Phosphorylation of Protein-tyrosine Phosphatase PTP-1B on Identical Sites Suggests Activation of a Common Signaling Pathway during Mitosis and Stress Response in Mammalian Cells*

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PTP-1B is a widely expressed non-transmembrane tyrosine-specific phosphatase. Previous studies indicated that, at mitosis, PTP-1B undergoes phosphorylation on two sites, Ser-352 and Ser-386. Although the Ser-386 site can be phosphorylated by Cyclin B/Cdc2 in vitro, the kinase for the Ser-352 site is unknown. We have found that these phosphorylation events are not unique to normal mitosis. Instead, treatment with many, but not all, stress stimuli, in particular osmotic shock and certain phosphatase and protein synthesis inhibitors, leads to phosphorylation of PTP-1B. Tryptic phosphopeptide and mutant analysis reveals that, as in mitosis, stress-induced PTP-1B phosphorylation involves both Ser-352 and Ser-386. Activation of the proline-directed kinases Erk1/2, JNKs, and p38 was neither necessary nor sufficient for stress-induced PTP-1B phosphorylation. Our data suggest the existence of a novel mitogen-activated protein kinase pathway in mammalian cells, which is activated at mitosis and in response to osmotic shock and other stresses and results in PTP-1B phosphorylation. This pathway may be similar to the recently described Spec1/Sty1 pathway in Schizosaccharomyces pombe.

Tyrosine phosphorylation controls cellular responses to a variety of environmental stimuli and contributes to cell cycle control. Although specific functions have been defined for many protein-tyrosine kinases (PTKs), less is known about the roles of individual protein-tyrosine phosphatases (PTPs). The non-transmembrane protein-tyrosine phosphatase PTP-1B, a major intracellular PTP (1–3), undergoes cell cycle-dependent phosphorylation (4, 5). In nocodazole-arrested cells, PTP-1B becomes phosphorylated on Ser-352 (within the sequence 352Ser-Pro-Leu-Asn) and Ser-386 (within the sequence 386Ser-Pro-Ala-Lys) (4). The Ser-386 site conforms to the consensus recognition sequence for the mitotic proline-directed kinase Cyclin B/Cdc2 (6), and Cyclin B/Cdc2 can phosphorylate PTP-1B on Ser-386 in vitro (4). These results suggest that Ser-386 may be phosphorylated at mitosis by Cyclin B/Cdc2. However, the Ser-352 site is not optimal for phosphorylation by Cyclin B/Cdc2 (6); moreover, Ser-352 is not phosphorylated by Cyclin B/Cdc2 in vitro (4). Presumably, during mitosis, a distinct proline-directed kinase phosphorylates this site. However, the identity of this kinase, as well as the components of the signaling pathway controlling mitotic phosphorylation of PTP-1B (at Ser-352), remains undefined.

Recent studies in the fission yeast Schizosaccharomyces pombe revealed an unexpected connection between cell cycle control, PTPs, and a signal transduction pathway regulating the cellular response to high osmolarity/nutrient starvation. Spec1/Sty1, an S. pombe MAP kinase family member activated by high osmolarity or nutrient limitation, also was found to be required for entry into mitosis (7, 8). Spec1/Sty1, which is most similar to the mammalian stress-activated kinase p38 (9–11) and the Saccharomyces cerevisiae osmo-sensing kinase Hog1 (12), is negatively regulated by two S. pombe PTPs, Pyp1 (13) and Pyp2 (8). Earlier genetic studies had identified Pyp1 and Pyp2 as mitotic inhibitors (8, 14, 15). However, in mammalian cells, no connection between stress responses and cell cycle control has been reported.

There are two general classes of proline-directed kinases in mammalian cells: cyclin-dependent kinases (Cdks) and MAP kinases. Since Cyclin B/Cdc2, the major Cdk activated at G2/M, is not responsible for Ser-352 phosphorylation, we explored the possibility that this site was the target of a MAP kinase(s). Therefore, we asked whether conditions that result in activation of various MAP kinases also evoke PTP-1B phosphorylation. We have found that mitotic-like phosphorylation of PTP-1B occurs during the cellular response to osmotic shock and several other stress stimuli. However, the known stress-activated kinases Erk1/2, JNKs, and p38 are neither necessary nor sufficient to induce PTP-1B phosphorylation. Our findings suggest the existence of a novel signaling pathway in mammalian cells, leading to PTP-1B phosphorylation, which operates in response to certain stresses and during the normal cell cycle.

