Supporting Information

Anken et al. 10.1073/pnas.0903036106

SI Methods

Cell Culture and Activation of B Cell Lymphomas. The I.29μ^+ lymphomas were cultured in suspension in RPMI (Life Technologies) supplemented with 10% LPS-free FCS (Hybond), 1 mM sodium pyruvate, and 50 mM 2-mercaptoethanol. For differentiation experiments, cells were incubated in the presence of 20 μg/mL LPS (Sigma). Cells were harvested before and after 1, 2, 3, 4, or 5 days of differentiation. HeLa cells were maintained in MEM supplemented with 10% FCS and nonessential amino acids. HT1080 cells were cultured in DMEM supplemented with 8% FCS; 293FT cells were cultured in DMEM supplemented with 10% FCS. All media were further supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM (or 1 mM for I.29μ^+ lymphomas) glutamax. Cells were cultured at 37 °C in 5% (vol/vol) CO_2.

The 2D Gel Electrophoresis and Proteomic Data Analysis. I.29μ^+ lymphomas were washed twice in ice-cold buffer (20 mM Mes/100 mM NaCl/30 mM Tris·HCl, pH 7.5) and lysed in a small volume of MNT, the same buffer solution, containing 1% Triton X-100, and protease inhibitor mixture (1 mM PMSF/1 mM EDTA/10 μg/mL each of chymostatin, leupeptin, antipain, and pepstatin). Cell lysates were centrifuged for 10 min at 17,000 $\times$ *g* to get rid of nuclei and debris. Protein concentrations of postnuclear supernatants were determined by DC Protein Assay kit (Bio-Rad). Approximately 200 μg of protein from postnuclear lysates was dissolved in reswelling solution [6.0 M urea (Boehringer), 1.9 M thiourea (Sigma), 4% Triton X-100, 15 mM DTT, and 0.5% (vol/vol) carrier ampholytes pH 3–10 (AP Biotech)]. After reswelling of 18-cm Immobiline DryStrips pH 3–10 Non-Linearized (AP Biotech) with samples, proteins were separated by IEF in the first dimension and by continuous 10% SDS/PAGE in the second dimension. Protein spots were visualized by silver staining. Signals from proteins were detected by densitometric scanning of silver stained gels. Spot detection and matching of spots between gels was performed with the PDQuest software package, version 8.0.1 (Bio-Rad). Relative abundance of proteins in the proteome was then determined from spot intensities as percentage of total signal of all spots in the gel. Spot identities were either determined before (1) or in the same manner (in the case of ERp46 and the novel 18-kDa protein). Protein identity was revealed by mass fingerprinting or by comparison of sequence tags from tandem MS analyses to the sequences of proteins or translated ESTs.

Profiling and Exact Mass Determination by MS. Lysates from I.29μ^+ lymphomas were purified and concentrated by using μZipTips C18 (Millipore) and either directly analyzed by nano-ESI-MS Q-ToF (Micromass) MS, or first mounted onto a target plate in a matrix of 10 mg/mL α-cyano-4-hydroxycinnamic acid (Fluka) in acetonitrile/water (50/50) with 0.1% TFA before analysis by MALDI-ToF (Voyager DE-STR Perseptive Biosystems) Reflectron MS. The exact mass determination was determined by analyzing the differences in *m*/*z* values between individual peaks within a series of peaks. Because the mass of the protein is constant, differences in *m*/*z* value correspond to differences in charge (protonation). From the equations [first peak = (m/z_1); second peak = $m/(z_1 - 1)$; third peak = $m/(z_1 - 2)$; etc.] the value of *m* was deduced.

Cloning. A cDNA clone, bearing pERp1 ORF, was obtained from Research Genetics, which we named pT7T3-pERp1. pERp1 ORF was subcloned into pcDNA3 (Invitrogen), yielding pcDNA-pERp1 and into pBlueScript KS$^+$ (Stratagene), yielding pBS-pERp1. This latter construct served as template to generate cysteine mutants by site directed mutagenesis using the Quickchange system (Invitrogen) according to the manufacturer's instructions with mutagenic oligos where the TGT and TGC cysteine encoding codons were changed into GCT, respectively, GCC alanine encoding codons with flanking sequences of 12–14 nt on either side. For double mutants, an additional cycle of site directed mutagenesis was performed with single mutants as template. An HA-tagged version of pERp1 was generated by inserting a linker encoding YPYDVPDYA into pBS-pERp1 between the G21 and D22 codons, i.e., directly downstream of the second signal peptide cleavage site. Using PCR we cloned the pERp1 sequence from the D22 codon onwards into pET15b (Novagen). This construct served to express the novel protein without its signal peptide in the bacterial cytoplasm. All constructs and mutations were verified by sequencing.

