ERO1-L, a Human Protein That Favors Disulfide Bond Formation in the Endoplasmic Reticulum*

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Oxidizing conditions must be maintained in the endoplasmic reticulum (ER) to allow the formation of disulfide bonds in secretory proteins. Here we report the cloning and characterization of a mammalian gene (ERO1-L) that shares extensive homology with the Saccharomyces cerevisiae ERO1 gene, required in yeast for oxidative protein folding. When expressed in mammalian cells, the product of the human ERO1-L gene colocalizes with ER markers and displays Endo-H-sensitive glycans. In isolated microsomes, ERO1-L behaves as a type II integral membrane protein. ERO1-L is able to complement several phenotypic traits of the yeast thermosensitive mutant ero1-1, including temperature and dithiothreitol sensitivity, and intrachain disulfide bond formation in carboxypeptidase Y. ERO1-L is no longer functional when either one of the highly conserved Cys-394 or Cys-397 is mutated. These results strongly suggest that ERO1-L is involved in oxidative ER protein folding in mammalian cells.

Protein folding inside living cells is facilitated by the presence of a vast array of molecular chaperones and enzymes (1). For many secretory proteins, the acquisition of the native structure requires the formation of intra- and inter-molecular disulfide bonds (2). This process generally takes place in the endoplasmic reticulum (ER) (3, 4). This organelle contains many oxidoreductases, belonging to the protein disulfide isomerase family, which share a characteristic CXXC motif (5). These enzymes are thought to catalyze the formation of native disulfides in cargo proteins. Protein disulfide isomerase is necessary for disulfide bond formation in isolated microsomes (6, 7) and can be cross-linked to folding or assembly intermediates within the ER lumen (8–10). The crucial function of protein disulfide isomerase in the isomerization of S-S bonds in folding intermediates has been firmly established (11).

In bacteria, disulfide bonds are formed in the periplasmic space through the assistance of a set of well characterized molecules (for review, see Ref. 12). DsbA, a soluble protein of the periplasmic space, transfers disulfide bonds onto folding polypeptides. DsbA is oxidized by DsbB, an integral membrane protein protruding into the periplasmic space, which in turn transfers electrons to the respiratory chain (13–15). In respiratory-deficient conditions, DsbA is accumulated in the reduced form while DsbB is trapped in a disulfide-linked intermediate with DsbA (13). Although the primary function of DsbA seems to be the oxidation of periplasmic proteins, DsbC, whose redox state is under the control of DsbD, catalyzes disulfide isomerization. All members of the Dsb family contain at least one CXXC motif.

Although sequence comparisons do not allow the identification of eukaryotic homologs of Dsb genes, the basic principles of disulfide bond formation in the ER of eukaryotic cells and the bacterial periplasmic space are likely to be similar. Indeed, mammalian protein disulfide isomerase has been shown to complement bacterial DsbA mutants, a feature that implies a dithiol oxidant activity for this enzyme (16). Thus, protein disulfide isomerase seems to be capable of the functions that in bacteria are performed by DsbA (oxidation) and DsbC (isomerization), respectively.

One aspect of disulfide bond formation which is still poorly understood is how a suitable redox potential is generated and maintained in the ER. The view that oxidized glutathione (GSSG) is the source of the oxidizing equivalents in the ER (17 and references therein) was challenged recently by the discovery of the Ero1p protein in Saccharomyces cerevisiae (18, 19). Ero1p is required for the oxidation of SH groups of both nascent proteins and glutathione in the ER (20). Decreased Ero1p function leads to exaggerated susceptibility of yeast cells to DTT, whereas overexpression confers resistance (18, 19).

The levels of functional Ero1p also correlate with the rates of maturation and intracellular transport of disulfide-containing secretory proteins such as carboxypeptidase Y (CPY) and Gas1p, which cannot exit the ER in the reduced form. It has been suggested that Ero1p may fulfill the role of DsbB in the ER of eukaryotic cells (18, 19) maintaining protein disulfide isomerase in an oxidized state.

Several observations suggest that in mammalian cells, the ER redox potential is tightly controlled so as to modulate protein secretion (21), degradation (22), and signaling (23, 24). Therefore, we undertook a study aimed at identifying and...
characterizing mammalian genes potentially involved in redox homeostasis within the ER. The existence of genes homologous to \textit{S. cerevisiae} \textit{ERO1} had been reported in several species (19).

