Herp, a New Ubiquitin-like Membrane Protein Induced by Endoplasmic Reticulum Stress

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Hyperhomocysteinemia, a risk factor for vascular disease, injures endothelial cells through undefined mechanisms. We previously identified several homocysteinereactive genes in cultured human vascular endothelial cells, including the endoplasmic reticulum (ER)-resident molecular chaperone GRP78/BiP. Here, we demonstrate that homocysteine induces the ER stress response and leads to the expression of a novel protein, Herp, containing a ubiquitin-like domain at the N terminus. mRNA expression of Herp was strongly upregulated by inducers of ER stress, including mercaptoethanol, tunicamycin, A23187, and thapsigargin. The ER stress-dependent induction of Herp was also observed at the protein level. Immunochemical analyses using Herp-specific antibodies indicated that Herp is a 54-kDa, membrane-associated ER protein. Herp is the first integral membrane protein regulated by the ER stress response pathway. Both the N and C termini face the cytoplasmic side of the ER; this membrane topology makes it unlikely that Herp acts as a molecular chaperone for proteins in the ER, in contrast to GRP78 and other ER stress-responsive proteins. Herp may, therefore, play an unknown role in the cellular survival response to stress.

Hyperhomocysteinemia, with both genetic and environmental components, is a common risk factor for thrombotic vascular events such as stroke, myocardial infarction, and venous thrombosis (1–3). Severe hyperhomocysteinemia is caused by homozygous deficiency of cystathionine β-synthase, 5,10-methylenetetrahydrofolate reductase, and so forth. Moderate hyperhomocysteinemia may be caused by heterozygous cystathionine γ-lysinetetrahydrofolate reductase, and so forth. Moderate hyperhomocysteinemia is caused by heterozygous deficiency of cystathionine β-synthase deficiency, homozygosity for a common C677T mutation in the 5,10-methylenetetrahydrofolate reductase gene, renal insufficiency, aging, and insufficient uptake of folic acid, vitamin B-6, or vitamin B-12. Despite accumulating epidemiological evidence, the pathogenic mechanism responsible for thrombosis in patients with hyperhomocysteinemia is still poorly understood (4).

To investigate the gene expression changes induced by homocysteine, we performed a differential display analysis that identified six up-regulated and one down-regulated genes in human umbilical vein endothelial cells (HUVECs) (5). GRP78/BiP, a gene up-regulated by homocysteine, is an endoplasmic reticulum (ER)-resident molecular chaperone. The induction of GRP78 is a consequence of the cellular response to perturbations of the ER, suggesting that homocysteine may serve to induce the ER stress response. Another group also showed that homocysteine caused ER stress and growth arrest in HUVECs (6). GRP78 mRNA levels in the livers of hyperhomocysteinemic mice lacking the cystathionine β-synthase gene were increased over wild-type mice (7).

The ER in eukaryotic cells is optimized for synthesizing, folding, and assembling membrane proteins and soluble proteins destined for secretion or trafficking to lysosomes. Environmental changes leading to the accumulation of unfolded proteins in the ER trigger a stress response, referred to as the ER stress response. As one aspect of the cellular response, the unfolded protein response (UPR) was characterized in terms of the transcriptional induction of a set of mRNAs encoding ER-resident molecular chaperones and folding enzymes including GRP78, GRP94, PDI, ERP72, and calreticulin. The signaling pathway from the ER to the nucleus has been extensively studied in the yeast, Saccharomyces cerevisiae. Perturbation of the ER is sensed by an ER-resident transmembrane kinase/endonuclease, Ire1p (8, 9). Activated Ire1p produces transcription factor, Hac1p, through unusual mRNA splicing (10–13). Hac1p binds to the promoter cis-acting element, UPRE, activating the transcription of ER-resident chaperones (10, 14, 15).

Accumulating evidence supports a similar mechanism for the ER stress response in mammalian cells. A cis-element, designated ERSE, is present in the promoter regions of mammalian ER-resident proteins (16, 17). ATF6 has been identified as a trans-acting ERSE-binding protein (16, 18). Mammalian orthologues for the yeast UPR sensor Ire1p, Ire1α (19) and Ire1β (20), have been cloned and characterized, although their substrates are not known. As recently reviewed (21, 22), however, the mammalian UPR is considerably more diverse than the yeast UPR. The mammalian ER stress response seems to coordinate both transcriptional and translational controls involved in the cellular survival, inflammation, immune response, and apoptosis.