Expression and Purification of Recombinant pERp1 and Generation of α-pERp1 antisera. A starter culture of 5 mL LB containing 100 μg/mL ampicillin, 15 μg/mL kanamycin, and 12.5 μg/mL tetracycline was inoculated with the *Escherichia coli* strain Origami (DE3; Novagen) containing pET15b-pERp1 and grown overnight at 37 °C. Cultures were then grown in 500 mL of the same medium to an OD_{600} of 0.6 at 37 °C, and were put for 15 min at 28.5 °C before induction. Expression of the protein was induced by addition of 0.8 mM IPTG. After overnight expression at 28.5 °C cells were harvested by centrifugation at 6,500 $\times$ *g* for 15 min at 4 °C. The cell pellet was resuspended in 2 mL buffer A (50 mM Tris·Cl, pH 7.5/300 mM NaCl/20 mM imidazole) with 1 mM PMSF, 10 μg/mL of chymostatin, leupeptin, antipain, and pepstatin each, and 100 μg/mL lysozyme. Cells were lysed by sonication, and cells debris removed by centrifugation. The soluble fraction was then loaded onto a Ni^{2+}-NTA column (2 mL chelating Sepharose of Amersham Bioscience, with immobilized Ni^{2+}) that was preequilibrated in buffer A. The column was washed with buffer A. The adsorbed protein was eluted by increasing the ratio of buffer B (50 mM Tris·Cl, pH 7.5/300 mM NaCl/250 mM imidazole) over buffer A. Fractions containing recombinant protein were used to raise pERp1-specific antisera in rabbits. The antibody recognizes reduced unfolded, as well as disulfide bonded native pERp1, in Western blotting, immunofluorescence, electron microscopy, and in immunoprecipitations.

Immunoblotting. Western blotting was performed as described (1). Briefly, proteins from cellular lysates or culture media were separated by SDS/PAGE and subsequently transferred onto nitrocellulose membranes. Membranes were saturated with 3% nonfat milk (Bio-Rad) for >1 h, and then incubated in TBST with rabbit antisera: α-pERp1, α-PDI (2), α-GAPDH (Fitzgerald Industries International) or α-IgM (Zymed), and, next, by secondary HRP-conjugated antibodies (Bio-Rad). Alternatively, we used HRP-conjugated α-IgM antibodies (Southern Biotech) that obviated the need of a secondary antibody. HRP-conjugated antibody binding was detected via ECL (Bio-Rad) and Biomax MR film (Kodak).

SDS/PAGE. Samples were analyzed by reducing or nonreducing SDS/PAGE as described (3). Signals were detected by exposure of Biomax MR films or storage phosphor screens (Kodak) to

dried gels. Signals were detected on phosphor screens by using the Personal Molecular Imager FX (Bio-Rad), and quantitation was performed with the Quantity One software (Bio-Rad).

In Vitro Transcription and Translation and Translocation in Semipermeabilized Cells. The pT7T3-pERp1 was linearized with EagI. pERp1 cDNA was transcribed in vitro, using the riboprobe system (Promega), according to the manufacturer's instructions. Semipermeabilized cells were generated as follows: Subconfluent HT1080 cells were trypsinized, their plasma membrane was permeabilized with 1 μg/mL digitonin, cells were washed, and endogenous mRNA was degraded with micrococcal nuclease, as described (4). The cytosol of the SP cells was reconstituted with reticulocyte lysate (Promega), labeling mix [0.02 mM of all amino acids except methionine, 8 mM KCl, 5 mM DTT and 4 μCi Redivue Promix L-[^{35}S] labeling mix (AP Biotech)], and in vitro transcribed pERp1 mRNA, to allow in vitro translation and translocation for 30 min at 30 °C. Conventional in vitro translation served as a control. Samples were analyzed by SDS/PAGE.

