

Efficient IgM assembly and secretion require the plasma cell induced endoplasmic reticulum protein pERp1

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Plasma cells daily secrete their own mass in antibodies, which fold and assemble in the endoplasmic reticulum (ER). To reach these levels, cells require pERp1, a novel lymphocyte-specific small ER-resident protein, which attains expression levels as high as BiP when B cells differentiate into plasma cells. Although pERp1 has no homology with known ER proteins, it does contain a CXXC motif typical for oxidoreductases. In steady state, the CXXC cysteines are locked by two parallel disulfide bonds with a downstream C(X)₆C motif, and pERp1 displays only modest oxidoreductase activity. pERp1 emerged as a dedicated folding factor for IgM, associating with both heavy and light chains and promoting assembly and secretion of mature IgM.

Key to an effective antibody-mediated immune system is that under no condition antibody “leaks” from nonactivated B lymphocytes, whereas on activation, massive secretion starts, and then only of completely assembled antibody. Although they do produce subunits, resting B cells do not secrete antibody. Only when cells are activated by antigen or mitogen do they differentiate into plasma cells, which secrete their own mass in antibody molecules per day (1). The conversion to an antibody-secreting plasma cell requires a “total makeover” of the lymphocyte: All cellular machineries are reorganized for the single purpose of bulk antibody production (2–4). Most striking is the change in volume of endoplasmic reticulum (ER), because this organelle accommodates the biosynthesis and assembly of antibody.

The ER is the first compartment of the secretory pathway; it supports disulfide bond formation, folding, and oligomerization of newly synthesized proteins. Efficiency in the folding process is accomplished through assistance by an abundance of both “generic” and tissue- or substrate-specific chaperones and folding enzymes (5, 6). The ER harbors a single prominent and highly conserved HSP70 family member BiP, but also contains a variety (>20) of PDI family oxidoreductases with CXXC active site motifs (7). They all seem to be involved in the oxidation, reduction, and/or isomerization of disulfide bonds, but how they divide or share these tasks and their substrates is largely unknown.

IgM is a challenging client for the plasma cell ER. The IgM subunits undergo oxidative folding and form interchain disulfide bonds during their stepwise assembly into mature secretory protein. In the end, IgM consists of at least 21 subunits [10 μ heavy (H) chains, 10 λ or κ light (L) chains, and a single J chain] and counts ≈ 75 intrachain and ≈ 25 interchain disulfide bonds (1). Besides the increase of “generic” ER folding factors that are present already in the resting B cell, specialized folding assistants may enrich the ER of plasma cells and even be required for efficient IgM maturation and secretion.

Here, we report on a previously undescribed dedicated folding assistant of IgM: the lymphocyte-specific ER-resident protein pERp1. In the course of B cell differentiation, pERp1 was up-

regulated more than any other protein: from nearly undetectable to abundance in the same range as GRP94 and BiP in the plasma cell. It associated with IgM H and L chains, promoted their assembly, and thereby, the secretion of mature IgM polymers.

Results

The Novel 18-kDa Protein Is Strongly Up-Regulated During B Cell Differentiation. Using a dynamic proteomic approach on LPS-activated murine I.29 μ^+ (IgM, λ) lymphomas as model B lymphocytes (8), we found that, next to IgM subunits, the ER-resident proteins dramatically increased (3). The relationship between function and expression pattern identified a candidate ER-resident protein of 18 kDa (Fig. 1A) with expression levels that rose to $\approx 5\%$ of total signal in the silver-stained gel after 5 days. Staining with SYPRO-Ruby gave similar results. This protein ranked among the most highly expressed proteins in plasma cells, with actin and the ER proteins GRP94, calreticulin, and BiP (Fig. 1B).

Sequencing of tryptic fragments by MS revealed the identity of the 18.4-kDa (188-residue) protein (Swiss-Prot: Q9D8I1; Fig. 1C). It was annotated before by homology with the human gene MGC29506, which frequently is down-regulated transcriptionally in intestinal-type gastric cancer (9), and as the PACAP protein, implicated in apoptosis (10). However, the latter protein was deviant from residue 59 because of a frame shift (9). No sequence homology existed with any known family of ER proteins.