EXPERIMENTAL PROCEDURES

Materials

Calcineurin A and okadaic acid were purchased from LC Laboratories; DAPI was purchased from Calbiochem; TNFα and IL-1 were from Genzyme; all other chemicals were purchased from Sigma. The MAP kinase inhibitor (MEK) inhibitor PD98059 (16, 17) was the gift of Dr. Alan Saltiel (Parke-Davis). The p38 inhibitor SB-203580 (10), the less potent inhibitor SKF-86002-A2, and the inactive compound SKF-106978 were purchased from Calbiochem.
provided by Dr. John Lee (SmithKline-Beecham). PTP-1B-specific rabbit antibodies have been described (3), phosphospecific antibodies against MAP kinases were from New England Biolabs, and monoclonal anti-HA antibodies were from Boehringer Mannheim.

SDS-PAGE and Immunoblotting

PTP-1B electrophoretic mobility was determined by immunoblotting of total cell lysates (10–25 μg) resolved by 10% SDS-PAGE, as described previously (3). Erk1/Erk2 activation was assessed by immunoblotting with phosphospecific antibodies using conditions recommended by the manufacturer. Protein concentrations were determined by the Bradford method (18).

Cell Stimulations and Inhibitor Studies

HeLa and 293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum (HeLa) or 10% fetal calf serum (293), 2 mM L-glutamine, and penicillin/streptomycin. Confluent HeLa cells were treated with various stress-inducing chemicals or physical agents as described in Fig. 1A. UV irradiation was performed using a Stratalinker (Stratagene). For heat shock, plastic dishes with cell monolayers were sealed with Parafilm and transferred to a 45°C water bath for 25 min, followed by a 60-min recovery period at 37°C. For monolayers were sealed with Parafilm and transferred to a 45°C water bath for 25 min, followed by a 60-min recovery period at 37°C. For hypoxo-osmotic shock, cells were incubated for 40 min in growth media diluted 1:2 or 1:4 with water.

To assess the requirement for Erk1/2 or p38 in osmotic shock-induced PTP-1B phosphorylation, cells were pretreated for 30 min with MEK or p38 inhibitors at the indicated concentrations, and then exposed to 1 M sorbitol for 1 h, washed with phosphate-buffered saline (PBS), and lysed in the continuous presence of the appropriate inhibitor, as described (10, 19, 20).

Cell Cycle Synchronization

Nocodazole-induced Mitotic Arrest—HeLa cells were synchronized in pro-metaphase by the addition of nocodazole (40 ng/ml) to the culture media for 18 h. Round, mitotically arrested cells (30–50% of the cells) were washed off the plate, washed twice with PBS, and either lysed or fixed for flow cytometric analysis (see below).

Synchronization at the G1/S Border Was Performed by Thyminidine-Aphidicolin Block (21)—Mitotic cells began to appear 10–11 h following release from the second block and, by 12–13 h post-release, constituted 25–50% of all cells.

Flow Cytometric Analysis of Cell Cycle Distribution

Cells that remained attached to plates after nocodazole treatment/wash-off were detached by incubation for 10 min in PBS containing 25 mM EDTA. These cells and the mitotic wash-off cells were washed twice with ice-cold PBS, fixed in 70% absolute ethanol (~20 °C), and stored at 4 °C. Immediately before analysis, cells were stained with DAPI (1 μg/ml) for 20 min at room temperature, washed twice with PBS, and analyzed (Becton-Dickinson flow cytometer with FACStar PLUS Software, version 2.01).

Phosphatase Treatment of Cell Lysates

Cell lysates (10 μl, containing 10–30 μg of protein) were added to 250 μl of reaction buffer (20 mM Tris, pH 8.5, 150 mM NaCl, 1 mM MgCl2, 1 mM dithiothreitol (DTT)) with calf intestinal alkaline phosphatase (Boehringer Mannheim), followed by incubation at 37 °C for 30 min. After acetone precipitation and SDS-PAGE, PTP-1B was analyzed by immunoblotting.

