascites fluid for 1 h at 4°C with gentle rocking. A total of 40 μl of 50% protein A-Sepharose (Sigma) was added, and the incubation was continued at 4°C with gentle rocking. After being washed three times in 500 μl of NP-40 buffer, the immunoprecipitates were eluted from the beads by boiling in SDS sample buffer. The samples were subjected to Western blot analysis after size separation on a 10% polyacrylamide gel, as described previously (Gamper *et al.*, 1996).

RESULTS

Patterns of STATa Nuclear Enrichment in Mutant PTP1 Strains

To test the possible role of *Dictyostelium* PTP1 in the regulation of STATa, the pattern of STATa nuclear enrichment was examined in a mutant strain in which *ptp1* had been disrupted by homologous recombination (Howard *et al.*, 1992). The strain was developed to the slug stage and stained using either the anti-STATa monoclonal antibody D4 (Araki *et al.*, 1998) or the pTyr-specific anti-STATa polyclonal antibody, SC9P, prior to examination by confocal microscopy (Fig. 1A). These two antibodies give essentially identical results, since any cytoplasmic STATa detected with D4 does not obscure the much more concentrated nuclear staining. The extent of nuclear enrichment can be correlated with the degree of STATa activation, since only STATa phosphorylated on tyrosine enters the nucleus.

In these experiments, we also used the wild-type axenic strain KAx-3 (Fig. 1A), the parent of the PTP1 overexpressing strains (see below), and JH10, a KAx-3 derivative that is the parent of the *ptp1* null strains (data not shown). No detectable differences were observed between KAx-3 and JH10, and the pattern of STATa nuclear enrichment in these strains closely resembled that previously described for another wild-type strain, Ax2 (Araki *et al.*, 1998). As shown in Fig. 1A, nuclear enrichment of STATa is only detected in a subset of pstA cells in the tip of the slug. When the staining pattern of *ptp1* null cells is compared to the wild-type strain, we see a clear increase in the number of cells in which STATa shows a strong nuclear enrichment, with staining cells extending into the pstO domain and in some scattered cells in the anterior of the prespore region (Fig. 1A). A striking feature of the *ptp1* null cell staining pattern is that STATa nuclear enrichment is no longer confined to the cortical cell layers, as seen for wild-type slugs posterior to the extreme tip of the slug (Araki *et al.*, 1998) (Fig. 1B). To further examine the role of PTP1 in the regulation of STATa, we expressed a mutant PTP1, PTP1[C310S], in which the essential, conserved cysteine residue in the active site was changed to a serine residue, in wild-type (KAx-3) cells. This mutation is known to abolish catalytic activity (Guan and Dixon, 1991) but still allows binding to substrates (Sun *et al.*, 1993; Milarski and Saltiel, 1994; Noguchi *et al.*, 1994) and can often sequester and protect a substrate from dephosphorylation by the endogenous PTP, as was demonstrated for *Dictyostelium* PTP3 (Gamper *et al.*, 1996, 1999). The nuclear STATa staining seen in the PTP1[C310S] strain was stronger than that observed in wild-type slugs (Fig. 1A), suggesting that STATa activation and hence its nuclear translocation have been potentiated. However, these experiments cannot distinguish between a direct effect on STATa or on an upstream kinase that regulates STATa tyrosine phosphorylation. Attempts to detect an interaction of the PTP1[C310S] protein and STATa
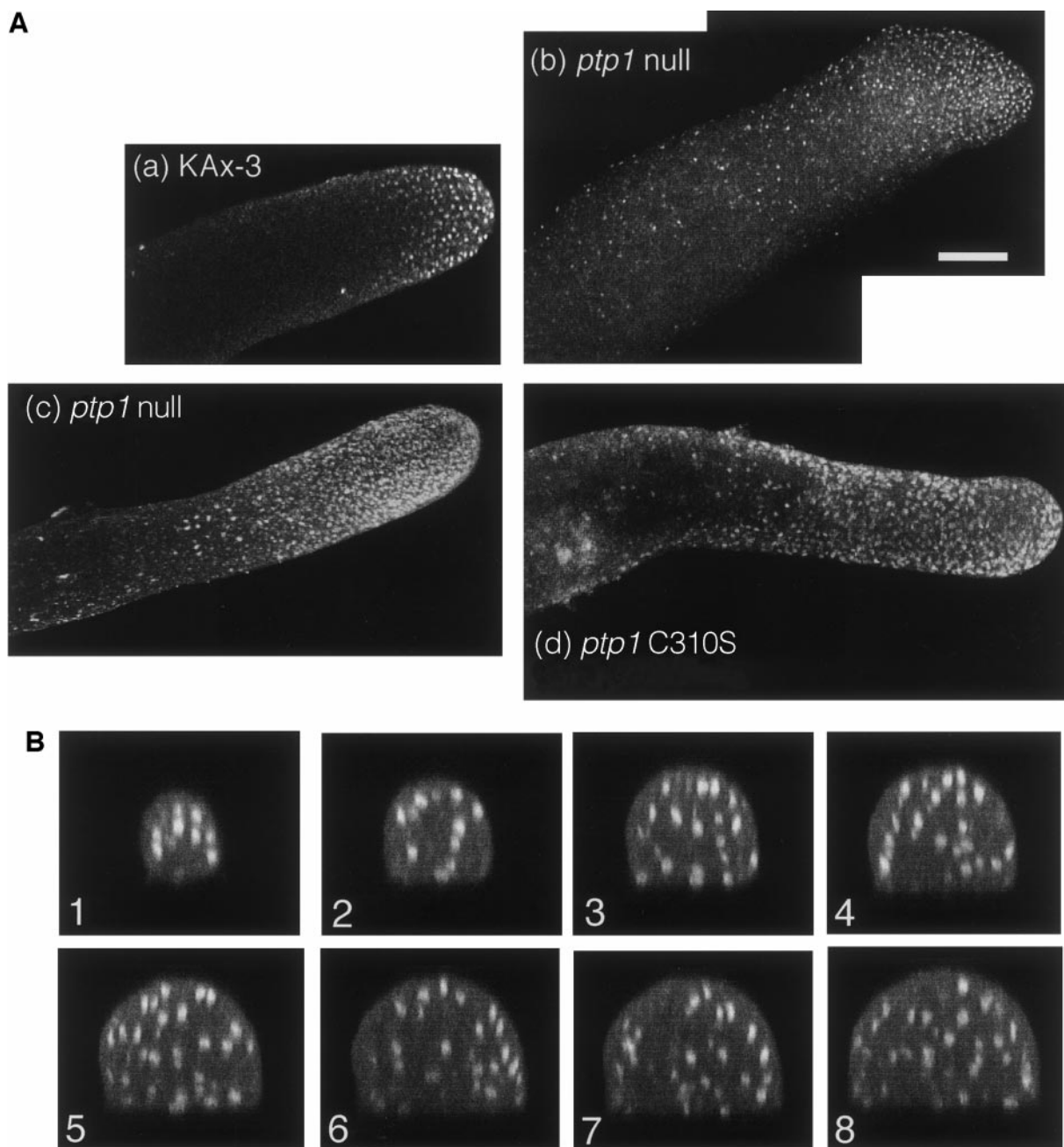
FIG. 1. Patterns of STATa nuclear enrichment in whole mounts of wild-type and PTP1 mutant slugs detected with anti-STATa antibodies. At least five slugs were examined for each strain, because in general STATa staining is dynamic and patterns observed can vary from slug to slug. This variation can depend on whether or not the slug tip is in contact with the substratum (T. Abe, personal communication). All examined slugs showed a similar pattern and a representative slug is shown. Newly formed slugs from water agar plates were fixed, stained, and mounted before viewing by confocal microscopy. (A) All slugs shown are projected images derived from Z series. (a) and (b), stained with the monoclonal D4, which was raised against a STATa C-terminal peptide and recognises total STATa (Araki *et al.*, 1998). (c) and (d), stained with the affinity purified polyclonal SC9P, which recognises tyrosine phosphorylated STATa. Both antibodies detect nuclear STATa, since it is known to be in the p-Tyr form. (a) KAx-3, a parental axenic strain, showing STATa nuclear enrichment in the tip only. (b) PTP1 null, showing much more widespread STATa nuclear enrichment. (c) PTP1 null, and (d) PTP1^{C310S}, showing a similar increase in nuclear STATa staining. The scale bar in (b) represents 50 μM. (B) Pattern of nuclear enrichment of STATa staining in cross sections of the prestalk region in a typical PTP1 null slug. Staining was detected using SC9P. Transverse sections (1–8) were scanned by the confocal microscope at 20-μm intervals from the tip of the slug to the rear of the pstO region, and in contrast to the lack of staining in the slug interior the extreme tip seen in wild-type slugs (Araki *et al.*, 1998), there is staining in the centre of the PTP1 null slugs in all sections.