In the present paper, we describe the isolation and characterization of a human homolog (\textit{ERO1-L}) encoding a membrane-associated N-glycoprotein of the ER which favors oxidative protein folding in this organelle.

\textbf{EXPERIMENTAL PROCEDURES}

\textbf{Isolation of Human ERO1-L cDNA Clones}—Approximately 3.4 \times 10^8 clones from a cDNA library derived from NT2-D1 human embryonal carcinoma cells (gift of H. P. Loeb, Boehringer Ingelheim, Verviers, Belgium) were screened using an EcoRI fragment of clone IMAGE: 0612711 (nucleotides 1–832). Positive plaques were purified and analyzed by standard procedures (25). The longest clone (clone F1) was sequenced entirely (accession no. AF081866).

\textit{ERO1} and \textit{ERO1-L} Expression Vectors—For expression in mammalian cells, the complete coding sequence of \textit{ERO1-L} was PCR amplified from the F1 clone with forward oligonucleotide GTGT TTC TAGAGGCG CAGGCTCGAATG and two different reverse oligonucleotides (GTGTG TAGCATGATATCTGTAAACAAGT and GTTGGTAGCTTAAATGTAATTCTTGA) to obtain clones with or without the myc-6his tag of pCDNA3.1 fused in-frame with \textit{ERO1-L} (pCDNA3.1\textit{ERO1-Lmyc} and pCDNA3.1\textit{ERO1-Lmyc-6his}, respectively). The inserts from the two clones were sequenced entirely. For expression in yeast, the inserts of pCDNA3.1\textit{ERO1-Lmyc} and of pCDNA3.1\textit{ERO1-L} were excised with XhoI and NotI and cloned into pCDNA3.1 (version A (Invitrogen, San Diego, CA). The inserts from the two clones were sequenced entirely. For expression in yeast, the inserts of pCDNA3.1\textit{ERO1-Lmyc} and of pCDNA3.1\textit{ERO1-L} were excised with XhoI and NotI and cloned into the XhoI and HindIII sites (the latter filled in by Klonev) of pVT102-U (26), to yield p\textit{VTERO1-L} and p\textit{VTERO1-Lmyc}. The \textit{S. cerevisiae ERO1} (a kind gift of A. Frand and C. Kaiser, MIT, Boston, MA) was cloned in the same vector as follows. The \textit{ERO1} gene fused to a triple myc tag was PCR amplified from plasmid pAF82 (18) by the following oligonucleotides: ye\textit{ERO1}-FW: GTGTGTGATCATGATATCTGTAATTCTTGA ye\textit{ERO1}-RV: GAAGATGGTGACCGCTGTAATCGTGCTACGATCTCTTGCG

The PCR product was digested with BamHI and NotI and cloned into BamHI and XhoI sites in pVT102-U as for \textit{ERO1-L}.

To generate the COOH-terminal truncated variant of yeast \textit{ERO1} (\textit{ERO1AC}), the region of the gene corresponding to the first 444 residues was amplified from pAF82 by oligonucleotides ye\textit{ERO1-FW} (see above) and CCACCTTAGAATCAATGATTTTGTGCTACCACTCTTAC as follows: ye\textit{ERO1AC}-FW: GTGTGTGATCATGATATCTGTAATTCTTGA ye\textit{ERO1AC}-RV: GAAGATGGTGACCGCTGTAATCGTGCTACGATCTCTTGCG

The PCR product was digested with BamHI and XhoI and cloned into pVT102-U to yield p\textit{VTERO1AC}.

To replace the three cysteines within the conserved CXXCXX motif with alanines, the 1306–1520 EcoRI fragment (sequence AF081863) was subcloned into pBluescript II SK+ (Stratagene). On this template, three PCRs were performed by using the following couples of oligonucleotides: ERO1-L variants were transferred into the yeast vector pCDNA3.1\textit{ERO1-Lmyc}. The \textit{ERO1-Lmyc} insert was excised with XhoI and NotI and cloned in pVT102-U as for \textit{ERO1-L}.

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The mutated EcoRI fragments were then reinserted into pCDNA3.1\textit{ERO1-Lmyc} and pCDNA3.1\textit{ERO1-L}. The presence of the proper mutation in all the clones was confirmed by sequencing. Mutated \textit{ERO1-L} variants were transferred into the yeast vector pVT102-U as described above for wild type (wt) \textit{ERO1-L}.