Immunoprecipitations, Phosphoamino Acid and Phosphotryptic Analysis

Metabolic labeling with [γ-32P]ATP, and PTP-1B immunoprecipitations were performed as described (3). Nocodazole, sorbitol, or calyculin A was included in the labeling media, where indicated. Phosphoamino acid analysis was performed by two-dimensional thin layer electrophoresis (22). Tryptic phosphopeptides were resolved by thin layer chromatography at pH 1.9 followed by ascending chromatography, as described (4, 22).

Transient Transfection Analysis

Vectors directing the expression of HA-tagged wild-type PTP-1B and its Ser-352 → Ala mutant (23) were introduced into 293 cells by the calcium phosphate method as described (3). Forty eight hours post-transfection, cells were treated with 1 M sorbitol for 1 h and PTP-1B mobility was determined.

Kinase Assays

JNK and p38 immune complex kinase assays were performed essentially as described (24) with the following modifications. Cells were washed several times with ice-cold PBS and lysed in cold JNK-lysis buffer (JLB: 20 mM Tris HCl, pH 7.4, 87 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM Na3VO4, 50 mM NaF, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml antipain, and 20 μg/ml PMSF). For immunoprecipitations, rabbit antisera against GST/p38 or GST/JNK1 fusion proteins were added (3 μl/250 μg of total protein) and incubated on ice for 2 h. Immune complexes were collected on Protein A-Sepharose (Pharmacia Biotech Inc.) for 20 min at 4 °C, washed 4 times with JLB and 2 times with JNK reaction buffer (JRB: 25 mM Hepes, pH 7.4, 25 mM β-glycerophosphate, 25 mM MgCl2, 2 mM DTT, 2 mM Na3VO4, and incubated in 40 μl of JRB containing 1 μCi of [γ-32P]ATP, 25 μM non-radioactive ATP, and 1 μg of GST-ATF2 fusion protein (25) for 10 min at 30 °C. Reactions were terminated with 40 μl of 2 × SDS-PAGE sample buffer (18), boiled for 5 min, and separated by 10% SDS-PAGE. GST-ATF2 phosphorylation was monitored by autoradiography and quantitated by scanning the film.
lease into the cell cycle for various periods of time and wash-off to obtain G2/M-enriched populations (see "Experimental Procedures"). The upper isoform of PTP-1B, characteristic of nocodazole-arrested cells (lane Noc, Fig. 1B), also appeared 14–15 h after release from S phase block, which correlated with the appearance of round, mitotic cells. Similar results were obtained when cells were synchronized in S phase by double thymidine block and released (data not shown). These results strongly suggest that PTP-1B phosphorylation is a bona fide mitotic event.

We next confirmed that the stress-induced PTP-1B mobility shift was caused by phosphorylation, as has been reported for the mobility shift in mitotic cells (4, 5). Lysates of sorbitol-treated cells were treated with various amounts of calf intestinal alkaline phosphatase (Fig. 2A). Phosphatase treatment reversed the osmotic shock-induced decrease in PTP-1B mobility. The action of alkaline phosphatase was prevented by inclusion of phosphatase inhibitors, indicating that it was a consequence of dephosphorylation rather than protease contamination. Phosphoamino acid analysis revealed that PTP-1B from osmotically shocked cells was phosphorylated exclusively on seryl residues (Fig. 2B), as is the case for mitotic PTP-1B (4, 5).²

We also examined whether the osmotic shock- and mitosis-induced PTP-1B mobility shifts result from phosphorylation of the same residue, Ser-352. ²⁹³ cells were transfected with plasmids expressing either HA-tagged wild type PTP-1B (HA-PTP-1B) or an HA-tagged Ser-352 → Ala mutant of PTP-1B (HA-PTP-1BS → A), and, following osmotic shock, the mobility of the respective HA-tagged proteins, as well as that of endogenous PTP-1B, was analyzed by immunoblotting (Fig. 2C). As expected, osmotic shock induced a decrease in the electrophoretic mobility of both endogenous PTP-1B (compare PTP-1B and phospho-PTP-1B (pPTP-1B) and the HA-tagged wild type protein (HA-PTP-1B) and phospho-HA-PTP-1B (pHA-PTP-1B)). Mutation of Ser-352 eliminated the osmotic shock-induced mobility shift. These results strongly suggest that, as in

² J. V. Frangioni, V. I. Shifrin, and B. G. Neel, unpublished data.
mitosis, Ser-352 becomes phosphorylated upon osmotic shock.

