Regulation of *Dictyostelium* Protein-tyrosine Phosphatase-3 (PTP3) through Osmotic Shock and Stress Stimulation and Identification of pp130 as a PTP3 Substrate*

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Osmotic shock and growth-medium stimulation of *Dictyostelium* cells results in rapid cell rounding, a reduction in cell volume, and a rearrangement of the cytoskeleton that leads to resistance to osmotic shock. Osmotic shock induces the activation of guanylyl cyclase, a rise in cGMP mediating the phosphorylation of myosin II, and the tyrosine phosphorylation of actin and the 130-kDa protein (p130). We present data suggesting that signaling pathways leading to these different responses are, at least in part, independent. We show that a variety of stresses induce the Ser/Thr phosphorylation of the protein-tyrosine phosphatase-3 (PTP3). This modification does not alter PTP3 catalytic activity but correlates with its translocation from the cytosol to subcellular structures that co-localize to endosomal vesicles. This translocation is independent of PTP3 activity. Mutation of the catalytically essential Cys to a Ser results in inactive PTP3 that forms a stable complex with tyrosine-phosphorylated p130 (pp130) in vivo and *in vitro*, suggesting that PTP3 has a substrate specificity for pp130. The data suggest that stresses activate several interacting signaling pathways controlled by Ser/Thr and Tyr phosphorylation, which, along with the activation of guanylyl cyclase, mediate the ability of this organism to respond to adverse changes in the external environment.

In order to survive, cells need to adapt rapidly to environmental stresses. New environmental conditions are sensed by plasma membrane-associated proteins, activating signal transduction cascades that, in turn, regulate metabolism, cytoskeletal changes, secretion, or uptake of compounds, and gene expression (1, 2). Recently, research has predominantly focused on the role of MAP1 kinase pathways in stress response regulation (3–14). Phosphorylation of myosin II on three Thr residues, the osin-actin interaction, and the relocalization of myosin play key roles in this process and are crucial for the cells to survive hyperosmotic stress (2). Exposure of the cells to 0.3 M glucose leads to an intracellular rise in cGMP (2, 14) which is required for the phosphorylation of myosin II (2). This rise in cGMP is thought to activate a cGMP-dependent protein kinase, which in mammalian cells, the MAP Jun N-terminal kinases (JNKs) or stress-activated protein kinases are activated by a diverse set of stimuli, leading to the phosphorylation and activation of transcription factors (1, 3, 4). UV irradiation and osmotic stress are believed to induce membrane perturbation or conformational changes in membrane proteins, which promote cell-surface receptor clustering, autophosphorylation, activation, and eventually, through a MAP kinase cascade, the activation of JNK (5). p38, another MAP kinase, is also activated by osmotic shock (6), but the signaling pathway seems to be at least partially different from the JNK pathway (3, 7). In yeast *Saccharomyces cerevisiae*, the pathway induced by hyperosmotic condition is very well elucidated. As in *Escherichia coli*, in which a two-component system composed of a histidine kinase (EnvZ) and a response regulator (OmpR) is involved in osmoregulation (8), hyperosmolarity in yeast is sensed by a transmembrane histidine kinase (SLN1; Ref. 9). Under normal, low osmotic conditions, SLN1 is active and autophosphorylated on histidine. The phosphate is transferred in three steps via YPD1 to an aspartic acid residue of the response regulator SSK1 (10). Phosphorylated SSK1 prevents the activation of the HOG1 MAP kinase cascade, whereas under high osmotic conditions, SLN1 is inactive, SSK1 is not phosphorylated, and the HOG1 MAP kinase cascade is active, leading to gene expression and glycerol production (10). From the above mentioned components, only a histidine kinase (DokA, see below) has been found in *Dictyostelium*. Other signaling pathways, activated in *Dictyostelium* in response to stress stimulation, are summarized below.

In this study, we examine stress responses and osmotic shock stimulation in *Dictyostelium* and the potential role of a protein-tyrosine phosphatase in mediating these responses. *Dictyostelium* grows as single-celled amoebae, but upon starvation the cells aggregate, differentiate, and form a multicellular organism (11). Within 5–10 min after single *Dictyostelium* cells are exposed to high osmolarity or growth medium, the cells round up and shrink to ~50% of their original volume (2, 12–14). Phosphorylation of myosin II on three Thr residues, the subsequent disassembly of myosin filaments, the reduced myosin-actin interaction, and the relocalization of myosin play key roles in this process and are crucial for the cells to survive hyperosmotic stress (2). Exposure of the cells to 0.3 M glucose leads to an intracellular rise in cGMP (2, 14) which is required for the phosphorylation of myosin II (2). This rise in cGMP is thought to activate a cGMP-dependent protein kinase, which in

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† The abbreviations used are: MAP, mitogen-activated protein; JNKs, Jun N-terminal kinases; PTP3, protein-tyrosine phosphatase-3; PBS, phosphate-buffered saline; GST, glutathione S-transferase; WT, wild type; IP, immunoprecipitation; FITC, fluorescein isothiocyanate.
turn activates a myosin II heavy chain-specific protein kinase C (15, 16). Similarly, extracellular cAMP induces guanylyl cyclase activity and myosin II phosphorylation during *Dictyostelium* aggregation, which mediates chemotaxis (15, 17). However, the kinetics of intracellular cGMP accumulation and the signal transduction pathway leading to guanylyl cyclase stimulation are different than after osmotic shock stimulation (2, 18).