\textbf{Transfection of Recombinant \textit{ERO1-L} in Mammalian Cells}—Cell lines were obtained from ATCC. COS-7 and 293T were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Paisley, Scotland) and Isevo’s modified Dulbecco’s medium (BioWhittaker, Boehringer Ingelheim, Verviers, Belgium), respectively, supplemented with 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, and 0.5 units/ml penicillin and streptomycin. Cells (2 \times 10^7) were transfected by Lipofectin (Life Technologies) as recommended by the supplier and analyzed by Western blotting with affinity-purified anti-myc antibodies (27).

\textbf{Endoglycosidase Treatment of Recombinant \textit{ERO1-L} in COS-7 and 293T Cells}—Cells were lysed in 10 mM Tris, pH 7.4, 100 mM NaCl, 10 mM iodoacetamide, 1% Nonidet P-40, supplemented with protease inhibitors (Roche Molecular Biochemicals). 30-μl aliquots of lysates were added to 30 μl of 2 × buffer and incubated overnight at 37 °C in the presence of 2 milimilliters of endoglycosidase-H (Endo-H, Roche Molecular Biochemicals) or 40 milliliters of endoglycosidase-F (Endo-F). Reactions were performed separately as controls (2 × Endo-H buffer: 150 mM sodium citrate, pH 5.5, 0.1 mM 2-mercaptoethanol; 2 × Endo-F buffer: 100 mM sodium acetate, pH 5.2, 0.1 mM 2-mercaptoethanol).

\textbf{RESULTS}

\textbf{Isolation of \textit{S. cerevisiae} Homologs of \textit{S. cerevisiae} \textit{ERO1}}—The yeast \textit{ERO1} nucleotide sequence (z50178) was used to search for related expressed sequence tags (ESTs) in the EST division of the GenBank database through the BLAST algorithm (21) accessed through the NCBI Web site. EST ID 14855-06, which presumably extended most 5′ (accession no. AF123887), encodes a protein similar to Ero1p but lacking a translation initiation sequence. The screening of a human cDNA library (see “Experimental Procedures”) led to the isolation of four clones, the longest of which, F1 (accession no. AF081866), contains a complete coding sequence for a human...
homolog of ERO1, which we called ERO1-L (for ERO1-Like). The putative translational start site was established based on the existence of a stop codon 155 base pairs upstream of an ATG located in a good initiation context (32).

The predicted human gene product is a 468-amino acid polypeptide (Fig. 1A), showing 48.5% similarity and 36.5% identity to yeast Ero1p. The algorithm SignalP (33) predicts a signal sequence for ER translocation at the NH2 terminus, with a potential cleavage site between residues 23 and 24. A putative EF-hand calcium binding domain (34) is present in positions 159–171. Two N-glycosylation sites are predicted at positions 280 and 384, respectively. Residues 391–397 contain the highly conserved CXXCXXC motif, suggested to be important for bioactivity (18, 19).

A murine EST clone was identified (EST IMAGE 1294317) and sequenced (accession no. AF144695) and appears to contain a full-length coding sequence 87.3% identical to human ERO1-L at the nucleotide level. The predicted mouse protein is 92.7% identical to the human sequence. Fluorescence in situ hybridization experiments revealed that ERO1-L is located on human chromosome 14 at q22.1 and in the synthetic 14 C–D region in the mouse (Fig. 1, A–C). To acquire a broader view of the ERO1 gene family in different species, we identified and sequenced an EST (cDNA clone LD02945) coding for a Drosophila melanogaster ERO1-L gene (accession no. AF125280). The sequence of the predicted Drosophila ERO1-L polypeptide is shown in Fig. 1A, together with the products of the Schizosaccharomyces pombe and Arabidopsis thaliana genes.

ERO1-L Is an ER Resident N-Glycoprotein—To determine the intracellular localization of the ERO1-L gene product, human ERO1-L was tagged and expressed in COS-7 cells. The pattern obtained with anti-myc largely overlapped with the distribution of ConA, a lectin that recognizes glycoproteins with terminal mannose residues, and therefore decorates primarily the ER of permeabilized cells (Fig. 2A). Similar results were obtained in 293T cells (not shown). As an additional marker of the ER we chose erGFP(S65T), a VH-CH1-green fluorescent protein chimeric molecule retained in the ER (35). The co-localization with ConA and erGFP indicates that exogenous ERO1-Lmyc accumulates primarily in the ER.