Immunofluorescence. HeLa cells were grown on cover slips in 35-mm dishes to a confluency of 80% before they were infected with VVT7 and transfected with 4 μg of pBS-HA-pERp1 per dish. Four hours after infection, cells were fixed with methanol for 10 min at −20 °C, washed with 0.3% BSA in PBS containing calcium and magnesium (PBS^{++}), and blocked with 3% BSA in PBS^{++} for 30 min. Cells were incubated with a mixture of primary antibody at a dilution of 1:50 (α-HA-tag, 12CA5 mouse monoclonal cell culture supernatant; Roche) and 1:750 (rabbit polyclonal α-PDI). After washing with 0.3% BSA in PBS^{++}, antibodies were detected with a 1:500 dilution of Cy2-conjugated goat anti-mouse and Cy3-conjugated goat anti-rabbit secondary antibodies (Brunschwig). Cells were washed and mounted with mowiol (Sigma). Microscopy was performed by using a Leitz Aristoplan microscope (Leica), a 3CCD color vision camera module (Donpisha) controlled with the Sony XC-003 software.

Immunoelectron Microscopy. I.29μ^+ lymphomas that were activated with LPS for 4 days were fixed by adding 4% freshly prepared formaldehyde and 0.4% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 to an equal volume of culture medium for 10 min, followed by postfixation in 2% formaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 without medium. Cells were stored until further processing in 1% formaldehyde at 4 °C. Processing of cells for ultra thin cryosectioning and immunolabeling according to the protein A-gold method was done as described (5) with α-pERp1 or α-KDEL antisera. In brief, fixed cells were washed with 50 mM glycin in PBS, scraped gently from the dish in PBS containing 1% gelatin and pelleted in 12% gelatin in PBS. The cell pellet was solidified on ice and cut into small blocks. For cryoprotection, blocks were infiltrated overnight with 2.3 M sucrose at 4 °C, and afterward mounted on aluminum pins and frozen in liquid nitrogen. To pick up ultra thin cryosections, a 1:1 mixture of 2.3 M sucrose and 1.8% methylcellulose was used (6).

Viruses and Mammalian Protein Expression. To drive expression of WT or mutant pERp1 under control of the T7 promoter, subconfluent HeLa cells grown in 35-mm dishes were infected with recombinant vaccinia virus expressing T7 polymerase (7) at a multiplicity of infection of ≈5. Cells were transfected with a mixture of complete medium diluted 1:1 with MEM, containing 2 μg of pBS-pERp1 mutant or WT and 5 μg polyethyleneimine 30 min after infection.

Pulse–chase. Pulse–chases were performed as described previously (3). Briefly, 4 h and 45 min postinfection cells were washed with HBSS (37 °C) followed by a 15- to 30-min starvation in methionine- and cysteine-free MEM (37 °C). Subsequently, cells were pulse-labeled for 5 min in the presence or for 2 min in the absence of 5 mM DTT with 100 μCi/mL Redivue Promix L-[^{35}S] labeling mix. Directly after the pulse or after various chase times, cells were transferred onto ice and washed <5 min in ice-cold HBSS, containing the alkylating agent 20 mM *N*-ethylmaleimide (NEM) to block free sulfhydryl groups and prevent any further disulfide bond formation and isomerization. Cells were lysed in ice-cold lysis buffer, containing protease inhibitor mixture, for HeLa cells MNT and for I.29μ^+ lymphomas 0.5% Nonidet P-40, 0.5% deoxycholate, 50 mM Tris·Cl at pH 7.4, 150 mM NaCl, 20 mM NEM with or without 1 mM thiol-cleavable cross-linker dithiobis(succinimidylpropionate) (DSP; Sigma). Lysates were cleared from nuclei and debris by 10-min centrifugation at 16,000 × *g* at 4 °C. Postnuclear supernatants were subjected to immunoprecipitation with protein A-Sepharose-coupled antibodies: α-pERp1, α-PDI, or α-IgM antisera. Immunoprecipitates were washed twice with 10 mM Tris·Cl, pH 8.6/300 mM NaCl/0.05% Triton X-100/0.05% SDS (pERp1) or with 0.5% Nonidet P-40/0.5% deoxycholate/50 mM Tris·Cl, pH 7.4/150 mM NaCl (IgM). Last, samples were heated in sample buffer in the presence (reducing) or absence (nonreducing) of DTT before SDS/PAGE analysis.

In Vitro Thiol Oxidoreductase Assays. Recombinant pERp1 was compared with *E. coli* DsbA and DsbC as catalyst of insulin reduction with DTT by measuring the increase in turbidity at 650 nm, as described (8). Briefly, the protein catalysts were preincubated in 50 mM KH_2PO_4/KOH, pH 6.9/1 mM DTT/1 mM EDTA for 5 min at 25 °C, and the reductase assay was started by addition of insulin to a final concentration of 130 mM. Isomerase activity was measured in an in vitro assay with scrambled RNase A as substrate, as described (8).