Cellular stresses such as ATP depletion, as well as the exposure of cells previously starved in non-nutrient buffer to growth medium, lead to rapid cell rounding and transient tyrosine phosphorylation of certain proteins, including actin and p130 (12, 19–22). Actin tyrosine phosphorylation, as with the activation of guanylyl cyclase, correlates with cell-shape change and a rearrangement of actin filaments and is affected by the level of the protein-tyrosine phosphatase PTP1 (12, 20). The tyrosine phosphorylation of p130, however, is affected in strains overexpressing wild-type or mutant forms of protein-tyrosine phosphatase PTP3 but not PTP1, suggesting it might be a substrate of PTP3 and play a different role in these response pathways (22). PTP3, determined to be a nonreceptor PTP by sequence analysis, was found to be transiently phosphorylated in response to growth medium stimulation, supporting the involvement of PTP3 in p130 regulation. PTP3 is expressed in growing cells, and its expression is induced to higher levels during multicellular development (22). Recently, a putative intracellular histidine kinase (DokA) was reported, and a *dokA* null strain appears to be less osmo-tolerant than wild-type cells, indicating a potential role of this enzyme in osmoregulation (13). Although it is likely that MAP kinase cascades are involved in *Dictyostelium* osmoregulation, no members of a stress-activated MAP kinase pathway have been identified.

In this report, we further investigate the role of PTP3. We find that PTP3 becomes phosphorylated on Ser and Thr residues after osmotic shock or other stress stimulations, which also lead to the tyrosine phosphorylation of actin and p130. However, by using different concentrations of osmotically active substances, we find that the signaling pathways mediating tyrosine phosphorylation of multiple components in *Dictyostelium* cultures were for 2–3 days in shaking cultures and the cells were washed, in either 12 mM sodium/potassium phosphate buffer (pH 6.1) or phosphate-free MES-PDF buffer (25). The cells were resuspended in 1.0 × 107 cells/ml 1.6 × 106 cells/ml and shaken for 3 h at room temperature at 150 rpm. Growth medium, osmotic shock, or other stress stimulations were performed as indicated in the figure legends. At different time points, total protein samples of 5.0 × 106 cells were taken and boiled in 80 μl of SDS sample buffer. Usually, 2–3 μl were loaded per lane on an 8% SDS gel.

**Antibodies, Western Blot, Immunoprecipitation, PP2A Assay, and Immunostainings**—The polyclonal, affinity purified anti-PTP3 antibody (22), the monoclonal anti-Tyr(P) antibody PY72 (26), and the monoclonal anti-FLAG antibody M2 (IBI/Kodak, New Haven, CT) were used for Western blot analysis and/or immunoprecipitation (IP). Western blots, IPs, and the PP2A assay were done as described previously (22), except that for the PP2A assay, the PP2A holoenzyme was used.

For the anti-FAg IPs, 2 μg of the M2 antibody were used per 2.0 × 107 lysed cells. For the immunostainings, the following antibodies at the indicated dilutions were used: polyclonal anti-PTP3 antibody (1:20) (22), anti-Myc (1:1000) (Invitrogen, La Jolla, CA), and anti-F, B antibody (27). Immunostainings were done as described by Araki et al. (28).

FITC-dextran was used to label endosomal compartments as described (29). Briefly, starved cells were placed on coverslips, placed in dishes, and flooded with either sodium/potassium phosphate buffer or HL5 growth medium containing FITC-dextran (2 mg/ml; Sigma) for 30 min.

**Phosphoamino Acid Analysis**—The *Dictyostelium* strains overexpressing PTP3Δ1(C649S) were starved for 2–4 h in MES-PDF buffer in the presence of 1 μCi/ml [32P]orthophosphate (35 mCi/70 μl; ICN Biomedicals, Costa Mesa, CA). After starvation, the gel was washed with the remaining cell culture. After an additional 15 min of shaking, 2.0 × 106 cells were taken for the second IP. The anti-PTP3 IPs were boiled in SDS sample buffer and loaded on a preparative SDS gel (10%) and stained with Coomassie blue. The proteins were transferred to an Immobilon-P membrane (Millipore) for 1 h as described previously (22). The membrane was exposed to a Kodak XAR film to detect the 32P incorporation and subjected to Western blot analysis by using the anti-PTP3 antibody. The hot spots were cut out, and after two washing steps in 100% methanol and H2O, the pieces of membrane were submerged in “constant boiling HCl” and incubated at 110 °C for 1 h. Afterward, the hydrolysates were lyophilized and dissolved in H2O containing markers for Ser(P), Thr(P), and Tyr(P). The phosphoamino acids were separated by two-dimensional electrophoresis (pH 1.9 and pH 3.5) as described previously (30).

**Guanosine Cyclase Assays**—Wild-type cells were starved in 12 mM sodium/potassium phosphate buffer as described above. After the addition of the osmotic active solution, the cells were kept in shaking culture. At the indicated time points, aliquots of 2.0 × 106 cells (usually 100 μl) were withdrawn (Fig. 4). The aliquots were diluted in 100 μl (1 vol) of 1.6 mM ATP and incubated on ice for 80–90 min with periodic vigorous shaking. The solution was neutralized by the addition of 45 μl of 50% saturated KHCO3, and incubated for another 60 min on ice with occasional vigorous shaking. After a final spin for 10 min at 4 °C, 100 μl of the supernatant was analyzed with the cyclic GMP 3H assay system (Amersham Pharmacia Biotech).

