Phosphorylation at Tyrosine 262 Promotes GADD34 Protein Turnover

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**Background:** Proteasomal degradation of GADD34 dictates cell sensitivity to ER stress.

**Results:** Phosphorylation at tyrosine 262 controls GADD34 protein turnover and cell fate following ER stress.

**Conclusion:** Tyrosine 262 phosphorylation controls the cellular levels of GADD34, a key component of the stress-activated eIF2α phosphatase.

**Significance:** Stress-induced GADD34 gene transcription and subsequent regulated GADD34 protein turnover control the feedback loop that ensures cell survival following ER stress.

In mammalian cells, metabolic and environmental stress increases the phosphorylation of the eukaryotic translational initiation factor, eIF2α, and attenuates global protein synthesis. Subsequent transcriptional activation of GADD34 assembles an eIF2α phosphatase that feeds back to restore mRNA translation. Active proteasomal degradation of GADD34 protein then reestablishes the sensitivity of cells to subsequent bouts of stress. Mass spectrometry established GADD34 phosphorylation on multiple serines, threonines, and tyrosines. Phosphorylation at tyrosine 262 enhanced the rate of the GADD34 protein turnover. Substrate-trapping studies identified TC-PTP (PTPN2) as an active proteasomal degradation of GADD34 dictates cell sensitivity to ER stress.

**Significance:** Stress-induced GADD34 gene transcription and subsequent regulated GADD34 protein turnover control the feedback loop that ensures cell survival following ER stress.

Mammalian cells possess complex mechanisms for detecting transient changes in their environment and respond to unfavorable conditions or stress by activating signaling pathways that ensure cell survival. Errors in the integrated stress response (ISR) have been implicated in many chronic human diseases (1–3). Stresses such as nutrient deprivation, viral infection, iron deficiency, radiation, hypoxia, and other insults activate one or more of four mammalian protein kinases, GCN2, PKR, HRI, and PERK, which phosphorylate serine 51 on the eukaryotic translational initiation factor, eIF2α. Phosphorylated eIF2α binds to eIF2B to inhibit its guanine nucleotide exchange function. This in turn prevents the recruitment of the initiator methionyl-tRNA and the ribosomal subunits to assemble the translation initiation complex and thereby inhibits general protein translation. This allows cells to focus their attention on expressing stress proteins and overcome or survive the stress. Among the stress-induced proteins that are translated in the presence of eIF2α phosphorylation are two transcription factors, ATF4 (4) and CHOP (5), which collaborate to induce the expression of GADD34, the protein product of a Growth Arrest and DNA Damage-inducible gene (6). GADD34 recruits the α-subunit of protein phosphatase-1 (PP1α) and assembles an active eIF2α phosphatase (7), which functions in a feedback loop to restore protein synthesis (6, 8). While ISR, and particularly eIF2α phosphorylation, allows cells to survive mild or transient bouts of stress, ISR also initiates programmed cell death in response to chronic stress eliminating damaged or dysfunctional cells.

GADD34 and its upstream regulators, ATF4 and CHOP, are transiently expressed in mammalian cells (9), and both their mRNAs and proteins are rapidly turned over following ER stress. Modeling their expression subsequent to ER stress hinted that the turnover of the GADD34 protein may be a particularly important determinant of cell fate. Our prior work established that GADD34 was polyubiquitinated and degraded by the 26S proteasome with a t½ ≈ 1 h (10). Later studies (11) established that human GADD34 was equally distributed between ER and cytosol and identified an N-terminal amphipathic helix that mediated its association with ER membranes. Mutations that precluded ER membrane localization increased the stability of the GADD34 protein. Fluorescence recovery after photobleaching studies suggested rapid shuttling of GADD34 on and off the ER membranes. However, the mechanisms that control trafficking of GADD34, its subsequent

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§ The abbreviations used are: ISR, integrated stress response; PTP, protein tyrosine phosphatase; TG, thapsigargin; TN, tunicamycin; eIF, eukaryotic translational initiation factor; PP, protein phosphatase; PI, propidium iodide; CA, calyculin A; MC, microcystin; CHX, cycloheximide.
polyubiquitination and proteasomal degradation are still not fully understood.

