Herp Stabilizes Neuronal Ca\(^{2+}\) Homeostasis and Mitochondrial Function during Endoplasmic Reticulum Stress*

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In response to endoplasmic reticulum (ER) stress, cells launch homeostatic and protective responses, but can also activate cell death cascades. A 54 kDa integral ER membrane protein called Herp was identified as a stress-responsive protein in non-neuronal cells. We report that Herp is present in neurons in the developing and adult brain, and that it is regulated in neurons by ER stress; sublethal levels of ER stress increase Herp levels, whereas higher doses decrease Herp levels and induce apoptosis. The decrease in Herp protein levels following a lethal ER stress occurs prior to mitochondrial dysfunction and cell death, and is mediated by caspases which generate a 30-kDa proteolytic Herp fragment. Mutagenesis of the caspase cleavage site in Herp enhances its neuroprotective function during ER stress. While suppression of Herp induction by RNA interference sensitizes neural cells to apoptosis induced by ER stress, overexpression of Herp promotes survival by a mechanism involving stabilization of ER Ca\(^{2+}\) levels, preservation of mitochondrial function and suppression of caspase 3 activation. ER stress-induced activation of JNK/c-Jun and caspase 12 are reduced by Herp, whereas induction of major ER chaperones is unaffected. Herp prevents ER Ca\(^{2+}\) overload under conditions of ER stress and agonist-induced ER Ca\(^{2+}\) release is attenuated by Herp suggesting a role for Herp in regulating neuronal Ca\(^{2+}\) signaling. By stabilizing ER Ca\(^{2+}\) homeostasis and mitochondrial functions, Herp serves a neuroprotective function under conditions of ER stress.

The endoplasmic reticulum (ER) is a unique cellular compartment simultaneously involved in the processes of protein synthesis and Ca\(^{2+}\) homeostasis. Various conditions, including oxidative and metabolic stress and Ca\(^{2+}\) overload can interfere with ER functions leading to the accumulation of misfolded proteins. Cells sense and respond to such ER stress by activating a signaling cascade termed the unfolded protein response, which results in the transcriptional up-regulation of stress proteins including members of the glucose-regulated protein (grp) family and other protein chaperones (calnexin, calreticulin, ERp72) that enhance the protein folding capability of the ER (1). ER stress has been documented in neurons in a variety of diseases including cerebral ischemia and severe epileptic seizures (2). However, despite the fact that disruption of cellular Ca\(^{2+}\) homeostasis contributes to the death of neurons in these conditions, it is not known how molecular responses to ER stress modify cellular Ca\(^{2+}\) homeostasis and the cell death process. Studies of cultured cells suggest that ER stress can stimulate the expression of cytoprotective genes such as protein chaperones (3) but may also trigger a form of programmed cell death called apoptosis (4), which may involve activation of ER-associated caspases and transcription factors such as Gadd153. A better understanding of ER stress and its links to cell survival/death decisions is therefore needed.

Recent findings suggest that ER stress is also implicated in several chronic neurodegenerative disorders including Alzheimer’s (5, 6), Parkinson’s (7), and Huntington’s (8) diseases. Alzheimer’s disease (AD) results from altered proteolytic processing of the amyloid precursor protein (APP), resulting in aggregation of neurotoxic forms of amyloid β-peptide (Aβ) (9). Exposure of cultured neurons to Aβ peptide, and metabolic and oxidative insults can induce an ER stress response (6, 10). Moreover, mutations in presenilin-1 (PS1) that cause early-onset familial AD perturb ER Ca\(^{2+}\) homeostasis (11, 12) and impair the ability of neurons to engage a cytoprotective ER stress response (20). The adverse effects of Aβ and PS1 mutations on ER function may sensitize neurons to excitotoxicity and apoptosis (11).

A novel 54 kDa protein called Herp (homocysteine-induced ER protein) was recently identified and characterized as a stress-responsive protein localized in the ER membrane; Herp contains a ubiquitin-like domain and resembles the human DNA excision repair protein hHR23 (13). The function of Herp is unknown. Sai et al. (14) showed that Herp binds to PS1 and alters APP processing in HEK-293 cells, although it is not known whether the interaction affects the PS1 role in regulating ER Ca\(^{2+}\) homeostasis, which might also contribute to altered APP processing in neurons (15). In the present study we demonstrate a role for Herp in stabilizing cellular Ca\(^{2+}\) homeostasis and preventing neuronal death following ER stress.

EXPERIMENTAL PROCEDURES

Neuronal Cell Cultures—Hippocampal and cortical cell cultures were established from 18 day Sprague-Dawley rat embryos as described previously (16). Briefly, intact hippocampi and neocortical fragments were trypsinized, and cells were dissociated by mild trituration using a...
Pasture pipette with a fire-polished tip. Cells were seeded into polylysine-coated plastic 35- or 60-mm diameter plastic dishes or 22 mm glass coverslips, and maintained at 37 °C in Neurobasal medium containing B-27 supplements (Invitrogen), 2 mM l-glutamine, 0.001% gentamycin sulfate and 1 mM HEPE (pH 7.2). All experiments were performed using 7–8-day-old cultures; greater than 90% of the cells in these cultures were neurons.

Generation of DNA Constructs and Stably Transfected PC12 Cell Lines—Plasmids containing the full-length human Herp cDNA or full-length human Herp with an N-terminal c-Myc tag and a C-terminal FLAG tag were constructed as described previously (13). Site-directed mutagenesis was performed to generate a PCR-based primer overlap extension method. In brief, same pair of primers and two different mers in ‘vernese priming’ primer were used. The PCR primers were 5′-CCGCGATCCCTTTTTTTTTTAATGAGTCCCGAGACC-3′ (forward) and 5′-CCGGATTACATCGTATGCGTTCGGG-3′ (reverse). The PCR products that contained the mutated sequence were cloned into the PCRII TOPO TA cloning vector (Stratagene), which was then amplified and digested with BamHI and EcoRII and subcloned into the pcDNA3.1 vector. The mutation was confirmed by automated DNA sequencing (ABI Prism 3700 DNA analyzer). Transfection of PC12 cells was carried out using the LipofectAMINE reagent using the manufacturer’s protocol (Invitrogen). Stably transfected clones were obtained after selection for growth in the presence of gentamicin (500 μg/ml) and characterized for Herp expression by immunoblot analysis.