**GST Fusion Protein Isolation and Adsorption of Cell Lysates**—The...
isolation of GST fusion proteins from E. coli strain BL21(DE3) was done as previously reported (22) except that the proteins were not eluted from the glutathione-Sepharose beads after the washing steps. The in vitro adsorption of Dictyostelium proteins was performed as follows. After starvation and 15 min of growth medium incubation, wild-type cells were lysed in lysis buffer (1× PBS (pH 7.4), 50 mM NaF, 1% Nonidet P-40, 2 mM EDTA (pH 7.2), 1 mM sodium pyrophosphate, 1.6 µg/ml leupeptin, 4 µg/ml aprotonin). Sodium orthovanadate (Na$_3$VO$_4$) was only added when indicated. After a cell lysis on ice for 5 min and a centrifugation at 4 °C for 10 min, the lysate of 2.0 µl was only added when indicated. A, response to different concentrations of osmotic active substances. B, response to other stresses, such as anoxia, heavy metal ions, and heat shock (33 °C). The arrows point to non-stimulated and modified PTP3. sorb., sorbitol; gluc., glucose.

RESULTS

PTP3 Is Phosphorylated in Response to Stress—When Dictyostelium cells were starved for 4 h in non-nutrient buffer and resuspended in growth medium, PTP3 became transiently phosphorylated. This modification was evident by anti-PTP3 Western blot analysis since it led to a slower migrating form of PTP3 on an SDS gel (22). We were interested in examining other conditions that might induce PTP3 phosphorylation. For this purpose, cells overexpressing an inactive form of PTP3 with an internal deletion of 116 amino acids (PTP3$_D$1(C649S) (22)) were used. The truncated version of PTP3 was used instead of the full-length protein of 989 amino acids because it gave a higher level of expression in Dictyostelium and a greater mobility shift on SDS gels. Thus, PTP3$_D$1(C649S) presumably contains the critical phosphorylation site(s) for the shift. Osmotically active small molecules, such as 0.3 mM glucose, 0.2 mM sorbitol, or 0.4 mM sodium chloride, induced a mobility shift of PTP3$_D$1(C649S) on SDS gels (Fig. 1A). Within the resolution of this assay, the shift was identical for the three active compounds at these concentrations and is similar to observations when starved cells are shifted to growth medium. In addition, stresses such as ATP depletion, heavy metal ions, or heat shock induce tyrosine phosphorylation of actin and p130 (20), as previously shown for growth medium addition to starved cells (12). Since pp130 is a potential PTP3 substrate (22), we tested whether the exposure of cells to 1 mM sodium azide (to deplete ATP), 100 mM cadmium chloride, or a heat shock at 33 °C induced a mobility shift of PTP3 on an SDS gel. All of the stresses led to PTP3$_D$1(C649S) phosphorylation (Fig. 1B). We observed mobility shifts to those shown in Fig. 1B when cells were taken from growth medium and exposed to the stresses mentioned above (data not shown). Our data suggest a possible general role for PTP3 in osmo- and stress regulation.

After Osmotic Shock Stimulation PTP3 Is Phosphorylated on Ser and Thr—When PTP3$_D$1(C649S) was immunoprecipitated from lysates of sorbitol-stimulated cells and treated with protein serine-threonine phosphatase PP2A, the mobility shift of PTP3$_D$1(C649S) was reversed. The PTP3$_D$1(C649S) migrated more rapidly on an SDS gel with the same mobility as PTP3$_D$1(C649S) from unstimulated cells (Fig. 2A), as we previously demonstrated for PTP3$_D$1(C649S) from growth medium-stimulated cells (22). In control experiments, incubation of phosphorylated PTP3$_D$1(C649S) with PP2A in the presence of microcystin LR, a potent inhibitor of PP2A, or with reaction buffer alone did not affect PTP3$_D$1(C649S) migration. Thus, both growth medium and osmotic shock stimulation resulted in a similar PTP3$_D$1(C649S) phosphorylation.

To determine the amino acids that were phosphorylated on PTP3, cells overexpressing PTP3$_D$1(C649S) were labeled in vivo with $[^{32}P]$orthophosphate. In vivo $^{32}$PO$_4$-labeled cell lysates were made from cells starved for 4 h and stimulated or not stimulated with 0.2 M sorbitol. PTP3$_D$1(C649S) was immunoprecipitated with anti-PTP3 antibodies, and the IPs were separated on an SDS gel and blotted onto a membrane. The membrane was exposed to a film and also subjected to Western blot analysis using anti-PTP3 antibodies (Fig. 2B). The Western blot confirmed that equal amounts of PTP3$_D$1(C649S) were immunoprecipitated in the samples and that the PTP3$_D$1(C649S) exhibited a mobility shift after sorbitol stimulation. The autoradiogram indicated that PTP3$_D$1(C649S) was phosphorylated before and after sorbitol treatment and the mobility shift correlated with an increase in the level of phosphorylation. Interestingly, both bands of the PTP3$_D$1(C649S) doublet were labeled in starved cells and the sorbitol stimulation led to a very broad, fuzzy series of bands. The labeled PTP3$_D$1(C649S) proteins were excised from the membrane and examined by phosphoamino acid analysis (30). In starved, unstimulated cells, only Ser(P) was detected, whereas after sorbitol induction the amount of label in the Ser(P) increased, and some Thr(P) was also detected (Fig. 2C). Since the 116-amino acid region that was deleted in PTP3$_D$1(C649S) does not contain any tyrosines and since neither anti-Tyr(P) Western analysis nor in vivo $^{32}$PO$_4$-labeling detected any PTP3 tyrosine phosphorylation (22), we conclude that PTP3 is phosphorylated exclusively on serines and threonines.

