Supporting Information

Shimizu, et al. 10.1073/pnas.0811591106

SI Materials and Methods

Cell Lines and Antibodies. The mouse plasmacytoma cell lines Ag8(8) (γ^+,κ^-) and Ag8.653, and human Burkitt's lymphoma cell line Ramos were maintained in complete RPMI-1640 medium containing 10% FBS (FBS), 2 mM l-glutamine, 100 units/mL penicillin-streptomycin, and 55 μM 2-mercaptoethanol (2ME). The I.29μ^+ murine B cell line was cultured in complete RPMI-1640 medium containing 20% FBS (FBS), 2 mM l-glutamine, 100 units/mL penicillin-streptomycin, 1x nonessential amino acids (Invitrogen), and 55 μM 2-mercaptoethanol (2ME), and 293T and HeLa cell lines were cultured in DMEM media supplemented with 10% FBS, 2 mM l-glutamine, and 100 units/mL penicillin-streptomycin. All cell lines were cultured at 37 °C under 5% CO_2, except for the I.29μ^+ cell line, which was cultured under 8% CO_2. 2.5 μg/mL tunicamycin (Sigma) and 1 μM thapsigargin (Sigma) were used for indicated times.

The anti-pERp1 antiserum was raised against recombinant human pERp1 (residues 75–190) fused to the amino-terminal GST-tag (GST-pERp1) and expressed in *E. coli*. The antiserum from an immunized rabbit was subjected to ammonium sulfate precipitation (50% saturation), followed by absorption of antiserum first with recombinant GST protein bound to Glutathione Sepharose 4 fast flow (Amersham), and subsequent affinity purified with amino-terminal His-tagged human pERp1 (residues 17 - 190) covalently coupled to CNBr-activated Sepharose 4 fast flow (Amersham). The monoclonal anti-HA (12CA5) antibody was kindly provided by Dr. Al Reynolds (Vanderbilt University), and the anti-ERp57 (M6/2A6) and anti-PDI (M5/3H1) monoclonal antibodies were gifts from Dr. Akio Tsuru (Nara Inst. Sci. Tech., Japan). Rabbit polyclonal anti-rodent BiP and anti-CHOP antiserum were described in refs. 1 and 2. All other antibodies were purchased from commercial sources; anti-mouse IgM, Ig κ, and Ig λ (Southern Biotech), anti-actin (Santa Cruz), anti-phosphorylated eIF2α, anti-total eIF2α (Biosource), and rabbit IgG (Jackson ImmunoResearch).

Identification and Cloning of pERp1. pERp1 was identified as part of chaperone complex bound to Ig HC in Ag8(8) cells, using mass-spectrometry and peptide sequencing of trypsin-digested fragments as described in ref. 3. Human and mouse pERp1 were cloned from Ramos and Ag8(8) cell lines, respectively, using the following primer pairs; 5′-CTACACTTGCTGAACTGGCTCCTGG-3′ and 5′-GAGGGTAGAGTCCAGGACTAGAGCTC-3′ for human pERp1, 5′-GCCATGAGACTGCCTCTGCCAC-3′ and 5′-CTAAAGCTCTTCTCTCTGGGCCAGG-3′ for mouse pERp1. The cysteine residue numbers are off by one in the human sequence compared with that of the mouse due to a single amino acid insertion in the human signal sequence (ref. 4; see also Fig. S6*A*).

Transfection of Cells and Detection of Proteins. Cells were lysed either by boiling in the presence of SDS for immunoblotting, or in Nonidet P-40 containing buffer for immunoprecipitation. For the SDS boiling method, cells were collected, washed with PBS and suspended in SDS lysis buffer [50 mM Tris·HCl (pH8.0), 0.6% SDS, 0.25 mM phenylmethylsulphonyl fluoride (PMSF), and the Complete protease inhibitor mixture (Roche)]. After incubating at 95 °C for 10 min, samples were diluted with a 4-fold volume of buffer containing 10 mM sodium phosphate (pH 7.2), 2 mM EDTA, 250 mM NaCl, 0.1% Nonidet P-40, 0.25 mM PMSF, and the protease inhibitor mixture, followed by centrifugation to remove debris. For the Nonidet P-40 extraction method, cells were collected, rinsed with PBS and suspended in Nonidet P-40 lysis buffer [50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 1 mM EDTA, 10% glycerol, 0.25 mM PMSF, and the protease inhibitor mixture]. Proteins were extracted on ice for 1 h and debris was removed by centrifugation. For expression in mammalian cells, full length human wild-type or mutant pERp1 was inserted into pCDNA3 (GE Healthcare), and a full length chimeric humanized mouse Igγ HC (5) was inserted into pQCXIH (Clontech). The truncated Igγ HC (V-C_H1: mini-HC) and hamster BiP were inserted into pSVL (Amersham) and pMT respectively as described in ref. 5. All constructs were introduced into 293T cells by the calcium phosphate precipitation method (6) or into HeLa and COS-1 cells using FuGENE6 reagent (Roche). The pSuper.retro.puro vector (Oligoengine) was used to produce shRNA directed against two different mouse pERp1 sequences. The targeting sequence for sequence 1 was: 5′-AAGAGAAGUACUCGGCUCAUA-3′, and for sequence 2 was 5′-AAUCAGCGUGAUGAUUUCUGG-3′. The vectors were introduced into Ag8(8) cells using a Gene Pulser (Bio-Rad) as described in ref. 7. Twenty-four hours after transfection, cells were selected with puromycin, and single cell clones were isolated. Cross-linking with the membrane permeable, thiol-cleavable cross-linker dithiobis(succinimidyl propionate) (DSP) was performed as described in ref. 3. To isolate proteins, the indicated antibodies were incubated with the cell lysate, and immune complexes were recovered using protein A-Sepharose (Sigma). Mouse γ HC expressed in Ag8(8) cells were isolated by direct binding to Protein A Sepharose beads. For immunostaining, transfected HeLa cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and stained with anti-pERp1 and a mouse monoclonal anti-ERp57. FITC-conjugated goat anti-rabbit IgG (Southern Biotechnology) and CY3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) were used as secondary antibodies. Signals were detected by fluorescence microscopy using a Nikon Eclipse E600 Microscope.