Mammalian GADD34 proteins are characterized by multiple PEST repeats in the central region flanked by the N-terminal ER membrane localization domain and the C-terminal PP1α-binding domain. While PEST sequences have been implicated in directing proteasomal degradation of many proteins (12), systematic deletion of the PEST repeats had no effect on GADD34 protein turnover (10). On other hand, metabolic labeling showed that cellular GADD34 was highly phosphorylated with majority of the covalent modification being in the PEST domains (10). Prior studies used phosphopeptide enrichment followed by electron transfer dissociation tandem mass spectrometry to analyze the phosphoproteome in HEK293T cells and identified a phosphopeptide containing phosphorytrosine 262 and phosphoserine 264, in human GADD34, also known as PPP1R15A (13). However, the role of covalent modification in GADD34 function or regulation has not been investigated.

In current studies, vanadate increased eIF2α (serine 51) phosphor- ylation in GADD34 expressing cells. Mass spectrometry of vanadate- or pervanadate-treated HEK1293T cells ectopically expressing human GADD34 identified numerous phosphorylated serines, threonines, and tyrosines. Site-directed mutagenesis established tyrosine 262 as the major phosphorylated tyrosine in human GADD34. Substitution of tyrosine 262 with a phenylalanine reduced the rate of turnover of GADD34 in cells. Utilizing substrate-trapping mutants of protein tyrosine phosphatases (PTPases) implicated in ER stress (14–17), we identified TC-PTP, as a candidate ER-associated GADD34 phosphatase. Consistent with this, levels of stress-induced GADD34 protein were significantly reduced in TC-PTP-null MEFs compared with WT MEFs, differences that were abolished by inhibition of the proteasome. Our results highlighted that the increased ER stress-induced apoptosis in TC-PTP-null MEFs could be ameliorated by the ectopic expression of GADD34. These studies suggested that ER-association and subsequent tyrosine phosphorylation determine the cellular abundance of the GADD34 protein and cell fate following ER stress.

MATERIALS AND METHODS

Cell Culture, Transfection, Reagents, and Antibodies—Human embryonic kidney epithelial (HEK) 293T, Human cervical cancer epithelial (HeLa) cells, COS-7 African Green Monkey kidney cells and TC-PTP WT (+/+) and KO (−/−) Mouse Embryonic Fibroblasts (MEFs) were maintained in the Dulbec- co’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen) unless otherwise stated. Transfections were undertaken using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. Transfections on TC-PTP MEFs were carried out by Neon® transfection system (Invitrogen). Thapsigargin, tunicamycin, calcyulin A, cycloheximide, Hoechst 33258 was obtained from Sigma, MG132 and okadaic acid from Tocris Bioscience and Na₃VO₄ from Santa Cruz Biotechnology. The following commercial antibodies were used: anti-GADD34 (Serotec); anti-eIF2α and anti-PTP1B (Santa Cruz Biotechnology); anti-phospho-eIF2α-S51 (Invitrogen); anti-FLAG (Sigma); anti-tubulin (Sigma); anti-CHOP, anti-Bi, anti-cleaved PARP, anti-cleaved-caspase3, and anti-phosphorytrosine PY100 (Cell Signaling), anti-phosphorytrosine pY20 (Millipore); anti-HA (Zymed Laboratories Inc.); anti-TC-PTP (R&D Systems). The anti-TRAPα antibody was provided by Christopher Nicchitta of Duke University. Immunoblot analyses were undertaken as previously described (11).

Site-directed Mutagenesis—The substitutions, Y262A, Y262E, and Y262F, and other point mutations were introduced into GADD34-FLAG using QuikChange® Site-directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions.