We then compared the proteome at various days of differentiation by direct profiling of lysates from I.29 μ^+ lymphomas by using MALDI-ToF-MS. This technique is complementary to 2D gel electrophoresis, as it allows detection of low molecular mass

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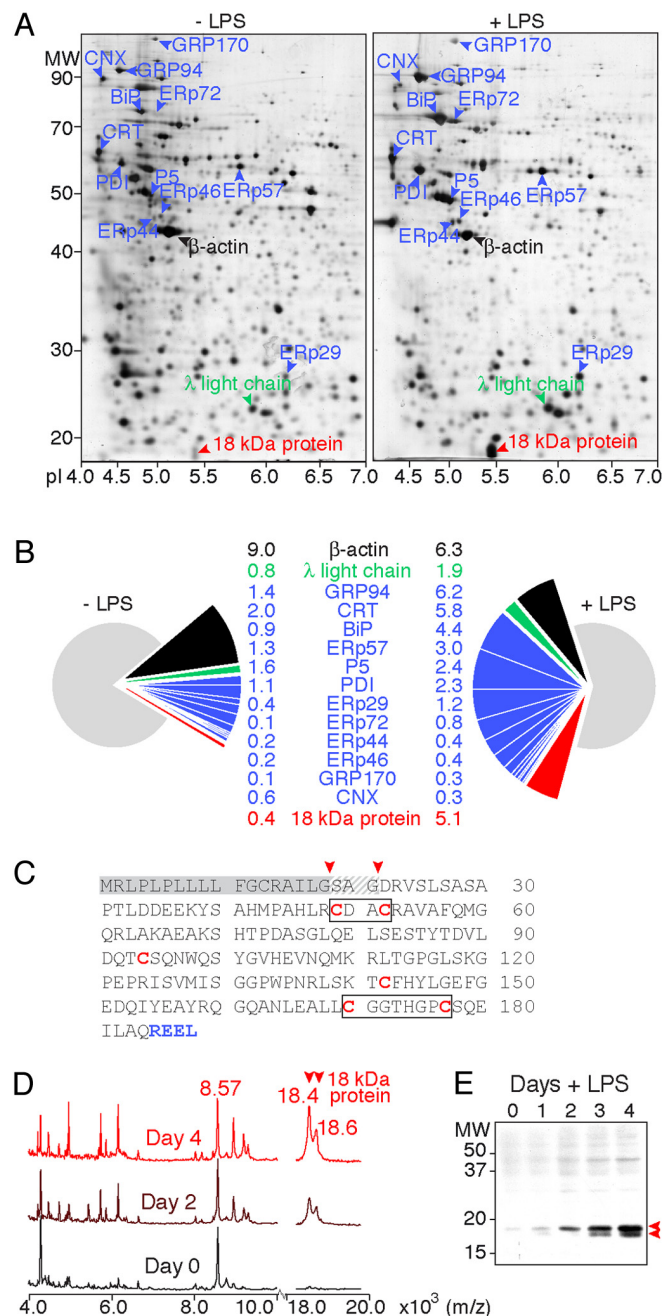


Fig. 1. A novel 18-kDa protein is heavily up-regulated during B cell differentiation. (A) Two-dimensional gel analysis of the *I.29* μ^+ B cell proteome before or after activation with LPS for 5 days. Proteins were first separated by pI using isoelectric focusing in the horizontal direction (x axis, pI) and next separated by molecular mass using standard 10% SDS PAGE in the vertical direction (y axis, MW). ER-resident chaperones and folding enzymes [blue, calnexin (CNX) and calreticulin (CRT)], λ L chain (green), the most abundant protein β -actin (black), and the novel 18-kDa protein (red) are indicated with arrowheads. (B) Relative abundance of proteins annotated in A as determined by densitometry of silver stained gels and quantitation by PDQuest. The percentage within the total proteome before or after activation is given and depicted in pie diagrams; color-coding as in A. (C) Sequence of the novel 18-kDa protein. Indicated are signal peptide (gray), cysteines (red), and the C-terminal peptide that conveys ER retention (blue). Arrowheads mark the two alternative signal peptide cleavage sites. The CXXC and C(X)₆C motifs are boxed. (D) Profiling of *I.29* μ^+ B lymphomas. Crude lysates were analyzed by using MALDI-ToF, either before or after stimulation with LPS for 2 or 4 days. Spectra were normalized to the highest peak in the spectrum (8.6 kDa). (E) Immunoblot analysis of lysates from *I.29* μ^+ B lymphomas, before or after activation with LPS for 1, 2, 3, or 4 days with an antiserum raised against the novel 18-kDa protein.