Isolation of Total RNA, Northern Analysis and Reverse Transcriptase-PCR (RT-PCR). Total RNA was isolated from cells using an RNeasy mini kit (Qiagen). Northern blot analysis was performed by standard methods (6). A human multitissue Northern blot used in Fig. 1*B* was purchased from Clontech (catalog number 7780–1). cDNA probes were prepared by RT-PCR using the specific primer pairs for human and mouse pERp1 that were described for cloning them (see above), for human β-actin as described in ref. 8, and for mouse 28S ribosomal RNA; 5′-CTCAGTACGAGAGGAACCGC-3′ and 5′-CGGATTCTGACTTAGAGGCG-3′. Probes were radiolabeled by using Prime-It II Random Primer Labeling Kit (Stratagene) following the manufacturer's instructions. The loading control used in Fig. 1*B Lower* was used from ref. 9 with permission. To detect splicing of mouse XBP-1 mRNA in I.29μ^+ cells, RT-PCR that only amplifies the spliced XBP-1 was carried out using following primer pair; 5′-GCTGAGTCCGCAGCAGGTG-3′ and 5′-CCTCCAGACTAGCAGACTCTG-3′. The PCR was conducted using the following protocol; 1 min at 94 °C and 35 cycles of 30 sec at 94 °C (denaturation), 30 sec at 56 °C (annealing), and 1 min at 72 °C (extension). An additional extension of 2 min followed after the 35 cycles. An aliquot of input RNA used in the

RT-PCRs was run on agarose gels and the 28S rRNA was detected by ethidium bromide staining for the loading control.

Isolation and Fractionation of Primary B Cells and Induction of Plasma Cell Differentiation. Mouse B cells were isolated from the bone marrow or spleen of 8- to 12-week-old female C57BL/6 mice by depleting non-B cells with surface markers (CD3, CD4, CD8, Mac1, GR1 and TER119) using autoMACS (Miltenyi Biotec). Individual B cell subpopulations were isolated by first gating on $B220^+$ cells, and further separated by immunofluorescence cell sorting using the FACS Vantage DiVa (BD Biosciences) as described (10, 11). In brief, pre-B ($CD43^+$, $CD19^-$), pro-B ($CD43^-$, $CD19^+$, IgM^-, IgD^-), immature-B ($CD43^-$, $CD19^+$, IgM^+, IgD^-), and mature-B ($CD43^-$, $CD19^+$, IgM^+, IgD^+) were separated from bone marrow B cells, and T_1 ($CD19^+$, $CD93^+$, $CD23^-$), T_2 ($CD19^+$, $CD93^+$, $CD23^+$), follicular-B ($CD19^+$, $CD93^-$, $CD23^+$), and marginal zone-B ($CD19^+$, $CD93^-$, $CD23^-$, $CD21^{High}$) were separated from splenic B cells. Plasma cell differentiation was induced from purified splenic B cells by either treating with 50 μg/mL LPS (Sigma), or with 20 μg/mL goat anti-Igμ $F(ab')_2$ fragment (Jackson ImmunoResearch), followed by incubating with 30 units/mL IL-4 and 10 units/mL IL-5 (PeproTech) as described in ref. 12. Cells were cultured as described for the I.29μ^+ cell line.

Metabolic Labeling and Pulse–Chase Experiments. Cells were incubated in methionine- and cysteine-free DMEM or RPMI MEDIUM 1640 labeling medium containing 10% dialyzed FBS with ^{35}S-TransLabel (MP Biomedicals) for the specified times. Chase was initiated by washing the cells twice with ice-cold PBS where indicated and then adding an excess of unlabeled methionine (2 mM) and cysteine (2 mM) to the chase media. Aliquots of cells were removed at the indicated times of chase, and cells were separated from the media and washed once with ice-cold PBS containing 10 mM NEM before lysing. The cell lysates were immunoprecipitated as indicated. To monitor the folding of the mini-HC, the intensity of each HC form was quantified on a phosphorimager, and the percentage of the fully oxidized form was calculated. Mean values and standard errors are calculated from at least two independent experiments.