Experimental Treatments—To induce ER stress, undifferentiated PC12 cells were treated with tunicamycin, thapsigargin, homocysteine, or 2-mercaptoethanol (Sigma) for various time points as indicated. At the end of each treatment, cultures were processed for biochemical analyses of levels of cytoplasmic and ER stress proteins, and for evaluating the extent of cell death. The caspase inhibitor z-VAD-fmk (BioMol) was prepared as a 500× stock in dimethyl sulfoxide. Amylloid β-peptide-(1–42) (Bachem) was prepared as a 1 mM stock in water, which was diluted 1:1000 overnight at room temperature prior to dilution into culture medium.

siRNA Preparation and Nucleofection—Several 19-base pair (bp) sequences were selected from the rat Herp sequence and synthesized with the siRNA Construction kit (Ambion). siRNA duplexes targeting Herp (siRNA_{Hep}) were prepared as described. Briefly, 100 nm sense primers (5′-AACACACCGGGCTTTTGTT-3′ for siRNA_{Hep}, 5′-AAUCUGACUUCUGGAAATdT-3′ for siRNA_{Hep}, 5′-AAUCUGACUCUGGAAATdT-3′ for siRNA_{Hep}) and corresponding antisense primers were mixed in 50 μM of the final concentration synthesized using scrambled sequences as described above. GenBank™ search revealed no other known genes exhibit sequence homology to the selected target sequences. After thorough mixing of the cells and 0.5–1 μg of siRNA duplexes, transfection was immediately carried out using the Cell Line Nucleofector Kit V according to the manufacturer’s protocol for PC12 cells. Mock-transfected cells were nucleofected with vehicle (water).

RNA Isolation and Reverse Transcriptase-PCR—Total RNA was extracted from primary neuronal cultures using TRIzol reagent (Invitrogen). 2 μg of RNA was used for synthesis of cDNA using random primers and the M-MLV reverse transcriptase kit (Invitrogen). 500 ng of the cDNA was used in the PCR reaction using the following pairs of primers: Herp 5′-GAGGAAGATGTAATAATCCGAGAT-3′ (forward) and 5′-TCAGTTGTGCATTGCGTTCGG-3′ (reverse); β-actin 5′-TGGTGAAGCCTGCGGTCGAC-3′ (forward) and 5′-ACAGCTTCTTCCTGATGTGACCG-3′ (reverse). The optimized PCR conditions were 2 min at 94 °C, 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, followed by a 1 h incubation in primary antibody diluted in blocking solution at room temperature. Membranes were then incubated in 1 h in secondary antibody conjugated to horseradish peroxidase, and bands were visualized using a chemiluminescence detection kit (ECL, Amer sham Biosciences). The primary antibodies included: rabbit polyclonal antibody against Grp78, Grp94, and Hsp60 (StressGen), β-tubulin, and α-actin (Sigma), Herp (13), JNK (C-17, Santa Cruz Biotechnology) and phospho-c-Jun (Ser63, Cell Signaling); mouse monoclonal antibodies against Hsp70 (Sigma), PS1 (Chemicon), p-JNK (G7, Santa Cruz Biotechnology), cytochrome c (PharMingen), and Bcl-2 (StressGen); chicken antibody against calreticulin (ABR); rat monoclonal antibody against α-tubulin from I. Yuan, Harvard University (goat antibody against c-Jun (sc-45, Santa Cruz Biotechnology).

Immunoprecipitation—A aliquots of cell lysates or brain homogenates containing 100 μg of total protein were incubated with mouse monoclonal anti-c-Myc, anti-FLAG tag, or rabbit polyclonal anti-Herb antibodies in immunoprecipitation buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Nonidet, 1% of 5 μg/ml leupeptin, 2 μg/ml pepstatin A, 0.25 mM phenylmethylsulfonyl fluoride). Antigen-antibody complexes were precipitated with protein A or G for 1 h at 4 °C, washed three times in immunoprecipitation buffer, and solubilized by heating by Lammli buffer containing 2-mercaptoethanol at 100 °C for 5 min.

In Vitro Protein Synthesis and Caspase Cleavage—Two oligonucleotides, 5′-GGGTTACATATGCTGGGAATGAGTCCGAGACC-3′ and 5′-GGATTACATGTTGCGAATGGCTGGG-3′, were used to PCR amplify the entire open reading frame of Herp. The product, digested with KpnI and EcoRII, was ligated into the corresponding site of a plasmid vector, pZeoSV2 (Invitrogen), with a T7 priming site. Using the resultant recombinant plasmid, pZeoSV2/Herp as a template, Herp was synthesized in vitro by the TnT T7 quick-coupled transcription/translation systems (Promega). [35S]Met-labeled full-length Herp protein was obtained by in vitro transcription and translation using the Promega Coupled kit (Promega) and subjected to cleavage by purified caspase-3 (gift from G. Salvesen, The Burnham Institute) as described (18).

Immunofluorescence Confocal Microscopy—Following experimental treatments, cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, permeabilized with 0.2% Triton X-100 in PBS for 10 min, followed by a 30-min incubation in the presence of 5% non-immune horse serum, and incubation in the presence of primary antibodies for 2 h or overnight. The primary antibodies included rabbit polyclonal antibodies against α-tubulin (1:1000, Grp94; StressGen), and mouse monoclonal antibodies against c-Myc (1:2000, Santa Cruz Biotechnology) and PS1 (1:500, Chemicon). Cells were then incubated for 1 h in PBS containing fluorescein isothiocyanate (FITC)-labeled horse anti-mouse IgG or Texas Red-labeled goat anti-rabbit secondary antibodies (1:50 dilution in PBS; Vector Laboratories). Cells were then washed with PBS, and images of fluorescence were acquired using the Zeiss LSM 510 confocal laser-scanning microscope.