This was confirmed further by endoglycosidase sensitivity assays. A mobility shift was observed when lysates from transfected COS-7 or 293T cells were treated with Endo-H or Endo-F (Fig. 2B). Hence, ERO1-Lmyc molecules are N-glycosylated and remain in an Endo-H-sensitive configuration, consistent with their localization in the ER.

In Vitro Translated ERO1-Lmyc Is Translocated across Microsomal Membranes and Has the Properties of an Integral Membrane Protein—To ascertain whether human ERO1-Lmyc is a soluble or membrane protein, in vitro transcription/translation assays were performed. Translation in rabbit reticulocyte lysate yielded a 58-kDa band (Fig. 2C, lane 1). In the presence of microsomes, an additional product of slower mobility became detectable (lane 3). This slower migrating polypeptide was sensitive to glycosidases (lane 2), indicating that a fraction of ERO1-Lmyc synthesized in the presence of microsomes underwent N-linked glycosylation. Translocation into microsomes was confirmed by protease protection experiments (Fig. 2D). The low mobility polypeptide synthesized in the presence of microsomes, which was sensitive to digestion with glycosidases, was not digested by proteinase K (lane 2) unless membranes were solubilized by detergent (Triton X-100, lane 3). The faster migrating polypeptides seen after translation in the presence of microsomes (lane 1) were sensitive to proteolysis (lane 2) and therefore correspond to untranslocated molecules.

**FIG. 1.** Isolation and mapping of mammalian ERO1-L. A, alignment of ERO1-Like polypeptides from different organisms. Identical residues are boxed, and conserved residues are shaded. The alignment was performed using the pileup algorithm of the Wisconsin Package version 9.0, Genetics Computer Group (GCG), Madison, WI. B, mapping of the human ERO1-L gene by fluorescence in situ hybridization. Clone F1 was used as a probe on human metaphase chromosomes, showing signals at 14q22. The chromosome 14 pair from a more elongated metaphase is shown in the upper right corner. C, mapping of the murine ERO1-L gene. With EST IMAGE 1294317 as a probe, a signal is detected at 14 C-D (synthetic with human 14q22). The mouse cell line 513, containing multiple well characterized Robertsonian translocations, was used because it allows an easy identification of the mouse chromosomes (39). Fluorescence in situ hybridization was performed as described (40).
The electrophoretic mobility of ERO1-Lmyc translated in the absence of microsomes was indistinguishable from that of the translocated and deglycosylated translation products (Fig. 2C), suggesting that the signal peptide of ERO1-Lmyc may not be cleaved. This uncleaved signal sequence could function as a transmembrane domain. To ascertain whether ERO1-Lmyc is indeed integrated into the membrane, primed microsomes were incubated with sodium carbonate alone or in the presence of salt or urea (Fig. 2E). In general, integral membrane proteins are not extracted by these treatments unless detergent is added and therefore fractionate with the pellets, whereas soluble and peripheral membrane molecules accumulate in the supernatants. Clearly, most glycosylated ERO1-Lmyc fractionated with the pellets (Fig. 2E, lanes 3, 5, 7, and 9), unless microsomes were solubilized with detergent (Triton X-100, lanes 11 and 12). Unexpectedly, the faster migrating band was poorly solubilized even in the presence of detergent, suggesting that untranslocated ERO1-Lmyc molecules may form insoluble aggregates.

Taken together, these findings indicate that glycosylated
ERO1-Lmyc is either itself an integral membrane protein or forms a strong interaction with an integral membrane protein.

**Functional Complementation of the Yeast ero1-1 Mutant by Human ERO1-L**—To explore the functional properties of ERO1-L, we expressed the human coding sequence, with or without a myc tag, in the thermosensitive ero1-1 yeast mutant strain (CKY559). These cells do not grow at 37 °C and display increased sensitivity to DTT and inefficient ER-Golgi transport of disulfide-rich proteins (18).