To confirm these observations, as well as to assess whether stress induces phosphorylation of other residues in PTP-1B, we performed phosphopeptide mapping studies. Osmotic shock (sorbitol) or calyculin A treatment produced 2 major tryptic phosphopeptides (labeled 1 and 2 on Fig. 2D), which co-migrated with the 2 major phosphopeptides in mitotic PTP-1B.

These peptides have been shown previously (4) to contain Ser-386 (spot 1) and Ser-352 (spot 2). A third peptide (spot 3), largely absent in PTP-1B from mitotic cells but strongly phosphorylated in sorbitol- and calyculin A-treated cells, probably contains Ser-378, a protein kinase C recognition site (4). Thus, during mitosis and osmotic shock, PTP-1B becomes phosphorylated on both Ser-352 and Ser-386. Since Ser-352 and Ser-386 are each phosphorylated under both conditions, it is possible that they are targets of the same kinase. Randomly growing cells display a distinctly different PTP-1B phosphorylation pattern (Fig. 2D, Control), which does not include Ser-352 or Ser-386. Moreover, inhibition of serine phosphatases with calyculin A does not enhance the phosphorylation pattern characteristic of randomly growing cells, but instead evokes new phosphorylation events (e.g. spots 1 and 2), yielding the pattern characteristic of mitotic and osmotically shocked cells.

These results suggest that calyculin A (and, by inference, osmotic shock and mitosis) promotes PTP-1B phosphorylation not simply by inhibiting a PTP-1B specific phosphatase(s) but by activating a PTP-1B specific kinase. This idea is in agreement with the ability of calyculin A to activate p38 and JNKs (data not shown), as well as with the ability of another Ser-Thr phosphatase inhibitor, okadaic acid, to induce a similar pattern of protein phosphorylation as TNF and IL-1 (33). Moreover, in preliminary studies, we have detected an increase in a kinase activity that phosphorylates PTP-1B on Ser-352 in membranes from osmotically shocked, calyculin A-treated, or mitotically arrested cells (data not shown).

Osmotic shock activates several proline-directed MAP kinases, including Erk1/2 (29, 30), p38 (9, 30, 34), and JNKs (35). We asked whether activation of any of these kinases correlated with the appearance of the upper PTP-1B isoform. Erk1/2, although activated by osmotic shock, are unlikely to be responsible for PTP-1B phosphorylation, because blocking their activation by inhibiting their upstream activator MEK with the specific inhibitor PD98059 (16, 17, 36, 37) did not affect PTP-1B mobility shift during the osmotic shock response (Fig. 3A). Moreover, several treatments that strongly activate Erk1/2 (e.g. growth factor or phorbol ester stimulation) did not result in PTP-1B phosphorylation on Ser-352 (data not shown).

The stress-activated kinase p38 also is an unlikely candidate for the PTP-1B kinase. First, activation of p38 was insufficient to cause PTP-1B phosphorylation. We observed that p38 becomes activated in nocodazole-treated HeLa cells (Fig. 3C, top panel). After 18 h of nocodazole treatment, about 50% of the HeLa cells became round and could be easily washed from the plates. These cells (noc. round cells in Fig. 3C) were mitotically arrested, as evinced by their 4n DNA content (Fig. 3C, bottom panel) and high level of Cyclin B/Cdc2 H1 kinase activity (data not shown). The remaining, adherent cells were in different stages of the cell cycle, as indicated by their DNA content similar to that of randomly growing cells (Fig. 3C, bottom panel) and their low levels of Cyclin B/Cdc2 H1 kinase activity (data not shown). However, both populations of nocodazole-treated cells had very similar levels of (elevated) p38 activity (Fig. 3C, top panel). These results suggest that nocodazole treatment causes activation of p38 in HeLa cells independently of mitotic arrest. More importantly, though, Ser-352-phosphorylated PTP-1B was present only in nocodazole-treated mitotic cells (Fig. 3C, middle panel). Therefore, activation of p38 per se is not sufficient to cause Ser-352 phosphorylation. Moreover, p38 activation is not necessary for Ser-352 phosphorylation, as treatment with the p38-specific inhibitor SB 203580 at concentrations known to inhibit p38-mediated events in vivo (10, 19, 20, 38) did not affect osmotic shock-induced phosphorylation of PTP-1B (Fig. 3B).
It also is unlikely that a JNK is the PTP-1B kinase. The levels of activation of JNKs caused by various stress treatments did not correlate with the ability of these treatments to induce the upper isoform of PTP-1B (Fig. 3D). For example, anisomycin and cycloheximide both evoke strong activation of JNKs, but only anisomycin treatment leads to significant PTP-1B phosphorylation. Conversely, the level of PTP-1B phosphorylation is comparable in TNF and anisomycin-treated cells, but these treatments result in dramatically different levels of JNK activity. JNKs are activated by treatment with IL-1 or peroxide, yet these agents have little effect on Ser-352 phosphorylation. Finally, in mitotic cells, Ser-352 phosphorylation is comparable in TNF and anisomycin-treated cells, but these treatments result in dramatically different levels of JNK activity. JNKs are activated by treatment with IL-1 or peroxide, yet these agents have little effect on Ser-352 phosphorylation. Therefore, activation of JNKs by stress treatments does not correlate with PTP-1B phosphorylation.