Here, we report the characteristics of a novel protein induced by the ER stress. In contrast to the previous features, this new
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UPR target protein, with an N-terminal ubiquitin-like domain, is present on the cytoplasmic face of the ER membrane.

EXPERIMENTAL PROCEDURES

Cell Culture—HUVECs (Clonetics) were cultured in MCD8131 medium (Life Technologies, Inc.) supplemented with 10 mM glutamine (Life Technologies, Inc.), 20 mM Hepes-NaOH (pH 7.4), 2% fetal bovine serum (Life Technologies, Inc.), and 10 ng/ml human basic fibroblast growth factor (R & D Systems) on dishes coated with type I collagen (Sumitomo Bakelite) in humidified air with 5% CO₂ at 37 °C. After treatment, cells were washed with Dulbecco’s PBS (Life Technologies, Inc.) and analyzed by Northern blot, Western blot, or immunocytochemistry.

cDNA Cloning of Herp—A cDNA library was constructed from homocysteine-treated HUEVs (5). Positive phages were cloned by PCR-based screening as described previously (5). Approximately 1.5 × 10⁹ phages were plated at a density of 300–400 plaque-forming units/dish with Escherichia coli XL1-Blue MRF² strain as the host. PCR was performed using 5'-CTGGGAACTTTGGTGGG-3' and 5'-CATGATG-TACTGTCTGTC-3' as primers with the plate lysates as templates. After several sequential dilutions, 10 positive phages were cloned, and their plasmid DNAs were prepared. Insert DNAs were sequenced in both directions using BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (PerkinElmer Life Sciences).

Many mouse expressed sequence tag clones similar to human Herp cDNA exist in public data bases. We purchased and sequenced five clones, I.M.A.G.E. Consortium CloneIDs: 388850, 425720, 439667, 479754, and 548945 (23), through Research Genetics, Inc.

Probe Synthesis for Nonradioisotopic Northern Blot Analysis—Specific probes were generated by PCR as described previously (5). Briefly, the reaction mixture (100 μl) contained 1× PCR buffer, 50 μM dATP, 50 μM dCTP, 50 μM dGTP, 10 μM dUTP, 50 μM fluorescein-12-dUTP (PerkinElmer Life Sciences), 10 ng of the plasmid DNA, 1 μM sense primer, 1 μM antisense primer, and 10 units of Taq polymerase. (Takara). The reaction was incubated in a 96-well plate at 94 °C (30 s), followed by 35 cycles of 94 °C (15 s), 55 °C (15 s), and a final incubation at 60 °C (5 min). The primers used for the Herp-specific PCR were 5'-GCCATGGATCGCAGAC-3' and 5'-CTGGGAACTTTGGTGGG-3' as primers for the first PCR and the PCR product, respectively. After inserting the PCR products into the plasmid vector, PCR II (Invitrogen), 10 clones were derived from each of the heart and brain cDNA libraries were sequenced.

To synthesize specific probes for stress proteins, we cloned partial cDNAs of GRP78, GRP94, PDI, ERp72, calnexin, HSP70, HSP90, and HSP60 from mouse liver cDNA libraries (23). We sequenced specific probes for GRP78, 5'-GCAGATCAGTGTC-3', and 5'-GAGGACCTGATGGAGTCCGAGACCGAAC-3', respectively.

Probes were hybridized to the membrane by capillary blotting techniques. Hybridization and detection procedures were performed according to the manufacturer’s specification. Chemiluminescence was detected by an image analyzer, LAS-1000plus (Fujifilm).

Preparation of Antiserum Specific to Herp—Two oligonucleotides, 5'-GCCATGCTAGGATTTCGACCAAC-3' and 5'-GCTAGCTGCAGACCC-3', were used to emulate the open reading frame of Herp. The PCR product digested with BglII and XhoI was ligated into the corresponding site of pGEKT-3 (Amer- sham Pharmacia Biotech), a Schistosoma japonicum glutathione S-transferase fusion expression vector. Glutathione S-transferase-Herp fusion protein was purified according to the manufacturer’s instructions. Purified protein following digestion of the fusion protein with thrombin. We raised anti serum against recombinant Herp in rabbits. Fusion protein (1 mg) was emulsified in adjuvant TiterMax (CytRx) and injected intradermally. Every 2 weeks, rabbits were boosted with 0.5 mg of fusion protein in the same adjuvant. The blood was taken 2 weeks after the third injection. Antibodies specific for Herp (anti-Herp) were purified by recombinant Herp-immobilized affinity chromatography.