Detection of Mixed Disulfides. Cells were washed with ice-cold PBS containing 10 mM NEM for 5 min and treated with 10% TCA to prevent postlysis disulfide bond formation/reduction. The resulting pellet was washed with 70% acetone twice, and proteins were dissolved in pH6 lysis buffer [40 mM sodium phosphate (pH 6.0), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 10% glycerol, 0.25 mM PMSF, and the complete protease inhibitor mixture (Roche)]. The immunoprecipitated samples from the lysate were subjected to two-dimensional SDS/PAGE under nonreducing (first-dimension), then reducing (second-dimension) conditions as described in ref. 3.

Purification of Bacterially Expressed Recombinant Proteins and ATPase Assay. Recombinant hamster BiP and human ERdj3 were expressed in bacteria and purified as indicated (13). Recombinant human pERp1 was inserted into the pQE30 vector (Qiagen), expressed in *Escherichia coli* M15 cells, isolated under nondenaturing conditions and purified on Ni–agarose beads (Qiagen). ATPase assay was performed in the presence of DTT as described in ref. 13 or in the absence of DTT, because pERp1 has three disulfide bonds that were found to be sensitive to even very low levels of DTT. To measure ATP hydrolysis, recombinant BiP (1 μM) was incubated at 37 °C for 20 min with ERdj3 (0.5 μM), and/or various concentrations of pERp1 proteins as indicated. After chromatography, the radioactive ATP and free phosphate signals were quantified by phosphorimaging analysis (Molecular Dynamics) with the use of Image Quant software (Invitrogen). The free phosphate signal was expressed as a percentage of the total phosphate signal. The mean values and standard errors were calculated from two independent experiments.

1. Brewer JW, Hendershot LM, Sherr CJ, Diehl JA (1999) Mammalian unfolded protein response inhibits cyclin D1 translation and cell-cycle progression. *Proc Natl Acad Sci USA* 96:8505–8510.
2. Hendershot LM, et al. (1995) In vivo expression of mammalian BiP ATPase mutants causes disruption of the endoplasmic reticulum. *Mol Biol Cell* 6:283–296.
3. Meunier L, Usherwood YK, Chung KT, Hendershot LM (2002) A subset of chaperones and folding enzymes form multiprotein complexes in endoplasmic reticulum to bind nascent proteins. *Mol Biol Cell* 13:4456–4469.
4. Katoh M, Katoh M (2003) MGC29506 gene, frequently down-regulated in intestinal-type gastric cancer, encodes secreted-type protein with conserved cysteine residues. *Int J Oncol* 23:235–241.
5. Lee YK, Brewer JW, Hellman R, Hendershot LM (1999) BiP and immunoglobulin light chain cooperate to control the folding of heavy chain and ensure the fidelity of immunoglobulin assembly. *Mol Biol Cell* 10:2209–2219.
6. Sambrook JF, Russell DW (2001) *Molecular Cloning* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), 3rd Ed.
7. Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, eds (1991) *Current Protocols in Immunology* (John Wiley & Sons, Hoboken, NJ).
8. Brewer JW, Cleveland JL, Hendershot LM (1997) A pathway distinct from the mammalian unfolded protein response regulates expression of endoplasmic reticulum chaperones in non-stressed cells. *EMBO J* 16:7207–7216.
9. Shen Y, Meunier L, Hendershot LM (2002) Identification and characterization of a novel endoplasmic reticulum (ER) DnaJ homologue, which stimulates ATPase activity of BiP in vitro and is induced by ER stress. *J Biol Chem* 277:15947–15956.
10. Hardy RR, Shinton SA (2004) Characterization of B lymphopoiesis in mouse bone marrow and spleen. *Methods Mol Biol* 271:1–24.
11. Rolink AG, Andersson J, Melchers F (2004) Molecular mechanisms guiding late stages of B-cell development. *Immunol Rev* 197:41–50.
12. Phillips C, Klaus GG (1992) Soluble anti-mu monoclonal antibodies prime resting B cells to secrete immunoglobulins in response to interleukins-4 and -5. *Eur J Immunol* 22:1541–1545.
13. Awad W, Estrada I, Shen Y, Hendershot LM (2008) BiP mutants that are unable to interact with endoplasmic reticulum DnaJ proteins provide insights into interdomain interactions in BiP. *Proc Natl Acad Sci USA* 105:1164–1169.

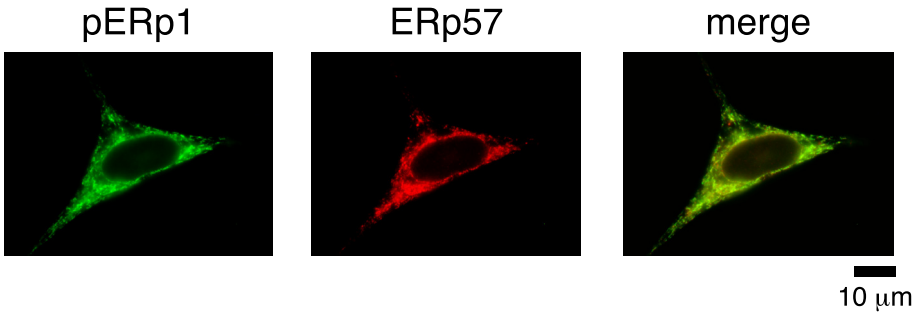


Fig. S1. Subcellular localization of pERp1 protein. HeLa cells were transiently transfected with full length human pERp1. pERp1 and ERp57 were visualized by immunofluorescence staining with the indicated antisera. (*Left*) pERp1. (*Middle*) Endogenous ERp57 as ER marker. (*Right*) Merged image of *Left* and *Middle*. This data indicates that pERp1 is a resident ER protein.