Immunoprecipitation—Plasmids expressing FLAG-tagged GADD34 proteins were transfected in HEK293T cells. Cells were harvested and lysed in 50 mM Tris-HCl pH 7.4 containing 100 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM EDTA, and supplemented with protease and phosphatase inhibitors. Lysates were subjected to centrifugation at 16,000 × g for 10 min to remove cell debris. The supernatants were incubated with FLAG-M2 beads for 3 to 5 h at 4 °C. The beads were washed with lysis buffer 3 to 5 times and following the addition of equal volume of 2 × SDS-PAGE buffer, heated at 95 °C for 10 min prior to SDS-PAGE and Western immunoblotting.

Substrate-trapping Experiments—HEK293T cells were transfected with plasmids expressing either FLAG-TC-PTP(C/S) or GST-PTP1B(C/S) and WT GADD34-FLAG or GADD34(Y262F)-FLAG. Cells were lysed and the lysates incubated with either anti-FLAG antibodies and FLAG-M2 beads or GST-Sepharose. The bound proteins were resolved by SDS-PAGE and detected by immunoblotting. In competition experiments, lysates were supplemented with 10 mM Na₃VO₄ prior to sedimenting the PTPase complexes as described above.

Immunocytochemistry—Cells were grown on coverslips in 6-well or 12-well plates, transfected with plasmids expressing GADD34-FLAG proteins. After 24 h, cells were fixed with 4% (v/v) formaldehyde. For immunostaining, the cells, permeabilized using 0.2% (v/v) Triton X-100, were incubated with goat serum, followed by the primary antibody and the fluorescent dye-conjugated secondary antibody. Coverslips were rinsed with PBS (phosphate-buffered saline) and stained with Hoechst 33258. The coverslips mounted on glass slides CRYSTAL/ MOUNT™ (Biomeda) were viewed using Confocal Scanning Microscope LSM710 (Zeiss) and the images processed by the ZEN 2009 software (Zeiss).

Analysis of Protein Turnover—HEK293T or HeLa cells expressing GADD34 proteins were treated with cycloheximide (30 µg/ml). Cells were harvested at 1 to 2 h intervals, lysed in 2 × SDS sample buffer, and subjected to SDS-PAGE and Western immunoblotting.

Real-time Quantitative Polymerase Chain Reaction—Total mRNA was extracted from cells using RNA easy mini kit (Qia- gen). The complementary cDNA were synthesized using iScript (Bio-Rad) and qPCR performed using SsoFast kit (Bio-Rad) on iQ5 thermocycler (Bio-Rad). The following primers were used in the PCR reactions: murine GADD34: 5'-gagagttctcaaatagagc-3' and 5'-cagggacgtcagggcagc-3' (9); murine CHOP: 5'-gcgacagacgccgataaca-3' and 5'-gtacaccccttggcaga-3'; murine ATF4: 5'-atgagtttgcgggtg-3' and 5'-catttttcacactcata-3'; murine β-actin: 5'-ctaaagcccaactgtgaaag-3' and 5'-acccagagccatacagggc-
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Tyrosine phosphorylation in regulating either the eIF2α/α20 (MEFs) treated with thapsigargin (1 mM) for 30 min. Cells were lysed in 20 mM HEPES (pH 7.4), 137 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, protease inhibitors (Roche), and 0.2 mM sodium orthovanadate or 0.5 mM sodium pervanadate for 30 min. Cells were lysed in 20 mM HEPES (pH 7.4), 137 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, protease inhibitors (Roche), and 0.2 mM sodium orthovanadate. The lysates were incubated with FLAG-M2 agarose beads for 2 h at 4°C, the beads were sedimented by centrifugation, washed with the above buffer and proteins eluted in SDS sample buffer. Following SDS-PAGE, the GADD34 band was excised from Coomassie Blue-stained gels and destained before incubating with trypsin or Glu-C protease. The peptides were separated by Prominence HPLC (Shimadzu) followed by mass spectrometry on LTQ-FT Ultra mass spectrometer (Thermo Fisher) as described (18). Mass Spectrometry analyses were undertaken in the Proteomic Core Facility of the Biological Research Centre in Nanyang Technology University.