Western Blot Analysis—HUVECs were incubated with a final concentration of 10 μM 2-mercaptoethanol, 10 μM tunicamycin, or 1 μM thapsigargin for 6 h. After washing, cells were lysed with SDS sample buffer (10 mM Tris-HCl, 2% SDS, 50 mM dithiothreitol, 2 mM EDTA, 0.02% bromophenol blue, 6% glycerol, pH 6.8) and boiled for 7 min. After SDS-polyacrylamide gel electrophoresis, proteins in the gel were transferred to an Immun-Blot polyvinylidene difluoride membrane (Bio-Rad). Remaining binding sites on the membrane were blocked with 3% skim milk in T-PBS, 150 mM NaCl, 0.05% Tween 20, pH 7.4) for 1 h. The membrane was then incubated with 1 μg/ml anti-Herp or anti-RDEL (StressGen) in 3% skim milk for 1 h. After washing with 3% skim milk, the membrane was incubated with 0.1 μg/ml peroxidase-labeled goat anti-rabbit IgG or anti-mouse IgG (Kirkegaard and Perry Laboratories) in 3% skim milk for 1 h. The membrane was thoroughly washed with T-PBS and with PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4), followed by chemiluminescent detection using the Renaissance Western blot Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). Chemiluminescence was detected by an image analyzer, LAS-1000plus.

In Vitro Synthesis of Herp—Two oligonucleotides, 5'-GGGGTACCA-TGGAGTCCGACGACCAAC-3' and 5'-CGGATCTCTTCAGTTTGGACG-GCTCGGG-3', were used to PCR amplify the entire open reading frame of Herp. The product, digested with KpnI and EcoRI, was ligated into the corresponding site of a plasmid vector, pZeoSV2+ (Invitrogen), with a T7 priming site. Using the resultant plasmid DNA, many mouse expressed sequence tag clones similar to human Herp cDNA were synthesized using an in vitro T7 Quick Coupled Transcription/Translation Systems (Promega).

The resultant RNA derived from these clones was synthesized by T7 transcription with a T7 priming site. Using the resultant RNA, we prepared an in vitro translation system using rabbit reticulocyte lysate. After incubation for 3 h at 4 °C, separated into supernatant and pellet fractions. The fractions were subjected to Western blot analysis using an anti-Herp antibody.

Fluorescent Immunocytochemistry—HUVECs were incubated with or without 1 μM thapsigargin for 6 h. Cells were rinsed with Dulbecco’s PBS, fixed in 2% paraformaldehyde for 15 min, and permeabilized with 0.05% Triton X-100, by centrifuging at 1000 × g for 3 h at 4 °C and separated into supernatant and pellet fractions. The fractions were subjected to Western blot analysis using an anti-Herp antibody.

UPR target protein, with an N-terminal ubiquitin-like domain, is present on the cytoplasmic face of the ER membrane.
RESULTS

Previously, we identified several transcribed fragments of novel genes that were responsive to a 4-h treatment of HUVEC with homocysteine (5). The CA13 fragment demonstrated the strongest induction following treatment (data not shown). The sequence, containing a typical polyadenylation signal near the 3’ terminus, is considered to be a 3’ region of cDNA. We termed the encoded protein Herp.

Induction of the mRNA Expression by Homocysteine—Northern blot analysis revealed a faint 2.2-kilobase mRNA in untreated HUVECs. mRNA levels increased dramatically following an incubation with homocysteine (Fig. 1). The mRNA induction peaked after 4 h of treatment increasing in intensity approximately 50-fold over levels seen in untreated cells. Previous work demonstrated the dependence of the induction of GRP78, an ER-resident molecular chaperone, on homocysteine (5). Enhanced expression of GRP78 is a consequence of the cellular responses to ER stress. To examine the relationship of homocysteine to ER stress induction, we analyzed the expression of various stress proteins after homocysteine treatment. Homocysteine treatment of HUVECs enhanced the mRNA abundance of multiple molecular chaperones or folding enzymes in the ER: GRP78, GRP94, PDI, ERP72, and calnexin (Fig. 1). mRNA induction was not observed for HSP70 and HSP90, molecular chaperones in the cytoplasm/nucleus, or HSP60, in mitochondria. These data suggest that homocysteine specifically induces the cellular ER stress response. Notably, mRNA induction of Herp is faster and of a greater magnitude than other ER-resident stress proteins examined.