in vitro were unsuccessful (data not shown), which could be due to STATa not being a direct substrate of PTP1 or the possible inaccessibility of this phosphorylated tyrosine under the conditions used.

The effect of overexpressing PTP1 was examined in strains (PTP1[OE]) in which PTP1 was expressed from the cloned *ptp1* promoter on a high-copy vector containing an actin 6-neomycin resistance cassette (Howard *et al.*, 1992). Cells were selected at two different levels of G418 (20 and 80 μg/ml) to obtain strains with low and moderate levels of overexpression, respectively (Fig. 2). It was previously shown that by increasing the G418 concentration, the copy number of the PTP1[OE] construct and the severity of the phenotype were also increased (Howard *et al.*, 1992). Therefore, the level of PTP1 activity is assumed to correlate with the G418 level used. No change in STATa nuclear staining was seen in slugs derived from cells selected with 20 μg/ml G418. However, in the cells selected with 80 μg/ml G418, there was a significant reduction in the intensity of STATa staining throughout the anterior region of the slug. Thus, abolishing or increasing the level of PTP1 has a reciprocal effect on STATa nuclear enrichment, with an inverse correlation between PTP1 activity and the degree of nuclear enrichment.

The Phosphorylation of STATa on Tyrosine Is Altered in the PTP1 Mutant Strains

STATa tyrosine phosphorylation is activated in aggregation-competent cells in response to high, continuous cAMP (Araki *et al.*, 1998). To examine if the kinetics and level of STATa tyrosine phosphorylation are altered in response to cAMP in PTP1 mutant strains, cells were first pulsed with 30 nM cAMP for 5 h to maximize the levels of cAMP receptor cAR1 and other components of the signaling pathways (Insall *et al.*, 1994: Schnitzler *et al.*, 1995) and then stimulated with 100 μM cAMP. Samples were taken at times varying from 30 s to 20 min after cAMP stimulation. Each sample was divided in two, and both halves were subjected to an immunoprecipitation reaction using ascites fluid derived from the anti-STATa monoclonal antibody, D4 (Araki *et al.*, 1998). The products were processed for Western blotting using either the anti-STATa polyclonal antibody SC7 (Araki *et al.*, 1998) or the anti-pTyr antibody PY72 (Glenney *et al.*, 1988) as a probe (Fig. 3). The anti-STATa polyclonal probe indicated that equal levels of STATa protein were loaded in all lanes. Using PY72 as a probe, only (p-Tyr)-STATa is detected. In wild-type cells, no tyrosine phosphorylated (p-Tyr)-STATa is visible at the zero time point. A faint p-Tyr-STATa band is detected after 30 s, which increases to a maximum level by 10 min. A second, more slowly migrating species is first clearly visible 10 min after the addition of cAMP in the control cells. It has been shown that this lower mobility form is the result of additional phosphorylation on serine by GSK-3 (Ginger *et al.*, 2000). The rapid nuclear translocation of STATa upon cAMP stimulation is coincident with the appearance of
