Complement-mediated glomerular injury is reduced by inhibition of protein-tyrosine phosphatase 1B

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Nezvitsky LV, Tremblay ML, Takano T, Papillon J, Cybulsky AV. Complement-mediated glomerular injury is reduced by inhibition of protein-tyrosine phosphatase 1B. Am J Physiol Renal Physiol 307: F634–F647, 2014. First published July 23, 2014; doi:10.1152/ajprenal.00191.2014.—The unfolded protein response and endoplasmic reticulum-associated degradation (ERAD) contribute to injury in renal glomerular diseases, including those mediated by complement C5b-9. In the present study, we address the role of protein-tyrosine phosphatase 1B (PTP1B) in complement-mediated glomerular injury and ERAD. In glomerular epithelial cells (GECs)/podocytes and PTP1B-deficient mouse embryonic fibroblasts exposed to complement, inhibition/deletion of PTP1B reduced ERAD, as monitored by the ERAD reporter CD36. Overexpression of PTP1B produced an effect similar to PTP1B deficiency on ERAD in complement-treated GECs. Complement-mediated cytotoxicity was reduced after PTP1B overexpression and tended to be reduced after PTP1B inhibition. PTP1B enhanced the induction of certain ERAD components via the inositol-requiring-1α branch of the unfolded protein response. PTP1B knockout mice with anti-glomerular basement membrane glomerulonephritis had decreased proteinuria and showed less podocyte loss and endoplasmic reticulum dysfunction compared with wild-type littermates. These results imply that endogenous levels of PTP1B are tightly regulated and that both overexpression and inhibition can affect ERAD. The cytoprotective effects of PTP1B deletion in cultured cells and in anti-glomerular basement membrane nephritis suggest that PTP1B may potentially be a therapeutic target in complement-mediated diseases.

PROTEIN-TYROSINE PHOSPHATASE 1B (PTP1B) is a nonreceptor protein-tyrosine phosphatase that is expressed ubiquitously (51). The enzyme is anchored to the cytosolic face of the endoplasmic reticulum (ER) membrane via a COOH-terminal tail (24). Despite being tethered to the ER, PTP1B is nonetheless capable of interactions with and dephosphorylation of various substrates, which are apparently not localized at the ER. Substrates such as activated receptor tyrosine kinases can undergo endocytosis, which may bring them in close proximity to PTP1B (51). Moreover, the ER membrane is potentially dynamic and flexible, thereby allowing PTP1B to contact substrates at the plasma membrane. Interactions between PTP1B and its substrates can also be mediated through adaptor proteins (51). PTP1B is involved in insulin and leptin signaling in diabetes and obesity (51). Mice with global knockout (KO) of PTP1B have mild phenotypic abnormalities, including decreased blood glucose and insulin levels, and are leaner and resistant to high-fat diet-induced weight gain (20). In recent years, several studies have implicated PTP1B as a mediator of ER stress (28).

Approximately one-third of all newly synthesized proteins translocate into the ER lumen, where they will acquire their proper tertiary structure (5, 10, 52). Upon entry into the ER, a protein will undergo N-glycosylation. The two outermost glucose residues are then removed by glucosidases I and II, allowing calnexin or calreticulin to bind and facilitate folding. Correctly folded glycoproteins will exit the ER, whereas those that have not achieved proper conformations will undergo another folding attempt (1, 6, 10, 27). Maturation of proteins equally relies on the oxidative environment in the ER and on appropriate levels of Ca2+, glucose, and ATP, and disruption of this homeostasis can lead to misfolded protein accumulation and ER stress (5, 26, 28, 29, 52). The ER can alleviate protein misfolding by activating the unfolded protein response and ER-associated degradation (ERAD). Activation of the unfolded protein response results in increased expression of folding chaperones and attenuation of protein translation, whereas ERAD leads to the removal of permanently misfolded proteins.

The unfolded protein response is initiated by three ER transmembrane protein sensors: activating transcription factor 6 (ATF6), inositol-requiring enzyme-1α (IRE1α), and protein kinase RNA-like ER kinase (PERK). In unstressed cells, the sensors are inactive through association with the chaperone Bip [glucose-regulated protein (Grp)78] (28, 29, 52). Upon accumulation of misfolded proteins, Bip binds to the unfolded proteins and dissociates from the sensors, allowing their activation. PERK phosphorylates and inactivates the eukaryotic translation initiation factor 2α (eIF2α), thereby decreasing the general rate of translation and protein load on a damaged ER (28, 29, 52). ATF6 transits to the Golgi, where it is cleaved by resident proteases, yielding a cytosolic fragment, which migrates to the nucleus and upregulates the expression of ER folding chaperones. IRE1α RNase removes an intron from X-box-binding protein-1 mRNA, yielding a potent transcription factor. After translation, X-box-binding protein-1 translocates to the nucleus, where it can induce expression of the unfolded protein response and ERAD genes (28, 29, 52).

Proteins that do not attain their native structures despite multiple folding attempts become substrates for ERAD (5, 6, 10, 26). Mannose is trimmed from misfolded glycoproteins by ER mannosidase I and ER degradation-enhancing α-mannosidase-like protein 1 (EDEM1) (11, 40, 41). The glycoprotein can then associate with lectins, including XTP3-B and OS-9, which bring the ERAD substrate to the retrotranslocation machinery (10). ERdj4, a protein upregulated during ER stress, and derlin-1 assist with the destruction of misfolded proteins (5, 34). The ERAD substrate meets the membrane-bound E3 ubiquitin ligase complex HRD1-SEL1L, becomes retrotranslo-
cated to the cytosol, ubiquitinated, and degraded by the proteasome (6, 10, 26). While removal of misfolded proteins is believed to facilitate cell survival, prolonged/excessive ER stress may be cytotoxic, via apoptosis. ER stress-induced apoptosis is mediated by C/EBP homologous protein-10 (CHOP), downstream of ATF6 and PERK, and IRE1α-mediated activation of JNK (28, 48).