To examine the regulation of Herp expression by the ER stress response, we investigated the effects on Herp mRNA levels of additional agents known to induce the ER stress response (Fig. 2). Herp mRNA increased in response to treatment with 2-mercaptoethanol (reducing agent), tunicamycin (N-glycosylation inhibitor), A23187 (calcium ionophore), and thapsigargin (ER-resident Ca2+-ATPase inhibitor). Thus, Herp expression is regulated by the ER stress response referred to as the UPR.

Nucleotide Sequence of Herp cDNA—We isolated 10 independent clones with full-length cDNAs containing the CA13 fragment sequence from a homocysteine-treated HUVEC cDNA library. All clones contained an identical open reading frame encoding the Herp protein. We performed cap site hunting to determine the 5’-terminal transcription start site of the full-length cDNA. All 20 clones sequenced contained the identical starting sequence of 5’-AGAGACG, which we concluded to be the 5’ terminus of the full-length Herp cDNA.

The complete nucleotide sequence of human Herp cDNA (Fig. 3) has been submitted to the GenBankTM/EMBL/DDBJ data bases under accession number AB034889. The 1,176-base pair open reading frame, beginning at an ATG start codon and ending at a TGA stop codon, produces a protein sequence of 391 amino acid residues (designated as Herp). The presumed initiating ATP-calcium ionophore, perfectly matches the consensus sequence for initiation of translation in vertebrates, GCCGCC(A/G)CCATGG (24). Homology searches of the GenBankTM/EMBL/DDBJ data bases revealed that human Herp cDNA is 99.9% identical to KIAA0025 (GenBankTM accession number D14695), a randomly sampled human cDNA clone (25), and 95.5% identical to a stretch of 0041AS (GenBankTM accession number M29512), a pig hepatic cDNA clone (26). Recently, van Laar et al. (27) identified human KIAA0025...
as Mif1 (GenBank™ accession number NM014685), a gene induced by methyl methanesulfonate. They also showed that Mif1 expression was induced by tunicamycin treatment, osmotic shock, and UV light irradiation (27).

Amino Acid Sequence of Herp—Many homologous clones were found when the human Herp nucleotide sequence was searched against the mouse expressed sequence tag data base. We have submitted the accurate sequence to the GenBank™/EMBL/DDBJ data bases under the accession number AB034991. The deduced amino acid sequences of human and mouse Herp share 88.7% identity (aligned in Fig. 4 A). Human Herp, with a calculated molecular mass of 43,719 Da, is predicted to be a membrane protein with one transmembrane domain (Ser285–Trp307; Fig. 4 A, underline) by the SOSUI, the secondary structure prediction system (28). A PATTERN search of the PROSITE data base (29) did not discover a motif match that might divulge the functional properties of Herp; a PROFILE search, however, demonstrated a significant match to ubiquitin (Fig. 4 B). Ubiquitin is a highly conserved, 76-amino acid protein with 100% identity between the human and the mouse. The stretch of amino acids between Val14 and Val85 of the human and mouse Herps share 32% and 33% identity with the Val5–Val70 stretch of ubiquitin, respectively.

Constitutive Expression of Herp in Human Organs—To examine the distribution of Herp in human organs, we performed a Northern blot analysis using a Herp-specific probe. Human Herp was constitutively expressed in all organs tested as a single species of mRNA at 2.2 kilobases (Fig. 5), consistent with the band appearing in cultured HUVECs (Fig. 1). Herp mRNA was most abundant in pancreas, suggesting a role for Herp in that organ.