Analyses of Cell Death and Mitochondrial Membrane Potential—Cell viability was assessed using the fluorescent DNA binding dye Hoechst 33342 or by the trypan blue exclusion method as described previously (18). Mitochondrial membrane potential was assessed using the fluorescent probe TMRE (Molecular Probes). Briefly, cells were incubated for 30 min in the presence of 100 nM TMRE, washed three times in fresh culture medium, and confocal images of cellular TMRE fluorescence were acquired using a confocal microscope (485 nm excitation and 510 nm emission). The average pixel intensity in individual cell bodies was determined using the software supplied by the manufacturer (Zeiss). Triplicate cultures were used for each condition, and images were coded and analyzed without knowledge of experimental treatment history of the cultures.

Cytochrome c Release—Cytochrome c release was analyzed by Western blotting. Proteins were extracted in lysis buffer containing 250 mM sucrose, 20 mM Hepes, pH 7.5, 10 mM KC1, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, and 100 μg/ml of the proteinase inhibitor set (Roche, Applied Science). Homogenates were centrifuged twice at 750 × g for 15 min at 4 °C, and the supernatant represents the cytosolic fraction. Both the mitochondrial and cytosolic fractions were subjected to immunoblot analysis. The remaining supernatant represents the cytosolic fraction. Both the mitochondrial and cytosolic fractions were subjected to immunoblot analysis.

Caspase Activity Measurement—Caspase activity was assessed using a method described previously (11). Briefly, after exposure of cells to tunicamycin for designated time periods, cell membranes were permeabilized by incubation of cells in a solution of 0.01% digitonin in PBS for 5 min. Cells were then incubated for 30 min in PBS containing biotinylated antivD-FITC (a subunit of CDV-DCHO) (a substrate of caspases 6, 7, 8, and 10) for 30 min. Cells were then fixed in a solution of 4% paraformaldehyde in PBS for 20 min, incubated for 5 min in PBS containing 0.2% Triton X-100, and then incubated for 30 min in PBS containing Oregon green-streptavidin (Molecular Probes). Images of fluorescence were...
FIG. 1. Herp is constitutively expressed in the brain where its levels change during development. A, immunoblot analysis showing relative levels of Herp and β-tubulin proteins in samples of cerebral cortex from mice of the indicated ages from embryonic day 11 (E11) to postnatal day 2 (P2) and a 3-month-old adult. B, immunoblot showing Herp protein levels in the cerebral cortex, hippocampus, striatum, and cerebellum of three different 3-month-old adult mice and rats. C, immunoblot showing Herp and grp94 protein levels in nuclear, microsomal, mitochondrial, and cytosolic fractions of cerebral cortex tissue from an adult mouse. D, immunoblots showing levels of Herp protein in cultured rat primary cortical neurons before and after exposure to 1 μM thapsigargin, 1 μg/ml tunicamycin, or 100 μM homocysteine. For each immunoblot each lane was loaded with 50 μg of protein. E, levels of Herp mRNA in cultured rat primary cortical neurons before and after exposure to 1 μM thapsigargin and 1 μg/ml tunicamycin.

captured using a confocal laser-scanning microscope, and the average pixel intensity in individual cell bodies was measured using software supplied by the manufacturer.

Calcium Imaging—Levels of intracellular free Ca^{2+} ([Ca^{2+}]_i) were quantified by fluorescence imaging of the calcium indicator dye fura-2 as described previously (18). Briefly, cells were incubated for 30 min in the presence of 2 μM acetoxyethyl ester form of fura-2 (Molecular Probes) and then washed twice in Locke’s buffer (mM: NaCl, 154; KCl, 5.6; CaCl_2, 2.3; MgCl_2, 1.0; NaHCO_3, 3.6; glucose, 10; Hepes 5, pH 7.2) and allowed to incubate an additional 20–30 min to allow complete deesterification of the dye. Measurement of ER Ca^{2+} content was performed by washing and imaging the cells in Ca^{2+}-free Locke’s buffer upon addition of 1 μM thapsigargin. Cells were imaged on a Zeiss Axioplan microscope (×40 oil immersion objective) coupled to an Attocslr imaging system. The average [Ca^{2+}]_i, in 40–60 cells per microscope field was quantified in four separate cultures per treatment condition.

Subcellular Fractionation—Pellets of frozen PC12 cells and cortical brain tissues were homogenized in ice-cold fractionation buffer pH 7.4 (20 mM HEPES, 10 mM KCl, 250 mM sucrose, 1.5 mM MgCl_2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and a protease inhibitor mixture). Nuclei were pelleted by a 10-min spin at 750 × g and the supernatant was recovered and centrifuged at 10,000 × g for 30 min. The mitochondrial pellet was resuspended in fractionation buffer, and the supernant was reconstituted for 1 h at 100,000 × g. The resulting supernatant contained the soluble cytosolic fraction, and the pellet constituted the microsomal fraction. The purity of the microsomal fraction was confirmed by the presence of grp94, an ER lumen protein.