Statistical Analysis—Statistical analysis was undertaken using the Student’s t test or ANOVA by GraphPad Prism software (GraphPad Inc).

RESULTS

Vanadate Stimulates eIF2α Phosphorylation in Cells Expressing GADD34—Endogenous GADD34 protein levels were elevated in cells following the pharmacological inhibition of the proteasome (10). HeLa cells, treated with vehicle (DMSO), displayed modest phosphorylation of the eukaryotic initiation factor, eIF2α. Induction of GADD34 by exposure to the proteasomal inhibitor MG132 (1 μM for 8 h) resulted in the reduction of basal eIF2α phosphorylation (Fig. 1A), reflecting GADD34’s ability to assemble an eIF2α phosphatase. Treatment with the protein serine/threonine phosphatase inhibitors, 100 nM calyculin A (CA) or 1 μM okadaic acid (data not shown) for 30 min, further increased eIF2α phosphorylation, consistent with the inhibition of the GADD34-bound PP1 (8). To our surprise, exposure of MG132-treated HeLa Cells to 1 mM Na3VO4, a PTPase inhibitor, also elevated eIF2α phosphorylation to levels similar to CA (Fig. 1A). This suggested a role for tyrosine phosphorylation in regulating either the eIF2α kinase or phosphatase.

Ectopic expression of C-terminal hemagglutinin (HA)-fused human GADD34 (GADD34-HA), which, like the endogenous GADD34, was degraded by the 26S proteasome (10), reduced basal eIF2α phosphorylation in HeLa cells (Fig. 1B). Treatment of GADD34-HA-expressing cells with 1 mM Na3VO4 for 30 min increased basal eIF2α phosphorylation above that seen in untreated cells (Fig. 1B). Using microcystin (MC), a protein serine/threonine phosphatase inhibitor, immobilized on agarose, we noted equivalent pulldown of PP1 catalytic subunit and GADD34 from lysates of the control and vanadate-treated cells (Fig. 1C). Neither PP1 nor GADD34 bound to control (C) agarose. Moreover, equivalent amounts of GADD34-FLAG and the associated PP1 catalytic subunit were communoprecipi-
tated from lysates of HEK293T cells, either untreated or treated with 1 mM Na$_3$VO$_4$ or 10 µM PP1, a Src tyrosine kinase inhibitor (Fig. 1D), suggesting that the increased basal eIF2α phosphorylation seen in vanadate-treated cells did not result from the disruption or disassembly of the GADD34/PP1 complex.

Tyrosine 262 Is the Major Phosphorylated Tyrosine in Human GADD34—Metabolic labeling of GADD34-overexpressing HeLa cells with $^{32}$P orthophosphate established that human GADD34 was highly phosphorylated with the majority of phosphate residing in a region encompassed by amino acids 250 to 450 (10). Global phosphoproteome analyses in HEK293T cells showed the phosphorylation of endogenous GADD34 on tyrosine 262 and serine 264 (13). Multiple Mass Spectrometry experiments analyzed either GADD34-FLAG or FLAG-GADD34 expressed in HEK293T cells and identified 32 phosphoserines, 21 phosphothreonines, and 3 phosphotyrosines (supplemental Fig. S1). However, peptides representing the region between residues 240 and 290 as well as 341 and 371 were not detected. No peptides containing phosphotyrosine 262 and phosphoserine 264 (13), were identified.