Identification of Endogenous Herp—We immunized rabbits against bacterial-expressed glutathione S-transferase-Herp fusion protein to obtain Herp-specific polyclonal antibodies (anti-Herp). Western blot analysis following the in vitro transcription/translation of the Herp expression plasmid, pZeoSV2Herp, revealed a single, 54-kDa protein (Fig. 6A, lane 1); no signal was observed using the mock vector, pZeoSV2 (data not shown). Anti-Herp recognized a single 54-kDa band by Western blot analysis of HUVEC lysates (lane 2), considered to be the endogenous Herp. The 10-kDa difference between the apparent mass of the endogenous 54-kDa protein and the calculated mass (43,719 Da) may be due to acidic characteristics resulting from a low pI of 5.0.

To examine the effects of ER stress on the expression of the 54-kDa protein, HUVECs were incubated with 10 mM 2-mercaptoethanol, 10 μM tunicamycin, or 1 μM thapsigargin for 6 h. When the lysates were subjected to Western blot analysis, the immunoreactive 54-kDa protein levels increased as a result of each treatment (Fig. 6A, lanes 3–5). This observation was consistent with the expression pattern of Herp mRNA (Fig. 2).
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These results suggest that the 54-kDa protein recognized by anti-Herp is the bona fide product of the Herp gene.

Membrane Association of Herp—To confirm the transmembrane nature of Herp, we separated HUVEC lysates into supernatant and pellet fractions by centrifugation. In isotonic buffer, Herp was observed only in the pellet (Fig. 6B, lanes 2 and 3). In the presence of 1 M NaCl, Herp was also present only in the pellet (lanes 4 and 5). This localization pattern indicated that Herp is strongly bound to membranes. A similar distribution was observed in the presence of 0.1 M Na2CO3 (pH 11) (lanes 6 and 7), which permeabilizes microsomal membranes to extract both peripheral and luminal proteins. In contrast, treatment with 1% Triton X-100 resulted in the efficient solubilization of Herp (lanes 8 and 9). These results suggest that Herp is an integral membrane protein.

Subcellular Localization of Herp—To elucidate the subcellular localization of Herp, we performed indirect fluorescent immunocytochemistry. We observed an intense perinuclear staining and a uniform staining of a peripheral lace-like network in thapsigargin-treated HUVEC stained with anti-Herp (Fig. 7D). Because the perinuclear staining and peripheral lace-like network suggested Herp may associate with the ER, we performed co-localization studies with marker proteins of the ER. The anti-KDEL staining pattern of Fig. 7F, reacting to GRP78 and GRP94, was nearly identical to the staining pattern of anti-Herp (Fig. 7D). These data suggest that Herp is associated with the ER. Therefore, Herp was named the homocysteine-responsive ER-resident protein.

Membrane Topology of Herp—Knowledge of the protein orientation of Herp in the ER membrane may facilitate an understanding of its function. We investigated the membrane topology of Herp by protease protection assay. We expressed a doubly epitope-tagged Herp, Myc-Herp-FLAG, in HUVECs by transient transfection of the expression plasmid, pcDNA3mHerpf. Following homogenization and centrifugation, both anti-Myc tag and anti-FLAG tag recognized a single 61-kDa protein in the membrane fraction (Fig. 8A, lanes 1 and 4), considered to be Myc-Herp-FLAG. This band was absent from microsomes prepared with the mock vector pcDNA3.1(+)-transfected cells (data not shown). As a control, we monitored endogenous calnexin, a type I transmembrane protein in the ER, by anti-Calnexin NT and CT, recognizing the luminal N terminus and the cytoplasmic C terminus of calnexin, respectively. Following digestion with levels of proteinase K that diminish the full-length calnexin band, anti-Calnexin NT (Fig. 8A, lane 9), not CT (lane 12), detected a lower band corresponding to the N-terminal luminal domain of calnexin. Therefore, polypeptides in the lumen were protected from proteinase K activities under these experimental conditions. In the same microsomes, neither the anti-Myc tag (lane 3) nor the anti-FLAG tag (lane 6) antibodies detected fragments derived from the parent 61-kDa band, suggesting that both the N and C termini are exposed to the cytoplasm.