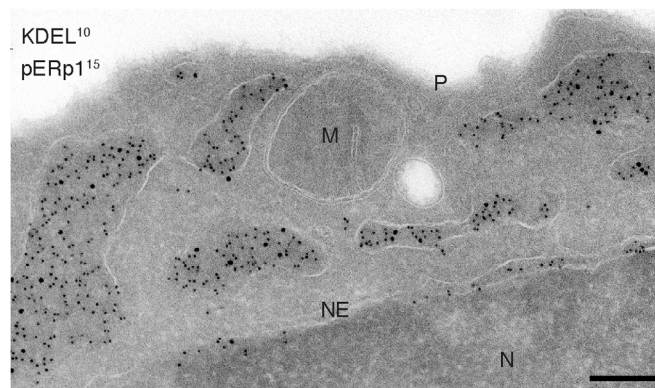


Fig. 2. The novel 18-kDa protein is resident to the ER. Subcellular localization of the 18-kDa protein by immunoelectron microscopy. *I.29* μ^+ B cells were activated for 4 days with LPS before fixation and ImmunoGold labeling with protein A-gold conjugated α -pERp1 (15 nm) and α -KDEL antisera (10 nm), which recognizes several ER-resident proteins (including BiP, PDI, and CRT). Cryosections were analyzed by electron microscopy; the nucleus (N), nuclear envelope (NE), mitochondrion (M), and plasma membrane (P) are indicated. The empty structure is an endocytic vesicle, the ER is dilated and labeled with the two sizes gold. (Scale bar, 200 nm.)

proteins only (11). The spectra displayed several ion signals that increased with time (Fig. 1D). In line with the novel protein's highly abundant expression, its signal rose from undetectable levels to the most prominent amid all proteins smaller than 20 kDa in the cell at 4 days after activation. Western blotting confirmed that the novel protein was heavily up-regulated in the course of B cell differentiation (Fig. 1E).

The Novel 18-kDa Protein Is ER Resident. The hydrophobic N terminus of the novel protein (Fig. 1C) was predicted (9) to be a cleavable signal peptide. Two partly overlapping fragments corresponding to residues D22-K38 and S19-R48 did not arise from trypsin digestion alone, because neither D22 nor S19 flanks a K or R residue. The hydrophobic N terminus of the protein, thus, was cleaved off at either of two sites (Fig. 1C), which explains why the "main" 18.4-kDa peak (Fig. 1D) was accompanied by an 18.6-kDa "shoulder" peak, and why the protein spot in the 2D gel was spread out in the vertical (mass) direction (Fig. 1A) and appeared as a doublet in Fig. 1E. Further confirmation came from the exact mass of the main and shoulder peaks as determined by ESI-Q-ToF MS (Fig. S1 and Table S1). To biochemically validate that the signal peptide mediates ER entry, we in vitro translated and radiolabeled the novel protein in the absence or presence of semipermeabilized HT1080 cells as a source of ER membrane (12). The novel protein indeed was translocated across the ER membrane, whereupon its signal peptide was removed by the signal peptidase (Fig. S2A).

Whereas its N terminus mediates entry, the C-terminal REEL sequence (Fig. 1C) may well serve as ER retention signal, like the canonical KDEL (13). Indirect immunofluorescence indeed showed colocalization with the ER marker PDI (Fig. S2B). We confirmed its ER residency in LPS treated *I.29* μ^+ cells by ImmunoGold labeling and electron microscopy. The novel protein exclusively populated ER cisternae, identified through its content of KDEL-containing resident ER proteins (Fig. 2). We named the novel protein plasma cell induced ER-resident protein 1 (pERp1).

