Protein-tyrosine phosphatase 1B (PTP-1B) is the prototypic tyrosine phosphatase whose function in insulin signaling and metabolism is well established. Although the role of PTP-1B in dephosphorylating various cell surface receptor tyrosine kinases is clear, the mechanisms by which it modulates receptor function from the endoplasmic reticulum (ER) remains an enigma. Here, we provide evidence that PTP-1B has an essential function in regulating the unfolded protein response in the ER compartment. The absence of PTP-1B caused impaired ER stress-induced IRE1 signaling. More specifically, JNK activation, XBP-1 splicing, and EDEM (ER degradation-enhancing α-mannosidase-like protein) gene induction, as well as ER stress-induced apoptosis, were attenuated in PTP-1B knockout mouse embryonic fibroblasts in response to two ER stressors, tunicamycin and azetidine-2-carboxylic acid. We demonstrate that PTP-1B is not just a passive resident of the ER but on the contrary has an essential role in potentiating IRE1-mediated ER stress signaling pathways.

Protein-tyrosine phosphatase 1B (PTP-1B) is a key regulator of metabolism and cell growth. This tyrosine phosphatase has been implicated in insulin, leptin, and growth hormone signaling pathways. Consequently, PTP-1B knock-out mice exhibit resistance to diet-induced diabetes and obesity, as well as enhanced growth hormone sensitivity (1–5). PTP-1B is ubiquitously expressed and localized to the endoplasmic reticulum (ER) via a C-terminal 35 amino acid sequence (6). It is not well understood how ER-localized PTP-1B could interact with cell surface receptors. The specific localization of PTP-1B in the ER compartment prompted us to investigate the possible role of PTP-1B in signaling pathways emanating directly from the ER membrane.

The ER is responsible for the folding and assembly of secretory proteins. Several cellular conditions such as high demand for protein secretion, nutrient/oxygen deprivation, viral infection, or the presence of certain genetic mutations lead to defects in protein folding in the ER (7). This results in the accumulation of improperly folded proteins in the ER lumen, which induces an adaptive signaling response known as the “unfolded protein response” (UPR) (8, 9). Two ER resident type I transmembrane protein kinases, IRE1 and PERK, mediate this stress response. Under basal conditions, they are maintained inactive by their association with the molecular chaperone Bip/Grp78 through their luminal domain. When improperly folded proteins accumulate in the ER, Bip dissociates from IRE1 and PERK, activating the transcription of EDEM (ER degradation-enhancing α-mannosidase-like protein) gene, a component of the ER-associated degradation machinery (17). Furthermore, following ER stress the basal association of IRE1 with Bip/Grp78 is disrupted, leading to ERK activation (27). Second, stress-induced oligomerization of the double-stranded RNA-activated protein kinase (PKR)-like kinase (PERK) leads to its activation and consequently to eIF-2α phosphorylation, thus attenuating protein translation to reduce the ER load (18, 19). A third component of the ER stress signaling is the transmembrane protein ATF6. The basic leucine-zipper transcription factor contained in its cytoplasmic domain is cleaved in the Golgi apparatus upon ER stress to activate the transcription of specific transcription factors, including XBP-1, ER chaperones, and folding enzyme (14, 20, 21). Together,
these different signaling pathways synergize to promote folding and/or degradation of the misfolded proteins. Prolonged and severe ER stress, however, will eventually lead to apoptosis and cell death through the activation of JNK, caspase-12 cleavage (except in human where caspase-12 is a pseudo-gene) and CHOP expression, downstream of IRE1, PERK, and ATF6 (13, 22, 23).