ER stress can be modulated by PTP1B (28). Deficiency of PTP1B impaired activation of the IRE1α pathway in mouse embryonic fibroblasts (MEFs) (25). Specifically, JNK activation, X-box-binding protein-1 splicing, EDEM gene induction, and apoptosis were attenuated in PTP1B KO MEFs (25). Alternatively, overexpression of PTP1B in insulinoma and apoptosis were attenuated in PTP1B KO MEFs (25).

Moreover, in adipocyte-specific PTP1B KO mice, there was enhanced activation of PERK/ERF2α and increased X-box-binding protein-1 mRNA (7). The anti-GFP antibody cross-reacts with yellow fluorescent protein (YFP) and is referred to as anti-GFP/YFP. Rabbit anti-PTP1B was purchased from Transduction Laboratories (Lexington, KY), and mouse anti-DsRed2 was purchased from Clontech (Palo Alto, CA).

Materials and Methods

Materials. Transfection reagents, Lipofectamine 2000 and OptimEM, were purchased from Invitrogen-Life Technologies (Burlington, ON, Canada). C8-deficient serum and purified C8 were from C8-deficient serum (80% [vol/vol]) in PBS. We have generally used heterologous (human) complement to facilitate studies with complement-deficient sera and to minimize possible signaling via complement-regulatory proteins; however, in earlier studies, we confirmed that homologous (rat) complement activated analogous signaling pathways in GECs. Although there was some variability in complement activity among batches of sera, we selected 1.5–2.5% as sublytic concentrations (concentration that induced minimal cytolysis). MEFs were incubated with antiserum, as described above, followed by 5% NS (sublytic concentration) (17, 18).

Measurement of complement-mediated cytotoxicity. Complement-mediated cytolysis was determined by measuring lactate dehydrogenase (LDH) release, as previously described (17, 18). Specific LDH release was calculated as follows: (NS – HIS)/(100 – HIS), where NS represents the percent total LDH released into cell supernatants in incubations with NS and HIS represents the percent total LDH released in incubations with HIS.

Immunoblot analysis. Techniques for immunoblot analysis and protein quantification by scanning densitometry [using ImageJ software, National Institutes of Health (NIH)] were as previously described (17, 18).

Measurement of PTP1B activity. GECs transfected with GFP-PTP1B were lysed with Nonidet P-40 (NP-40). GFP-PTP1B was immunoprecipitated using anti-GFP/YFP antibody, or normal mouse IgG in controls, followed by 5% NS (sublytic concentration) (17, 18).
RNA extraction and analysis. RNA extraction and cDNA analysis were done using the RNeasy Mini Kit and QuantitiTect Reverse Transcription Kit from Qiagen. Real-time quantitative RT-PCR was performed with a pair of target-specific primers, and detection was with iTaq SYBR Green Supermix with ROX from Bio-Rad Laboratories (Applied Biosystems 7300 Real-Time PCR System). Primers for HDRI/SYVN1 were 5′-CAGTCCGGGTAGTGGAC-3′ (forward) and 5′-AAGGCAAAGGCTGAGTG-3′ (reverse). Primers for ERdj4/DnaJ9 were 5′-GTTTGGAGAAGCTGCGTC-3′ (forward) and 5′-AICTGACTTGAGTGCTCCCA-3′ (reverse). Primers for EDEM1 were 5′-GACCTCGAGAACCCTTCAA-3′ (forward) and 5′-ACTGCTTTCTGAACTCGGA-3′ (reverse). Standardization of gene expression measurements was done against hypoxanthine ribosyltransferase; the primers were 5′-AGTCCCAGCCTGTTAATGAT-3′ (forward) and 5′-GAGCAAGTCTTTCATGCTGG-3′ (reverse).

Induction of anti-GBM nephritis in mice. Anti-GBM nephritis was induced in 12- to 20-wk-old PTPIB+/− or PTPIB+/− BALB/c mice by a single intravenous injection of sheep anti-rat GBM antiserum (5 μl diluted in 0.1 ml sterile PBS) (42). Glomeruli were isolated using a differential sieving technique (42). All experiments were approved by the Animal Care Committee of McGill University.

Immunofluorescence microscopy. Kidney sections were snap frozen using Histoprep (Fisher Scientific, Ottawa, ON, Canada) in isopentane (cooled on dry ice) and stored at −80°C. Cryostat sections (4 μm) were fixed in cold acetone and blocked with 5% normal rabbit serum (for sheep IgG) or goat serum (for C3) in 5% BSA or in 5% BSA alone (for Wilm’s tumor-1 and nephrin antibodies). Sections were then stained with FITC-conjugated rabbit anti-sheep IgG or FITC-conjugated goat anti-mouse C3 (MP Biomedical, Santa Ana, CA) or Cy3-conjugated goat anti-rabbit IgG (Cell Signaling Technology) diluted in 5% BSA. Sections were examined under a Zeiss AxioObserver fluorescence microscope with visual output connected to an AxioCam digital camera. Fluorescence intensity was quantified using ImageJ software (NIH).

Urine albumin/creatinine measurements. Urine samples were collected before the administration of antisemir and before mice were euthanized. Urinary albumin was determined using a Mouse Albumin ELISA Quantification Kit (Bethyl Laboratories, Montgomery, TX), and urinary creatinine was determined using a Creatinine Assay Kit (Cayman Chemical, Ann Arbor, MI), both according to the manufacturers’ instructions. Values obtained from albumin and creatinine assays were used to calculate albumin-to-creatinine ratios for each mouse.

Statistics. Data are presented as means ± SE. The t-statistic was used to determine significant differences between two groups. One-way ANOVA was used to determine significant differences among groups. Where significant differences were found, individual comparisons were made between groups of interest using the t-statistic and adjusting the critical value using the Bonferroni method. Two-way ANOVA was used to determine significant differences in multiple measurements among groups.