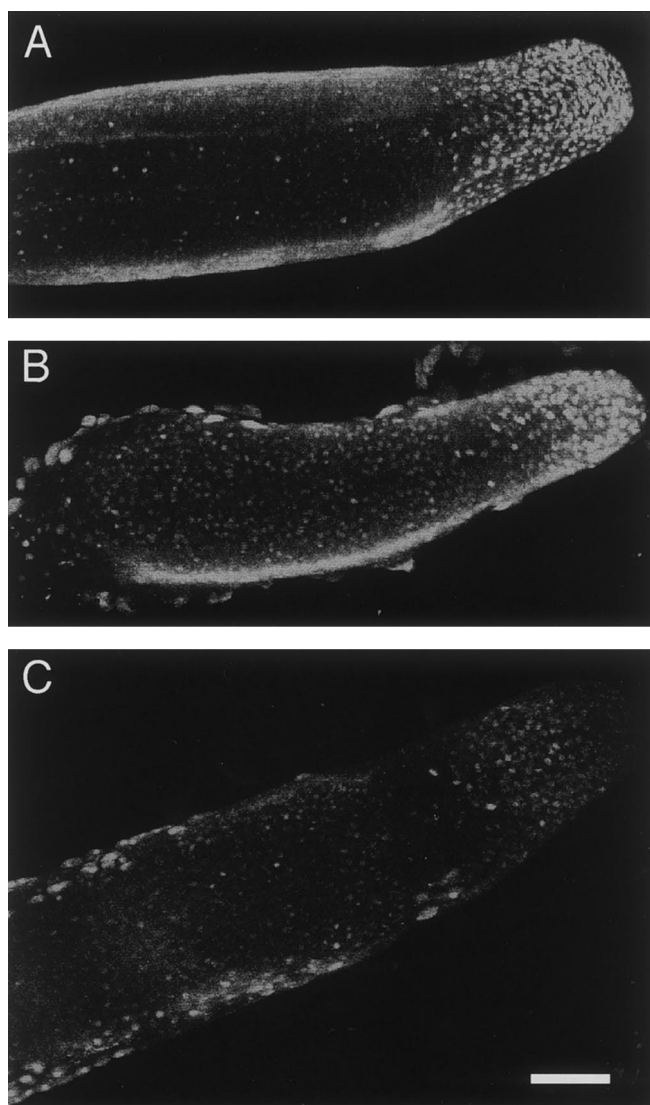

FIG. 2. STATa nuclear enrichment in strains overexpressing PTP1, detected using SC9P. All images shown are projected Z series, recorded using identical confocal settings. The laser power was increased relative to that used for the images in Fig. 1 to enable signal detection in C. (A) KAx-3 slug. (B) PTP1[OE] slug, selected at 20 μg/ml G418, showing a wild-type level and pattern of staining. (C) PTP1[OE] slug, selected at 80 μg/ml G418, showing significantly reduced but not absent staining. The staining cells attached to the slugs in B and C are not part of the slugs but are among those cells that have failed to aggregate and have been left on the substratum. PTP1 expression was driven by its own promoter in conjunction with an actin 6 promoter-driven, neomycin-selectable marker (Howard *et al.*, 1992). The scale bar in (C) represents 50 μM.

tyrosine phosphorylation rather than of this later modification (Araki *et al.*, 1998). The results obtained for the *ptp1* null cells and the cells overexpressing PTP1[C310S] were very similar to each other. The lower band was visible in
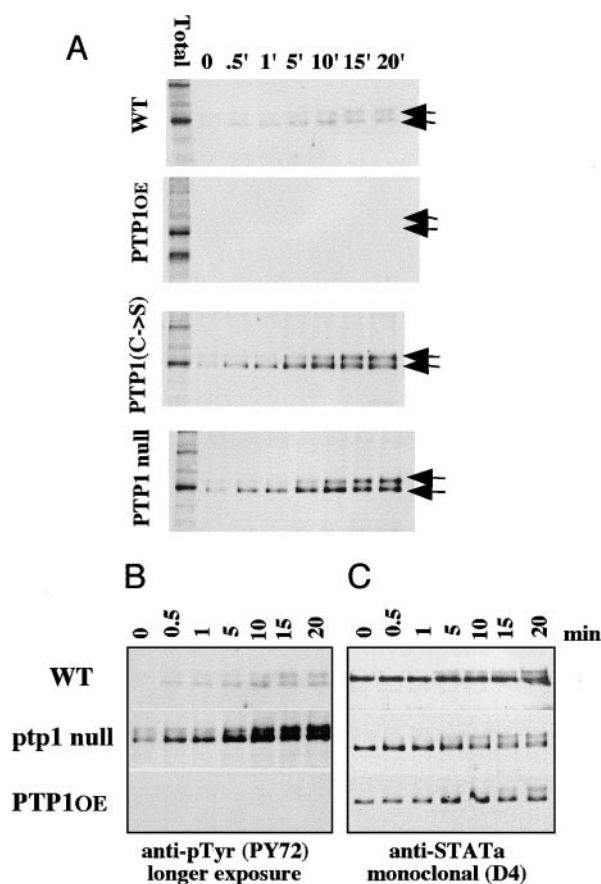
FIG. 3. Comparison of tyrosine phosphorylation of STATa in response to added cAMP in PTP1 mutant and wild-type cells. Cells of the various strains shown were developed in shaking suspension for 5 h, after which the zero time point was harvested and 100 μM cAMP added to the remainder of the cells. Further time points were removed 30 s, 1 min, 5 min, 10 min, 15 min, and 20 min after the addition of 100 μM cAMP. The time points were subjected to immunoprecipitation using ascites fluid derived from the anti-STATa monoclonal antibody D4 (Araki *et al.*, 1998), and the products were analysed by Western blotting using the anti p-Tyr monoclonal PY72 (Glenney *et al.*, 1988) (A and B) or the anti-STATa polyclonal SC7 (Araki *et al.*, 1998) as a probe (C). The longer exposure of the blot of samples obtained from the PTP1[OE] cells, WT cells, and *ptp1* null cells shown in B demonstrates the extent of signal reduction for the PTP1[OE] strain. C illustrates that equal levels of STATa protein were loaded in all lanes. The two arrows indicate the two STATa bands that are detected on Western blots. Both are tyrosine phosphorylated and the additional modification that gives rise to the upper band has been shown to be serine phosphorylation mediated by GSK-3 (Ginger *et al.*, 2000).