In the current report, we investigated the role of PTP-1B in ER stress. The absence of PTP-1B caused impaired IRE1-dependent JNK activation as well as XBP-1 splicing and EDEM transcription, which were reduced in immortalized (MEFs) or primary (PMEFs) PTP-1B knock-out mouse embryonic fibroblasts. The effect of PTP1B on signaling through IRE1 was dependent on its tyrosine phosphatase activity. Finally, ER stress-induced apoptosis was attenuated upon prolonged stress in PTP-1B knock-out cells. Our results thus demonstrate that PTP-1B has an active physiological role in the ER compartment and that it has an important function in potentiating IRE1-mediated ER stress signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Reagents were from Fisher Biotech unless otherwise indicated. Antibodies against TC-PTP were described previously (5). Calnexin antibody was a gift from Dr. John Bergeron (McGill University). Other antibodies were purchased from Upstate Biotechnologies (PTP-1B), Cell Signaling (phospho-SAPK/JNK T183/Y185, phospho-p38MAPK T180/Y182, phospho-eIF2α S51, p38MAPK), Santa Cruz Biotechnologies (JNK-1). Azetidine-2-carboxylic acid (Aze) was from Sigma. Tunicamycin (Tun), 1,2-bis(2-aminophenoxy)ethane-N,N,N,N'-tetraacetate (BAPTA), and anisomycin were from Calbiochem.

**Cell Culture and Isolation of Primary Embryonic Fibroblasts**—Sporadically immortalized PTP-1B and CT-PTP WT MEFs (PTP-1B+/−MEFs and CT-PTP+/−MEFs, respectively) and corresponding knockout MEFs (PTP-1B−/−MEFs and CT-PTP−/−MEFs, respectively) were cultured in Dulbecco’s modified Eagle’s medium + 10% fetal bovine serum (Invitrogen) as described (5). PTP-1B “rescued” cell lines were obtained by re-introducing a Myc-tagged murine PTP-1B either WT (“rescue” R3 and R5) or bearing a D181A mutation (“rescue” D181A) in a FACscan flow cytometer (BD Biosciences). Cells were analyzed using the manufacturer’s recommendations (BIOSOURCE). Cells were grown and treated with Azc (2.5 mM) and Tun (10 μg/ml). PTP-1B knock-out PMEFs were isolated from the same litter from the heterozygous parents. Embryos were genetically typed by Southern blot.

**ER Stress Treatment, Preparation of Cell Lysates, and Immunoblotting**—Cells were grown to ~80% confluence. Aze (10 μM) or Tun (10 μg/ml) was added for the indicated period of time, and cells were washed once in phosphate-buffered saline and lysed on ice in 150 mM NaCl, 50 mM Tris-HCl, 1% Nonidet P-40, 0.25% Na deoxycholate, 1 mM Na3VO4, 50 mM NaF, and Complete protease inhibitors (Roche Applied Science). Protein concentration was measured using the Bradford method, and ~25 μg of proteins were loaded on SDS-PAGE and analyzed by immunoblot with specific antibodies.

**RT-PCR**—After the same ER stress treatment as described above, the expression of Bip, CHOP, XBP-1, EDEM, and GAPDH was assessed by semiquantitative RT-PCR as described previously (27). The following oligonucleotides used were: 5'-GGGAAAGGTCTTACCAGGAC 3'-GACATTATGACCCACCAACAGG for Bip, 5'-CCCCTGCTTTTGCGTTGG for TGG and 5'-CCCGCTCGTTTCTCCCTG for CHOP, 5'-AATCTCAGCTTAAATTACAG and 5'-CCATGTGGAAGAGCTTCTCGG for XBP-1, 5'-GGTTAAACCGCTAAGGACGCCCTG for EDEM, and 5'-AACCATTGGAAGGGCCTG and 5'-CTCACTGATGCCAACAGTG for GAPDH.

**RESULTS**

**Impaired ER Stress-mediated Stress Kinase Activation in PTP-1B Knock-out Fibroblasts**—PTP-1B associates with the ER membrane via its C-terminal 35 amino acids and exposes its tyrosine phosphatase domain on the cytoplasmic face of the ER membrane (6). Although most of the substrates of PTP-1B localize at the plasma membrane, PTP-1B was not known to relocate to this compartment, neither at steady state nor upon EGF, PDGF, or insulin stimulation (24, 25). In agreement with these findings, we did not observe any noticeable change in ER localization of PTP-1B during growth hormone stimulation (supplementary Fig. 1).