RESULTS

Inhibition of ERAD in GECs reduced the degradation of CD36-YFP upon exposure to complement. In certain glomerular diseases, C5b-9 assembly induces GEC injury and proteinuria, impairs ER membrane integrity, and induces ER stress (18). In the first set of experiments, we characterized ERAD in GECs undergoing complement attack using kifunensine, a drug that interrupts glycoprotein processing and ERAD through inhibition of ER mannosidase I. We used a well-characterized ERAD reporter, CD36-YFP (33). CD36 is a T cell receptor subunit that assembles with other subunits in the ER before secretion to the plasma membrane in T cells (50). However, in cells that do not express all components of the T cell receptor, ectopic CD36 is targeted for degradation via ERAD; thus, in these cells, CD36-YFP undergoes a time-dependent decrease in expression, the rate of which reflects the ERAD capacity for this substrate (50). GECs transiently transfected with CD36-YFP were incubated with anti-GEc antisemir followed by sublytic NS (to assemble C5b-9) or HIS in controls with or without kifunensine. In this context, activation of complement induces ER protein misfolding and ER stress (18). In the absence of kifunensine, the level of CD36-YFP decreased at 24 h compared with 4 h, reflecting ongoing degradation of the ERAD reporter, and there were no significant differences between cells exposed to complement (NS) and control (HIS; Fig. 1, A and B). As expected, in control (HIS-treated) GECs incubated with kifunensine to inhibit ERAD, the level of CD36-YFP increased (i.e., there was a reduction in the degradation of CD36-YFP) at 4 and 24 h (Fig. 1, A and B). In complement (NS)- and kifunensine-treated GECs, there was a further increase in the level of the CD36-YFP reporter compared with HIS-treated cells at 4 and 24 h (Fig. 1, A and B). The most likely explanation for the further increase in CD36-YFP in complement- and kifunensine-treated cells is that complement increased the amount of misfolded proteins in the ER, and all of these misfolded proteins were competing with CD36-YFP for ERAD. Certain steps in ERAD, e.g., interactions with specific ERAD mediators or retrotranslocation from the ER to the cytosol, may be rate limiting; consequently, the increased misfolded proteins in the ER would be competing with CD36-YFP and would lead to an increase in CD36-YFP, particularly in the context of impaired ERAD capacity. The most likely reason why complement did not increase CD36-YFP in the absence of kifunensine is that there was excess ERAD capacity to handle all of the misfolded proteins in the ER.

Next, we assessed if modulation of ERAD affects complement-mediated cytotoxicity. In vivo, complement-mediated GEC injury is associated with proteinuria. In cultured cells, complement-induced injury (lysis) can be monitored by the release of LDH, a cytosolic enzyme that is released into the medium upon cell membrane damage. GECs were incubated with anti-GEc antisemir followed by NS at serially increasing concentrations or HIS in controls with or without kifunensine. There was a tendency toward less complement-mediated cytotoxicity in the presence of kifunensine compared with untreated (5–10% NS) cells, although the change did not reach statistical significance (Fig. 1C).

PTPIB modulates ERAD in GECs exposed to complement. The role of PTPIB in modulating ERAD was studied during complement attack by monitoring the degradation of the ERAD reporter CD36-YFP after inhibition or overexpression of PTPIB. In the first set of experiments, GECs were transiently transfected with CD36-YFP. GECs were then incubated with antisemir followed by sublytic NS or HIS in controls with or without a specific allosteric inhibitor of PTPIB at a concentration of 50 μM. This concentration has been previously shown to inhibit PTPIB activity in a cell-based assay (4). The specificity of this inhibitor relies on a unique allosteric site in PTPIB, which is poorly conserved among other phosphatases (46). Compared with controls, incubation with complement markedly decreased the degradation of CD36-YFP in GECs treated with the PTPIB inhibitor (Fig. 2, A and B). To confirm...
that the effect of complement was actually due to the assembly of C5b-9, GECs were incubated with antibody and C8-deficient serum (to assemble an inactive C5b-7 complex) or with C8-deficient serum reconstituted with purified C8 (which assembles C5b-9). By analogy, there was reduced CD3-YFP degradation after complement attack (results not shown). In summary, both inhibition and overexpression of PTP1B decreased complement-mediated degradation of the C3d ERAD reporter.

PTP1B modulates complement-mediated cytotoxicity in GECs. To investigate the effect of PTP1B on complement cytotoxicity, we overexpressed PTP1B by transfection or in-

To substantiate the results obtained with chemical inhibition of PTP1B, we blocked PTP1B using a dominant negative cDNA, GFP-PTP1B D181A. In this substrate-trapping mutant, conserved aspartic acid 181 in the catalytic domain is replaced by alanine, which abolishes the catalytic activity of the enzyme but does not modify its affinity for substrate. This allows the D181A mutant to compete with endogenous PTP1B and act as an inhibitor (23). After transfection, the apparent expression of GFP-PTP1B D181A was comparable with endogenous PTP1B (Fig. 2E). However, the transfection efficiency of GECs is ~30% (i.e., ~30% of GECs were GFP positive, as assessed by visual counting); therefore, in cells cotransfected with GFP-PTP1B D181A and CD3-YFP, the expression of GFP-PTP1B D181A is actually approximately threefold greater than endogenous PTP1B. GECs cotransfected with CD3-YFP and GFP-PTP1B D181A (empty vector in controls) were incubated with antibody and complement for 4 or 24 h. Incubation with complement decreased the degradation of CD3-YFP in GECs that expressed the PTP1B dominant negative mutant, and the effect was significant at 4 h compared with controls (Fig. 2, E and F). At 24 h, CD3-YFP was also greater in complement-treated cells that expressed dominant negative PTP1B, although the difference did not reach statistical significance. These experiments are in keeping with the results obtained with chemical inhibition of PTP1B.