We next performed immunocytochemistry on permeabilized HUVECs transiently transfected with the Myc-Herp-FLAG expression plasmid. SLO is a bacterial, pore-forming toxin that requires cholesterol binding in the bilayer, selectively permeabilizing the plasma membrane without affecting intracellular membranes. Thus, the immunoreactivity of a given epitope is dependent upon both the accessibility of the antibody and the orientation (e.g. cytosolic or luminal) of a membrane protein. Without the SLO treatment, fluorescence could not be detected after staining with anti-Myc tag, anti-FLAG tag, or anti-KDEL (Fig. 8B, panels a, d, and g). Following treatment with Triton X-100, permeabilizing both the plasma membrane and intracellular membranes, anti-KDEL, recognizing the ER-luminal proteins GRP78 and GRP94, stained a lace-like pattern (panel i). Although we detected nuclear staining with propidium iodide in SLO-treated cells (panel h), anti-KDEL signals were not observed under these conditions, indicating an intact ER membrane. Under the same condition, both the anti-Myc tag (panel b) and anti-FLAG tag (panel e) antibodies stained the ER. Therefore, both the N and C termini of Herp are exposed to the cytoplasm.

DISCUSSION

Perturbations of the ER in mammalian cells result in the activation of a stress signaling pathway out of the ER, leading to transcriptional and translational regulation. Following the accumulation of unfolded proteins in the ER, two protein kinases, Ire1α/β (19, 20) and PERK (30), are activated. Active Ire1 is believed to be the most proximal factor for the transcriptional induction of molecular chaperones and folding enzymes in the ER. Activated PERK contributes to ER stress-induced translational attenuation by phosphorylating the α-subunit of eukaryotic initiation factor 2. In this report, we demonstrate a new target gene for UPR-induced transcription, Herp. Herp is localized to the ER (Fig. 7) in a manner similar to other genes induced by ER stress: GRP78, GRP94, GRP170, calreticulin, FKBP13, PDI, and ERP72. In contrast to the proteins described above present in the ER lumen, Herp is an integral membrane protein (Fig. 6B).
Although calnexin is a transmembrane protein in the ER, it demonstrates weak induction by the UPR (Fig. 1). UPR-induced ER-luminal soluble proteins have an ER-retrieval signal motif, KDEL or KDEL-like sequence, at the C terminus (31, 32), absent from the Herp sequence. Herp also lacks a C-terminal K(X)KXX motif, facilitating the interaction of the protein with the coatomer (COP1) complex for the retrograde transport of type I membrane proteins back to the ER (33). Other motifs such as a diphenylalanine sequence (34, 35), a DL sequence (36), and transmembrane domain structure (37–39) may act as the signal for ER retrieval or retention; the mechanism by which Herp is localized to the ER, however, remains unknown.

At present, all UPR-inducible proteins in the ER are molecular chaperones or folding enzymes assisting protein folding. It is doubtful that Herp conforms to this category of ER stress-induced protein judging from the membrane topology demonstrated from several experiments: 1) The protease protection assay shown (Fig. 8A). 2) Immunocytochemistry of selectively permeabilized cells shown (Fig. 8B). 3) The lack of N-glycosylation despite a potential site for N-glycosylation (Asn 141). In vitro synthesized or tunicamycin-induced Herp was the same size as other stimulant-induced Herp (Fig. 6A); PNGase F treatment did not alter the size of Herp on SDS-PAGE (data not shown). 4) When the activation peptide sequence of blood coagulation factor IX, known to be glycosylated (40), was added to either the N or C terminus of Herp, glycosylation did not occur (data not shown). Although the tagged protein may have inserted in the membrane incorrectly, these data strongly suggest that both the N and C termini of Herp face the cytoplasm. The SOSUI system secondary structure prediction (28) suggests that Herp is a membrane protein with one transmembrane domain (Ser285–Trp307). If this is correct, the topology of Herp is depicted like model 1 in Fig. 9. A hydrophathy profile

**Fig. 7.** Subcellular localization of Herp. Untreated (A–C) or thapsigargin-treated (D–F) HUVECs were fixed and stained with both anti-Herp and anti-KDEL antibodies. Red signals in (A and D) show the Herp localization, whereas green signals in (C and F) show the localization of GRP78 and GRP94. B and E are the merged images of A and C and of D and F, respectively, and yellow signals indicate the co-localization of Herp and GRP78/GRP94. Bar, 20 μm.