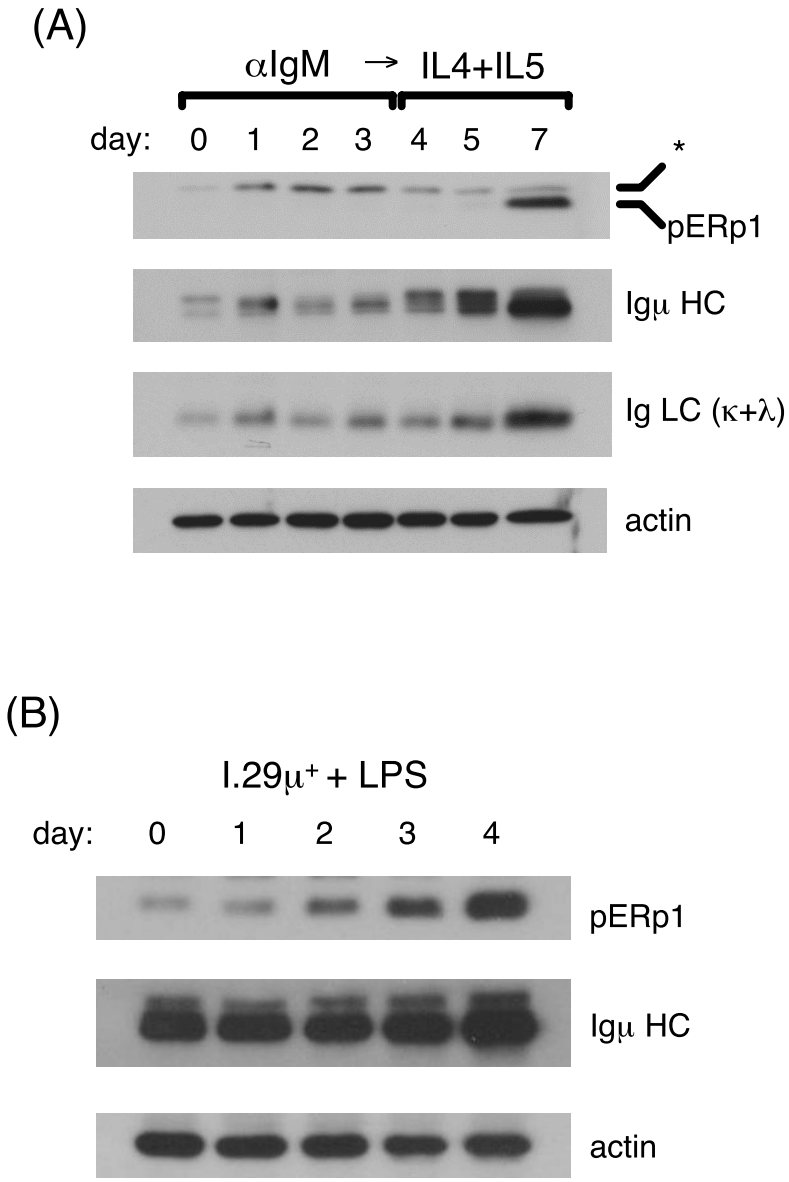


Fig. S2. Expression of pERp1 during plasma cell differentiation of mouse splenic B cells and the I.29μ^+ murine cell line. (*A*) Mouse splenic B cells were isolated as described in the Materials and Methods and treated with goat anti-Igμ F(ab′)$_2$ fragment. After three days, cells were washed to remove the anti-IgM antibody and then treated with IL-4 and IL-5 for up to 4 additional days (7 days total). At each of the indicated time points, an aliquot of cells was collected, and lysed. Each sample was normalized for total protein and electrophoresed for analysis by immunoblotting with the indicated antibodies. The asterisk denotes a nonspecific band recognized by our antiserum. Actin serves as a loading control. We found that pERp1 expression was induced in splenic B cells not only when they were stimulated through the toll receptor by LPS, but also when they were activated through the Ig receptor, indicating that the induction of pERp1 is generally linked to plasma cell differentiation. (*B*) I.29μ^+ cells were treated with LPS for the indicated times to induce plasma cell differentiation. Cells were collected, lysed, and proteins were analyzed by immunoblotting as indicated. Actin serves as a loading control. We found that similar to splenic B cells, pERp1 expression is induced in I.29μ^+ cells in response to terminal differentiation signals.