RNA Silencing. MicroRNA-based shRNA lentiviral vectors (miR30) were produced as follows. Using Lipofectamine 2000 (Invitrogen), we cotransfected 293FT cells (Invitrogen) with the HIV-1 packaging plasmid, psPAX2 (Addgene), with the VSV-G expression plasmid, pMD2.G (Addgene), and with transfer vectors (Addgene) harboring the puromycin-resistance gene and shRNAs targeting pERp1 or as control the reporter luciferase. A day later, the transfection medium was replaced with fresh medium, and the day after, viral particles were harvested from the culture medium by centrifugation (5 min at 200 × *g*), and filtration through a 0.45-μm syringe filter (Sarstedt). The resulting shRNA lentiviral vectors were used to transduce I.29μ^+ lymphomas on two consecutive days. Via clonal selection of puromycin-resistant cells, we obtained I.29μ^+ lymphoma cell lines that were depleted of pERp1 to different levels as judged by immunoblotting with the α-pERp1 antiserum. Sequences of shRNAs targeting pERp1 were ts1: 5′-ggctcacacactggatgatgaa-3′ and ts2: 5′-agcctcgaaagtgctgcattta-3′. We validated efficiency of the complete RNA silencing procedure by producing isogenic control cells in parallel, targeting the built-in reporter luciferase instead of pERp1. All procedures were identical for control and pERp1-silenced cells, including the puromycin concentrations used.

1. van Anken E, et al. (2003) Sequential waves of functionally related proteins are expressed when B cells prepare for antibody secretion. *Immunity* 18:243–253.
2. Benham AM, et al. (2000) The CXXCXXC motif determines the folding, structure and stability of human Ero1-Lalpha. *EMBO J* 19:4493–4502.
3. Braakman I, Hoover-Litty H, Wagner KR, Helenius A (1991) Folding of influenza hemagglutinin in the endoplasmic reticulum. *J Cell Biol* 114:401–411.

4. Wilson R, Allen AJ, Oliver J, Brookman JL, High S, Bulleid NJ (1995) The translocation, folding, assembly and redox-dependent degradation of secretory and membrane proteins in semi-permeabilized mammalian cells. *Biochem J* 307:679–687.
5. Slot JW, Geuze HJ (2007) Cryosectioning and immunolabeling. *Nat Protoc* 2:2480–2491.
6. Liou W, Geuze HJ, Slot JW (1996) Improving structural integrity of cryosections for immunogold labeling. *Histochem Cell Biol* 106:41–58.
7. Fuerst TR, Niles EG, Studier FW, Moss B (1986) Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc Natl Acad Sci USA* 83:8122–8126.
8. Frickel EM, et al. (2004) ERp57 is a multifunctional thiol-disulfide oxidoreductase. *J Biol Chem* 279:18277–18287.