RESULTS

Herp Is Present in the ER in the Developing and Adult Brain and Its Levels Are Markedly Increased in Neurons Subjected to ER Stress—We first examined Herp protein levels in the mouse brain during development. Herp was present at relatively high levels in the cerebral cortex during embryonic development from ages E11 to E15, but levels decreased between E15 and postnatal day 2, and were decreased further in adult mice (Fig. 1A). A developmental decrease in constitutive expression of Herp similar to that seen in the cerebral cortex also occurred in the hippocampus and cerebellum (data not shown). The differential expression of Herp from E11 to postnatal day 2 suggests that Herp may play an important role during CNS development. In the adult rat and mouse, Herp protein levels were similar in the cerebral cortex, hippocampus and cerebellum (Fig. 1B). Next, we investigated the subcellular localization of Herp in adult brain cells. Brain homogenates were fractionated into nuclear, mitochondrial, microsomal, and soluble fractions and analyzed by immunoblotting (Fig. 1C). Herp was predominantly found in the microsomal fraction along with grp94 suggesting that, as is the case in non-neuronal cells (13), Herp is also an ER-resident protein in neural cells. To determine whether Herp is induced by ER stress in neurons, we examined the time course of increase in Herp mRNA and protein levels in cultured rat cortical neurons after treatment with agents that induce ER stress. Basal levels of Herp mRNA and protein were very low in the cultured cortical neurons, but increased rapidly within 6 h of exposure to a subtoxic concentration tunicamycin (an agent that inhibits N-glycosylation) or thapsigargin (an inhibitor of the ER Ca^{2+}-ATPase that induces depletion of ER Ca^{2+}) (Fig. 1D and E). Homocysteine (Fig. 1D) and 2-mercaptoethanol (data not shown), agents that modify the redox environment of the ER, also induced an elevation in Herp protein within 6 h. The up-regulation of Herp was specific for ER stress, because Herp levels did not increase in neurons subjected to serum deprivation, which induces apoptosis (data not shown).

Overexpression of Herp Protects PC12 Cells against ER Stress-induced Cell Death—To determine the consequences of
Herp used as positive control (CON). For each immunoblot each lane was loaded with an equal amount (50 μg) of total protein as confirmed by the actin band.

an increase in Herp protein levels during the ER stress response, we overexpressed Herp, or Herp-tagged with an N-terminal c-Myc tag and a C-terminal FLAG tag, in PC12 cells (Fig. 2A). Several clones stably overexpressing Herp were selected, and the level of Herp overexpression and its subcellular localization were analyzed by immunoblot and immunocytochemical analyses. The levels of Herp and tagged Herp (t-Herp) proteins in extracts of stably transfected PC12 were 4–6-fold higher than the endogenous level of Herp in VT cells (Fig. 2B). Confocal images of Herp immunoreactivity revealed localization to reticular structures in the cytoplasm, which were also stained with the anti-grp94 antibody demonstrating that Herp was mainly localized in the ER (Fig. 2C) as previously reported (13). The tags did not affect protein localization, because the staining pattern of t-Herp was identical to that of the untagged protein. The levels of ectopic expression of Herp protein were approximately 2–6-fold higher than the total inducible Herp protein levels achieved in cells following exposure to 5 and 20 μg/ml tunicamycin, respectively, for 12 h (Fig. 2D), a time point at which greater than 80% of the cells exposed to 20 μg/ml tunicamycin were still viable (see also Fig. 3A). The effect of increased Herp levels on the vulnerability of cells to ER stress-induced death was determined by exposing Herp-overexpressing and VT cells to increasing concentrations of tunicamycin. Compared with VT cells, Herp-overexpressing cells were significantly more resistant to death induced by tunicamycin (Fig. 3A). After 24 h of exposure to a toxic concentration of tunicamycin (20 μg/ml), 77% of the VT cells were apoptotic compared with 42% of cells overexpressing Herp. Herp-overexpressing cells were also more resistant to ER stress-induced apoptosis following a prolonged exposure to a subtoxic concentration of tunicamycin (5 μg/ml). Similar results were obtained when thapsigargin (100 nM) was applied as the ER stressor (Fig. 3B). We also tested several other clones overexpressing Herp, and they were consistently more resistant to cell death induced by ER stress but were not more resistant to death induced by serum withdrawal (data not shown).

siRNA Inhibition of Herp Expression Increases Vulnerability of Neurons to ER Stress-induced Cell Death—If induction of Herp expression during the ER stress response is critical for maintaining neuronal survival, then suppressing Herp expression should have adverse effects on viability. To suppress Herp we used RNA interference (RNAi), a process of posttranscriptional gene silencing that inhibits, with high specificity, the expression of genes in mammalian cells (19). The transfection of small interfering RNA duplexes (siRNA) into naïve PC12 cells was optimized using the nucleofection method as described “Experimental Procedures.” Among the three siRNAs targeting Herp (siRNAHerp1–3; see under “Experimental Procedures” for sequence and target mRNA of each siRNAHerp), siRNAHerp2 appeared to more effective in reducing endogenous Herp protein accumulation. Though basal expression of Herp is very low, siRNAHerp2 achieved detectable reduction of Herp protein level 24 h after transfection (Fig. 3C, upper panel). ER stress-induced expression of Herp protein was effectively suppressed in cells transfected with siRNAHerp and its levels 6 h following exposure to tunicamycin was ~6-fold lower compared with that in mock-transfected cells or cells transfected with the non-silencing control siRNA (siRNACont). Under these conditions, basal and ER stress-induced expression of two other ER stress proteins, grps78 and 94, were unaffected by the siRNA treatments (Fig. 3C, bottom panel). Next, we examined the effect of Herp inhibition on the survival of naïve PC12 cells exposed to ER stress.