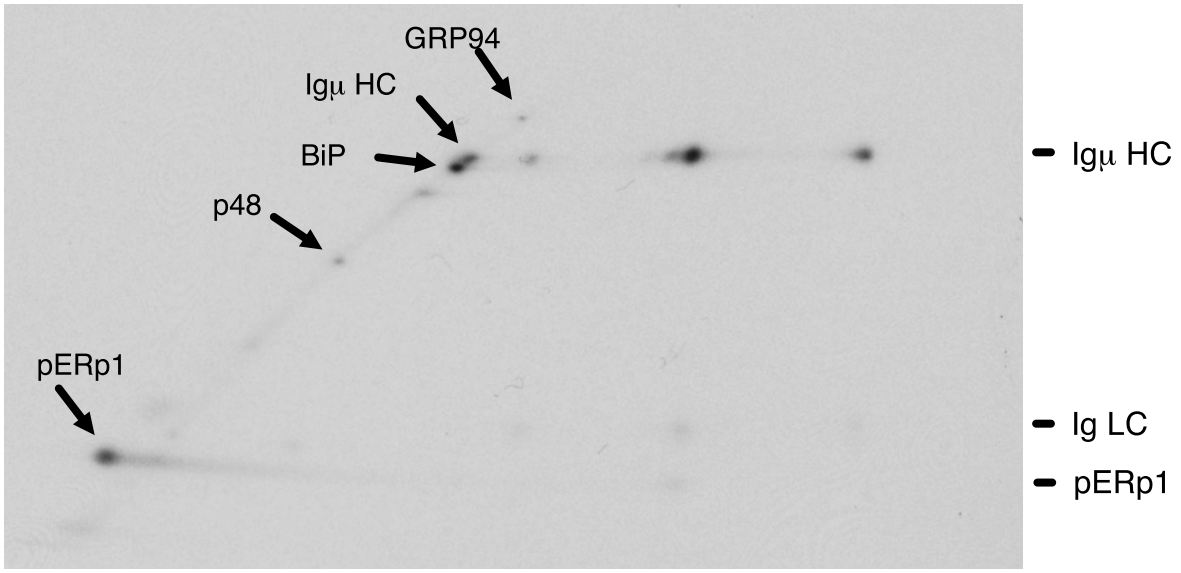


Fig. S3. pERp1 associates with BiP and other members of the ER chaperone complex in addition to assembling IgM intermediates. A lighter exposure of the gel used in Fig. 4*B* is shown. It is clear that there are two closely located spots along the diagonal, which represents BiP and μ HC as indicated. Other members of the ER chaperone complex (3) were also detected and are indicated.

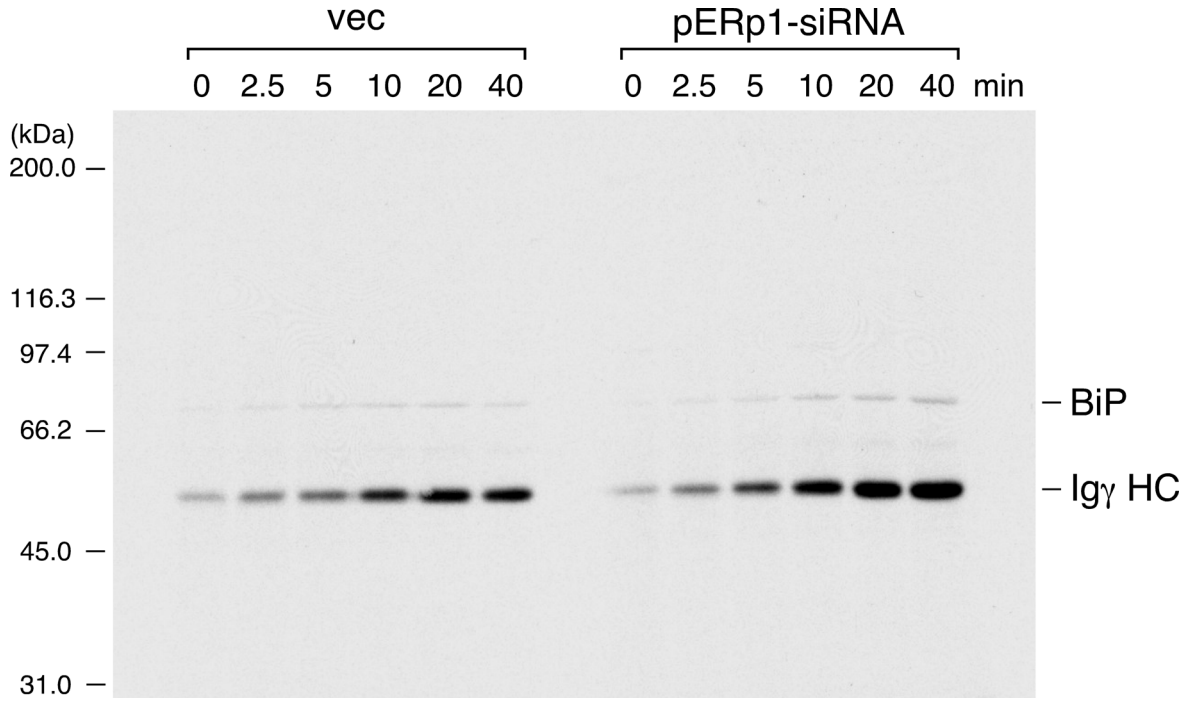


Fig. S4. Only γ HC and BiP are observed in Ag8(8) control and pERp1 deficient cells when isolated HC are analyzed under reducing conditions. Pulse–chase experiments were performed as in Fig. 5*B*, except that proteins were separated by SDS/PAGE under reducing conditions.