First, we tested the ability of human ERO1-L to rescue the temperature sensitivity of the ero1-1 mutant. Thus, ero1-1 was transformed with different vectors and grown at either 24 °C or 37 °C. Clearly, the human gene allowed growth at 37 °C (Fig. 3A). A striking difference between the human and yeast genes is the presence in the latter of a long COOH-terminal region missing from the human sequence (see Fig. 1). The ability of the human sequence to rescue the ero1-1 growth defect suggests that the COOH-terminal domain is not essential for viability. In agreement with this, a mutant of the S. cerevisiae ERO1 gene truncated at position 444 (ERO1ΔC) was able to rescue ero1-1 viability at 37 °C.

The high conservation of the CXXCXXC motif in all members of the ERO-1 family suggests that it may play an important functional role. To test this hypothesis, variants of ERO1-L were generated in which the three conserved cysteines were replaced individually by alanines. Mutating cysteines 394 and 397 was detrimental to the ability of ERO1-L to rescue ero1-1 thermosensitivity, whereas the mutant C391A behaved like wt ERO1-L (Fig. 3A). As determined by Western blot analysis (not shown), the expression levels of the three mutants were comparable to those of wt ERO1-Lmyc.

In agreement with a role for Ero1p in maintaining an oxidative environment in the ER, the ero1-1 mutant is more sensitive to DTT than the wt strain (18, 19). The expression of human ERO1-L partially alleviated ero1-1 DTT sensitivity (Fig. 3B). Also in this assay, no differences were observed between ERO1 and ERO1ΔC, suggesting that the COOH-terminal region is not crucial for the protective effects toward DTT. As observed in the thermosensitivity assays, mutations in the second and third cysteines of the CXXCXXC motif were detrimental to the activity of ERO1-L, whereas the phenotype of the C391A mutant was indistinguishable from wt ERO1-L.

Lastly, we tested the ability of ERO1-L to complement Golgi transport of CPY. CPY offers a convenient tool for monitoring the redox conditions in the ER because it contains intrachain S-S bonds, whose formation is required for folding and export from the ER (36). After transit through the Golgi, CPY is targeted to the vacuole where it is proteolytically cleaved, yielding the mature (m) form of the polypeptide.

When ero1-1 was transformed with the vector alone and grown under nonpermissive conditions, CPY accumulated in...
the ER in the precursor (p) form (Fig. 4A, lanes 1–4). Transformation with human ERO1-L (lanes 5–8) allowed the transport of CPY along the exocytic pathway, although at a slower rate than yeast ERO1 (lanes 9–12).

Thus, ERO1-L allows intracellular transport of CPY, otherwise retained in the ER. To determine whether this reflects intrachain disulfide bond formation, we exploited a gel mobility assay that allows discrimination of oxidized and reduced CPY. When ero1-1 was transformed with the vector alone, no differences in the electrophoretic mobility of CPY were observed, suggesting that CPY was retained in the ER in the reduced state (lanes 1 and 2). In contrast, a mobility shift was evident in sec18 (compare lanes 7 and 8), a mutant in which, because of a general ER-Golgi transport defect (37), CPY accumulates in the ER in the oxidized form. As described previously (18), transformation of ero1-1 with S. cerevisiae ERO1 allowed CPY oxidation and cleavage (lanes 5 and 6). When ero1-1 was transformed with ERO1-L, some CPY remained reduced in the ER. However, most of the protein accumulated in the oxidized vacuolar form (lanes 3 and 4), indicating that human ERO1-L is able to promote oxidative protein maturation in the ER.

**DISCUSSION**

The striking homologies among different species suggest that the ERO1 gene is endowed with an important function maintained across evolution. The human and mouse genes are 87.3% identical at the nucleotide level and 92.7% identical at the protein level. Because they map to synthenic regions (Fig. 1, B and C), they are likely to be orthologous. Blocks of identical residues are distributed throughout the sequences (Fig. 1). The yeast sequence is unique in having a long COOH-terminal tail protruding for 139 residues with respect to the human and mouse sequences. The A. thaliana sequence also displays a COOH-terminal portion, shorter than the yeast tail and unrelated in sequence. Because a truncated ERO1 mutant appears to be active in both thermosensitivity and the DTT sensitivity assays, the function of this COOH-terminal tail remains to be clarified. One of the largest blocks of sequence identity revolves around the CXXC motif. Less conserved are the NH2-terminal portions of the sequences. However, all share hydrophobicity and are predicted to act as ER-targeting signal sequences. Therefore, like the S. cerevisiae Ero1p (18), it is likely that the other members of the family are also localized in the ER.