Expression of pERp1 Is Lymphocyte Specific. When comparing levels of pERp1 by tissue immunoblotting, we found that pERp1 expression was restricted to spleen and (to a far lesser extent) thymus, lung, and uterus (Fig. 3A). Spleen and thymus typically harbor

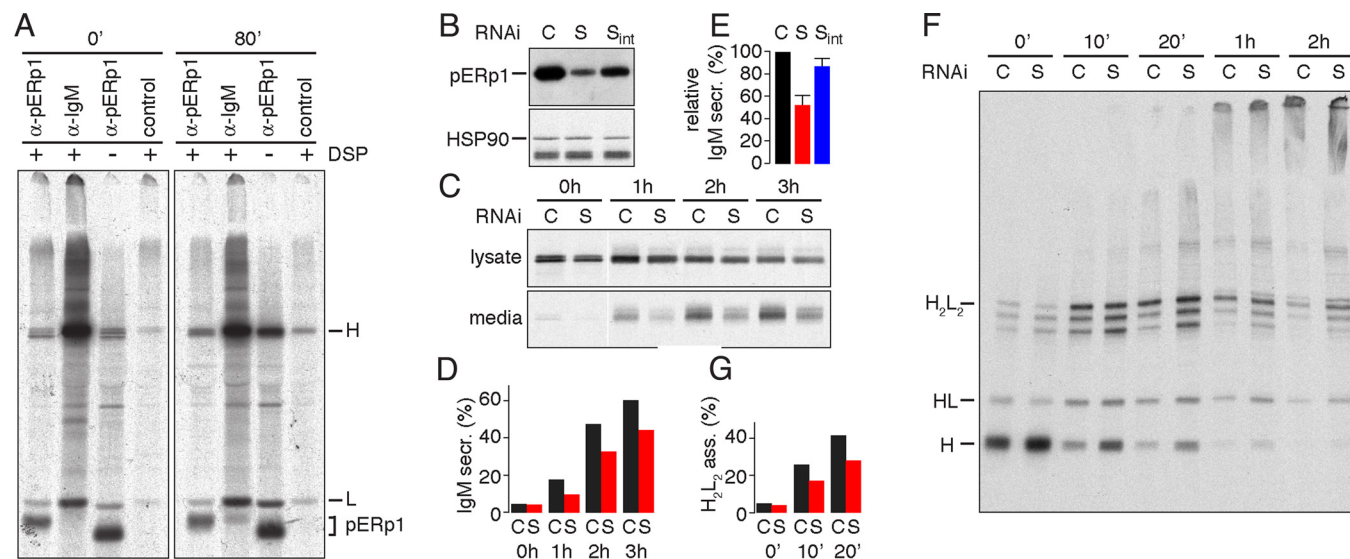


Fig. 6. pERp1 interacts with H and L chains and promotes assembly and secretion of IgM. (A) pERp1 associates with IgM subunits in I.29 μ^+ cells. Cells were activated with 20 mg/mL LPS for 3 days, pulse-labeled for 5 min, and lysed before or after a chase of 80 min in the presence or absence of cross-linker DSP. Lysates were immunoprecipitated with α -pERp1, α -IgM, or control antibodies. pERp1, IgM H and L chain are indicated. Note that the covalent association of pERp1 with the cross-linker causes a slight mobility shift. (B) Down-regulation of pERp1 in I.29 μ^+ lymphomas by using RNAi. Immunoblot analysis with α -pERp1 or, for reference, with α -HSP90 antibodies of lysates from 3-day activated I.29 μ^+ cells in which pERp1 expression was stably silenced at two different levels: (S) and (S_{int}). A control cell line (C) was generated in parallel with luciferase as target gene instead of pERp1. Selection and puromycin concentrations were identical for all cell lines. (C) Decreased IgM secretion from I.29 μ^+ lymphomas with stably silenced pERp1. Control cells and the cells with strongest silencing (S) were pulse labeled for 5 min and chased for indicated periods (hours). Culture media were harvested and cells were lysed. Equivalent samples from culture media and lysates were immunoprecipitated with α -IgM. Samples were analyzed by reducing 10% SDS/PAGE. (D) Quantitation of IgM secretion. Signals from C were quantified, and the percentage of secretion per sample is represented in a histogram showing quantization for control cells in black and for pERp1 silenced cells in red. (E) Decrease of IgM secretion by pERp1 silencing. Percentage of secretion of the silenced clone (S) was compared with that of the control cell at identical time points, setting the control at 100% (black bar). Samples from 30 min, 1, and 2 h chase from experiments shown in D and Fig. S3, as well as from replicate experiments were used for the analysis. Means and SEM are shown; $n = 6$ (S) in red; $n = 2$ (S_{int}) in blue. (F) Decrease in IgM assembly in I.29 μ^+ cells with stably silenced pERp1. Pulse-chase analysis as in C for indicated chase periods (minutes). Immunoprecipitates from lysates were analyzed by nonreducing 4–20% gradient SDS/PAGE. Assembly intermediates HL and H₂L₂ are indicated. (G) Quantitation of H₂L₂ assembly. Signals from F, showing the early phases of IgM assembly (0-, 10-, and 20-min chase), were quantified, specifically the monomeric H₂L₂ and submonomeric intermediates H, HL, and the two other bands below H₂L₂ that likely represent H₂ and H₂L. Percentage of H₂L₂ among this collection of subunits and intermediates is shown in a histogram; color-coding as in D.