unstimulated cells; after 30 s, the intensity of this band was greater than seen at any stage in wild-type cells, and the levels increased to a maximum level 5 min after cAMP addition. The upper band was also detected earlier and was of greater intensity than in wild-type cells, so that the ratio

of the upper to lower band was similar to that in the control cells. Overall, there was an eightfold increase in the level of p-Tyr-STATa. The opposite result was obtained for the PTP1[OE] cells: no p-Tyr-STATa could be detected at any time point. Taken together, these data clearly demonstrate a correlation between the activity of PTP1 and the rate and overall extent of STATa tyrosine phosphorylation.

Moderate Overexpression of PTP1 Partially Phenocopies the STATa Null Mutant

The developmental phenotype of PTP1[OE] strains depends on the degree of overexpression (Howard *et al.*, 1992). When PTP1 is very highly overexpressed, aggregation is blocked. This phenotype is more severe than that of the STATa null, suggesting that PTP1 has additional functions beyond that of regulating STATa and/or that at very high levels of protein, PTP1 may affect pathways that it normally does not regulate in wild-type cells. When PTP1 is moderately overexpressed (cells selected at 80 μg/ml G418), slugs are formed, enabling the consequences of the misregulation of STATa by PTP1 at the multicellular stage to be studied. As the tyrosine phosphorylation and nuclear enrichment of STATa is reduced in this strain, an obvious prediction was that aspects of the STATa null phenotype would be seen. A key feature of the STATa null phenotype is premature expression of the *ecmB* gene in the anterior prestalk region of slugs as detected using the reporter *ecmB/lacZ* (Jermyn and Williams, 1991; Mohanty *et al.*, 1999) To examine the effect of PTP1 overexpression on *ecmB* expression, an Ax-2 background was used, as it is the strain in which *ecmB* repression by STATa was previously demonstrated. However, there was no ectopic expression of *ecmB/lacZ* in the PTP1[OE] slugs (Fig. 4), suggesting that the reduced, but detectable, level of nuclear STATa present is sufficient to mediate *ecmB* repression.

In contrast, abnormal patterning was seen in the PTP1[OE] strain using *ecmAO/lacZ*, which is expressed in all prestalk cells (Jermyn and Williams, 1991), and the two prespore reporters, *pspA/lacZ* and *SP60/lacZ* (Early *et al.*, 1988; Dingermann *et al.*, 1989; Haberstroh and Firtel, 1990). A large domain that does not stain with prespore markers but does stain with *ecmAO/lacZ* was observed at the rear of a subset of the slugs, in contrast to the KAx-3 control (Fig. 4). A similar staining pattern is observed in STATa null slugs that have migrated for a period of time (Fig. 4; Mohanty *et al.*, 1999). It is thus likely that the change in spatial patterning of prestalk and prespore cells when PTP1 is overexpressed is a consequence of a reduction in nuclear STATa.