Several lines of evidence indicate that this is indeed the case for the human ERO1-L gene product. First, immunofluorescence experiments reveal that a transfected myc-tagged ERO1-L expressed in simian COS-7 or human 293T cells colocalizes with two ER markers, a chimeric VHC-CH1-GFP (35) and ConA, a lectin that preferentially reacts with N-glycans bearing terminal mannos, characteristic of ER glycoproteins. Second, ERO1-Lmyc undergoes N-glycosylation, a modification restricted to proteins synthesized in the ER. Consistent with an ER localization, the same mobility shift is observed upon treatment with Endo-F and Endo-H, two enzymes that cut all or only immature N-glycans, respectively. Third, in vitro translated ERO1-Lmyc is translocated into microsomal membranes when the latter are present during synthesis and a mobility shift is observed, which is abolished by endoglycosidase digestion, confirming that the newly synthesized polypeptide is N-glycosylated.

Unexpectedly, the mobility of the newly glycosylated product is indistinguishable from the protein synthesized in the absence of added microsomal membranes. This suggests that the signal sequence is not cleaved and could act as a transmembrane domain. In support of this, carbonate washing of the membranes in the presence of urea or high salt failed to extract ERO1-Lmyc. Because no other candidate transmembrane regions are detectable in the ERO1-L sequence, the conservation of the hydrophobic leader could therefore explain the association of ERO1-L with microsomal membranes. Alternatively, this association might be established by strong interactions with membrane protein(s). Further investigation is required to clarify how ERO1-L associates with membranes and is retained in the ER.

ERO1-L is expressed, in varying amounts, in all cell lines and tissues examined (data not shown), suggesting that this gene may be involved in a general cellular function. The yeast complementation experiments indicate that this function is likely to be related to oxidative protein folding in the ER. Indeed, ERO1-L is able to complement several phenotypic traits of the ero1-1 mutant. ERO1-L completely restores the ability of ero1-1 to grow at 37 °C and partially confers resistance to DTT. A clear recovery of function is also observed for the oxidative maturation of CPY in the ero1-1 mutant (Fig. 4). Therefore, ERO1-L can perform, although with reduced efficiency, the oxidative function of Ero1p in yeast cells. The possibility that the human protein may act by rescuing the activity of the defective endogenous ERO-1 yeast gene cannot be formally ruled out. However, the observation that an intact CXXC motif (394–397) is required for ERO1-L activity argues against a simple “in trans” reactivation of the endogenous ERO1 gene. Substitutions to alanine of the second or the third cysteine of the ERO1-L CXXC motif (cysteines 394 and 397) are detrimental to ERO1-L activity. In contrast, we were unable to detect loss in activity for the C391A ERO1-L mutant with respect to the wt molecule in any of the assays performed. This is somewhat surprising in view of the conservation of this residue in different species (see Fig. 1A). A prediction of ERO1-L secondary structure with the PREDATOR algorithm (not shown) locates Cys-394 in a loop and Cys-397 at the beginning of an a-helix. This arrangement is also found in thioredoxin, DabA, and protein disulfide isomerase. This observation, together with the mutagenesis data, is consistent with the possibility that cysteines 394 and 397 may constitute a classical CXXC motif (38), crucial for the redox activity of ERO1-L. Beyond the three residues of the CXXC motif, ERO1-L contains additional cysteines, some of which are also highly conserved, which may contribute to the redox activity. Further mutagenesis will be required to elucidate the functional relevance of these residues.

In conclusion, our data suggest that the molecular machine responsible for oxidizing the ER might be remarkably similar in yeast and humans. It will be important to determine whether also in eukaryotes the oxidation of ERO1-L is linked to the respiratory chain as the ultimate electron acceptor system.