The ER localization of PTP-1B prompted us to examine its possible involvement in ER stress signaling. We induced ER stress using either Aze, a proline analogue containing a 4-carbon ring, or Tun, an inhibitor of N-glycosylation that prevents transfer of the oligosaccharide moiety from dolichol phosphate to newly synthesized protein. PTP-1B+/− and PTP-1B−/− MEFs were treated with Aze and Tun for the indicated periods of time. Immunoblot analysis using phospho-specific antibodies against JNK and p38MAPK showed a strong activation of JNK and p38MAPK in WT cells after 3–6 h of Aze treatment (Fig. 1A) or after 12–16 h of Tun treatment (Fig. 1B). However, the activation of JNK and p38MAPK was almost undetectable in the PTP-1B−/− MEFs treated with either drug (Fig. 1, A and B). Furthermore, anisomycin, a potent JNK agonist stimulated JNK and p38MAPK phosphorylation in the PTP-1B knock-out cells to the same extent as in WT cells, showing that there is no intrinsic defect in JNK or p38MAPK activation in the absence of PTP-1B (Fig. 1B). As a control of specificity, we postulated that the absence of the closely related phosphatase TC-PTP should not reduce JNK or p38MAPK activation (26). Indeed, when TC-PTP−/− MEFs were treated with Aze or Tun, JNK and p38MAPK activation was indistinguishable from the TC-PTP+/− MEFs (Fig. 1C), thus demonstrating that PTP-1B, but not TC-PTP, is involved in ER stress kinase activation (Fig. 1C). To confirm that the inhibition of JNK and p38MAPK activation was indeed caused by the absence of PTP-1B, Myc-tagged PTP-1B was re-expressed in PTP-1B−/− MEFs using retroviral infection (rescue). Rescued cells had lower expression levels of PTP-1B than WT cells, but this amount was sufficient to restore JNK and p38MAPK activation in response to Aze. This result was observed in two different rescued cell lines R5 and R3 (Fig. 1D). Overall, our data indicate that PTP-1B is specifically involved in the activation of stress kinases in response to ER stress.

**Impaired IRE1 Signaling in Primary PTP-1B Knock-out Cells**—Immortalization of MEF cells often involves uncontrolled mutations and deletions, resulting in indirect phenotypes. To rule out these effects, we isolated primary fibroblasts derived from PTP-1B+/− and PTP-1B−/− mouse embryos (PMEFs). Two different pairs of WT and knock-out PMEFs were isolated and treated with Aze and activation of JNK and p38MAPK in WT PMEFs was detected after 3–6 h, similar to what was observed in the immortalized cells (Fig. 2A). However, JNK and p38MAPK activation were largely reduced in both pairs of PTP-1B knock-out PMEFs after the same treatment (Fig. 2A). These results confirmed the direct role of PTP-1B in stress kinase activation. Since JNK activation is induced downstream of IRE1 activation, our results suggest a role of PTP-1B in IRE1 signaling pathway.

Analysis of gene expression profiles by RT-PCR revealed that Grp78/Bip and CHOP mRNA expression levels increased 3–6 h after Aze and Tun treatments in both WT and PTP-1B knock-out PMEFs (Fig. 2B). This suggested no obvious defect in the transcriptional activation of these two genes in the absence of PTP-1B. In contrast, after 3–6 h of Aze or Tun treatment, although the total XBP-1 mRNA expression level (unspliced + spliced forms) increased to a similar extent in both WT and PTP-1B knock-out PMEFs, XBP-1 mRNA splicing was dramat-
FIG. 1. JNK and p38MAPK activation are inhibited in immortalized PTP-1B knock-out fibroblasts. A, immortalized PTP-1B+/− and PTP-1B−/− MEFs were treated with 10 mM Azc for the indicated periods of time. Cell lysates were analyzed by immunoblot using the indicated antibodies. B, same as in A, except that cells were treated with 10 μg/ml Tun. WT (+/+) and knock-out (−/−) cells were also treated with anisomycin (Ani) for 6 h for JNK activation. C, TC-PTP−/− and TC-PTP+/− MEFs were treated with Tun or Azc for 3 or 6 h, and lysates were analyzed for JNK and p38MAPK activation as in A and B. D, PTP-1B+/− MEFs and two different clones of rescued cells re-expressing myc-PTP-1B (rescue “R5” and rescue “R3”) were treated with Azc for 3 h, and immunoblotting was done as described for A–C.