To study the effects associated with PTP1B overexpression, GECs were cotransfected with CD3-YFP and WT GFP-PTP1B (empty vector in controls). Cells were incubated with antibody and complement (NS). Surprisingly, degradation of CD3-YFP was decreased in complement-stimulated GECs that overexpressed PTP1B compared with controls (Fig. 3, A and B). By analogy, when the experiment was carried out using C8-deficient serum with or without reconstitution with purified C8, there was reduced CD3-YFP degradation upon C5b-9 assembly in GECs that overexpressed PTP1B, confirming that the observed effect is due to assembly of C5b-9 but not C5b-7 (Fig. 3, C and D). It should be noted that ectopic GFP-PTP1B was localized predominantly in the perinuclear region of GECs (Fig. 3E). This pattern is similar to the localization of the ER protein calnexin in GECs (21) and is in keeping with the known localization of PTP1B at the ER. Moreover, the result implies that overexpression did not induce mislocalization of PTP1B.
hibited PTP1B with the chemical inhibitor. GECs were incubated with antiserum followed by NS at serially increasing concentrations or HIS in controls. Complement-mediated cytotoxicity (LDH release) was reduced significantly after PTP1B overexpression (Fig. 4A). There was a tendency toward reduced cytotoxicity after inhibition of PTP1B, although the difference was of borderline statistical significance (Fig. 4B).

Inhibition of PTP1B decreases tunicamycin-induced increases in HRD1 and ERdj4 mRNA. Activation of IRE1α leads to the induction of certain ERAD components (29). Since PTP1B modulated ERAD during complement attack, and PTP1B has been shown to be involved in IRE1α signaling (25), we proceeded to assess if PTP1B can modulate specific mediators of ERAD that have been shown to be downstream of IRE1α (i.e., HRD1, ERdj4, and EDEM1) (5, 29–31, 37, 49). GECs were incubated with or without the PTP1B inhibitor and were treated with tunicamycin for 1, 3, or 7 h. (Tunicamycin was used as it specifically activates the unfolded protein response via inhibition of N-glycosylation, leading to extensive protein misfolding.) mRNA was extracted, and expression of HRD1, ERdj4, and EDEM1 mRNA was determined using real-time quantitative RT-PCR. Tunicamycin induced robust increases in HRD1 and ERdj4 (at 3 and 7 h), and these increases were attenuated by 45–80% after inhibition of
PTP1B staining demonstrated the localization of nuclei. Bar, 6.6-Diamidino-2-phenylindole (representative fluorescence micrograph). PTP1B was localized predominantly in the perinuclear region of GECs (endothelial cells). Three experiments were performed in duplicate.

To determine if other unfolded protein response pathways were affected by PTP1B inhibition, immunoblot analysis for Grp94 and CHOP was performed. Increases in Grp94 induced by tunicamycin (at 24 h), which reflect mainly ATF6 activation, were comparable both with and without PTP1B inhibition (Fig. 5, D and E). Induction of CHOP by tunicamycin, which occurs downstream of PERK activation and eIF2α phosphorylation, was reduced ~20–30% after inhibition of PTP1B (at 6 and 24 h), but this change was not statistically significant (Fig. 5, D and F). The PTP1B inhibitor did not independently increase Grp94 and CHOP. Therefore, PTP1B enhanced the induction of the IRE1α-dependent ERAD genes HRD1 and ERdj4, but did not affect the ATF6 pathway, and may have had only a modest stimulatory effect on PERK activation.

Fig. 3. Complement decreased the degradation of CD3δ-YFP in GECs that overexpress PTP1B. GECs were cotransfected with CD3δ-YFP and wild-type (WT) GFP-PTP1B or vector (control). GECs were incubated with antiserum followed by 1.5% NS or HIS (control) for 4 or 24 h in duplicate. Lysates were immunoblotted with antibodies to GFP/YFP (CD3δ-YFP), PTP1B (ectopic and endogenous PTP1B), and actin. A: representative immunoblots. B: densitometry of CD3δ-YFP. *P < 0.0001, NS vs. HIS (GECs transfected with GFP-PTP1B, 4 and 24 h). Four experiments were performed in duplicate. Transfected, antibody-sensitized GECs were incubated with 2.5% C8-deficient serum reconstituted with purified C8 (C8DS + C8) or C8-deficient serum alone (C8DS) for 4 h. Lysates were immunoblotted with antibodies to GFP/YFP (CD3δ-YFP and GFP-PTP1B) and actin. C: representative immunoblots. D: densitometry of CD3δ-YFP. *P < 0.0001, C8DS + C8 vs. C8DS (GFP-PTP1B). Three experiments were performed in duplicate. E: ectopic GFP-PTP1B was localized predominantly in the perinuclear region of GECs (representative fluorescence micrograph). 4',6-Diamidino-2-phenylindole (DAPI) staining demonstrated the localization of nuclei. Bar = 20 μm.