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**REFERENCES**

1. Gething, M. J., and Sambron, J. (1992) Nature 355, 33–45
2. Huppa, J. B., and Ploegh, H. L. (1998) Cell 92, 145–148
3. Braakman, I., Helenius, J., and Helenius, A. (1992) Nature 356, 260–262
4. Helenius, A., Marquardt, T., and Braakman, I. (1992) Trends Cell Biol. 2, 227–231
5. Martin, J. L. (1995) Structure 3, 245–250
6. Bulleid, N. J., and Freedman, R. B. (1988) Nature 335, 649–651
7. Bulleid, N. J., and Freedman, R. B. (1990) EMBO J. 9, 3527–3532
8. Roth, R. A., and Pierce, S. B. (1987) Biochemistry 26, 4179–4182
9. Otani, M., Onnur, F., Yoshinori, T., and Kikuchi, M. (1994) J. Biol. Chem. 269, 6874–6877
10. Reddy, P., Sparvoli, A., Fagioli, C., Fassina, G., and Sitta, R. (1996) EMBO J. 15, 2077–2085
11. Weissman, J. S., and Kim, P. S. (1993) Nature 365, 185–188
12. Missiakas, D., and Raina, S. (1997) J. Bacteriol. 179, 2465–2471
13. Kobayashi, T., Kishigami, S., Sone, M., Inokuchi, H., and Ito, K. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1857–1862
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14. Kobayashi, T., and Ito, K. (1999) *EMBO J.* 18, 1192–1198
15. Bader, M., Muse, W., Ballou, D. P., Gassner, C., and Bardwell, J. C. A. (1999) *Cell* 99, 217–227
16. Jonda, S., Huber-Wunderlich, M., Glocshuber, R., and Mosner, E. (1999) *EMBO J.* 18, 3271–3281
17. Hwang, C., Sinskey, A. J., and Lodish, H. F. (1992) *Science* 257, 1496–1502
18. Frand, A. F., and Kaiser, C. A. (1998) *Mol. Cell* 1, 161–170
19. Pollard, M. G., Travers, K. J., and Weissman, J. S. (1998) *Mol. Cell* 1, 171–182
20. Cusso, J. W., and Kaiser, C. A. (1999) *Nat. Cell Biol.* 1, 130–135
21. Saita, R., Neuberger, M., Alberini, C., Bet, P., Fra, A., Valetti, C., Williams, G., and Milstein, C. (1990) *Cell* 60, 781–790
22. Torturella, D., Story, C. M., Huppa, J. B., Wertz, E. J., Jones, T. R., Bacik, I., Bennett, J. R., Yewdell, J. W., and Ploegh, H. L. (1998) *J. Cell Biol.* 142, 365–376
23. Bauskin, A. R., Alkalay, I., and Ben-Neriah, Y. (1991) *Cell* 66, 685–696
24. Pahl, H. L., and Baeuerle, P. A. (1995) *EMBO J.* 14, 2580–2588
25. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., pp. 2.60–2.63, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Vernet, T., Dignard, D., and Thomas, D. Y. (1987) *Gene (Amst.)* 52, 225–233
27. Evan, G., Lewis, G., Ramsay, G., and Bishop, J. (1985) *Mol. Cell. Biol.* 5, 3610–3616
28. Gurevich, V. V., Pokrovskaya, I. D., Obukhova, T. A., and Zozulya, S. A. (1991) *Anal. Biochem.* 195, 207–213
29. Austen, B. M., Hermon-Taylor, J., Kaderbhai, M. A., and Ridd, D. H. (1984) *Biochem. J.* 224, 317–325
30. Guthrie, C., and Fink, G. R. (1991) *Methods Enzymol.* 194, 1–863
31. Autschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* 215, 403–410
32. Kozak, M. (1987) *Nucleic Acids Res.* 15, 8125–8148
33. Nielsen, H., Engelbrecht, J., Brunak, S., and Von Heijne, G. (1997) *Protein Eng.* 10, 1–6
34. Kawasaki, H., and Kretsinger, R. H. (1995) *Protein Profile* 2, 297–490
35. Rizzuto, R., Pinto, P., Carrington, W., Fay, F. S., Fogarty, K. E., Lifshitz, L. M., Tuft, R. A., and Pozzan, T. (1998) *Science* 280, 1763–1766
36. Jamsa, E., Simonnen, M., and Makarow, M. (1994) *Feast* 10, 355–370
37. Kaiser, C. A., and Schekman, R. (1990) *Cell* 61, 723–733
38. Chievers, P. T., Laboissiere, M. C. A., and Raines, R. T. (1996) *EMBO J.* 15, 2659–2667
39. Zerrig, M., Klett, C., Lovec, H., Hameister, H., Wickiing, H., Adolph, S., and Moroy, T. (1995) *Cytogenet. Cell Genet.* 71, 37–40
40. Lichter, P., Tang Chang, C. J., Call, K., Hermanson, G., Evans, G. A., Housman, D., and Ward, D. C. (1990) *Science* 247, 64–69
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