Suppression of Herp expression with siRNA significantly increased sensitivity to tunicamycin-induced apoptosis. Reduction of basal Herp protein levels appeared to increase spontaneous apoptosis suggesting a central role for Herp in cell homeostasis (Fig. 3D). Cells transfected with the non-silencing control siRNA exhibited normal morphology (data not shown) and were not more sensitive to ER stress-induced apoptosis when compared with mock-transfected cells (Fig. 3D).
Overexpression of Herp Attenuates Activation of Downstream Caspases during ER Stress—In many cell types, including neurons, ER stress-induced death involves an apoptotic cascade involving activation of caspases (20). We found that tunicamycin induced an increase in caspase 3 activity in PC12 cells, and that the magnitude of caspase 3 activation was significantly...
decreased in cells overexpressing Herp (Fig. 4A). Apoptosis triggered by ER stress involves mitochondrial alterations that include membrane depolarization and release of cytochrome c (4, 21). Because Herp overexpression suppressed ER stress-induced caspase 3 activation, a caspase activated in response to cytochrome c release from mitochondria, we determined the effects of Herp overexpression on mitochondrial membrane potential and release of cytochrome c. TMRE fluorescence, an indicator of mitochondrial membrane potential, decreased in PC12 cells following exposure to tunicamycin for 24 h, and this decrease was significantly attenuated in cells overexpressing Herp (Fig. 4B). Cytochrome c release from the mitochondria, a central step in the apoptosis induced by many death stimuli, was also reduced in Herp-overexpressing cells compared with VT cells (Fig. 4C). Thus, Herp can maintain mitochondrial function and inhibit downstream events associated with ER stress-induced apoptosis such as mitochondrial depolarization and cytochrome c release.

**Herp Counteracts the Induction of the ER Stress Apoptotic Signaling Cascades Mediated by JNK and Caspase 12**—Next, we determined how Herp in the ER may influence cellular responses to ER stress upstream of mitochondria. To exclude the possibility that the protective action of Herp is mediated by changes in the level of other stress proteins, we analyzed levels of several ER and non-ER stress proteins in cells overexpressing Herp, and in VT cells. Basal levels of grp78, calreticulin, hsp70 and Bcl-2 in VT cells were comparable to those in several clones overexpressing Herp (Fig. 5A), indicating that Herp overexpression alone does not induce a stress response. In addition, Herp overexpression did not affect the magnitude or time course of induction of several ER stress proteins, including grp78, grp94 and calreticulin, following exposure to tunicamycin (Fig. 5B). On the other hand, levels of the pro-apoptotic protein gadd153 were slightly reduced in cells overexpressing Herp following exposure to 5 but not 20 μg/ml tunicamycin (Fig. 5B). Thus, the Herp-mediated neuronal protective effect is not mediated by up- or down-regulation of proteins known to regulate neuronal survival upon ER stress induction. At present, we cannot rule out the possibility that Herp overexpression may affect the induction of other genes including Bbc/PUMA (22) and BAR (23), or the redistribution of members of the Bcl-2 family of proteins such as Bax and Bak during ER stress (24).

We also determined whether Herp affects apoptosis-associated ER stress-induced signaling cascades involving c-Jun N-terminal kinase (JNK) (25) and caspase 12 (10). Recent evidence suggests that activation of the JNK/c-Jun pathway plays an important role in triggering neuronal apoptosis and that inhibition of JNK or c-Jun activity is sufficient to prevent apoptosis (26, 27). JNK pathway activation was assessed by immunoblotting using phosphorylation site-specific antibodies that recognize the phosphorylated and activated form of JNK or c-Jun (Fig. 5C). Tunicamycin-
Fig. 6. Herp stabilizes ER Ca\(^{2+}\) homeostasis during ER stress. A, representative recording showing levels of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) before and after addition of 10 \(\mu\)M bradykinin (BK; arrow indicates time of addition) to vector-transfected (VT) and Herp-overexpressing PC12 cell clones. Values are the mean and S.D. of determinations made in four separate cultures (40–60 cells assessed/culture). B, representative recording showing levels of [Ca\(^{2+}\)]\(_i\), prior to and after exposure of VT and Herp-overexpressing cells to 1 \(\mu\)M thapsigargin (Thap; arrow indicates time of addition). Values are the mean and S.D. of determinations made in four separate cultures. C, quantitative data from three different experiments showing the peak amplitude of the Thap-induced rise in [Ca\(^{2+}\)]\(_i\) in vehicle-treated cells (Con) and cells treated with 20 \(\mu\)g/ml tunicamycin for 8 and 16 h. *, \(p < 0.01\); **, \(p < 0.005\) (ANOVA with Scheffe post-hoc tests). D, quantitative data showing the resting levels of [Ca\(^{2+}\)]\(_i\), and the peak amplitude of the Thap-induced rise in [Ca\(^{2+}\)]\(_i\), in mock-transfected PC12 cells (vehicle) or cells transfected with the siRNA\(_{Hep2}\) or siRNA\(_{CTRL}\). All cultures were exposed to 5 \(\mu\)g/ml tunicamycin for 8 h prior to measurements of [Ca\(^{2+}\)]\(_i\). *, \(p < 0.01\); **, \(p < 0.005\) (ANOVA with Scheffe post-hoc tests).

In addition to the pro-apoptotic activation of p54 (JNK isoform 2) and p46 (JNK isoform 1) occurred within 6 h and was sustained in VT cells whereas the activation of this kinase was only transient in Herp-overexpressing cells. One of the best characterized targets of JNK is the c-Jun proto-oncogene; JNK phosphorylates specific serine residues in the transactivation region of c-Jun and thereby enhances its transcriptional activity. As shown in Fig. 5C, the time-dependent increase in c-Jun phosphorylation at Ser\(^{63}\) was reduced in cells overexpressing Herp.

In addition to the pro-apoptotic activation of JNK, ER stress induced by tunicamycin also promotes the activation of the ER-localized pro-caspase 12 (20). Fig. 5D shows that Herp attenuated the proteolytic activation of pro-caspase 12 which, compared with caspase-3 activation (Fig. 4A), also occurs very rapidly in VT cells. Sai et al. (14) recently showed that Herp physically interacts with PS1, an ER integral membrane protein that has been implicated in ER Ca\(^{2+}\) homeostasis (11, 12). Since mobilization of the ER Ca\(^{2+}\) store and increase in the intracellular Ca\(^{2+}\) concentration could play a role in the activation of JNK (28) and caspase 12 (29), we performed a series of studies to determine whether Herp affects ER Ca\(^{2+}\) homeostasis under conditions associated with ER stress.