**Fig. 8.** Determination of the membrane topology of Herp. A, protease protection assay. Epitope-tagged Herp, Myc-Herp-FLAG, was expressed in HUVECs and microsomal membranes were collected. After incubation with increased amounts of proteinase K (0 μg for lanes 1, 4, 7, and 10; 0.1 μg for lanes 2, 5, 8, and 11; and 0.4 μg for lanes 3, 6, 9, and 12), Western blot analysis was performed using anti-Myc tag, anti-FLAG tag, anti-Calnexin NT, and anti-Calnexin CT antibodies. Positions of molecular mass standard proteins are shown on the left with their molecular masses. B, immunocytochemistry of selectively permeabilized HUVECs with SLO. HUVECs were transiently transfected with the Myc-Herp-FLAG expression plasmid. Before (panels a, d, and g) or after permeabilization with SLO (panels b, e, and h) or SLO/Triton X-100 (panels c, f, and i), cells were stained with anti-Myc tag, anti-FLAG tag, and anti-KDEL antibodies. The immunoreactivities are shown in green, and the nuclear staining by propidium iodide is in red. Bar, 20 μm.
based on the algorithm of Kyte and Doolittle (41), however, predicts an additional hydrophobic stretch at the extreme C terminus (Fig. 9). If this region is embedded in the lipid bilayer, Herp may assume a multi-pass topology with both termini exposed to the cytoplasm. The Arg^311–Ser^365 loop would face the ER lumen (Fig. 9, model 2), with the majority of the molecule exposed to the cytoplasm. Thus, Herp may play a role separate from the molecular chaperones for proteins in the ER. Herp may interact with other ER-resident chaperones to assist their function in the UPR.

Herp has an unusual N-terminal domain similar to ubiquitin (Fig. 4B). Ubiquitin is a small, highly conserved protein present in all eukaryotic cells. The modification of cellular proteins with ubiquitin targets them for degradation by a large, multisubunit protease, the 26 S proteasome. Many proteins with a structural similarity to ubiquitin present in cells, Ubs (ubiquitin-like proteins), are divided into two subclasses: small, type-1 Ubs, such as SUMO-1 and NEDD8, that are ligated to target proteins in a similar manner to ubiquitin, and type-2 Ubs containing ubiquitin-like structures within a variety of large proteins having distinct functions, such as Elongin B, Rad23, and Parkin. Although ubiquitin and type-1 Ubs are central players in post-translational protein modification, the significance of type-2 Ubs remains obscure (42, 43). The lack of a diglycine motif at the C-terminal end of the ubiquitin domain suggests that Herp is a type-2 Ubl. The ubiquitin-like domain of Elongin B serves a chaperone-like function, facilitating the assembly and enhancing the stability of the Elongin A/B/C complex (44, 45). Rad23 interacts with the 26 S proteasome through an N-terminal ubiquitin-like domain (46, 47). ER-resident molecular chaperones and folding enzymes participate in ER-associated degradation in a manner dependent on the proteasome (48). From the viewpoint of quality control (49), it is interesting to consider that UPR-inducible Herp could possibly interact with the proteasome. The identification of molecules interacting with the Herp ubiquitin-like domain may promote a functional understanding of Herp.

The proximal promoter region of UPR target genes such as GRP78, GRP94, and calreticulin contains an ER stress response cis-element, ERSE (16, 17). van Laar et al. (27) recently demonstrated a functional ERSE in the 5′-flanking region of Mif1/KIAA0025, a gene identical to Herp. To obtain the high levels of Herp induced by the UPR, an additional cis-element may be involved in Herp mRNA up-regulation. In addition to transcriptional regulation, cells under ER stress immediately control the cellular, translational capacity, protecting against further accumulation of unfolded proteins in the ER. This translational inhibition occurs mainly through phosphorylation of the α-subunit of eukaryotic initiation factor 2, catalyzed by PERK (30). It remains unknown how UPR-inducible proteins such as Herp and GRP78 can be efficiently translated during transcriptional repression. GRP78 mRNA can be translated in poliovirus-infected cells when general, cap-dependent translation of host cell mRNAs is inhibited, indicating that cap-independent translation initiated at an internal ribosome-binding site is utilized (50). Interestingly, the induction of Herp at the protein level is greater than GRP78 in our experiments (Fig. 6A). It will be interesting to address the molecular mechanism facilitating the translation of UPR-inducible proteins.

At present, the role of the activation of the UPR response in patients with hyperhomocysteinemia remains unknown. Development of a sensitive method to monitor ER stresses is required. The UPR-sensitive transcriptional regulation and undefined translational system of Herp may be useful in the understanding and treatment of this disease condition.

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