Our data demonstrate that PTP-1B is phosphorylated on the same sites, Ser-352 and Ser-386, in both mitotic and stress-treated cells, but these treatments result in dramatically different levels of JNK activity. JNKs are activated by treatment with IL-1 or peroxide, yet these agents have little effect on Ser-352 phosphorylation. Consequently, the level of PTP-1B phosphorylation is comparable in TNF and anisomycin-treated cells, but these treatments result in dramatically different levels of JNK activity. JNKs are activated by treatment with IL-1 or peroxide, yet these agents have little effect on Ser-352 phosphorylation. Therefore, activation of JNKs by stress treatments does not correlate with PTP-1B phosphorylation.

These properties distinguish the PTP-1B kinase activity from known members of the MAPK family, as well as from multiple other serine/threonine kinases, including Cyclin/Cdkks, cAMP-dependent protein kinase, and protein kinase C. Studies in fission yeast (see introduction) provide a precedent for the involvement of the same MAP kinase family member, the S. pombe homolog of p38, in both mitotic stress response and control of mitotic entry (7, 8). However, although human p38 does not phosphorylate PTP-1B in vitro (data not shown) and its inhibition does not affect phosphorylation of PTP-1B in vivo (Fig. 3B), we favor the hypothesis that another MAP kinase analog, activated in response to both stress and mitosis, is responsible for PTP-1B phosphorylation (Fig. 4). Validation of this hypoth-

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esis will require identifying the kinase that phosphorylates Ser-352 of PTP-1B at mitosis and demonstrating that it is identical to the PTP-1B kinase activated in response to stress treatments. Identification of this kinase could then provide a means for uncovering the mammalian pathway analogous to that in *S. pombe*.

The biological consequences of PTP-1B phosphorylation for the stress response, as well as its mitotic role, remain to be defined. It is possible that PTP-1B functions analogously to Pyp1 or Pyp2 to inactivate stress- and mitosis-induced MAP kinases. However, it should be noted that when introduced into *S. pombe* (39), PTP-1B behaves more like Pyp3, a mitotic entry stimulator (40), than the mitotic inhibitors Pyp1 or Pyp2 (8, 14, 15). The effect of phosphorylation on PTP-1B enzymatic activity is controversial. Flint et al. (4) reported a 30% decrease in PTP-1B activity, even a 30% decrease in its activity might result in a significant increase in Tyr(P) content of cellular proteins. Thus, the increase in tyrosine phosphorylation in HeLa cells (41), even a 30% decrease in its activity of various PTKs (43) but also from inactivation of PTP-1B and PTP-1B may result in a significant increase in Tyr(P) content of cellular proteins. Thus, the increase in tyrosine phosphorylation in response to UV irradiation (42) and to various other stresses, such as during mitosis, may result not only from activation of various PTKs (43) but also from inactivation of PTP-1B and perhaps other PTPs. Interestingly, several proteins of similar molecular weight become phosphorylated on Tyr during mitosis and stress-response. The identities of these proteins, which could be PTP-1B targets, are currently under investigation.

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