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*

Ying Shen‡§, Laurent Meunier‡, and Linda M. Hendershot‡§¶

From the ‡Department of Tumor Cell Biology, St. Jude Children’s Research Hospital, Memphis, Tennessee 38105 and the §Department of Molecular Sciences, University of Tennessee, Health Science Center, Memphis, Tennessee 38163

The activity of Hsp70 proteins is regulated by accessory proteins, which include members of the DnaJ-like protein family. Characterized by the presence of a highly conserved 70-amino acid J domain, DnaJ homologues activate the ATPase activity of Hsp70 proteins and stabilize their interaction with unfolded substrates. DnaJ homologues have been identified in most organisms where they are involved in nearly all aspects of protein synthesis and folding. Within the endoplasmic reticulum (ER), DnaJ homologues have also been shown to assist in the translocation, secretion, retro-translocation, and ER-associated degradation (ERAD) of secretory pathway proteins. By using bioinformatic methods, we identified a novel mammalian DnaJ homologue, ERdj4. It is the first ER-localized type II DnaJ homologue to be reported. The signal sequence of ERdj4 remains uncleaved and serves as a membrane anchor, orienting its J domain into the ER lumen. ERdj4 co-localized with GRP94 in the ER and associated with BiP in vivo when they were co-expressed in COS-1 cells. In vitro experiments demonstrated that the J domain of ERdj4 stimulated the ATPase activity of BiP in a concentration-dependent manner. However, mutation of the hallmark tripeptide HPD (His → Gln) in the J domain totally abolished this activation. ERdj4 mRNA expression was detected in all human tissues examined but showed the highest level of the expression in the liver, kidney, and placenta. We found that ERdj4 was highly induced at both the mRNA and protein level in response to ER stress, indicating that this protein might be involved in either protein folding or ER-associated degradation.

The endoplasmic reticulum (ER) is the site of synthesis and maturation of secretory pathway proteins, which include resident proteins of the endocytic and exocytic organelles as well as surface and secreted proteins. Approximately one-third of all cellular proteins are translocated into the lumen of ER, which possesses a unique oxidizing and Ca$^{2+}$-rich environment, where post-translational modification, folding, and oligomerization of nascent proteins occur. ER molecular chaperones and folding enzymes associate with the newly synthesized proteins to prevent their aggregation and help them fold and assemble correctly. Through a process called ER quality control, proteins that do not mature properly are retained in the ER and are eventually targeted for ER-associated degradation (ERAD) through the action of the chaperones (1).

BiP, also known as GRP78, is the mammalian ER member of the Hsp70 family and was the first component of the ER quality control apparatus to be identified (2). Hsp70 family members exist in all organisms and in all organelles, where they aid in the folding and assembly of nascent proteins and prevent their aggregation during conditions of physiological stress (3, 4). Like other Hsp70 proteins, BiP plays an essential role in the biosynthesis of proteins. In addition, BiP maintains the permeability barrier of the ER translocon during early stages of protein translocation (5), targets misfolded proteins for proteosomal degradation (6, 7), serves as a sensor for ER stress (8), and contributes to ER calcium stores (9). At least some of these other roles also require the ATPase activity of BiP (10, 11). Hsp70 proteins bind and hydrolyze ATP through a highly conserved N-terminal 44-kDa ATPase domain, which is essential for their chaperoning activity. The ATPase cycle alternates between two states: the ATP-bound state, which binds and releases peptide rapidly, and the ADP-bound state that binds and releases peptide much more slowly. Hsp70s first bind to unfolded substrate proteins in the ATP-bound form and then hydrolyze ATP to ADP, which stabilizes their binding to the unfolded substrate. The exchange of ADP to ATP triggers the release of the substrate allowing it to fold. This cycle is tightly controlled by cofactors that stimulate the rate of ATP hydrolysis, like members of the DnaJ family, or proteins that regulate nucleotide exchange, like GrpE in bacteria and mitochondria (12, 13), and Hip (14), Hop (15), and Bag-1 (16) in mammals. BiP undergoes the same ATP/ADP cycle to bind and release substrates as demonstrated by in vivo and in vitro studies (3, 17); however, very few mammalian BiP regulators have been identified.