Polyclonal and monoclonal antibodies generated against synthetic peptides encompassing phosphotyrosine 262 in human GADD34 recognized the antigen phosphopeptide with >20,000-fold selectivity over the corresponding unphosphorylated peptide but failed to show any immunoreactivity with the full-length human GADD34 protein. To establish the GADD34 phosphorylation on tyrosine 262, FLAG-GADD34 and the corresponding mutant protein, Y262F, substituting tyrosine 262 with a non-phosphorylatable amino acid, phenylalanine (F), were expressed in HEK293T cells. FLAG-GADD34, immunoprecipitated from control and vanadate-treated cells, was subjected to immunoblotting with an anti-phosphotyrosine antibody. Cells treated with orthovanadate showed an ~1.4-fold increase in tyrosine phosphorylation of WT FLAG-GADD34. The ratio of phosphotyrosine (P-Tyr) immunoreactivity to that for the FLAG epitope suggested that FLAG-GADD34 (Y262F) was associated with ~80% reduction in tyrosine phosphorylation compared with WT FLAG-GADD34 (Fig. 2A), highlighting that tyrosine 262 was the major phosphorylated tyrosine in human GADD34.

Immunoprecipitation of WT FLAG-GADD34 and mutant FLAG-GADD34 (Y262F) established that both proteins bound PP1 (Fig. 2B). Pulldowns using microcystin-coupled agarose (MC) also confirmed the equivalent association of PP1 with both mutant GADD34 proteins containing either Y262F or the phosphomimetic substitutions, Y262E or Y262D (Fig. 2C). Together with the data shown in Fig. 1D, this demonstrated that the covalent modification at tyrosine 262 did not impair PP1 binding to GADD34.

Tyrosine 262 Phosphorylation Enhances GADD34 Protein Turnover—GADD34 is a short-lived protein (10, 11). To investigate the potential contribution of tyrosine 262 phosphorylation in GADD34 protein turnover, the decay rate for GADD34 was analyzed following cycloheximide (CHX) treatment of HEK293T expressing either WT GADD34-FLAG or mutant GADD34(Y262F)-FLAG. Unlike the fusion of N-terminal epitopes, the C-terminal FLAG epitope did not alter the rate of GADD34 protein turnover (10). However, compared with WT GADD34-FLAG with $t_{1/2} < 1$ h, GADD34(Y262F)-FLAG displayed a significantly increased $t_{1/2} > 2$ h (Fig. 3, A and B). Vanadate treatment of HEK293T cells expressing WT GADD34-FLAG had no modest effect on the rate of GADD34 degradation (Fig. 3C) that was not significant in multiple experiments (Fig. 3D). Finally, analysis of the GADD34 mutants e.g. Y118F or S264A, the latter shown to be phosphorylated in cells (13), had no effect on the rate of protein turnover of GADD34-FLAG (Fig. 3E). These data highlighted the unique contribution of tyrosine 262 phosphorylation in enhancing GADD34 protein turnover.

Tyrosine 262 Phosphorylation Is Enhanced by GADD34 Association with ER Membranes—An N-terminal amphipathic helix-mediated GADD34 association with ER membranes and the substitution, V25R, redistributed the mutant GADD34 protein to the cytosol, slowing its degradation by the proteasome
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Analysis of COS7 cells expressing WT GADD34-GFP or GADD34 (Y262F)-GFP established that both proteins were localized at ER, immunostained with anti-TRAPα (Fig. 4A). Thus, subcellular distribution could not account for the increased stability of GADD34 (Y262F)-GFP protein.

Comparison of WT GADD34-GFP and GADD34(Y25R)-GFP, immunoprecipitated from HEK293T cells, suggested the reduced tyrosine phosphorylation of the largely cytosolic GADD34(Y25R)-GFP (Fig. 4B). Vanadate increased the tyrosine phosphorylation of WT GADD34-FLAG and to a lesser degree, GADD34(Y25R)-FLAG. However, no change in tyrosine phosphorylation of WT GADD34-FLAG and to a lesser degree, GADD34(Y25R)-FLAG was observed following vanadate treatment (Fig. 4C). These data suggested that ER association promotes the vanadate-mediated tyrosine phosphorylation of GADD34.