**Herp Stabilizes ER Ca\(^{2+}\) Homeostasis in Neural Cells Subjected to ER Stress**—The ER plays a critical role in maintaining and regulating levels of intracellular Ca\(^{2+}\), a messenger critical for diverse cellular functions and regulator of neuronal cell survival and death (6, 30). Recent findings suggest that ER Ca\(^{2+}\) stores are also targets of diverse apoptotic stimuli, such that stress-induced dysregulation of ER Ca\(^{2+}\) homeostasis plays a major role in triggering apoptosis (2, 6). We therefore determined if and how ER stress affects cellular Ca\(^{2+}\) homeostasis and whether Herp modifies Ca\(^{2+}\) homeostasis and signaling in neurons. First, we investigated the effects of overexpressing Herp on ER Ca\(^{2+}\) release and content in PC12 cells. The basal [Ca\(^{2+}\)]\(_i\), in VT and Herp overexpressing cells was similar, averaging ~50–60 nM (Fig. 6A). Bradykinin (BK), an agonist that activates cell surface receptors coupled to IP\(_3\) production, evokes a rapid and transient increase of [Ca\(^{2+}\)]\(_i\), the amplitude of which was significantly decreased by 44% in cells overexpressing Herp compared with VT cells (Fig. 6A). The ER Ca\(^{2+}\) store was measured as the rapid increase in [Ca\(^{2+}\)]\(_i\), on addition of thapsigargin to cells incubated in Ca\(^{2+}\)-free medium. The peak [Ca\(^{2+}\)]\(_i\) elevation induced by thapsigargin in VT cells (vehicle) was significantly decreased by 49% in cells overexpressing Herp, suggesting that Herp reduces the total pool of ER Ca\(^{2+}\) available for release (Fig. 6B). Next, we examined the effects of Herp overexpression on ER Ca\(^{2+}\) stores under conditions associated with ER stress. In cells stressed for 8 and 16 h with tunicamycin, the total pool of ER Ca\(^{2+}\) available for release was decreased by 62 and 40%, respectively, in cells overexpressing Herp compared with VT cells (Fig. 6C). This is the first report showing that tunicamycin caused a significant increase in ER
Ca\(^{2+}\) storage and release in a manner analogous to overexpression of sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) (31) and calreticulin (32) proteins, and that Herp overexpression effectively attenuated this increase. Lastly, we examined the effect of Herp inhibition on ER Ca\(^{2+}\) release induced by thapsigargin. All procedures were performed as in prior experiments except that PC12 cells were transfected with either siRNA\(_{\text{Herp}}\) or siRNA\(_{\text{CTRL}}\) 24 h prior to induction of ER stress with tunicamycin. The resting [Ca\(^{2+}\)]\(_i\) and the peak [Ca\(^{2+}\)]\(_i\) elevation induced by thapsigargin was significantly higher in ER-stressed PC12 cells transfected with the siRNA\(_{\text{Herp}}\) compared with that in mock-transfected cells or cells transfected with the siRNA\(_{\text{CTRL}}\) (Fig. 6D). Collectively, these data suggest that Herp regulates total ER Ca\(^{2+}\) load and release under conditions associated with ER stress.