DnaJ was first identified as a cofactor of DnaK, the bacterial hsp70 homologue, which stimulated the ATPase activity of DnaK and helped replicate λ phage DNA in host cells (18). Since then a large number of DnaJ homologues have been identified and exist in all species and organelles. They can be divided into three subgroups based upon the degree of domain conservation with Escherichia coli DnaJ. Type I DnaJ proteins...
possess all three domains, including the N-terminal, highly conserved ~70-amino acid J domain, the glycin/phenylala-
mine-rich domain, and a cysteine-rich Zn^{2+}-binding domain. Type II DnaJ-like proteins have an N-terminal J domain and the Gly/Phe-rich domain but lack the C-terminal Zn^{2+}-binding domain. Type III proteins possess only a J domain, which can occur anywhere in the protein. The J domain contains the hallmark His-Pro-Asp (HPD) motif, which is essential for interac-
ting with Hsp70s (19, 20). This interaction stimulates the hydrolysis of ATP bound to Hsp70, thus stabilizing Hsp70 binding to unfolded substrate proteins and facilitating its ability to aid protein folding. Some type I DnaJs proteins bind directly to unfolded substrates through their zinc finger and C-terminal domains and may serve to target Hsp70s to these substrates. This has been demonstrated for E. coli DnaJ (21), the cytosolic yeast DnaJ protein, Ydj1p (22), and the mamma-
lian mitochondrial DnaJ protein, Mdp1p (23).

DnaJ homologues have been identified in organisms ranging from bacteria to yeast to humans to plants. Organelle-specific DnaJs work as cofactors to cooperate with their specific Hsp70 partners and, unlike the hsp70s, appear to be specific to different functions. The yeast ER contains three DnaJ-like proteins: Sec63p, Jem1p, and Scj1p. Sec63p is an essential membrane protein that assists BiP in translocating nascent proteins into the ER lumen (24). Jem1p and Scj1p are soluble ER luminal proteins that are not essential for cell viability under normal growth conditions. Scj1p cooperates with yeast BiP to fold and assemble proteins in the ER lumen (25), and Jem1p interacts with BiP to mediate nuclear membrane fusion during mating (26). Recent studies show that both Scj1p and Jem1p may facilitate the retro-translocation of ERAD substrates to the cytosol by preventing their aggregation in the ER lumen (10) and that Sec63p is a component of the retrograde translocon (27).

Recently, three mammalian ER DnaJ homologues Mj1 (28), hSec63 (29), and HEDJ (30) have been cloned. Based on structural predictions, Mj1 and hSec63 appear to be homologues of yeast Sec63, and hSec63 was shown to be associated with translocon components (31). HEDJ shows some sequence homology to yeast Scj1 and can bind to BiP in vitro (32). However, little functional data are available for any of the mammalian ER Dnas. Because BiP has multiple functions and a second Hsp70 homologue, GRP170/OPR150 (33), exists in the ER, we anticipate that more mammalian ER DnaJ homologues will be discovered. For simplicity and clarity, we propose they be named ERdjs (ER-localized DnaJ homologues) according to their order of discovery. Thus, Mj1 would be referred to as ERdj1, hSec63 as ERdj2, and HEDJ as ERdj3. We have identified a fourth mammalian ER-localized DnaJ type II homologue, ERdj4. In this report, we demonstrated that ERdj4 was a membrane protein with its J domain facing the ER lumen and that it interacted with BiP when they were co-expressed. In vitro assays showed that the J domain of ERdj4 could activate the ATPase activity of BiP, whereas a J domain mutant (His^{34} → Gln) failed to do so. We found that ERdj4 was expressed at the highest level in tissues that are highly active in synthesiz-
ing secretory pathway proteins and was potently up-regulated in response to ER stress, indicating that this novel protein may play a role in either ER protein folding or ERAD to diminish the accumulation of unfolded proteins.