creased (Fig. 6E) because of secretion of mature IgM, in particular from control cells. Because secretion confounds assessment of the fraction H₂L₂ assembled from its subunits, we calculated values only for the earlier time points (Fig. 6G). We concluded that pERp1 promotes IgM assembly and secretion, because pERp1 down-regulation significantly impeded these processes.

Discussion

We identified pERp1, a lymphocyte-specific ER-resident protein that reaches levels on a par with the most abundant known ER-resident chaperones once B lymphocytes differentiate into plasma cells. Its up-regulation is >15-fold, which is at least 3-fold higher than that of any other detectable protein, indicative of its importance in plasma cells. pERp1 associates with Ig H and L chains and stimulates disulfide-linked assembly and secretion of mature IgM.

Consistent with the notion that pERp1 is key for IgM production in plasma cells, it appears to be lymphocyte specific. Although we found highest protein expression in the spleen of mice, Shimizu et al. (14) found the highest expression of pERp1 transcripts in thymus of humans. Irrespective of differences being due to species or protein versus mRNA, pERp1 expression is highest in lymphocyte rich tissues. Consistent with lymphocyte-specific expression and a role in acquired immunity, pERp1 has closely related homologs only in mammals or birds, but not in species with a less sophisticated immune system (data not shown; ref. 9). Accordingly, pERp1 expression in human cancers was almost exclusive to leukemia and lymphoma (data not shown), but down-regulated in stomach cancers (9). pERp1 could well serve as diagnostic marker for these

immunological cancers, considering its abundant, but exclusive expression.

Tissue specificity for ER-resident folding factors is not unprecedented. The PDI family member PDIP is exclusively expressed in the pancreas (19) and PDILT, in testis (20). As a lymphocyte-specific protein, pERp1 likely is under control of a transcription factor specific for lymphocyte lineages. Interestingly, in B cells, pERp1 is a target of the UPR, similar to generic ER-resident proteins. This finding is compatible with a model in which pERp1 has UPR-specific promoter elements that are repressed in nonlymphoid cells, and that become responsive to ER stress only when the repression is lifted in lymphoid cells, although the fact that another UPR inducer, thapsigargin, did not up-regulate pERp1 (14) suggests that its regulation is likely to be quite complex. Other tissue-specific ER proteins may be similarly regulated as conditional UPR targets, i.e., via the interplay of tissue-specific signaling pathways with the UPR.