Caspase-mediated Cleavage of Herp during ER Stress-induced Cell Death—The cytosolic domain of Herp contains several putative caspase cleavage sites near the transmembrane and C-terminal regions (Fig. 7A), which are also present in many other ER-resident proteins (33, 34). To determine whether Herp is cleaved by caspases during ER-stress-induced apoptosis, we examined whole cell lysates from PC12 cells overexpressing Herp and VT cells treated with tunicamycin for 12 or 24 h and analyzed by immunoblotting (Fig. 7B). Levels of full-length Herp protein decreased, and amounts of smaller Herp fragments of 48 and 30 kDa, sizes consistent with their being N (48 kDa) and N (30 kDa) termini caspase-3 cleavage products of Herp. These data suggest that Herp is a substrate of caspases activated in response to ER stress. A, schematic diagram of Herp showing the locations of putative caspase cleavage sites near the transmembrane and C-terminal regions. B, immunoblot (50 μg protein/lane) showing time-dependent decrease of full-length Herp protein levels and corresponding increase in levels of the 30- and 48-kDa Herp fragments (*) in PC12 cells following induction of ER stress with 20 μg/ml tunicamycin (Tun). C, immunoblot showing inhibition of Tun-induced Herp proteolysis by the pan-caspase inhibitor zVAD-fmk (100 μM). As control, the samples were immunoblotted using an antibody against PARP (PARP is cleaved by caspase 3 into a 55-kDa fragment). D, left panel, autoradiogram showing the time course of in vitro generation of the 48- and 30-kDa Herp cleavage fragments (indicated by asterisks) from \(^{13}S\)-labeled Herp by purified recombinant caspase-3. Right panels, immunoblots (50 μg protein/lane) showing cross-reactivity of the 30-kDa Herp cleavage fragment (indicated by arrowhead) with the anti-Herp and anti-cMyc antibodies (top). Immunoblots showing the presence of the 30-kDa Herp fragment (indicated by arrowhead) in immunoprecipitated materials, which immunoreacted with the anti-cMyc but not anti-FLAG antibodies (bottom). IgG H and IgG L denote the heavy and light chains of the immunoglobulin G protein, respectively. E, double labeling confocal images showing release of Herp fragments from the ER into the cytosol during ER stress induced by 20 μg/ml Tun. PC12 cells stably expressing t-Herp were stained with mouse anti-c-Myc (red fluorescence) and rabbit anti-presenilin (green fluorescence), as the ER marker, before and 24 h following exposure to 20 μg/ml Tun. F, comparison of the time course of Herp protein degradation and appearance of Herp cleavage fragments (indicated by asterisks) in cells stably expressing either mutagenized D266E or wild-type Herp following a short (left panel) and prolonged (right panel) exposure to 20 μg/ml Tun. G, reduced vulnerability of cells expressing mutagenized D266E Herp to death following prolonged exposure to 5 and 20 μg/ml Tun. Cultures of wild-type and mutagenized D266E Herp overexpressing PC12 cells were treated with Tun for 12 and 24 h (short ER stress) or for 48 and 72 h (prolonged ER stress), and cell viability was assessed by the trypan blue dye exclusion method. Values are the mean and S.D. of three independent experiments. *, p < 0.05 (ANOVA with Scheffe post-hoc tests).
Herp, respectively, increased in stressed cells (Fig. 7B). Appearance of these proteolytic fragments occurred after caspase 3 activation (see Fig. 3D) and was blocked by the caspase inhibitor zVAD-fmk (Fig. 7C), suggesting that cleavage of Herp in cells subjected to ER stress requires activation of a caspase(s). Next, we assayed the ability of purified recombinant caspase 3 to cleave [35S]labeled, in vitro transcribed/translated Herp. The results shown in Fig. 7D (left panel) indicate that the full-length Herp protein was cleaved in vitro by caspase-3 into the homologous fragments detected in lysates from ER stressed cells (Fig. 7D, right top panels). Of these proteolytic fragments, only the 30-kDa Herp fragment was immunoprecipitated from the soluble fraction of ER-stressed cells suggesting that the 48-kDa Herp fragment most likely remains ER-associated following cleavage by caspase 3. The released 30-kDa Herp fragment immunoreacted with the anti-c-Myc but not the anti-FLAG antibody, which further confirms that it represents the N-terminal portion of the Herp protein (Fig. 7D, right bottom panels). Double immunofluorescence labeling further confirms the increased cytosolic localization of the released 30-kDa Herp fragment during ER stress induced death (Fig. 7E). The localization of the small C-terminal cleavage fragment of Herp was difficult to ascertain due to its low concentration and high instability. To determine whether the level of full-length Herp is critical in maintaining survival, we generated cells stably expressing Herp with a point mutation in which the aspartic acid residue in the P1 position of the consensus caspase 3 cleavage site located proximal to the transmembrane region (D266E) was changed to glutamic acid (Herp D266E). A time course study showed that, despite similar levels of overexpressed full-length Herp, the overall reduction in full-length Herp protein is attenuated in stressed cells stably expressing mutant Herp D266E when compared with that in stressed cells expressing wild-type Herp (Fig. 7F). The Herp fragment detected in cells expressing mutant Herp resulted from cleavage of endogenous Herp as this band was not detected with the anti-c-Myc antibody (data not shown). As a result of the increased level of full-length Herp protein detected after prolonged ER stress, cells stably expressing mutant Herp were protected from tunicamycin-induced death (Fig. 7G).

**Overexpression of Herp Protects Neuronal Cells against Aβ Peptide-induced Toxicity**—In addition to tunicamycin, a chemical inducer of ER stress, we explored the neuroprotective action of Herp against a physiologically relevant insult known to induce ER stress. Aβ, the major component of amyloid deposits in AD, has been shown to promote ER stress-induced apoptosis by activating caspase-12 (10) and JNK (27) signaling pathways. Aβ also...
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disrupts ER calcium homeostasis and causes elevation of cytosolic calcium (6, 9, 11). The effect of increased Herp levels on the vulnerability of PC12 cells to cell death induced by Aβ was determined by exposing VT and Herp-overexpressing PC12 cell clones to Aβ-41–42 (Fig. 8A). To determine whether endogenous Herp might play a role in modifying neuronal vulnerability to Aβ toxicity, we exposed primary rat cortical neurons to Aβ-41–42 and then assessed Herp levels by immunoblot analysis of cell lysates. The increase in Herp protein was only transient in cells exposed to Aβ-41–42, as this was followed by a rapid decrease in the full-length protein and an increase in Herp fragments (Fig. 8B). When cells were pretreated with Aβ-41–42 and then challenged with either bradycardin or thapsigargin, the elevation of intracellular Ca2+ levels induced by each agent was reduced in cells overexpressing Herp (Fig. 8C). Collectively, these data suggest that Herp can protect neurons against insults relevant to the pathogenesis of AD by a mechanism involving stabilization of ER and cellular Ca2+ homeostasis.

DISCUSSION

There is a growing body of evidence that the ER can play pivotal roles in regulating cell survival and apoptosis in a variety of cell types including neurons. Our findings provide evidence that the novel ER membrane protein Herp is induced and protect neurons against death under conditions associated with ER stress. Induction of Herp expression protected neural cells against death induced by Aβ and tunicamycin, two insults that trigger apoptosis by inducing ER stress (10, 20). Partially inhibiting this induction by RNA interference significantly increased neuronal vulnerability to ER-stress induced apoptosis. Interestingly, increased basal levels of Herp protein were also detected during early brain development consistent with the importance of ER stress during neuronal development (34).

The ability of Herp to prevent ER stress-induced death was correlated with its ability to stabilize cellular Ca2+ homeostasis. Dysregulation of ER Ca2+ homeostasis occurs as an early event during many forms of apoptosis and has been implicated in the pathophysiology of several neurodegenerative diseases including Alzheimer’s, Huntington’s, and prion diseases (6, 34, 35). Several different agents that evoke ER stress including Ca2+ ionophores and thapsigargin have been shown to disrupt intracellular Ca2+ homeostasis, suggesting a central role for Ca2+ in ER stress-induced apoptosis. ER stress increases the basal ER Ca2+ content (36), which is associated with a marked increase in Ca2+ fluxes across the ER membrane, decreased mitochondrial membrane potential and increased vulnerability of the cells to apoptosis (37, 38). In this regard, several members of the pro-apoptotic Bcl-2 family such as Bax and BIK, act on the ER to promote ER Ca2+ mobilization into the mitochondria (39, 40). Indeed, dantrolene and other agents that block ER Ca2+ release effectively suppress apoptosis induced by tunicamycin, oxidative stress and other apoptotic stimuli (3, 11, 18). Collectively, the available data suggest that increased Ca2+ release from the ER could serve as the primary trigger for ER stress-induced apoptosis. Our findings show that Herp plays a pivotal neuroprotective role under conditions of ER stress and exposure to Aβ, and that this beneficial function of Herp is mediated, in part, by stabilization of ER Ca2+ homeostasis.