**Experimental Procedures**

**DNA Constructs**

EST clone AI316972 encoding mouse ERdj4 cDNA was obtained from Incyte Genomics Systems Inc. (St. Louis, MO) and was found to contain the complete cDNA by DNA sequencing. ERdj4 cDNA was removed from its original vector and subcloned into Bluescript-SK downstream of the T7 promoter for in vitro transcription assays. To create an HA epitope-tagged version of ERdj4, the stop codon was removed by PCR amplification with Taq polymerase (Roche Molecular Biochemicals) using 5′-CGGCTGAGATCTCGGCACAGTCT-3′ as the forward primer and 5′-GAAGATCTCTTGCTCAAGGTTAAG-3′ as the reverse primer. The PCR product was subcloned into the 3HA-DSL vector, which is a kind gift from Dr. Michael Kastan (34), which was modified from the original pSG5 vector (Neupogen) by adding multiple cloning sites and an HA sequence encoding mouse ERdj4 (35). This PCR product was cut into pSG5 vector to produce ERdj4 in eucaryotic cells without an epitope tag. In order to make the HA-tagged chimeric DnaJ α1-antitrypsin protein, the α1-antitrypsin sequence was PCR-amplified using 5′-GGGGATCCCTAGTCGACACCGTGCGGCTCGA-3′ as the forward primer and 5′-GAAGATCTTTTGGGTGGGATTCACCACT-3′ as the reverse primer. The PCR product contained the full-length α1-

**Protein Expression and Purification**

The recombinant proteins were expressed in E. coli M15 cells according to the manufacturer’s protocol (Qiagen QIAexpress System). Cells were harvested after a 2-h induction period for recombinant BiP (35), WT J-ERdj4, and Mu J-ERdj4. The recombinant proteins were purified using immobilized metal-affinity chromatography using Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen) as described (35). The final J domain proteins were tested for residual ATPase activity and found to be negative, demonstrating that they were not contaminated with co-purifying ATPases or kinases. All three proteins were stored at −20 °C in 50 mM sodium phosphate, pH 7.0, containing 150 mM NaCl and 50% glycerol.

**In Vitro Translation and Proteinase K Treatment**

ERdj4 mRNA was transcribed from the T7 promoter of pBS-ERdj4 using the mCAPkit RNA Capping kit (Stratagene) and translated using [35S]methionine (Amersham Biosciences) and TNT Coupled Reticulocyte lysate (Promega) in the presence of rat liver microsomes produced as described (36). The protein product was either left untreated or digested with 150 μg/ml proteinase K in the presence or absence of 1% Nonidet P-40. Yeast α-factor precursor mRNA (Promega catalog number Y4070) was used as a positive control for protein translation.

**Cell Lines and Eucaryotic Expression**

Cell Lines—B16 murine melanoma cells were maintained in mini-
mum Eagle’s medium; NIH3T3 murine fibroblast cells, COS-1 monkey kidney fibroblast cells, and HeLa human epithelial cells were main-
tained in Dulbecco’s modified Eagle’s medium; and HepG2 human hepatocarcinoma cells and Ag8.653 murine plasmacytoma cells were maintained in RPMI 1640. All media were supplemented with 10% fetal calf serum, 2 mM l-glutamine, and 1% Fungizone (Biowhittaker, Walkersville, MD).