Tyrosine Phosphorylation of Actin and p130 Is Induced at Different Concentrations of Osmotic Active Substances—When Dictyostelium cells were starved for 2–4 h in non-nutrient buffer and then incubated with growth medium, we observed several distinct changes in the tyrosine phosphorylation pattern of certain proteins (Fig. 3A) (12, 22). p130 was fully phosphorylated within 5 min, whereas actin phosphorylation was first detected at 10 min and was maximal at 25 min after stimulation. When the cells were shifted back to low osmotic phosphate buffer, both proteins became dephosphorylated (Fig. 3A). Because growth medium stimulation and osmotic shock led to phosphorylation of PTP3, we tested whether osmotic conditions induced changes in protein tyrosine phosphorylation. Surprisingly, the results varied with the stimulant. Lower concentrations of sorbitol (0.10 or 0.15 M) resulted in a strong p130 phosphorylation (Fig. 3B; Table I), whereas higher sorbitol concentrations (≥0.20 M) resulted in weak phosphorylation (Fig. 3C; Table I). By a GST-PTP3(C649S) interaction (pull-down) assay (Fig. 5B), we verified that the faint Tyr(P) bands at 130 kDa visible after 0.20 and 0.30 M sorbitol stimulation represent tyrosine-phosphorylated p130 (data not shown). In some experiments, a strong Tyr(P) signal at 130 kDa in un-
stimulated cells (Fig. 3C) is visible. Since this Tyr(P) protein never showed any interaction with PTP3 in (GST-PTP3(C649S)) interaction assays (Fig. 5B) or co-immunoprecipitation assays with PTP3(C649S) (Fig. 6A), data not shown), it presumably is a protein other than pp130, or it is pp130 phosphorylated on another tyrosine that is not recognized by PTP3 (see Fig. 3B). The tyrosine phosphorylation of actin was regulated differently than that of p130; 0.10 mM sorbitol produced only a low level of actin tyrosine phosphorylation (data not shown); intermediate osmotic concentrations (0.15 M (Fig. 3B) and 0.20 M) led to strong actin phosphorylation, and high osmolarity (0.30 M and above) had only a minor effect (Table I; Fig. 3C). Analysis of osmotically active substances showed that ionic and non-ionic molecules had equal responses with respect to differential p130 and actin tyrosine phosphorylation and were dependent on the osmotic concentration (Table I). As the osmolarity response curves of actin and p130 tyrosine phosphorylation are different, we suggest the responses may be regulated, at least in part, by different signaling pathways.

**0.30 M Glucose or Sorbitol, But Not Growth Medium, Leads to a Strong Transient Accumulation of cGMP**—The activation of guanylyl cyclase and the tyrosine phosphorylation of actin were maximal 5–25 min after osmotic stress induction (Figs. 3A, and 4A; Table I; see Refs. 2, 12, and 14). However, despite these similar slow activation kinetics, actin phosphorylation was maximal at osmolarities of 0.15 and 0.20 M (Table I). For guanylyl cyclase activation, maximal stimulation was observed at osmolarities of 0.20 M (Fig. 4A; Table I; see Ref. 14). Stimulation with 0.20 M glucose or growth medium produced only a small increase in cGMP, whereas stimulation with 0.20 M sorbitol had little effect (Fig. 4, A and B). These data suggest that a distinct signaling pathway is responsible for the strong guanylyl cyclase activation. Overexpression of PTP3(WT) or the deletion of one of the two chromosomal PTP3 genes in Dictostelium did not affect guanylyl cyclase activation (data not shown). 0.20 and 0.15 M sorbitol stimulation led to cell rounding, with kinetics similar to growth medium stimulation. 0.10 M sorbitol produced cell rounding, but the initiation of the rounding was delayed by 5–10 min (data not shown).

**Specific Interaction of Tyrosine-phosphorylated p130 with a Catalytically Inactive Form of PTP3 in Vitro**—Since our preliminary data suggested that pp130 might be a PTP3 substrate (22), we further investigated the potential interaction between the two proteins. For this purpose, two nearly identical 22-kDa fusion proteins were designed in which the N-terminal 242 amino acids of PTP3 were replaced by GST. One protein had an inactive catalytic site (GST-PTP3(WT), pMG24; see Ref. 22), whereas in the other protein, a Ser was substituted for the Cys characteristic for PTPs, HCXXGXXRS(T) (31, 32). These conserved amino acids were found in the tyrosine phosphate, and in the initial step of the catalysis, the cysteine thiolate acts as a nucleophile yielding a covalent thiol phosphate intermediate (33). The Cys-to-Ser mutation still allows substrate recognition and binding, but the inability to hydrolyze the phosphate is reported to give a prolonged and more stable interaction with the substrate (34).