The Prestalk to Prespore Cell Ratio Is Altered in the ptp1 Null Strain

Cell-type-specific markers were similarly used in the *ptp1* null strain to examine cellular differentiation and patterning. Somewhat unexpectedly, in view of the absence
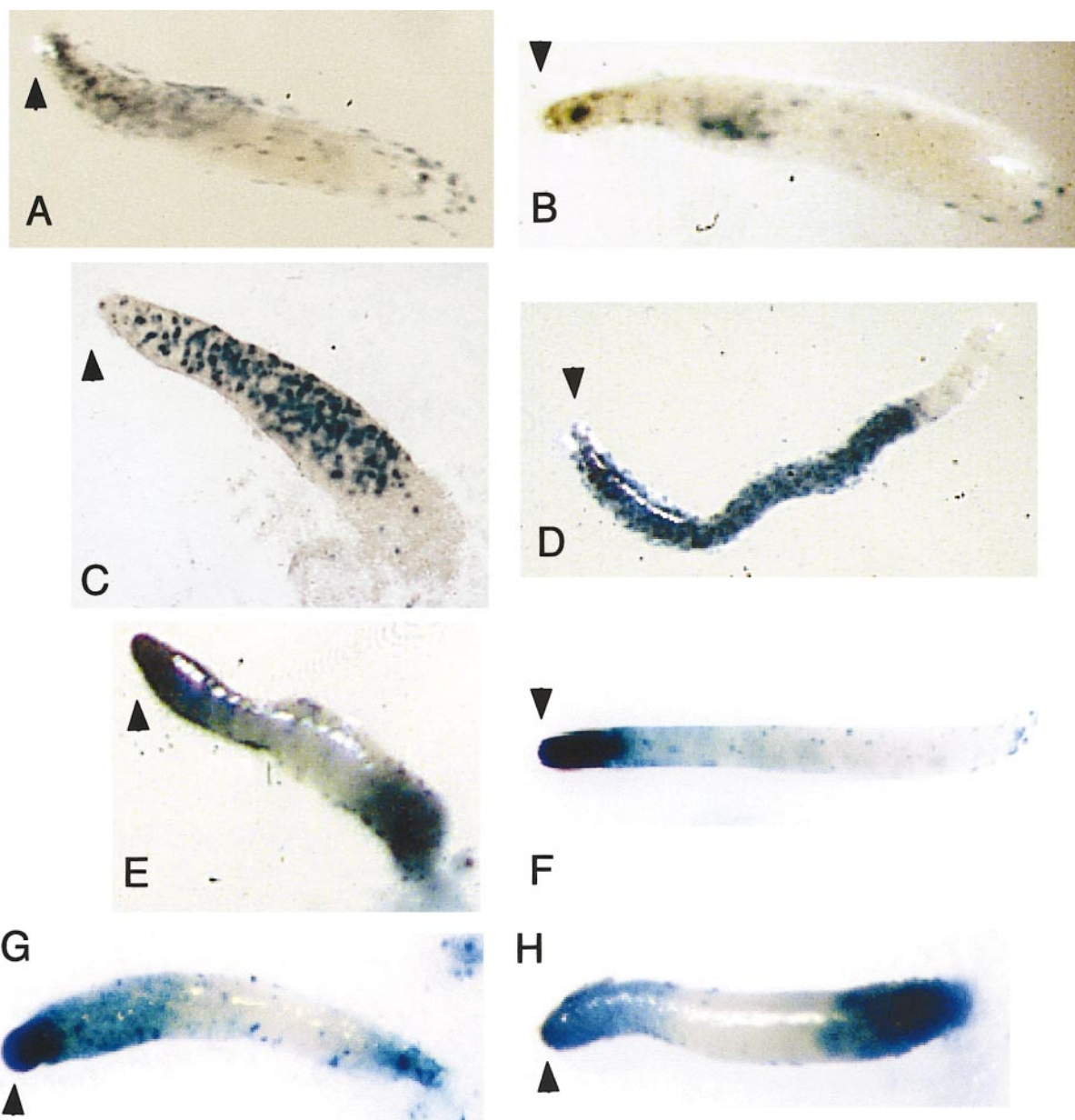
FIG. 4. Expression of cell-type-specific markers in PTP1[OE] slugs and in STATa null slugs, showing a similar expansion of prestalk staining at the rear of slugs. (A) and (B) Ax-2 slugs or (C–F) KAx-3 slugs, either overexpressing PTP1 or lacking PTP1[OE], were analysed 24 h after the cells were plated on water agar plates exposed to unidirectional light, i.e., after 6–8 h of slug migration. The cells were transformed with the following constructs: (A) *ecmB/lacZ* (wild-type control, no PTP1[OE]); (B) PTP1[OE] and *ecmB/lacZ*; (C) PTP1[OE] and SP60/*lacZ* (for control without PTP1[OE] see Fig. 5C); (D) PTP1[OE] and *pspA/lacZ* (for control without PTP1[OE] see Fig. 5E); (E) PTP1[OE] and *ecmAO/lacZ*; (F) *ecmAO/lacZ* (wild-type control, no PTP1[OE]). (G) and (H), STATa null cells transformed with *ecmAO/lacZ*. (F), 24 h, (G), 40 h after onset of development. In all cases, the prestalk region is oriented towards the left of the picture and indicated with an arrow. [N.B. In the case of C, the prestalk region is stained with the prespore *pspA-lacZ* marker due to the stability of the marker, combined with the forward movement of cells from the prespore to the prestalk region and accompanying transdifferentiation that occurs during slug migration (Detterbeck *et al.*, 1994).]

of any morphological abnormality in *ptp1* null slugs, a clear alteration in cellular differentiation was observed. There was a significant expansion in the size of the pstO domain and a corresponding large decrease in the size of the prespore zone using *pspA/lacZ* or *SP60/lacZ* as reporters (Fig. 5). Quantitation of the number of prespore cells in the
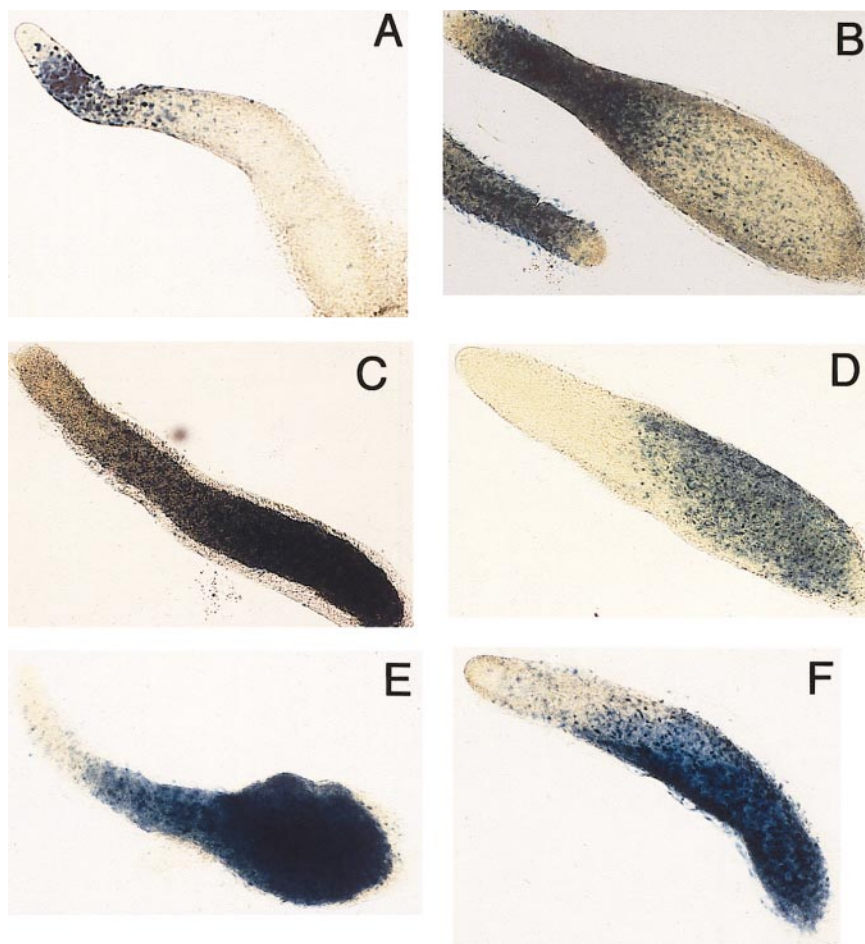
FIG. 5. Comparison of the expression of cell-type-specific markers in wild-type and *ptp1* null slugs demonstrates an altered prestalk (pst) O to prespore ratio in *ptp1* null slugs. (A), (C), and (E), newly formed KAx-3 slugs; (B), (D), and (F), newly formed *ptp1* null slugs. The following cell-type specific markers were used: (A) and (B), *ecmO/lacZ*; (C) and (D), SP60/*lacZ*; (E) and (F), *pspA/lacZ*. SP60/*lacZ* and pspA/*lacZ* are both prespore markers. For each marker, the staining reaction was continued for the same length of time for the mutant and wild-type slugs.