The list of novel chaperones and folding assistants in the ER is growing, in particular the PDI family (7). Although pERp1 does have a CXXC motif, which is the signature motif in the thioredoxin-like redox-active domains of PDI, pERp1 is no PDI/thioredoxin family member, since pERp1 is mainly α -helical (our unpublished observations; ref. 14) unlike the thioredoxin fold. No relation was found with any other known protein either. Instead, pERp1 is the founding member of a previously undescribed protein family.

The complexity of IgM subunit folding and multimeric assembly is mirrored by the intricacy of its assembly cascade, which is lined with many chaperones and folding assistants in the ER: BiP (21), GRP94 (22), PDI, ERp72 (23), which together form a complex with

P5 (also known as CaBP1), ERdj3, cyclophilin B, and UDP-glucosyltransferase (24). Shimizu et al. (14) identified pERp1 as part of this complex. Recently, also ERp44 and ERGIC53 were added to the list of assistants of IgM assembly (25). During folding and assembly of the IgM chains, all these ER or ERGIC proteins bind transiently, some shorter and some for longer periods, either simultaneously or in sequence. For example, early in the process, GRP94 acts after BiP (22), by promoting folding of H chains and assembly with L chains to HL “hemimers,” whereas much later, ERGIC53 acts after ERp44, when they assist assembly of H₂L₂ monomers into multimers (25).

IgM does mature in the absence of pERp1, but the reduction of IgM secretion on pERp1 down-regulation is impressive for any ER folding factor. For example, maturation of few glycoproteins is affected by deletion of either calnexin or calreticulin. A severe loss in ER function only becomes manifest once both lectins are absent (26). The absence of ERGIC53 leads to deficiencies in clotting factors V and VIII, but not to Ig deficiency (27). A link between pERp1 and disease has not been found, but its deficiency may lead to selective IgM deficiency, a condition linked to high susceptibility to persistent and life-threatening infections.

The question is how pERp1 exerts its effects. It may serve the IgM maturation process as a molecular chaperone, because it associates noncovalently to most assembly intermediates. As alternative scenario, or in addition, pERp1 may act as oxidoreductase. It has modest oxidoreductase activity, does form an interchain disulfide with IgM monomers and stimulates oxidation of Ig domains (14). It may have a role in the formation of the ≈100 disulfide bonds in IgM during folding and assembly, or in the B-cell-specific redox switch that releases the μ tailpiece cysteines from ER-resident oxidoreductases like PDI and Erp57 in the differentiating B cell (23, 28). In either case, the modest basal oxidoreductase activity of pERp1 may become enhanced by the unique conditions in the plasma cell. The preservation of activity in the SXXC mutant (14) shows that this protein is far from a standard oxidoreductase.

The interaction of pERp1 with many assembly intermediates from the moment of synthesis (our results; ref. 14) suggests pERp1 to be among the longest interacting folding assistants up to now, together with BiP. Although BiP is absolutely required for IgM folding (1), as it is for ER function in general (29), pERp1 may sustain the efficiency in IgM production that the plasma cell requires as a dedicated antibody factory.

Methods

Detailed methods for all approaches used are provided in the [SI Methods](#).

B Cell and Protein Analyses. I.29 μ^+ cells were activated with 20 μ g/mL LPS and cells and harvested before or after different days of differentiation for various analyses ([SI Methods](#)). Cells lysates were analyzed by immunoblotting or 2D gel electrophoresis, and proteins were identified as described (3). Immunoelectron microscopy was done as described (30).

Pulse-Chase Analyses. Pulse-chases were performed as described (31) in I.29 μ^+ cells or in HeLa cells expressing pERp1 or its mutants by using the vaccinia virus T7 infection-transfection system (32). Detergent lysates of radiolabeled cells or media were subjected to immunoprecipitation with protein A-Sepharose-coupled antisera. Immunoprecipitates were washed and heated in sample buffer in the presence (reducing) or absence (nonreducing) of DTT before SDS/PAGE analysis.

Silencing. I.29 μ^+ cells were transduced with shRNA lentiviral vectors to silence pERp1, with an isogenic control targeting the reporter luciferase.

In Vitro Thiol Oxidoreductase Assay. 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 (33).

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