Fig. 4. Effect of PTP1B on complement-mediated cytotoxicity (LDH release). A: GECs were transfected with GFP-PTP1B or vector (control). GECs were incubated with antiserum followed by 2.5%, 5%, or 10% NS or HIS (control) for 1 h. P = 0.008, PTP1B vs. vector (by two-way ANOVA). Eight experiments were performed. B: GECs were incubated as described above with or without PTP1B inhibitor (50 μM). P = 0.097, PTP1B vs. vector (by two-way ANOVA). Twelve experiments were performed.
Deletion of PTP1B in MEF impairs tunicamycin-induced activation of JNK. Having demonstrated that PTP1B modulates the effect of complement on ERAD in GECs, we assessed if these results were unique to GECs or if analogous effects can occur in other cell lines. For these experiments, we selected MEFs, since PTP1B has been deleted in these cells, and, furthermore, PTP1B has been shown to modulate the IRE1α branch of the unfolded protein response in MEFs (25). To verify the role of PTP1B and IRE1α in ER stress, we treated WT and PTP1B KO MEFs with tunicamycin and monitored the phosphorylation/activation of JNK, which reflects activation of the IRE1α pathway in these cells (25). Tunicamycin stimulated phosphorylation of JNK in WT MEFs at 24 h; however, phosphorylation was weak in KO MEFs (Fig. 6, A and B). In contrast, induction of the ER chaperone Grp94 by tunicamycin (reflecting ATF6 activation) was comparable in the two cell lines (Fig. 6, A and C). Furthermore,
Fig. 6. PTP1B and ER function in mouse embryonic fibroblasts (MEFs). A–D: Tm-induced activation-specific phosphorylation of JNK was reduced in PTP1B knockout (KO) MEFs compared with WT MEFs. MEFs were incubated with Tm (10 μg/ml, +) or medium alone (control, −) for 6 or 24 h at 37°C in duplicate. Cell lysates were immunoblotted with antibodies to JNK, phospho-JNK, PTP1B, Grp94, CHOP, and α-tubulin. A: representative immunoblots. B: densitometric quantification of phospho-JNK (both bands are included). *P < 0.0001, PTP1B+/+ vs. PTP1B−/− MEFs for 24 h with Tm vs. without Tm. Six experiments performed in duplicate. C: densitometric quantification of Grp94. *P < 0.0001, Tm-treated vs. untreated PTP1B+/+ and PTP1B−/− MEFs (24 h). Four experiments were performed in duplicate. D: densitometric quantification of CHOP. *P < 0.0001 and **P < 0.0003, Tm-treated vs. untreated PTP1B+/+ and PTP1B−/− MEFs (6 and 24 h). Three experiments were performed in duplicate. E and F: complement decreased the degradation of the ERAD reporter CD3δ-YFP in PTP1B KO MEFs. MEFs were transfected with CD3δ-YFP and incubated with 5% anti-GEC antiserum followed by 5% NS (to assemble C5b-9) or HIS (control) for 4 or 24 h at 37°C in duplicate. Cell lysates were immunoblotted with antibody to GFP/YFP (CD3δ-YFP). E: representative CD3δ-YFP immunoblot (the amido black-stained band is the loading control). F: densitometry of CD3δ-YFP. *P < 0.0001, NS vs. HIS in PTP1B−/− MEFs (4 h). Five experiments were performed in duplicate. G: complement-mediated cytotoxicity (LDH release) was reduced in PTP1B KO MEFs compared with WT MEFs. MEFs were incubated with 5% anti-GEC antiserum (40 min, 22°C) followed by 5%, 10%, or 15% NS (to assemble C5b-9) or HIS (control) for 1 h at 37°C. P = 0.017, WT vs. KO MEFs (by two-way ANOVA). Four experiments were performed in duplicate.
induction of CHOP by tunicamycin (reflecting PERK activation) actually tended to be greater in PTP1B KO MEFs (Fig. 6, A and D). Thus, by analogy to GECs, PTP1B enhanced the activation of the IRE1α pathway but not ATF6 or PERK.

Deletion of PTP1B in MEFs reduces complement-mediated degradation of CD36-YFP and cytotoxicity. Having shown that deletion of PTP1B in MEFs impairs the IRE1α-dependent activation of JNK (Fig. 6, A and B), we examined if ERAD would also be affected by PTP1B deletion. WT and PTP1B KO MEFs were transiently transfected with CD36-YFP and were incubated with antiserum followed by sublytic NS (HIS in controls) for 4 or 24 h. In WT MEFs, complement did not affect the level of CD36-YFP significantly (Fig. 6, E and F). In contrast, complement increased the level of CD36-YFP in KO MEFs (at 4 h), reflecting decreased degradation of the ERAD reporter. These results are in keeping with the findings in GECs (Fig. 2).

To address complement-mediated cytotoxicity, WT and PTP1B KO MEFs were incubated with antiserum followed by NS at serially increasing concentrations (to assemble C5b-9) or HIS in controls. Complement-mediated cytotoxicity was significantly lower in KO MEFs compared with WT MEFs (Fig. 6G). This reduction in cytotoxicity is consistent with the trend observed in GECs (Fig. 4).

Complement induces a minor increase in PTP1B activity. In the experiments described above, changes in PTP1B activity were achieved by overexpression and inhibition/deletion of PTP1B. We also considered the possibility that complement can alter PTP1B activity. To address this possibility, we assessed dephosphorylation-induced activation of Src, a known substrate of PTP1B. Dephosphorylation of the Src inhibitory phosphorylation site, Tyr527, by PTP1B leads to autophosphorylation of Tyr116 and Src activation (9, 34). GECs were incubated with antiserum and sublytic NS for 1 or 4 h with or without the PTP1B inhibitor. In the absence of the inhibitor, complement did not affect Src activation significantly, but after PTP1B inhibition, complement decreased Src activation (Fig. 7, A and B). The PTP1B inhibitor did not independently affect Src activation in control incubations. This result suggests that complement reduces the activation of Src directly but simultaneously stimulates PTP1B activity, which counteracts the reduction in Src activity. Although statistically significant, the effect of complement on PTP1B activity in this assay was small.

PTP1B activity was also studied by an in vitro phosphatase assay. This assay uses p-nitrophenyl phosphate, a substrate of protein tyrosine phosphatase, which is converted into p-nitrophenol, a chromogenic product, whose production can be monitored by absorbance at 405 nm. GECs transfected with GFP-PTP1B were incubated with antiserum followed by sublytic NS or HIS in controls. PTP1B was then immunoprecipitated from cell lysates and was incubated with p-nitrophenyl phosphate. Complement induced a small increase in PTP1B activity, in the range of 20% (Fig. 7C).