TC-PTP is a Candidate GADD34 (Tyrosine 262) Phosphatase—

TC-PTP (T cell protein tyrosine phosphatase) and PTP-1B (protein tyrosine phosphatase-1B) are structurally related enzymes that are broadly expressed in mammalian tissues. Both PTPases associate with ER and have been implicated in modulating ER stress (14, 16, 17). WT GADD34-GFP but not the mutant GADD34(Y262F)-GFP bound the substrate-trapping mutant, FLAG-TC-PTP(C/S) and was co-immunoprecipitated from HEK293T lysates. Moreover, the binding of WT GADD34-GFP to FLAG-TC-PTP(C/S) was reduced in the presence of vanadate, a competitive active site inhibitor (Fig. 5A). This identified TC-PTP as a potential or candidate GADD34(Y262) phosphatase. By contrast, the substrate-trapping mutant, GST-PTP1B(C/S), did not bind WT GADD34-GFP or GADD34(Y262F)-GFP (Fig. 5B) although some nonspecific binding of GADD34-GFP proteins was observed to glutathione-agarose. This suggested that PTP-1B did not recognize the tyrosine 262 phosphorylated GADD34.

Reduced GADD34 Induction by Stress in TC-PTP-null Cells—

GADD34 protein was undetected in WT (+/+ ) and TC-PTP(−/−) MEFs in the presence of vehicle (DMSO). Exposure to thapsigargin (TG), arsenite (As), and the eIF2α phosphatase inhibitor, Sal003 (19) induced GADD34 in these cells within 24 h. Much lower levels of GADD34 protein were induced in
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TC-PTP(−/−) compared with WT (+/+ ) MEFs (Fig. 6A). In numerous independent experiments, thapsigargin (TG) induced ~70% less GADD34 in TC-PTP-null MEFs compared with WT MEFs. By contrast, the levels of GADD34 protein induced by the proteasome inhibitor, MG132, were comparable in the two cells (Fig. 6, B and C). These data hinted at a potential role for TC-PTP in modulating proteasomal degradation of GADD34.

Despite the reduced levels of stress-induced GADD34 protein seen in the TC-PTP(−/−) MEFs at 24 h (Fig. 6A), the early phases of ER stress (0 to 6 h following thapsigargin (TG) treatment), exemplified by eIF2α phosphorylation and expression of ATF4, CHOP, and GADD34, were not significantly different between WT (+/+ ) and TC-PTP(−/−) MEFs (Fig. 7A). The ER stress-induced changes in mRNA levels for GADD34, ATF4, CHOP, and Xbp1 analyzed in the two cells using RT-qPCR were also not significantly different (Fig. 7B). Finally, no differences in Xbp1 splicing were noted in the two cells (Fig. 7C). Thus, the initiation of ER stress signaling by PERK and IRE1 was not impaired in the TC-PTP-null cells.

Loss of TC-PTP Sensitizes Cells to ER Stress-induced Cell Death—Thapsigargin (TG) and tunicamycin (TN) induced greater rounding and detachment from the culture dish with TC-PTP(−/−) than WT(+/+ ) MEFs. MG132, which resulted in equivalent induction of GADD34 protein in both cells, had no effect on their cell number or morphology (Fig. 8A). However, TC-PTP(−/−) MEFs displayed significantly reduced viability to exposure to thapsigargin (0.5 μM or 1 μM) and tunicamycin (5 μg/ml or 10 μg/ml) compared with WT(+/+ ) MEFs at 12 and 24 h (Fig. 8B).

Thapsigargin-treated TC-PTP(−/−) cells showed increased Annexin V and propidium iodide (PI) staining (Fig. 9A), DNA fragmentation (Fig. 9B), cleaved PARP (Fig. 9C) and cleaved or activated caspase-3 (Fig. 9D) compared with WT(+/+ ) MEFs. These data suggested the increased activation of apoptosis in TC-PTP(−/−) MEFs by ER stress. Ectopic expression of WT GADD34-GFP in TC-PTP(−/−) MEFs significantly attenuated the programmed cell death induced by thapsigargin (TG) and tunicamycin (TN) (Fig. 9E).