The mechanism by which Herp maintains ER Ca2+ homeostasis appears to be different from the proposed anti-apoptotic action of Bcl-2. Bcl-2 may reduce ER Ca2+ content and release (41–43), either directly by enhancing the permeability of the ER membrane to Ca2+ (44) or indirectly by down-regulating the transcription of genes encoding for proteins that are involved in ER Ca2+ re-uptake and sequestration such as SERCA2b and calreticulin (45). In addition, Bcl-2 transfected cells also exhibit reduced capacitative Ca2+ entry (46), a process whereby the depletion of Ca2+ from intracellular stores activates plasma membrane Ca2+ channels. However, in our study both the basal and ER stress-induced levels of Bel-2 and calreticulin in Herp and VT cells were not significantly different (Fig. 5, A and B) and capacitative Ca2+ entry was not reduced in Herp-overexpressing cells.2

Activation of ER stress-induced apoptotic signaling cascades has been associated with dysregulated Ca2+ release from the ER (6, 29, 34). Agents that induce ER stress including tunicamycin and Aβ can specifically activate ER-associated caspase 12 (10, 20). Activation of caspase 12, which may occur very early prior to any detectable changes in mitochondrial function, appears to be required for ER stress-induced apoptosis, because neurons from caspase 12-deficient mice are resistant to apoptosis induced by Aβ (10). One mode of caspase 12 activation is by cleavage of procaspase 12 by calpain in response to elevation of intracellular Ca2+ levels (29). Calpain is one of the most abundant neutral proteases in the nervous system and its expression is increased in neuro-pathological conditions such as AD and Parkinson’s disease (47), suggesting a role for this Ca2+-activated protease in stress responses associated with neurodegenerative disorders. Our data showing that Herp overexpression can normalize ER Ca2+ homeostasis and prevent Ca2+ overload during ER stress suggest that Herp can counteract the activation of calpain/caspase 12 and downstream executor caspases. Another pro-apoptotic signaling cascade induced by ER stress is the activation of JNK/c-Jun, which is attenuated by Herp. Since activation of these cascades is involved in Aβ-induced neurotoxicity (10, 27, 49), Herp may protect cells exposed to Aβ by suppressing caspase 12 and JNK activation.

Herp maintains mitochondrial function during ER stress. The Ca2+ released from the ER is rapidly redistributed into the mitochondria (38) and accumulation of Ca2+ in this organelle may cause mitochondrial membrane depolarization and release of cytochrome c, which induces caspase 3 activation (4, 21). By limiting this pro-apoptotic ER mitochondrial Ca2+-dependent cross-talk, Herp may attenuate mitochondrial membrane depolarization, cytochrome c release, and activation of caspase 3 (Fig. 4).

We found that Herp protein is rapidly cleaved by caspase 3 activated during ER stress-induced apoptosis. Cleavage of Herp releases the N-terminal fragment that contains the ubiquitin-like domain into the cytosol. Site-directed mutagenesis of the main caspase cleavage site prevented generation of the N-terminal fragment and significantly enhanced the neuroprotective function of Herp. Although the present study did not address possible cytotoxic properties of the released Herp fragments, it is more likely that cleavage reduces its intrinsic anti-apoptotic function at the ER. In this regard, it is noteworthy that the N-terminal ubiquitin-like domain of Parkin, a cytosolic protein, which also confers cytoprotection toward ER stress (50), is similarly cleaved and removed by caspases (51). As Herp has no demonstrable E3 ubiquitin-protein ligase activity, it is unlikely that Herp plays a direct role in protein substrate ubiquitination. On the other hand, the N-terminal ubiquitin-like domain of Herp may function as a proteasome-interacting domain, as has recently been demonstrated for Parkin (52) and other ubiquitin-like domain-containing proteins (53). Our findings that ER-stressed cells overexpressing Herp exhibited reduced ER Ca2+ release suggest that Herp may bind to and facilitate targeting of ER resident proteins.

2 S. Chan and M. P. Mattson, unpublished data.
involved in ER calcium regulation such as SERCA and the ER Ca\(^{2+}\) release channels for proteasome-mediated degradation (54–56). Studies employing specific proteasomal inhibitors will further elucidate possible mechanisms by which Herp stabilizes ER calcium homeostasis under conditions associated with ER stress. Alternatively, Herp may inhibit the sustained ER Ca\(^{2+}\) release during ER stress by inhibiting the association of the ER Ca\(^{2+}\) release channels with pathophysiological ligands including the huntington-associated protein 1 (35) and cytochrome c (57).

A recent study by Sai et al. (14) showed that Herp physically interacts with PS1, an ER integral membrane protein that has been linked to APP metabolism, ER Ca\(^{2+}\) regulation, and apoptosis (6). The contribution of this interaction to ER Ca\(^{2+}\) homeostasis may be dependent on the expression of APP as its processed carboxyl APP derivatives have been shown to regulate the expression of genes involved in ER Ca\(^{2+}\) homeostasis (58). In this study, we did not examine levels of the APP derivatives nor their downstream nuclear signaling activities (59). Clearly, further insight into Herp function will emerge from a better understanding of its interaction with PS1 and other ER resident proteins and how these interactions affect ER Ca\(^{2+}\) homeostasis and downstream signaling.

REFERENCES