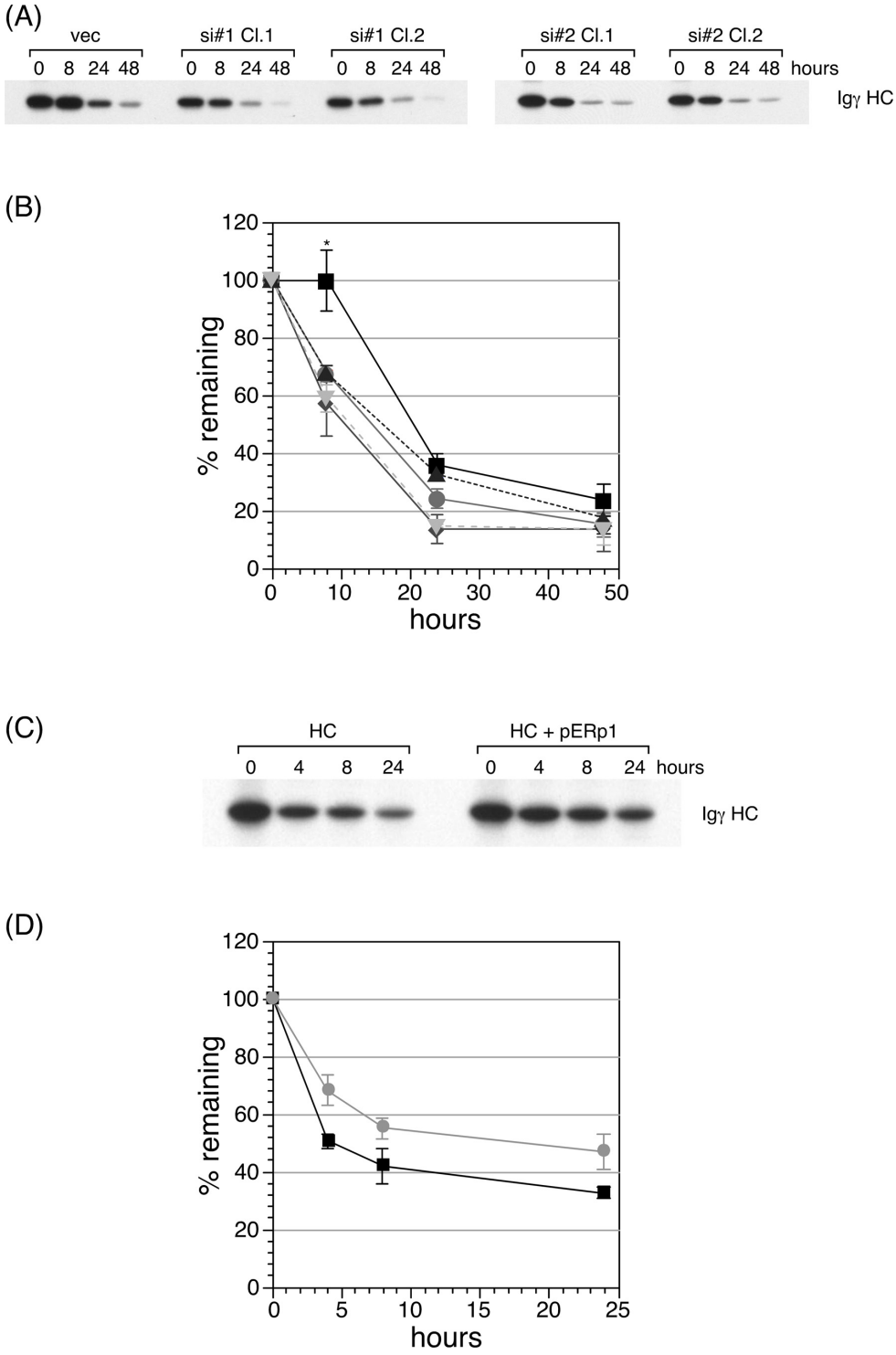
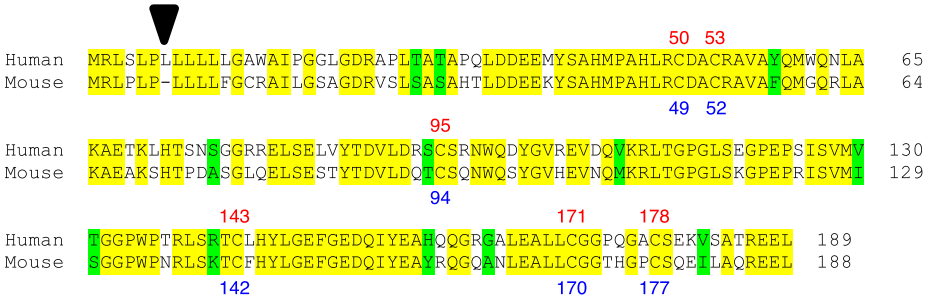
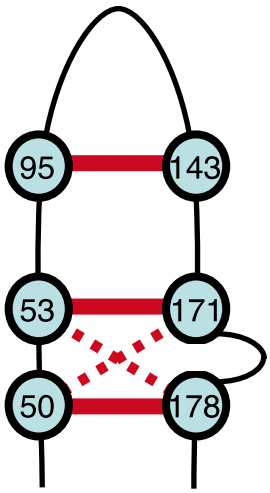