The two GST-PTP3 fusion proteins and the GST protein alone were expressed in *E. coli* and isolated using glutathione-
Fig. 3. Different concentrations of osmotic active substances regulate the tyrosine phosphorylation of actin and pp130. Anti-Tyr(P) Western blots are shown. A and B, KAx-3 wild-type cells were starved in 12 mM sodium/potassium phosphate buffer for 2–4 h, and a first protein sample was taken. The cells were resuspended in growth medium (A) or 0.15 m sorbitol (B), and subsequently, every 5 min, another total protein sample was taken. After 25 min, the cells were washed and resuspended in sodium/potassium phosphate buffer, and again, samples were taken every 5 min for 25 min. C, essentially the same experiments were performed as in A and B, but only two protein samples were taken, one after starvation (unstimul.) and the other 35 min after the different osmotic stimulations (as indicated). Note that 35 min after stimulation with growth medium or 0.15 m sorbitol, high levels of pp130 and actin phosphorylation were found (data not shown). The results shown in C were confirmed with full time courses as presented in A and B. In some gels, the ~130-kDa band migrates as two distinct bands as seen in B. The ~130-kDa phosphotyrosine band is seen as two bands, a faster mobility, lighter band observed in unstimulated cells that disappears with a stronger, slower mobility band (pp130) appearing within 5 min. After removal of the sorbitol, the slower mobility band disappears and the faster mobility band reappears.

Table I

Comparison of actin and p130 tyrosine phosphorylation and cGMP accumulation after different stimulation

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Actin/Tyr(P)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p130/Tyr(P)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>cGMP accumulation&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na/K phosphate buffer</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Growth medium&lt;sup&gt;c&lt;/sup&gt;</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>(HL5 + 56 mM glucose)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>0.10 m sorbitol</td>
<td>+</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>0.15 m sorbitol</td>
<td>++</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>0.20 m sorbitol</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>0.30 m sorbitol</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>0.40 m NaCl</td>
<td>–</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>0.10 m NaCl</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>0.20 m glucose</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>0.30 m glucose</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>0.01 M NaCl</td>
<td>–</td>
<td>–</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cell lysates taken 25 min after stimulation were analyzed by anti-Tyr(P) Western blot and Tyr(P)-levels of actin and p130 were compared.

<sup>b</sup>cGMP levels of cell lysates taken 10 min after stimulation were analyzed by anti-Tyr(P) Western blot and Tyr(P)-levels of actin and p130 were compared.

<sup>c</sup>The calculated osmolarity of HL5 is ~0.10 m.

Sepharose beads. As expected, GST-PTP3(WT) dephosphorylated p-nitrophenyl phosphate and a tyrosine-phosphorylated peptide; GST-PTP3(C649S) had no detectable activity toward these substrates (see Ref. 22; data not shown). To identify tyrosine-phosphorylated Dictyostelium proteins that interact with PTP3, wild-type Dictyostelium cells were lysed after starvation in non-nutrient buffer or after a subsequent stimulation with growth medium, and the lysates were incubated with the GST fusion proteins coupled to glutathione-Sepharose beads. After washing the resin, the retained proteins were eluted with SDS sample buffer, separated by polyacrylamide gel electrophoresis, and blotted onto a membrane. Anti-Tyr(P) Western blot analysis revealed that one tyrosine-phosphorylated 130-kDa protein bound very specifically to GST-PTP3(C649S). Because this protein had the same mobility as pp130 and was only detectable after growth medium stimulation (Fig. 5B), it is very likely that the protein is pp130. The active GST-PTP3(WT) did not bind stably to pp130, presumably because it dephosphorylated and released this substrate. From these results, we can conclude that GST-PTP3(C649S) interacts with the tyrosine-phosphorylated p130 specifically through the PTP3 catalytic domain. This interaction was quite strong, since the treatment of the adsorbed beads with 0.5 m NaCl did not decrease pp130 binding (Fig. 5C). In addition to pp130, two other bands were detected in the anti-Tyr(P) Western blots. The band at ~110 kDa corresponded to the very abundant GST-PTP3 protein that was bound to the glutathione-Sepharose (Fig. 5C) and results from a very weak binding of the antibody to this highly abundant protein on the blot. The band at ~60 kDa (pp60) is another tyrosine-phosphorylated protein that was present in lysates before and after medium stimulation and was not dephosphorylated by GST-PTP3(WT) (Fig. 5B). Since glutathione-Sepharose beads carrying GST alone did not bind pp60 (Fig. 5B), the interaction of pp60 with PTP3 is specific but most likely not mediated through the catalytic active site. Other
strongly tyrosine-phosphorylated proteins, among them actin and a protein of ~200 kDa, did not interact with the GST-PTP3 fusion proteins.

Recent structural data for *Yersinia* PTP Yop51 indicates that sodium orthovanadate inhibits PTPs through a covalent bond between vanadate and the active site Cys (35). One mM vanadate did not inhibit the interaction between GST-PTP3(C649S) and pp130, presumably because the active site cysteine thiolate was absent. In fact, 1 mM vanadate increased the amount of bound pp130 to the GST-PTP3(C649S) resin, possibly because it inhibited endogenous PTP activities present in the cell lysate. A higher concentration (10 mM) of vanadate did prevent the interaction GST-PTP3(C649S) with pp130 (data not shown), as was also observed for the interaction of PTP-PEST(C231S) with its substrate p130cas (36).