PTP1B deletion reduces proteinuria and podocyte loss in anti-GBM nephritis. Our experiments in cell culture demonstrated a role for PTP1B in ERAD and cytotoxicity during complement attack. To address the role of PTP1B in vivo, we induced anti-GBM nephritis in WT and PTP1B KO mice. C5b-9-induced GEC/podocyte injury and proteinuria are hallmark signs of heterologous mouse anti-GBM nephritis. In vivo, anti-GBM antibody binds in part to GEC antigens, and heterologous phase proteinuria is abolished by C6 deficiency, indicating a key role of C5b-9 (13). While anti-GBM nephritis may have an inflammatory component, this tends to be negligible in the heterologous phase (38). Mouse urine samples were collected just before the administration of anti-GBM antiserum and after 24 h, and kidney tissue was examined at 24 h. Both WT and PTP1B KO mice developed albuminuria 24 h after the induction of nephritis; however, PTP1B KO mice exhibited significantly lower levels of albuminuria compared with WT mice (Fig. 8A). The reduced proteinuria in PTP1B KO mice occurred despite equal glomerular anti-GBM antibody and C3 deposition in KO and WT mice (Fig. 8, B and C). By immunoblot analysis, there were no significant differences in PTP1B expression between WT control mice and WT mice with anti-GBM nephritis (Fig. 8D). To determine if PTP1B activity was altered in anti-GBM nephritis, glomerular lysates were immunoblotted with antibody to nonphospho-Src (Tyr527), as shown in Fig. 7A. There were no significant differences in nonphospho-Src
Fig. 8. PTP1B deletion reduces proteinuria and podocyte injury in anti-glomerular basement membrane (GBM) nephritis. WT and PTP1B KO mice received an injection of sheep anti-GBM antiserum. A: urine albumin and creatinine were measured before (control) and 24 h after injection (anti-GBM). After the induction of anti-GBM nephritis, PTP1B KO mice had significantly less albuminuria compared with WT mice (*P = 0.027, WT vs. KO mice (anti-GBM). n = 8 WT control mice, 8 KO control mice, 5 WT mice with anti-GBM, and 5 KO mice with anti-GBM. B: kidney sections (at 24 h) were stained with anti-sheep IgG or anti-mouse C3. There was bright glomerular staining for sheep IgG and mouse C3 in mice with anti-GBM nephritis. Staining was absent in untreated mice, although these mice showed periglomerular C3 staining, probably in Bowman’s capsule. (Untreated PTP1B WT mice are shown; however, untreated PTP1B KO mice and WT mice with anti-GBM (aGBM) nephritis (anti-PTP1B antibody immunoblot). As expected, PTP1B was not detectable in KO mice. C: quantification of anti-GBM antibody and complement C3 deposition (fluorescence intensity) in arbitrary units. There were no significant differences between groups. D: there were no apparent differences in PTP1B expression between WT control (Ctrl) and WT mice with anti-GBM (aGBM) nephritis (anti-PTP1B antibody immunoblot). As expected, PTP1B was not detectable in KO mice. E and F: WT mice with anti-GBM nephritis showed a greater reduction in podocyte number compared with PTP1B KO mice. Kidney sections were stained with anti-Wilm’s tumor-1 (WT-1) antibody, and the number of Wilm’s tumor-1-positive cells was quantified by visual counting. E: representative Wilm’s tumor-1 staining. F: quantification of Wilm’s tumor-1-positive cells. *P = 0.0005, PTP1B WT vs. KO mice (anti-GBM). The number of glomeruli quantified was 26 in WT control mice, 29 in KO control mice, 69 in WT mice with anti-GBM, and 59 in KO mice with anti-GBM. G–I: nephrin glycosylation and immunofluorescence staining. Glomeruli were isolated from WT and PTP1B KO mice treated with anti-GBM antibody or controls at 24 h. G: lysates were immunoblotted with anti-nephrin antibody. In the first lane (lane E), a control lysate was incubated with endoglycosidase H. In glomeruli, nephrin migrated as a doublet (bands 1 and 2). Endoglycosidase H induced a loss of the lower nephrin band (band 2) and the appearance of a faster-migrating band (band 3); however, band 1 was unaffected. H: kidney sections were stained with anti-nephrin antibody. Representative nephrin immunofluorescence is shown. I: quantification of nephrin immunofluorescence intensity (in arbitrary units). WT mice with anti-GBM nephritis showed a greater reduction in nephrin immunofluorescence compared with PTP1B KO mice. PTP1B WT PTP1B KO

signals between WT control mice (0.82 ± 0.06 densitometry units) and WT mice with anti-GBM nephritis (0.81 ± 0.09 densitometry units, 4 mice/group; immunoblot not shown).

In addition to albuminuria, glomerular injury may be associated with loss of podocytes. The number of podocytes in the glomerulus was assessed by visual counting of Wilm’s tumor-1-positive cells. Anti-GBM antibody reduced the number of podocytes substantially (by ~15%) in WT mice compared with PTP1B KO mice (Fig. 8, E and F). In contrast, podocyte numbers were not significantly different between anti-GBM-treated and control PTP1B KO mice (Fig. 8, E and F). Taken together, the results indicate that deletion of PTP1B confers a protective effect on the podocyte in glomerular injury in vivo.

PTP1B deletion ameliorates defective posttranslational modification of nephrin in anti-GBM nephritis. Nephrin is a key component of the podocyte filtration slit diaphragm and undergoes important posttranslational modifications in the ER before export to the plasma membrane. Underglycosylation of nephrin leads to ERAD (19). To address ER function in vivo,
we examined nephrin glycosylation in isolated glomeruli by SDS-PAGE. In control WT and PTP1B KO mice, nephrin migrated as a doublet of ~180 kDa (Fig. 8G, bands 1 and 2). Endoglycosidase H is a specific endoglycosidase that primarily cleaves asparagine-linked mannose-rich oligosaccharides but not highly processed complex oligosaccharides from glycoproteins. Endoglycosidase H induced a complete loss of the lower nephrin band (band 2) and the appearance of a faster-migrating band (band 3); however, the upper nephrin band (band 1) was unaffected (Fig. 8G, lane 1). Based on this experiment and on an earlier study (19), it can be concluded that band 1 most likely represents the fully mature form of nephrin, carrying complex oligosaccharide and localized in the plasma membrane. Band 2 is high mannose, an immature form of nephrin, found in the ER. Quantification of nephrin expression in control mice (n = 6) showed that there was two- to threefold greater expression of the mature form of nephrin (band 1) compared with the immature form (band 2). In anti-GBM nephritis, there was a reduction in the amount of mature nephrin compared with the immature form; however, the reduction was more pronounced in WT mice compared with KO mice (densitometric quantification of the mature/immature bands was 1.03 ± 0.07 in WT mice, n = 5, vs 1.37 ± 0.13 in KO mice, n = 5, P < 0.05). These results imply that in anti-GBM nephritis, there is defective glycosylation of nephrin, at least in part, in the ER. The disruption is less severe when PTP1B is deleted, implying that the action of PTP1B is associated with ER function.