Fig. S5. Expression of pERp1 stabilizes Ig HC. (*A* and *B*) The Ag8(8) vector control and pERp1 knockdown lines used in Fig. 5 *A* and *B* were metabolically labeled with [^{35}S] methionine and cysteine for 20 min and chased for the indicated times. Igγ HC were isolated from cells and visualized by autoradiography (*A*), and the intensities of the individual bands were quantified by phosphorimager. The mean values and standard errors are calculated from at least two independent experiments and plotted as a percentage of the signal at T = 0 (*B*). Square: vector control; circle: sequence 1, cl. 1; upward triangle: seqquence 1, cl. 2; diamond: sequence 2, cl. 1; downward triangle: sequence 2, cl. 2. *, $P < 0.005$. (*C* and *D*) 293T cells transfected with HC alone or with pERp1 were metabolically labeled with [^{35}S] methionine and cysteine for 20 min and chased for the indicated times. Igγ HC were isolated from cells and visualized by autoradiography (*C*), and the intensities of the individual bands were quantified by phosphorimager. The mean values and standard errors at each time point are calculated from at least two independent experiments and plotted as a percentage of the signal at $T = 0$ (*D*). Square and circle represent HC and HC+pERp1, respectively. *, $P < 0.001$; **, $P < 0.02$.

(A)



(B)



(C)

residue number	50	53	95	143	171	178
WT	C	C	C	C	C	C
C50S	S	C	C	C	C	C
C53S	C	S	C	C	C	C
C95S	C	C	S	C	C	C
C143S	C	C	C	S	C	C
C171S	C	C	C	C	S	C
C178S	C	C	C	C	C	S
C50S-C178S	S	C	C	C	C	S
C53S-C171S	C	S	C	C	S	C
C95S-C143S	C	C	S	S	C	C
C50-C178 only	C	S	S	S	S	C
C53-C171 only	S	C	S	S	C	S
C95-C143 only	S	S	C	C	S	S
C50S-C53S	S	S	C	C	C	C
C171S-C178S	C	C	C	C	S	S
C50-C53 only	C	C	S	S	S	S
C171-C178 only	S	S	S	S	C	C
no cys	S	S	S	S	S	S

Fig. S6. Identification of conserved cysteine residues in pERp1. (*A*) Alignment of human and mouse pERp1 amino acid sequences. Downward arrowhead indicates the single amino acid insertion in the human pERp1 signal sequence, which results in the residue numbers being off by one between human and mouse pERp1. Cysteine residues numbers are indicated in red and blue for human and mouse, respectively. (*B*) Schematic model of intrachain disulfide pattern of pERp1. van Anken et al. [(2009) *Proc Natl Acad Sci USA*, 10.1073/PNAS.0903036106] determined the disulfide bond pattern of recombinant mouse pERp1, but could not distinguish between C50-C171 and C53-C178 (shown in dotted line) or C50-C178 and C53-C171 (shown in solid line), because there is no trypsin cleavage site between either C50 and C53 or C171 and C178. However, because the C50S, C178S. and C50–178S mutants all retain activity whereas neither the C53S or C171S mutant does (Fig. 6), we believe it is most likely that the intrachain disulfide bonds are formed between C50-C178 and C53-C171. (*C*) A list of all of the cys → ser mutants used in this study (Fig. 6) is shown in the diagram.