**Specific Interaction of Tyrosine-phosphorylated pp130 with**

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**FIG. 4. Activation of guanylyl cyclase in response to different osmotic shocks.** Wild-type cells were starved for 2.5 h in sodium/potassium phosphate buffer and stimulated with hypertonic solutions as indicated. At different time points, samples were withdrawn and subjected to a guanylyl cyclase assay. The curves presented are representative of repeated experiments. **A**, the cell culture was split after starvation and stimulated with different osmotic active solutions. **B**, these results were obtained on a different day. In contrast to this slow rise in cGMP, cAMP-stimulated induction of guanylyl cyclase, which is required for chemotaxis, is much more rapid and cGMP levels peak at ~10 s (18).
**PTP3(C649S) in Vivo**—To determine whether the observed in vitro interaction of PTP3 with pp130 is biologically relevant, we tried to co-immunoprecipitate these two proteins from *Dictyostelium* cell lysates. For this purpose, the FLAG tag (DYKD-DYKD) was fused in-frame at the C terminus to full-length PTP3(C649S) or the truncated version PTP3Δ1(C649S). *Dictyostelium* cells expressing the FLAG-tagged proteins were lysed before and after medium stimulation, and the lysates were precipitated with an anti-FLAG antibody. The IPs were first incubated in the presence of dithiothreitol in the IP buffer (1 mM dithiothreitol, 1.6 mM sodium pyrophosphate, 1.6 g/ml leupeptin, 4 mM EDTA (pH 7.2), 1 mM sodium pyrophosphate, 1.6 μg/ml leupeptin, 4 μg/ml apro- tin) to keep the catalytic Cys of PTP3 reduced and active (39), then centrifuged, and supernatants were subjected to Western blotting to ensure that the proteins were precipitated. Samples were then taken from the reaction mixture, and the free phosphate was measured by scintillation counting (38). In the presence of 1 mM dithiothreitol in the IP buffer (1× PBS (pH 7.4), 50 mM NaF, 1% Nonidet P-40, 2 mM EDTA (pH 7.2), 1 mM sodium pyrophosphate, 1.6 μg/ml leupeptin, 4 μg/ml apro- tin) to keep the catalytic Cys of PTP3 reduced and active (39), similar PTP3 activities were found before and after stimulation (data not shown). In the absence of dithiothreitol, the PTP3 activity after starvation was significantly higher (−5-fold for the PTP3(WT) overexpressor strain; −2.5-fold for the wild-type strain) than after subsequent growth medium addition (data not shown). These results suggest that Ser/Thr phosphorylation does not affect PTP3 activity but possibly results in a conformational change of PTP3 that makes the active center more accessible to oxidation during protein isolation. In vivo, this conformational change could lead to altered substrate interaction or subcellular localization.

Second, we examined the intracellular localization of PTP3 before and after growth medium stimulation of wild-type cells overexpressing PTP3(WT) and PTP3(C649S). For these immunostaining experiments, two antibodies were used, the monoclonal anti-Myc antibody, directed against a C-terminal Myc-tagged PTP3(WT), and the polyclonal anti-PTP3 antibody, directed against PTP3(WT) and PTP3(C649S). After starvation, staining was visible throughout the cell for both forms, and cytoplasmic membranes remained unstained (Fig. 7A). In some experiments, nuclei whose localizations were determined by DNA (Hoechst dye) staining appeared as dark spots in the immunofluorescence experiments using the anti-PTP3 or Myc antibodies (data not shown). After growth medium addition, we observed a dramatic change in the PTP3-staining pattern. With both antibodies and the PTP3(WT) and PTP3(C649S) overexpressor strains, we found a scattered, dot-like staining throughout the cell after 15 min of stimulation (Fig. 7B, data for PTP3(C649S)). After a more extended period, PTP3 accumulates in larger domains (Fig. 7, C and D).

The staining pattern suggested that PTP3 may be associated with an organelle. We excluded the possibility that these dot-like structures are mitochondria by transforming the Myc-tagged PTP3(WT) into the *cluA* null strain (23). In this strain, all mitochondria are clustered near the cell center (23). After 30 min stimulation with growth medium, the mitochondria, as visualized by immunostaining the mitochondrial protein F1β, were found localized near the center of the cell (Fig. 7C), whereas PTP3 accumulated in domains that excluded the mitochondria (Fig. 7Ca). We examined whether the PTP3 may associate with an endosomal compartment. Cells were stabbed for 4 h and stimulated with growth medium containing FITC-labeled dextran to label endosomal compartments. As shown in Fig. 7D, there was a direct correlation between the distribution of dextran-containing compartments and PTP3 staining after stimulation. Non-stimulated cells show a random distribution of dextran (data not shown).
Stress Regulation and Substrate Specificity of PTP3