FIG. 2. PTP-1B tyrosine phosphatase activity is essential for IRE1 signaling. A, primary fibroblasts isolated from #2 WT and #7 knock-out embryo from one litter together with #5 WT and #7 knock-out embryo from a second litter were treated with 10 mM Azc for 3 or 6 h. Cell lysates were analyzed by immunoblot using the indicated antibodies. B, Bip, CHOP, XBP-1, and EDEM transcriptions were measured by semiquantitative RT-PCR after 3 or 6 h treatment of 10 mM Azc or 10 μg/ml Tun on #2 WT and #7 knock-out primary MEFs. C, as described for A, except that cells were treated 30 min with 10 μg/ml Tun or 2.5 μM BAPTA for 30 min, and cell lysates were analyzed by immunoblot with phospho-specific antibody against eIF2α and other indicated antibodies. D, immortalized PTP-1B+/− MEFs, PTP-1B−/− MEFs, and PTP-1B−/− MEFs re-expressing a Myc-PTP-1B carrying a point mutation in PTP-1B tyrosine phosphatase domain (PTP-1B−/− rescue D181A) were treated with Azc for the indicated periods of time. Cell lysates were analyzed by immunoblot using the indicated antibodies. E, XBP-1 transcription was measured by semiquantitative RT-PCR after treatment of immortalized PTP-1B+/−, PTP-1B−/− and PTP-1B D181A mutant rescued PTP-1B−/− MEFs with 10 mM Azc or 10 μg/ml Tun for 6 h.
ially reduced in the PTP-1B<sup>−/−</sup> cells (Fig. 2B). The expression product of the spliced XBP-1 is a transcription factor whose major target is EDEM (17). Because of the reduced level of XBP-1 splicing in PTP-1B knock-out primary fibroblasts, we measured EDEM mRNA expression levels in both WT and PTP-1B knock-out cells. While EDEM mRNA expression was induced in response to Azc or Tun treatment in WT cells, no induction was observed in PTP-1B knock-out cells (Fig. 2B). A similar defect in XBP-1 splicing was also obtained in immortalized PTP-1B knock-out MEFs (supplementary Fig. 2). The unconventional splicing of XBP-1 is an event directly triggered by IRE1 endoribonuclease activity (15). Together, these experiments led us to conclude that PTP-1B is involved in activation of IRE1 signaling upstream of stress kinase activation and XBP-1 splicing in response to ER stress.

To establish whether PTP-1B was also involved in other ER stress-mediated signaling pathways, we assessed PERK activation by measuring the phosphorylation of eIF2α on Ser<sup>51</sup> in response to ER stress in both PTP-1B<sup>+/+</sup> and PTP-1B<sup>−/−</sup> PMEFs. Phosphorylation of eIF2α was activated quickly (30 min) after treatment with Tun or the calcium chelator BAPTA, and no difference was observed between cell types (Fig. 2C). This result is in agreement with the fact that we did not observe a significant difference in Bip and CHOP gene transcription, which are indirectly controlled by PERK activation (Fig. 2B). These results indicate that PTP-1B specifically participates in IRE1-mediated but not in PERK-mediated signaling pathways. Moreover, the similarity in the induction of total XBP-1 transcription in WT and PTP-1B knock-out cells suggests that the ATF6 pathway is not significantly altered in the absence of PTP-1B. Primary cells therefore provide direct and physiologically relevant evidence that PTP-1B is specifically involved in IRE1 signaling pathways in response to ER stress.

To test whether the inhibition of ER stress-induced JNK and p38<sup>MAPK</sup> activation as well as the reduction of XBP-1 splicing were caused by PTP-1B tyrosine phosphatase activity, PTP-1B<sup>−/−</sup> MEFs were subjected to retroviral infection with a virus encoding a Myc-tagged PTP-1B carrying an inactivating point mutation in the tyrosine phosphatase domain (rescue D181A). The PTP-1B D181A rescued cells, along with PTP-1B WT and knock-out MEFs, were treated with 10 mM Azc for up to 9 h. The reduced activation of JNK and p38<sup>MAPK</sup> that was observed previously in the PTP-1B<sup>−/−</sup> MEFs (Fig. 1, A and B) was not reversed by re-expression of the PTP-1B D181A mutant in these cells (Fig. 2D). Since similar expression levels of WT PTP-1B in PTP-1B<sup>−/−</sup> cells were sufficient to rescue the impaired activation of both stress kinases (Fig. 1D), the absence of a similar effect in PTP-1B D181A rescued cells is likely due to the mutation itself. In addition, these cells were as inefficient as the PTP-1B<sup>−/−</sup> MEFs in mediating XBP-1 splicing upon Azc or Tun stress (Fig. 2E). Taken together, these results indicate that in response to ER stress PTP-1B tyrosine phosphatase activity is required for the activation of IRE1 signaling leading to XBP-1 splicing and activation of JNK and p38<sup>MAPK</sup>.