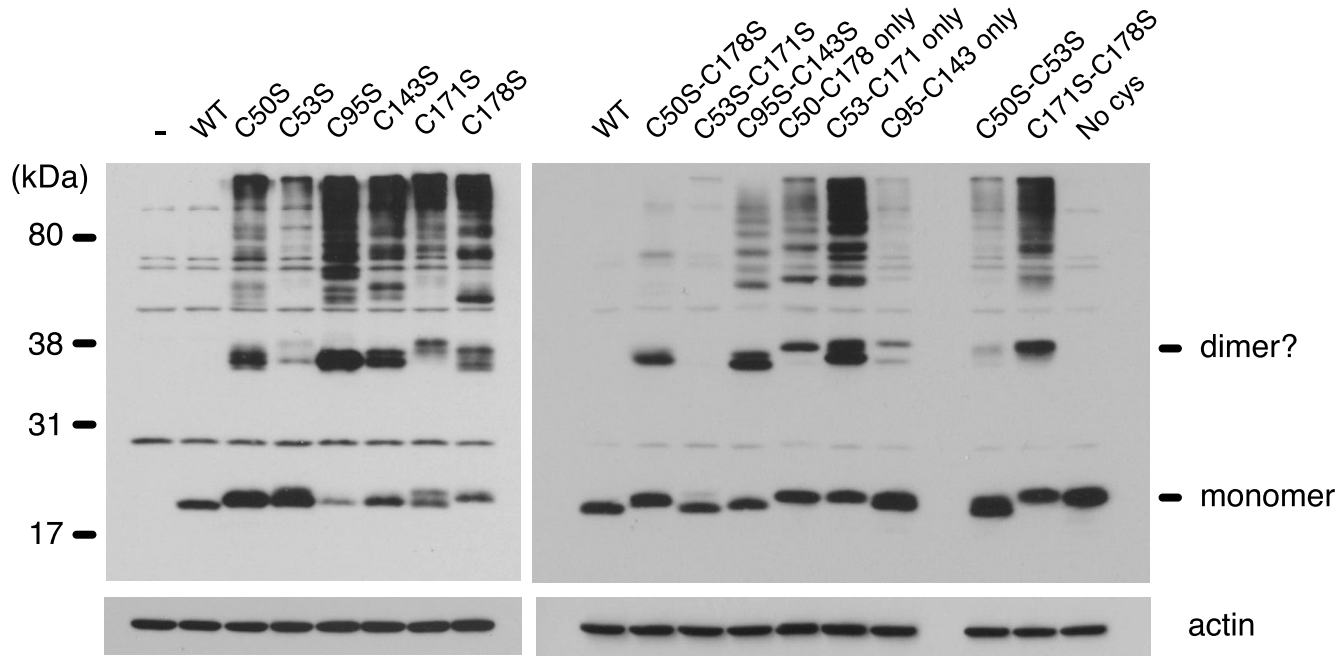


Fig. S7. Expression of pERp1 cysteine mutants in 293T cells. WT and all of the pERp1 mutants (Fig. S6C) were expressed in 293T cells, and the cell lysates were analyzed with nonreducing SDS/PAGE followed by immunoblotting. Actin serves as a loading control. It should be noted that unlike wild-type pERp1, many of the mutants formed dimers or higher order disulfide linked oligomers, suggesting that the mutations were likely to be affecting the overall structure of pERp1.

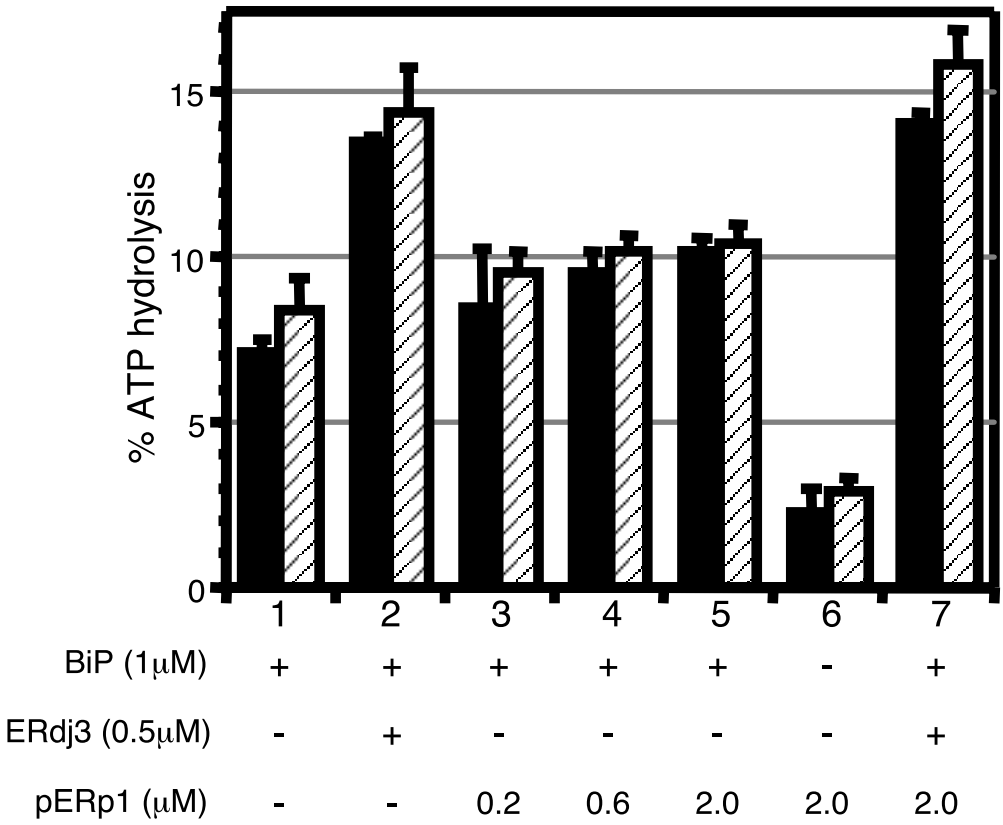


Fig. S8. pERp1 does not stimulate BiP's ATPase activity. Recombinant protein was isolated for wild-type hamster BiP, human ERdj3, and human pERp1. The ATPase activity was measured as indicated in the presence (hatched boxes) or absence (black boxes) of 10 mM DTT. There was a slight but reproducible stimulation of BiP's ATPase activity by pERp1 (compare lanes 1 and 3–5). However, we observed very similar pattern when assay was performed in the presence of 10 mM DTT, which completely reduces recombinant pERp1 protein (data not shown) and would be expected to inhibit its function. Thus, it is likely that our preparation of pERp1 has a very small amount of a contaminant, which is in keeping with this preparation having a very modest ATPase activity under both reducing and nonreducing conditions. Thus, it appears that pERp1 does not stimulate oxidative folding indirectly via interactions with BiP.