Multiple, Discrete Pathways Are Activated in Response to Stress—In this study, we analyzed stress responses in Dictyostelium in general and the regulation and role of PTP3 in these pathways in particular. We have shown that different osmorilities lead to different intracellular responses, suggesting that subtle regulatory mechanisms exist for the adaptation of cells to small changes in the extracellular environment. Considering the changes in the natural environment that Dictyostelium cells may experience, such mechanisms guarantee the ability of the cells to respond appropriately and to survive. Since p130 phosphorylation, actin phosphorylation, and the maximum activation of guanylyl cyclase are induced by different osmotic conditions, we suggest that the pathways leading to these events are, at least in part, different. A knock-out of the histidine kinase DokA or a mutation that reduces guanylyl cyclase activity leads to an osmosensitive phenotype (2, 13). However, cGMP accumulation is not affected in dokA null strains, indicating that DokA acts downstream of guanylyl cyclase or in another pathway (13). We have not observed an altered osmosensitivity for any PTP3 mutant, including the partial ptp3 null strain lacking one copy of PTP3 or the wild-type strain overexpressing active or inactive PTP3. Moreover, PTP3Δ1(C649S) expressed in the dokA null background was phosphorylated in response to stress. This most likely excludes the possibility that DokA lies upstream of PTP3. Instead, stress induced PTP3 translocated in response to osmotic stress, the translocation is independent of PTP3 activity. We suggest that PTP3 translocation is regulated through Ser/Thr phosphorylation. Our data suggest that PTP3 translocates to an endosomal compartment, although our analysis cannot distinguish between the compartments. As the response is transient when cells are placed in growth medium and can also be readily reversed by placing the cells in starvation medium, we suggest that the association with endosomal vesicles is probably on the outside of the structures. The functional reason for this translocation is not known, although we note that PTP3 is more resistant to oxidation under these conditions. Whereas this property is observed upon cell lysis and may not be an in vivo property of PTP3 in osmotically stressed cells, it is an indication of a change in the property of PTP3 that is associated with its phosphorylation and/or subcellular localization. In this process, the activity and expression level of PTP3 are induced by growth medium, which has a calculated osmolarity of 0.16 M. The results of cells stimulated with HL5 lacking the 0.056 M glucose support this possibility, as the tyrosine phosphorylation is similar to that of 0.1 M sorbitol induction (Table I).

Stress-induced Phosphorylation of PTP3 Correlates with a Translocation of PTP3—In response to high osmolarity, we found PTP3 to be hyperphosphorylated on Ser and Thr. PTP3 is a large protein (989 amino acids) with 153 (15.4%) Ser and 64 (6.5%) Thr residues. The broad fuzzy band that is observed after sorbitol stimulation (Fig. 2B) can be explained by differential Ser/Thr phosphorylation at multiple sites. Analysis of the PTP3 sequence by eye or by the psearch program (EMBL Data Library) identifies the following potential PTP3 phosphorylation sites for known protein kinases: MAP kinase, 14 minimal proline-directed recognition sites (Ser-Thr-Pro; see Ref. 40); protein kinase A and cGMP-dependent protein kinase, 1 recognition site (Lys-Arg-Arg-Ser); protein kinase C, 16 recognition sites (Ser-Thr-Xaa(hydrophobic)-Arg/Lys); and casein kinase II, 12 recognition sites (Ser-Thr-Xaa-Xaa-Asp/Glu).

The Ser/Thr phosphorylation of PTP3 correlated with a translocation of PTP3 from the cytoplasm to subcellular structures, but it did not affect PTP3 activity toward a phosphopeptide substrate. Since both wild-type PTP3 and the catalytically inactive PTP3(C649S) translocated in response to osmotic stress, the translocation is independent of PTP3 activity. We suggest that PTP3 translocation is regulated through Ser/Thr phosphorylation. Our data suggest that PTP3 translocates to an endosomal compartment, although our analysis cannot distinguish between the compartments. As the response is transient when cells are placed in growth medium and can also be readily reversed by placing the cells in starvation medium, we suggest that the association with endosomal vesicles is probably on the outside of the structures. The functional reason for this translocation is not known, although we note that PTP3 is more resistant to oxidation under these conditions. Whereas this property is observed upon cell lysis and may not be an in vivo property of PTP3 in osmotically stressed cells, it is an indication of a change in the property of PTP3 that is associated with its phosphorylation and/or subcellular localization and thus suggests some change in the in vivo properties of PTP3. There are other examples of intracellular translocation of PTPs upon stimulation as follows: phospholipid 12-myristate 13-acetate induces the differentiation of human HL-60 cells to macrophages. In this process, the activity and expression level of PTP1C increase 2–3 times; PTP1C is Ser-phosphorylated and translocates from the cytoplasm to the plasma membrane (41). In thrombin-activated platelets, SH-PTP1 translocates to the cytoskeleton (42).

pp130 Is a Substrate of PTP3—The catalytically inactive PTP3(C649S) binds tyrosine-phosphorylated pp130 in vivo and in vitro. These results show that PTP3 per se has a substrate specificity for pp130. Because pp130 did not associate with active PTP3(WT) in the in vitro binding experiments and because high vanadate concentrations inhibited PTP3(C649S) association with pp130 in vitro, the interaction between PTP3 and pp130 is presumably mediated through the catalytic site of PTP3.