ptp1 null strain and the wild-type control strain was carried out by staining disaggregated slug cells with MUD-1, the monoclonal antibody that recognises the product of the prespore-specific gene *pspA* (Krefft *et al.*, 1983). The number of pstO cells was also quantitated by counting stained cells after disaggregation of slugs expressing *pstO/lacZ*. The number of prespore cells decreased from ~70% in wild-type slugs to ~55% in the *ptp1* null slugs (Table 1), in good agreement with the observed patterns of *lacZ* staining. Conversely, the pstO population increased almost twofold from 19.9 to 38.3%. The size of the pstA region was apparently unaltered compared to wild-type slugs, and *ecmB/lacZ* staining continued to be restricted to the core of the pstA domain of the slug (data not shown). The latter result was as expected for slugs with increased levels of nuclear STATa, in view of the role of STATa as a negative regulator of *ecmB* (Mohanty *et al.*, 1999).

TABLE 1

Quantitation of the Prespore–Prestalk Ratio in Wild-Type and PTP1 Null Slugs, Either by Using the Mouse Monoclonal Antibody MUD-1, which Recognises the Product of the Prespore-Specific Gene *pspA* (Krefft *et al.*, 1983) to Stain Disaggregated Cells from Newly Formed Slugs, or by Counting Positive Cells after Disaggregating Fixed and Stained Newly Formed Slugs Marked with *ecmO/lacz*

Strain	KAx-3	*PTP1* null
%MUD1 +ve	69.5% (1869)	55.3% (1331)
%*ecmO-lacZ* +ve	19.9% (1203)	38.3% (1255)

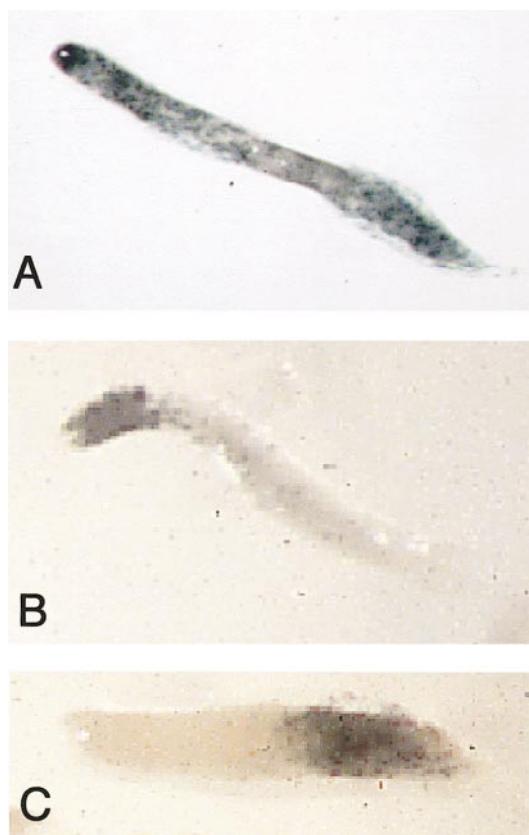The total number of cells counted for each strain is shown in parentheses.

FIG. 6. Patterns of marker gene expression in newly formed slugs composed of a mixture of JH10 wild-type cells and *ptp1* null cells. (A) 75% unmarked JH10 cells mixed with 25% *ptp1* null cells expressing the ubiquitous marker *Act15/lacZ*, showing that complete mixing has occurred. (B) 75% unmarked JH10 wild-type cells, 25% *PTP1* null cells expressing *ecmO/lacZ*. It was necessary to use 25% *ecmO/lacZ* marked cells to obtain detectable levels of staining in the chimera. (C) 90% JH10 cells, 10% *PTP1* null cells expressing SP60/*lacZ*.