Nephrin expression was examined by immunofluorescence microscopy and was quantitated. By analogy to albuminuria, the anti-GBM antibody induced a decrease in nephrin expression; however, nephrin expression was significantly lower in PTP1B WT mice (~30% reduction) compared with KO mice (~12% reduction; Fig. 8, H and I). By SDS-PAGE, there was a tendency toward reduced expression of mature nephrin in anti-GBM nephritis compared with control (in WT and KO mice), but the differences did not reach statistical significance (results not shown).

Certain signaling properties of nephrin are believed to be regulated by phosphorylation of cytoplasmic domain tyrosine residues, and phosphorylated nephrin has been reported to be a substrate of PTP1B, i.e., PTP1B can dephosphorylate Tyr1232 (4). In kidney sections of mice with anti-GBM nephritis stained with anti-phospho-nephrin (Tyr1232) antibody, there were no detectable differences in the pattern or intensity of staining between PTP1B KO and WT mice (results not shown). Therefore, these results suggest that the effect of PTP1B on proteinuria is most likely unrelated to nephrin phosphorylation.

DISCUSSION

C5b-9-mediated GEC injury and proteinuria, in association with ER stress, plays an important role in the pathogenesis of glomerular disease. Previously, we and others (17, 18) have demonstrated that assembly of C5b-9 leads to damage to the ER, protein misfolding, and activation of the unfolded protein response in GECs. In the present study, we used the ERAD reporter CD36-YFP to show that inhibition of ERAD delays the degradation of misfolded proteins, particularly during complement attack (Fig. 1). Furthermore, complement-mediated degradation of CD36-YFP was reduced in GECs treated with a PTP1B inhibitor or transfected with dominant negative cDNA (Fig. 2). Overexpression of WT PTP1B also reduced CD36-YFP degradation in GECs exposed to complement (Fig. 3). Therefore, both overexpression and inhibition of PTP1B can affect ERAD detrimentally, suggesting that the level of PTP1B activity may require tight regulation to ensure optimal ERAD function. While the involvement of PTP1B in unfolded protein response signaling has been previously demonstrated (7, 8, 25), to our knowledge this is the first demonstration of the involvement of PTP1B in ERAD.

After showing that ERAD is modulated by PTP1B during complement-mediated attack in GECs, we proceeded to ascertain if PTP1B can enhance the expression of ERAD mediators during ER stress. The upregulation of ERAD mediators during ER stress is a mechanism for enhancing ERAD capacity (5, 6, 49). PTP1B has been shown to modulate the IRE1α branch of the unfolded protein response (25), and the IRE1α pathway signals to upregulate certain genes involved in ERAD, including Erdj4 and HRD1 (29–31, 37, 49). Erdj4 is a component of ERAD that associates with substrates targeted for degradation, and HRD1 is part of the ubiquitin-ligase complex that is responsible for the retrotranslocation of ERAD substrates (6, 10, 26, 34). We observed that during tunicamycin-induced ER stress, increases in HRD1 and Erdj4 were significantly blunted after inhibition of PTP1B (Fig. 5), indicating that PTP1B was involved in the upregulation of IRE1α-dependent ERAD mediators. The effect of PTP1B was directed at the IRE1α branch of the unfolded protein response, since the induction of Grp94 and CHOP, reflecting the ATP6 and PERK pathways, respectively, were comparable in the presence or absence of PTP1B inhibition, although we cannot exclude a minor effect of PTP1B on PERK (Fig. 5). Therefore, it is reasonable to propose that the effect of PTP1B on ERAD was, at least in part, dependent on IRE1α; however, delineation of the precise mechanism will require further investigation. There may also be other ERAD pathway components that are regulated by phosphorylation and that might be targets of PTP1B, e.g., AAA+ ATPase p97 (53). This will require further study.

Since complement induces protein misfolding, enhancement of ERAD is potentially a mechanism for ameliorating protein homeostasis. Surprisingly, impairment of ERAD in GECs, either with kifunensine or by altering PTP1B activity, did not exacerbate complement-mediated cytotoxicity and actually tended to reduce cytotoxicity (Figs. 1 and 4). This result is distinct from the earlier observation that downregulation of the ER chaperone Bip exacerbated complement-mediated cytotoxicity in GECs (18) and that deletion of PERK aggravated complement-mediated cytotoxicity in MEFs (17). Most likely, these other protective mechanisms that were induced in GECs (upregulation of chaperones or reduced mRNA translation) compensated for the reduction in ERAD, such that global complement-induced cytotoxicity was not exacerbated after ERAD inhibition (also see below).