DISCUSSION

Accumulation of misfolded proteins activates the ER sensors, PERK, ATF6, and IRE1. ATF6 by increasing chaperone expression and IRE1 by enhancing ERAD (ER-associated protein degradation) together precondition cells to better tolerate subse-
quent bouts of stress (20). By contrast, PERK-mediated eIF2α/H9251 phosphorylation transiently attenuates general protein synthesis while enhancing expression of the transcription factors, ATF4 (4) and CHOP (21). Emerging evidence points the cooperation of ATF4 and CHOP in activating numerous genes including GADD34 (4, 21), which assembles an eIF2α/H9251 phosphatase that restores mRNA translation. GADD34 overexpression is also proapoptotic (22), possibly triggering oxidative stress (23, 24). Overexpression of its upstream activators, ATF4 and CHOP, suggested that GADD34-dependent and -independent mechanisms result in increased protein synthesis and oxidative stress to promote apoptosis (Fig. 10) (24). Thus, cells possess robust mechanisms for eliminating ATF4, CHOP and GADD34 mRNAs and proteins following the resolution of ER stress (10).

Focusing on GADD34, we previously showed that its short half-life (less than 1 h) resulted from polyubiquitination and degradation by the 26S proteasome (10). Moreover, its dynamic trafficking from cytosol to ER accelerated GADD34 protein turnover (11).

Reversible protein phosphorylation also plays a role in modulating protein turnover (25). In this context, our prior work showed that GADD34 is highly phosphorylated in cells, with the region between by amino acids 180 to 460 representing the major site of covalent modifications (10). Current work utilized mass spectrometry of GADD34 ectopically expressed in HEK293T cells to identify 32 phosphoserines, 21 phosphothreonines, and 3 phosphotyrosines (supplemental Fig. S1). While the identified peptides accounted for 80% of the primary sequence of GADD34, no peptides representing amino acids 240 to 289 were detected despite the digestion of GADD34 with different proteases and the phosphorylation of GADD34 at tyrosine 262 (or serine 264) previously shown using electron transfer dissociation (ETD) mass spectrometry (13) could not be confirmed. Efforts to generate phosphospecific antibodies against peptides encompassing Y262 also failed to resolve this issue.

The observation that vanadate, a PTPase inhibitor, enhanced basal eIF2α phosphorylation similar to okadaic acid, an inhib-
The N-terminal substitution, V25R, prevented ER localization of this mutant GADD43 and reduced its rate of protein turnover (11). GADD43(Y262F)-GFP, like WT GADD43-GFP, was however targeted to ER membranes. The cytoplasmic protein, GADD43(V25R), showed much reduced tyrosine phosphorylation compared WT GADD43-GFP, suggesting that association with the ER membranes may enhance tyrosine phosphorylation of GADD43. To identify potential ER-bound GADD43 regulators, we analyzed two PTPases, PTP1B and TC-PTP, previously shown to modulate ER stress (16, 17). Comparison of substrate-trapping mutants of TC-PTP(C/S) and PTP-1B(C/S) established that TC-PTP(C/S) but not PTP1B(C/S) bound WT GADD43. This binding was reduced by the Y262F substitution and by the addition of vanadate, a catalytic site-directed PTPase inhibitor (26). Consistent with TC-PTP's role in regulating Y262 phosphorylation and in turn, GADD43 protein turnover, the levels of GADD43 protein induced by stressors in TC-PTP-null MEFs were significantly reduced compared with either WT or PTP1B-null MEFs. The reduced GADD43 levels likely reflected its enhanced protein turnover in the TC-PTP-null cells as proteasome inhibition with MG132 induced similar levels of GADD43 in both WT and TC-PTP-null MEFs.

The TC-PTP-null MEFs were more sensitive than WT MEFs to ER stress-induced apoptosis, characterized by DNA fragmentation, Annexin V labeling, caspase-3 cleavage, and PARP cleavage. However, the early steps in ER stress signaling were indistinguishable in the WT and TC-PTP-null MEFs. Regardless, the ectopic expression of GADD43 protected the TC-PTP(--/-) cells from ER stress-induced cell death, suggesting a GADD43-mediated mechanism for cell survival (Fig. 9). In contrast, earlier studies used shRNA-mediated knockdown of TC-PTP to show that the loss of TC-PTP expression protected MIN6 cells from ER stress-induced apoptosis (16). This may simply highlight that TC-PTP regulates substrates other than GADD43 to control ER stress in different cell types.