To investigate the mechanism underlying this altered prespore-pstO ratio, we performed cell mixing experiments in which the *ptp1* null cells expressing either the pstO or a prespore-driven *lacZ* marker were mixed with an excess of the nontagged parental strain (JH10) and allowed to coaggregate and form chimeric slugs (Fig. 6). *ptp1* null cells marked with a reporter expressed in all cells, *Act15/lacZ*, were mixed with wild-type cells to demonstrate that the mutant cells were capable of contributing to all parts of the slug. With a mixture of 25% pstO-marked *ptp1* null cells and 75% JH10 cells, the pattern of pstO staining in the chimeric slugs is indistinguishable from that seen in wild-type slugs. We cannot rule out the possibility that the *ptp1* null cells continue to overproduce pstO cells, however, since the *ptp1* null cells now make up only a minority of the total cells. Consequently, any increase in the pstO region would be more difficult to detect than in pure

pstO-marked *ptp1* null slugs. In contrast, the prespore-marked *ptp1* null cells clearly still exhibit aberrant spatial patterning. When mixed with an excess of parental cells (in the ratio 25:75 or 10:90), *ptp1* null *SP60/lacZ* cells are confined to the rear 50% of the slug, as opposed to occupying the rear ~75% of wild-type slugs. Thus, the *ptp1* null cells seem to have a cell-autonomous defect in forming the prespore cells that occupy the anterior prespore region.

DISCUSSION

We have investigated the role of *Dictyostelium* PTP1 in the regulation of STATa. Our results provide clear evidence that PTP1 is a negative regulator of STATa function. First, there is an inverse correlation between the levels of PTP1 protein and the extent of tyrosine phosphorylation of STATa in response to cAMP stimulation of aggregation-competent cells. In strains overexpressing PTP1, STATa tyrosine phosphorylation is, within the limits of detection of the assay, noninducible by cAMP. In *ptp1* null cells, peak levels of STATa tyrosine phosphorylation are significantly increased (eightfold) compared to the peak levels in wild-type cells. Moreover, overexpressing a catalytically inactive version of PTP1, PTP1[310S], in a wild-type background had a presumed dominant negative effect and increased the tyrosine phosphorylation of STATa, similar to that observed in *ptp1* null cells. From these results, we conclude that PTP1 is required to attenuate the tyrosine phosphorylation of STATa induced by extracellular cAMP.

In addition, we found that there is a low, basal level of STATa tyrosine phosphorylation in unstimulated *ptp1* null cells. A similar effect has been observed with STATs in other species, for example, with Stat6 in mammalian cells briefly treated with the PTP inhibitor pervanadate. In these cells, this treatment inhibits a receptor-associated PTP activity, which leads to an increase in the tyrosine phosphorylation and activation of JAK1 (Haque *et al.*, 1997). In the case of *ptp1* null cells, the absence of PTP1 activity could lead to increased basal activity of the as yet unknown STATa kinase and hence a basal level of (p-Tyr)-STATa activation in the absence of ligand stimulation. We believe it likely that PTP1 inhibits STATa function by acting on an upstream component such as a tyrosine kinase, possibly by removing an activating tyrosine phosphate, rather than by dephosphorylating STATa directly. This is for three reasons: first, PTP1 has a putative N-terminal myristoylation signal and the protein is preferentially associated with the plasma membrane (M. Gamper and R. A. Firtel, unpublished observations), although we cannot exclude the presence of PTP1 in other cellular compartments; second, *in vitro* experiments using the "substrate-trapping" mutant PTP1[C310S] have not provided any evidence for direct interaction of PTP1 with STATa; third, PTP1 does not dephosphorylate p-Tyr-STATa *in vitro* under the conditions tested (data not shown). The absence of any detectable p-Tyr-STATa in PTP1[OE] cells even at early times after stimulation

is consistent with inhibition of the initial phosphorylation of STATa by PTP1, rather than direct dephosphorylation. We cannot, however, rule out the possibility that the STATa p-Tyr residue is inaccessible to PTP1 *in vitro* and/or that STATa is so rapidly dephosphorylated by PTP1 in the PTP^{OE} cells that no pTyr-STATa is observed. Thus, although these data are consistent with the model that STATa is not a PTP1 substrate, the evidence is not definitive. Also, since there is no evidence that any of the eight known *Dictyostelium* tyrosine kinases is a STATa kinase (Tan and Spudich, 1990; Adler *et al.*, 1996; Nuckolls *et al.*, 1996; Kim *et al.*, 1999; R. Firtel and J. G. Williams, unpublished data), the model that PTP1 dephosphorylates such a component cannot be tested at present.

When STATa is activated by tyrosine phosphorylation in response to cAMP signaling, it very rapidly translocates into the nucleus (Araki *et al.*, 1998). Therefore, if PTP1 is involved in attenuating levels of STATa tyrosine phosphorylation *in vivo*, it should regulate the level of nuclear STATa. Analysis of slugs of PTP1 mutant and wild-type strains using immunohistochemical methods revealed an inverse correlation between predicted PTP1 activity and levels of STATa in the nucleus. In contrast, changes in the pattern of STATa nuclear accumulation are not seen in mutants that lack or overexpress the two other known PTP genes, *ptp2* and *ptp3* (A. Early and R. A. Firtel, unpublished data). The changes in the pattern of STATa nuclear enrichment in the PTP1 mutant strains suggest that the anterior half of the slug is where PTP1 acts to attenuate STATa activation in response to cAMP. This is consistent with the observation that a *ptp1/lacZ* reporter is preferentially expressed in prestalk cells in the multicellular stages (Howard *et al.*, 1992). In contrast to the phosphorylation of STATa on tyrosine, the phosphorylation of STATa on serine by GSK3 promotes its export from the nucleus (Ginger *et al.*, 2000). In *ptp1* null cells in shaking culture treated with cAMP, the apparent level of GSK-3-mediated serine phosphorylation of STATa increases in line with tyrosine phosphorylation. However, this observation can be reconciled with the increased nuclear staining seen in the prestalk region of *ptp1* null slugs, since STATa regulation by GSK-3 seems to operate prior to tip formation only (Ginger *et al.*, 2000).