The effects of PTP1B on ERAD were not unique to GECs. By analogy, incubation of PTP1B KO MEFs with complement reduced the degradation of the CD36-YFP ERAD reporter, whereas degradation of CD36-YFP was not affected in WT MEFs (Fig. 6). Deletion of PTP1B in MEFs provided a cytoprotective effect in complement-mediated cytolysis, implying that PTP1B enhances cytotoxicity (Fig. 6). Impairment of ERAD related to changes in PTP1B expression was associ-
ated with reduced complement-mediated cytotoxicity in MEFs. However, as noted above in GECs, the presence of misfolded proteins in the ER can also induce cytoprotective signaling in MEFs, including PERK activation (17). In keeping with results in GECs and those of a previous study (25), we confirmed that deletion of PTP1B in MEFs affected the function of the IRE1α pathway; moreover, we demonstrated that the effect of PTP1B was restricted to the IRE1α branch of the unfolded protein response, since the induction of Grp94 and CHOP (reflecting the ATF6 and PERK pathways, respectively) was comparable in both WT and KO MEFs (Fig. 6). Similarly, it has been shown that mice with liver-specific PTP1B deletion have decreased X-box-binding protein-1 splicing upon the induction of ER stress by high-fat diet feeding or tunicamycin stimulation compared with controls, supporting the involvement of PTP1B in the IRE1α pathway (2).

While cultured GECs are a physiologically relevant model for the study of mechanisms of injury, it is important to determine if effects observed in cell culture are applicable to disease pathogenesis in vivo. To address this, we used heterologous anti-GBM nephritis, which features complement-mediated GEC/podocyte injury. PTP1B KO mice with anti-GBM nephritis showed decreased albuminuria compared with WT littermates (Fig. 8). In addition, mice with anti-GBM nephritis showed reduced numbers of podocytes; however, the loss of podocytes was attenuated in PTP1B KO mice compared with WT mice (Fig. 8). Reductions in podocyte number may be due to apoptosis (43). A pattern similar to albuminuria and podocyte number was also observed with the expression of the podocyte slit diaphragm protein nephrin (Fig. 8). Demonstrating protein misfolding in the ER, and ER dysfunction in models of injury in vivo, is complex, as is the in vivo use of reporter proteins such as CD3Δ-YFP. Therefore, to address ER function in anti-GBM nephritis, we examined the glycosylation of nephrin. The results show that nephrin glycosylation was disrupted in anti-GBM nephritis; however, the degree of impairment was lower in PTP1B KO mice compared with WT mice (Fig. 8). Impaired glycosylation of nephrin results in reduced export from the ER, disruption in processing of complex oligosaccharides, and reduced plasma membrane expression as well as an increase in ERAD of underglycosylated nephrin (19). Taken together, the results in the present study indicate that complement-mediated injury and albuminuria in vivo are associated with ER dysfunction and that deletion of PTP1B is protective to ER function.

The doses of anti-GBM antibody used in this study were relatively low, allowing us to demonstrate differences in albuminuria between PTP1B KO and WT mice. At these doses of antibody, we were not able to observe changes in the expression of glomerular ER chaperones. When higher doses of antibody were administered, mice with anti-GBM nephritis showed increased glomerular expression of Grp94 and Bip and increased phosphorylation of eIF2α (unpublished observations), in keeping with ER stress. However, at these higher doses, albuminuria was massive and did not allow for the study of PTP1B effects. Nevertheless, the unfolded protein response was probably activated in anti-GBM nephritis at the lower dose of antibody, even though the ER biochemical markers were below the levels of detectability. PTP1B deletion in mice did not alter tyrosine phosphorylation of nephrin, suggesting that this potential mechanism was not responsible for albuminuria in anti-GBM nephritis. Further studies will be required to determine how the in vivo protective effect of PTP1B is associated with ER dysfunction and if the IRE1α pathway is involved. Enhanced glomerular injury in PTP1B-sufficient mice may reflect more robust activation of the IRE1α pathway and IRE1α-mediated proapoptotic signaling in podocytes, leading to podocyte loss. This hypothesis would be consistent with our observation that deletion of PTP1B in cultured cells reduced complement-mediated cell death (Fig. 6) and the dominant proapoptotic signaling caused by the PTP1B-mediated regulation of IRE1α under conditions of excessive ER stress (25).

An interaction of PTP1B with the IRE1α pathway is supported by the present and previous studies (28, 29). Since IRE1α activity does not appear to be mediated by tyrosine phosphorylation, the interaction between PTP1B and IRE1α is more likely indirect. For example, PTP1B can form a stable complex with the adaptor protein Nck to facilitate the PTP1B-mediated dephosphorylation and inactivation of the insulin receptor in human embryonic kidney 293 cells (47). Nck and PTP1B KO mice display phenotypic similarities in glucose and insulin homeostasis (20, 35). Moreover, Nck has been shown to upregulate the IRE1α and PERK branches of the unfolded protein response in various cell lines (32, 36, 39). Therefore, Nck could potentially modulate IRE1α and PTP1B interactions (35). This and other potential interactors will require delineation in future experiments.

Both overexpression and inhibition of PTP1B showed similar effects on ERAD. Although these findings would seem to be contradictory, there are a number of examples where excessive or insufficient expression of a protein can have similar consequences. For example, both over- and underexpression of VEGF in podocytes results in podocyte injury and proteinuria (22). Expression of constitutively active and dominant negative Rho A GTPases in podocytes leads to proteinuria (45). Both overexpression and knockdown of Ste20-like kinase leads to apoptosis (3). Thus, tight regulation of expression or signaling by a number of proteins, including PTP1B, is critical for their biological functions.

In conclusion, the results of our study demonstrate a link between PTP1B and ERAD and show that the PTP1B-mediated regulation of ERAD may occur through IRE1α. Currently used treatment modalities of glomerular diseases, including those associated with complement-mediated podocyte injury, involve nonspecific immunosuppression, are only partially effective, and have significant toxicity. The cytoprotective effect of PTP1B deletion in cell culture and in anti-GBM nephritis suggests that PTP1B should be considered as a potentially novel and mechanism-based therapeutic target in complement-mediated glomerular diseases. PTP1B inhibitors that are effective and nontoxic in humans are in development and in early phase clinical trials. Further studies will be required to determine if the effect of PTP1B on IRE1α signaling and ERAD in the context of complement-mediated injury is applicable to multiple cell types and if PTP1B inhibition could also be a useful therapeutic approach in other diseases.

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