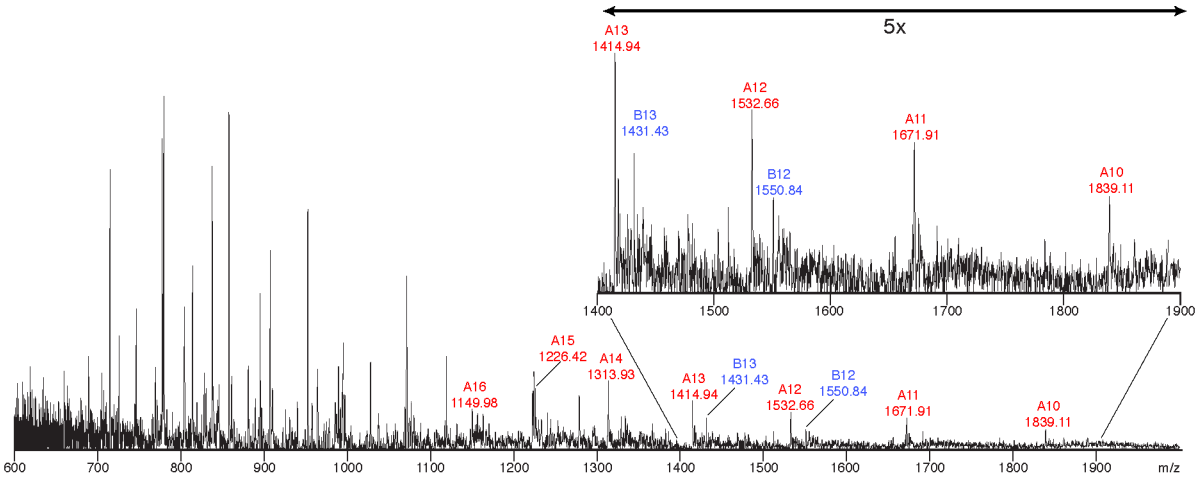

Fig. S1. Exact mass determination of pERp1. We analyzed by ESI-Q-ToF MS the lysate from 4-day activated I.29μ^+ lymphomas that we also used for direct profiling (Fig. 1*C*). By using home-made nanospray needles, lysate was infused into the Q-ToF mass spectrometer, which we calibrated with a mixture of NaI and CsI. Like the direct profiling approach, ESI-Q-ToF is biased toward smaller proteins. A prominent series of signals (red) and a second series of signals with lower intensity (blue) gave high *m/z* values and corresponded with the ''main,'' respectively, the ''shoulder'' peak in Fig. 1*C*. Mass differences between signals within series were in accordance with a charge state distribution from 10^+ to 16^+, from which we derived exact molecular masses of the intact protein as summarized in Table 1.

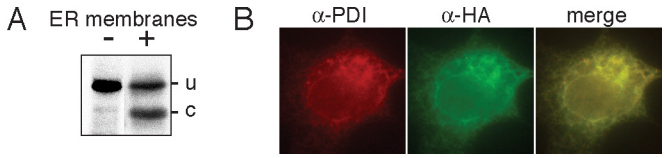

Fig. S2. pERp1 enters the ER and colocalizes with PDI. (*A*) In vitro translation and radiolabeling of the novel 18-kDa protein in the absence or presence of ER membranes. The bands correspond to the protein from which the signal peptide was removed (c, cleaved) or not (u, uncleaved). (*B*) Subcellular localization of the 18-kDa protein by immunofluorescence. HeLa cells transiently transfected with HA tagged pERp1 were fixed and costained with a mouse monoclonal antibody against the HA tag and with the rabbit α-PDI antiserum, followed by a Cy2-conjugated anti-rabbit secondary antibody and a CY3-conjugated anti-mouse secondary antibody. Cells were visualized by immunofluorescence microscopy.

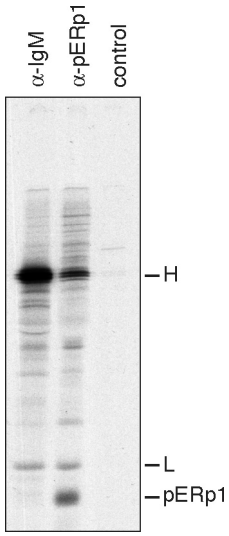

Fig. S3. pERp1 associates with IgM subunits in I.29μ^+ lymphomas in the presence of DTT. Cells were activated, pulse-labeled, and lysed as described in Fig. 6*A*, except that the pulse medium contained 5 mM DTT and cross-linker was not used. Lysates were immunoprecipitated with α-pERp1, α-IgM, or control antibodies, as indicated. IgM heavy (H) and light (L) chain and pERp1 are indicated.

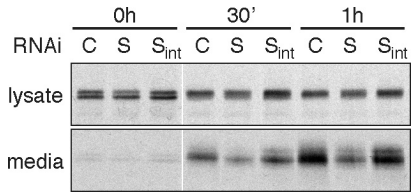

Fig. S4. Moderate silencing hardly diminishes IgM secretion. Intracellular and secreted IgM of all three cell lines as shown in Fig. 6*B*, control cells (C), cells with pERp1 silencing (S), or cells with intermediate silencing (S_{int}), were analyzed as in Fig. 6*C*. Note that a 5-fold excess of the IgM immunoprecipitate from the media was loaded.

Table S1. Mass balance sheet of the novel 18-kDa protein

Profiling spectra, kDa	Exact mass, Da	Sequence	Prediction, Da	Difference, Da
Main peak 18.4	18,380.77 ± 0.77	S19-L188	18,386.50	5.73 ± 0.77
Shoulder peak 18.6	18,596.28 ± 1.02	D22-L188	18,601.75	5.47 ± 1.02

The exact masses corresponding to the ″main″ and ″shoulder″ peaks in the MALDI-ToF spectra (Fig. 1*C*) were determined by ESI-Q-ToF (Fig. S1) and compared with the predicted masses of the novel protein with its N-terminal signal peptide cleaved off before either residue D22 or residue S19. Differences in experimentally determined and predicted mass both amounted to ≈6 Da, corresponding to the loss of 6 protons and electrons as a result of thiol oxidation.