Overexpression of PTP1 phenocopies some of the aspects of the STATa null mutant. The residual functions could be due to the reduced, but finite, levels of p-Tyr-STATa present in PTP1^{OE} cells during the multicellular stages or possible as yet uncharacterised roles for nontyrosine-phosphorylated STATa. STATa is believed to function, in part, as a transcriptional repressor that prevents premature entry of prestalk cells into the stalk cell pathway of differentiation. This has been inferred from the fact that in the STATa null mutant the *ecmB* gene is ectopically expressed in prestalk cells, prior to their entry into the stalk tube (Mohanty *et al.*, 1999). When PTP1 is overexpressed, the level of nuclear STATa in tip cells, although reduced, seems to be sufficient for correctly regulated *ecmB* expression.

However, there is another aspect of the PTP1^{OE} phenotype that may be the consequence of a reduction in nuclear STATa levels. In both STATa null and PTP1^{OE} slugs, a zone of *ecmAO*-positive, non-prespore cells accumulates at the rear. One possibility is that prestalk cells with either absent or reduced nuclear STATa drop back to the rear of the slug during migration. This is an attractive explanation because several lines of evidence have shown that the prestalk STATa null cells are impaired in cell movement (Mohanty *et al.*, 1999). An alternative explanation is that STATa activity is required at the rear of slugs to prevent prestalk cell differentiation and/or promote prespore cell differentiation. Although nuclear enrichment of STATa is not observed in the rear of slugs (Araki *et al.*, 1998), it is known to be present at some level in all slug nuclei and so its function here is not precluded.

The most striking characteristic of the *ptp1* null slugs is an alteration in the prespore/pstO ratio, with an expansion of the pstO cell population at the expense of the prespore population. There is no decrease in the prestalk region when PTP1 is overexpressed, but the absence of PTP1 compared with an increase in its activity is not necessarily expected to have directly opposing effects. A reciprocal expansion of the pstO cell population and decrease in the prespore cell population has been previously described for the *Wariai* (*Wri*) null mutant (Han and Firtel, 1998). *Wri* encodes a homeobox-containing gene and seems to function cell-nonautonomously to regulate the size of the pstO compartment. Cell mixing experiments suggest that in this case, a cell-autonomous process is responsible for the reduction in prespore cells in the *ptp1* null slug. When *ptp1* null cells were mixed with an excess of unmarked cells of the parental strain, a prespore marker was expressed only within the posterior prespore zone, despite the ability of the *ptp1* null cells to contribute to all cell types and the apparent absence of an expanded pstO region. Therefore, we suggest that PTP1 activity is required for the differentiation of the subset of prespore cells that reside in the anterior prespore zone. Previous evidence indicating differences in the pathways activating prespore gene expression in the anterior and posterior of the prespore zone has been obtained by analysing deleted versions of the *SP60* promoter (Haberstroh and Firtel, 1990). More recently, this has been shown, in part, to be due to a transcriptional activation gradient mediated by the interaction of the RING domain/ leucine zipper protein rZIP and cAMP-dependent protein kinase (Balint-Kurti *et al.*, 1998). Therefore, it is possible that PTP1 has a role in regulating this process.

There is evidence for involvement of PTPs in developmental processes in other systems. For instance, study of null mutants in mice has indicated that the receptor PTP LAR subfamily is likely to be involved in neuronal and epithelial development (Yeo *et al.*, 1997; Elcheby *et al.*, 1999; Wallace *et al.*, 1999). In *Drosophila,* receptor PTPs, including Dlar, have been shown to be involved in the control of axon guidance (Desai *et al.*, 1996, 1997; Kreuger *et al.*, 1996). LAR in mice also has a role in mammary

development (Schaapveld *et al.*, 1997). However, the ligands and downstream signalling components are little known in these processes. Homologs of the mammalian nonreceptor PTP SHP2 are present in *Caenorhabditis elegans* (Gutch *et al.*, 1998) and *Drosophila* (Perkins *et al.*, 1996). The *C. elegans* SHP2 homolog is involved in oogenesis and vulval development via EFGR signal modulation. Work from *Xenopus* using dominant-negative SHP2 has indicated a role in gastrulation and mesoderm induction through FGFR signalling (Tang *et al.*, 1995; O'Reilly *et al.*, 2000). This model is supported by studies of chimaeric SHP2 null mice, created to overcome the embryonic lethal effect of the null mutation (Saxton and Pawson 1999; Saxton *et al.*, 2000). These authors have found that SHP2 is required for gastrulation and limb development. The defect in cell movement in gastrulation may be due to the inability of cells to chemotax to FGF, shown *in vitro*. In limb development, it has been concluded that SHP2 is involved in signalling during ectodermal–mesenchymal interactions. The phenotype of mutants expressing truncated SHP2 is similar to that of the FGFR null mutant chimaera, again suggesting a mechanistic link (Saxton *et al.*, 1997). However, specific SHP2 interactions have not been characterised. A target has been defined for the SHP2 homolog Csw in *Drosophila* using substrate trapping, namely the scaffolding/adaptor protein Dos (Herbst *et al.*, 1996). Acting through Dos, Csw has a positive effect on Sevenless RTK signalling but the precise mechanism is not known. The role of SHP2 in developmental processes has not been linked to its regulation of STAT molecules to date.

In our studies, we have shown that similar to SHP2, PTP1 has a role in regulating both STAT activation and signalling pathways controlling developmental processes. Unlike SHP2, however, PTP1 does not contain SH2 domains, and therefore the details of the mechanisms will differ. As PTP1 contains an N-terminal myristoylation site and associates with the plasma membrane (M. Gamper, S. Mohanty, and R. A. Firtel, unpublished observations), it is possible that PTP1 is constitutively associated with the subcellular domain that would contain the activated STATa kinase. As we propose that with respect to STATa, PTP1 acts on an upstream component such as the STATa kinase rather than on STATa directly, identification of the *Dictyostelium* STATa kinase is of crucial importance in helping to resolve how PTP1 mediates STATa function.

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