Expressions of PTP3-Myc(WT) after starvation and growth medium addition. Ka-3 cells overexpressing PTP3-Myc(WT) after starvation and growth medium addition. Kb, anti-F, B staining (mitochondrial protein) of the same cells. Ka, anti-Myc staining of Ka-3 cells overexpressing PTP3-Myc(WT) after starvation and growth medium addition. Kb, anti-Myc staining of Kb-3 cells overexpressing PTP3-Myc(WT) after starvation and growth medium addition. D, FITC-dextran staining of endosomal compartments in the same cell.
PTP3 and the Tyr(P) and surrounding residues of pp130. Similarly, inactive PTP-PEST(C231S) selectively binds tyrosine-phosphorylated p130<sup>tyr<sup>phospho</sup></sup> in vitro and in vivo, whereas inactive PTP1B has no substrate specificity in in vitro binding assays and binds practically any tyrosine-phosphorylated protein present in the cell lysate (36).

It is possible that PTP3 substrates in addition to pp130 exist. Such substrates could be present only in low amounts or they may not be efficiently recognized by our anti-Tyr(P) antibody. Since PTP3 is also expressed during Dictyostelium multicellular development with a maximal expression at 8 h (22) as well as during growth, it is probable that during the multicellular stages, PTP3 interacts with proteins other than pp130 and functions in different pathways. At the moment, the molecular identity of p130 is unknown. Preliminary data from pp130 adsorbed in vitro to GST-PTP3(C649S) did not reveal any obvious autokinase activity under the conditions used.²

Possible Association of PTP3 with Stress-response Pathways—We have no direct proof that p130 or PTP3 plays a regulatory role in stress response, but from the data presented in this paper it is intriguing to speculate that they do. We observed a correlation between the phosphorylation of PTP3 and an intracellular translocation of PTP3 after stress stimulation, as well as an interaction of PTP3 with pp130. PTP3 isolated from growing cells migrated with a mobility on SDS gels that was similar to its migration in starved cells before stimulation. Similarly, PTP3 staining in growing cells looked like PTP3 staining in starved cells (data not shown). Assuming that pp130 is also cytoplasmically localized, our accumulated data could lead to the following hypothetical model (Fig. 8A). Under normal, non-hyperosmotic conditions during growth and development, PTP3 is in the cytoplasm and acts to keep pp130 in the unphosphorylated state. Stress induction stimulates PTP3 phosphorylation and may directly stimulate pp130 tyrosine phosphorylation. We propose that PTP3 phosphorylation leads to a conformational change exposing a site for endosomal docking and a subsequent translocation from the cytoplasm, which allows tyrosine-phosphorylated pp130 to accumulate in the cytoplasm. Although p130 could be a structural protein, it is intriguing to speculate that tyrosine phosphorylation of p130 has a positive or activating effect on stress-induced signal transduction pathways, and PTP3 plays a negative role in modulating these pathways. The co-immunoprecipitation experiments (Fig. 6) do not necessarily contradict this model. Because of the high overexpression of PTP3(C649S) it is likely that, although the translocation from the cytoplasm is apparent (Fig. 7), some PTP3(C649S) remains in the cytoplasm and associates with pp130. The model in Fig. 8B summarizes the known pathways outlining Dictyostelium stress regulation. Fast stress responses are observed within minutes after stimulation and include the phosphorylation of PTP3, p130, and DdMEK1. Slow responses are detected 10–20 min after the stress signal in wild-type cells and result in the phosphorylation of actin and myosin, the rearrangement of the cytoskeleton, and cell rounding.

Other PTPs are known to negatively regulate pathways induced by hyperosmolarity or other stresses. In S. cerevisiae, Spc1 is in the osmosensor SLN1 histidine kinase resulted in a non-phosphorylated downstream SSK1 response regulator, which is responsible for the lethal, constitutive activation of the HOG1 MAP kinase cascade. Overexpression of PTP2 rescued this lethal phenotype, and it was proposed that PTP2 directly dephosphorylates and inactivates HOG1 (9). In fission yeast Schizosaccharomyces pombe, the Spc1 MAP kinase pathway is activated by various cytotoxic stresses such as high osmolality, oxidative stress, and high temperature. spe1 null cells are unable to grow in high osmolality medium (43, 44). Spe1 is also required for the initiation of mitosis, meiosis, and mating (44–46). Two PTPs, PTP1 and PYP2, negatively regulate this pathway by dephosphorylating Spc1 (43, 44). Furthermore, PTP2 is a target gene of the Spc1-stimulated transcription factor Atf1, indicating a negative feedback mechanism (45, 46). In mammalian cells, arsenite ions (As³⁺) are toxic and highly carcinogenic. As³⁺ is thought to directly inhibit a phosphatase containing an essential Cys. In the absence of cellular stresses, this phosphatase activity is believed to maintain low JNK and p38 MAP kinase activities (47). Recently, PTP1B has been reported to be phosphorylated on Ser in response to stress and osmotic shock, but neither the function of the phosphorylation nor the upstream kinase have been identified (48). Because no members of a stress-regulated MAP kinase pathway have been identified in Dictyostelium, we cannot test whether PTP3 is phosphorylated by such a pathway or acts as a negative regulator of a MAP kinase as discussed in the examples above.
Purification and sequence analysis of p130 are likely to provide the data necessary to define its function and the function of PTP3 in regulating stress response pathways.

In Dictyostelium, osmotic and stress response regulation appears to be complex. The data presented here indicate different pathways control different aspects of the overall response. The identification of pp130 as a specific PTP3 substrate characterizes PTP3 as a highly selective PTP. The concomitant PTP3 phosphorylation and translocation in response to stress suggests that PTP3, perhaps through its inhibition of pp130 activation, may function to negatively regulates stress response pathways.

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