Enhanced Resistance to ER Stress-induced Apoptosis in PTP-1B Knock-out Cells—The activation of pro-apoptotic JNK

FIG. 3. PTP-1B knock-out fibroblasts are more resistant to ER stress induced apoptosis. A, PTP-1B<sup>+/+</sup> and PTP-1B<sup>−/−</sup> MEFs were treated with 10 μg/ml Tun or 10 mM Azc for 24 h. Phase contrast pictures of the cells were taken. B, primary fibroblasts #5 (PTP-1B<sup>−/−</sup>) and #1 (PTP-1B<sup>+/+</sup>) were treated with 10 μg/ml Tun or 2.5 mM Azc for 12 h, and apoptosis was analyzed by flow cytometry after cells were stained with fluorescein isothiocyanate-conjugated Annexin V and propidium iodide. The average of the percentage of early apoptotic (Annexin V-positive and propidium iodine-negative) population as well as other cell populations were calculated from more than three experiments, and standard deviations are presented.
DISCUSSION

In this study, we demonstrate that the absence of PTP-1B impairs IRE1 signaling in response to ER stress. This was reflected by the attenuation of JNK and p38MAPK activation, the defect in XBP-1 splicing, and the subsequent absence of EDEM transcriptional responses in both PTP-1B knock-out fibroblasts and primary cells isolated from PTP-1B knock-out embryos. Since JNK activation and XBP-1 splicing are triggered by activated IRE1 through its serine/threonine kinase and endoribonuclease activities, our results indicate that PTP-1B is involved in IRE1 signaling. In addition, our results suggest that a functional tyrosine phosphatase domain is required for PTP-1B to mediate this effect. IRE1, however, is not known to be directly regulated by tyrosine phosphorylation. Therefore, our data point to a mechanism in which PTP-1B tyrosine phosphatase activity indirectly promotes the activation of IRE1 in response to ER stress. Indeed, we have observed the presence of tyrosine-phosphorylated proteins associated with the ER under basal and stress conditions. Moreover, signaling molecules such as Ras and c-Abl were found to be associated with the ER membrane (28, 29). The possible role of these proteins in ER stress signaling is not well understood, but PTP-1B may be required to maintain the correct phosphorylation state of these, or other, targets on the ER membrane during IRE1 signaling. On the other hand, PTP-1B contains two proline-rich domains that have the potential to interact with Src homology 3 domain-containing proteins. Recently, the Src homology 3-containing adaptor protein Nck has been shown to directly interact with IRE1 and may therefore represent a potential binding partner for PTP-1B (27). It will be interesting to determine whether PTP-1B, in addition to its catalytic function, could contribute to IRE1 signaling by acting as a scaffold, assembling components of a signaling complex at the ER membrane.

Surprisingly, although the ER stress-mediated adaptive response (i.e. XBP-1 splicing and EDEM transcriptional response) was reduced in PTP-1B−/− cells, these cells were more resistant to ER stress-induced apoptosis. This phenomenon may result from unbalanced signaling of pro-survival and pro-apoptotic pathways in PTP-1B−/− cells when exposed to prolonged and severe stress. Indeed, we believe that although the XBP-1/EDEM axis is defective, PERK- and ATF6-mediated UPR pathways remain functional allowing PTP-1B knock-out cells to cope with the early phases of ER stress. Our results suggest that the reduced activation of major pro-apoptotic stress kinases (JNK/p38MAPK) finally results in a dominant effect on cell survival in PTP-1B knock-out cells.

In carrying out these studies, we have made a first step in the elucidation of an active role for PTP-1B in mediating signaling pathways emanating from the ER membrane. We hope that more studies will follow to elucidate the molecular mechanisms of its involvement in IRE1-mediated ER stress signaling.

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