The current studies did not identify the GADD43 (Y262) kinase but cell treatments with a broad specificity Src family kinase inhibitor PP1, reduced GADD43 tyrosine phosphorylation (data not shown) pointing to member(s) of this family as potential GADD43 kinases. Interestingly, the four tyrosines, Y262, Y391, Y434, and Y512, phosphorylated in human GADD43, reside within similar primary sequences with the consensus, Wv/iYr/qPGE, perhaps suggesting a single or common tyrosine kinase. Thus it is noteworthy that prior studies reported GADD43 phosphorylation in B-lymphocytes by Lyn, an ER-localized Src family tyrosine kinase (27). However, these studies did not identify the modified tyrosines and did not investigate the role of tyrosine phosphorylation in Lyn-mediated inhibition of GADD43 function.

The ability of ER stress to modulate tyrosine phosphorylation of ER stress signaling proteins and more specifically, alter the levels and/or activity protein tyrosine phosphatases, has only recently been apparent. For example, prolonged ER stress increased the expression of PTP1B in pancreas (16), muscle (28), and liver (29) while reducing TC-PTP levels in the same cells (16). Moreover, PTP1B and TC-PTP appeared to have opposing effects on ER stress (16). The antagonism between
PTP1B and TC-PTP has been highlighted in the regulation of Src. Specifically, PTP1B activates Src, reversing the inhibitory phosphorylation on tyrosine 527 (30, 31). By contrast, TC-PTP inhibits Src by dephosphorylating phosphotyrosine 416, an activating modification (32). The ER stressors, tunicamycin (31) and thapsigargin (33), both activate Src tyrosine kinase to phosphorylate the transcription factor, TFII-I and enhance transcription of the gene, encoding CHOP, and ATF4 and CHOP collaborate to promote the expression of GADD34 as well as many autophagy genes (35). Finally, GADD34 assembles an eIF2α phosphatase restores general protein synthesis. ATF4 and CHOP also collaborate to turn on numerous genes that may promote both GADD34-dependent and GADD34-independent protein synthesis (23, 24) and the ensuing oxidative stress plays a key role in triggering programmed cell death (24). CHOP, possibly in conjunction with ATF4, enhances the transcription of numerous proapoptotic genes (36). Thus, the current evidence suggests that both the phosphorylation and dephosphorylation of eIF2α is a key regulatory mechanism in the control of cell fate following ER stress.

Recent studies showed that ER stress also regulates PTPase activity. Specifically, tunicamycin and thapsigargin promoted the transient sulfydration of the catalytic cysteine, conserved in all PTPases, to inhibit PTP1B (34). This activated PERK, facilitating its autophosphorylation on phosphotyrosine 615, and increased eIF2α phosphorylation (34). Paradoxically, in the PTP1B-null mice, ER stress suppressed eIF2α phosphorylation in liver (29) and muscle (28) but increased this modification in adipose tissue (14). This suggests significant tissue-specific differences, potentially involving other PTP1B substrates, in controlling eIF2α phosphorylation. Current studies demonstrated, for the first time, that a key component of the stress-activated eIF2α phosphatase, GADD34, was also subject to tyrosine phosphorylation and identified TC-PTP as a candidate GADD34 phosphatase. Our studies suggested that TC-PTP by reversing the phosphorylation of Y262 increased cellular GADD34 protein levels. While further studies are needed to establish the utilization of this regulatory mechanism in mammalian tissues, our data provided an example of potential cooperation between PTP1B, which inhibits PERK activity, and TC-PTP, which stabilizes the GADD34 protein, to control eIF2α phosphorylation (Fig. 10) and fine-tune downstream signaling that determines cell fate following ER stress.

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