Antibodies—Anti-HA monoclonal antibody was kindly provided by Dr. Al Reynolds (Vanderbilt University). Anti-ERdj4 antisera was produced against the full-length recombinant mouse ERdj4. Anti-calnexin antisera was raised against a recombinant protein correspond-
ing to the cytosolic tail of mouse calnexin as described (37). Polyclonal
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anti-BiP and anti-GRP94 antisera have been described (37, 38). Rabbit anti-α-antitrypsin antisemur was purchased from Cappel Corp. (West Chester, PA). Unlabeled goat anti-mouse λ, FITC-labeled goat anti-mouse Ig, and TRITC-labeled goat anti-rabbit Ig antibodies were purchased from Southern Biotechnology Associates (Birmingham, AL). Twenty-four hours after transfection, cells were metabolically labeled for 16 h with [35S]methionine (ICN). Cell lysates were immunoprecipitated with protein A-Sepharose alone (negative control) or with anti-HA, anti-α-antitrypsin, or anti-mouse λ followed by protein A-Sepharose. Precipitated proteins were analyzed on 10% SDS gels under reducing conditions, and the signal was enhanced with Amplify (Amersham Biosciences) for radiographic visualization. For in vivo binding experiments, COS-1 were transfected with the indicated empty vector, untagged ERdj4, hamster BIP, or ERdj4 + hamster BIP using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals). Thirty hours after transfection, cells were starved in methionine-free medium for 30 min then metabolically labeled with [35S]methionine (ICN) for 2.5 h. Labeled cells were trypsinized and incubated in the presence of 150 μg/ml of a membrane-permeable cross-linking reagent DSP (Sigma) for 1 h at 4 °C before lysis and immunoprecipitation with anti-ERdj4, anti-BiP antisera or protein A-Sepharose.

Endoglycosidase H Digestion and Tunicamycin Treatment—For the studies on glycosylation of ERdj4, after immunoprecipitation protein samples were denatured by adding 15 μl of freshly made denaturing buffer (0.5% SDS, 1% 2-mercaptoethanol) and heated to 95 °C for 15 min. The denatured samples were diluted with 10 μl of 0.5 mM sodium citrate, pH 5.5, 50 μl of H2O, 2 μl of 100 mM phenylmethylsulfonyl fluoride, and 3 milliunits of Endo-H (Roche Molecular Biochemicals) and then incubated at 37 °C for 2 h. De novo glycosylation was inhibited by labeling cells in the presence of 1 μg/ml tunicamycin.

Microsome Preparation and Solubilization of ER Proteins—Microsomes were produced by Dounce homogenization as described (39, 40), and crude homogenates were centrifuged at 500,000 × g for 3 h to separate the cell debris and nuclei. The supernatant containing microsomes and cytosol was aliquoted into four samples that were centrifuged at 10,000 × g to pellet the microsomes. The ER microsomes were then resuspended in 100 μl of PBS buffer alone or PBS containing either 0.1% digitonin, 0.2% digitonin, or 1% deoxycholate. After rocking at 4 °C for 1 h, samples were centrifuged at 10,000 × g for 5 min to sediment all membranes (32). The supernatants and pellets were separated and prepared for SDS-PAGE and Western blotting.

Cellular Localization and Immunofluorescence Staining—COS-1 cells were seeded on a glass cover slide and transfected with HA-tagged ERdj4 using the FuGENE 6 transfection reagent. Twenty-four hours after transfection, cells were fixed on the cover slide with alcohol-acid (36). Cells were blocked with PBS containing 10% fetal calf serum for 1 h at room temperature and then stained with the anti-HA mouse monoclonal antibody and rabbit anti-GRP94 polyclonal antibody. After washing with PBS, the cover slides were incubated with FITC-labeled goat anti-mouse Ig and TRITC-labeled goat anti-rabbit Ig. The slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and visualized by fluorescence microscopy (Olympus BX50). Photos were taken with a Sensys camera (Photometrics, Tucson, AZ), and images were processed by V + + Digital Imaging software (Roper Scientific, Auckland, New Zealand) and Photoshop (Microsoft).

ATPase Assay

ATPase assays were performed as described previously (41, 42). The purified WT J-ERdj4 or Mu J-ERdj4 was added to recombinant BIP (0.5 μM) at concentrations ranging from 0.25 to 4 μM. Each reaction contained 20 μl of reaction buffer (50 mM Tris-HCl, 50 mM NaCl, 5 mM MgCl2, 1 mM DTT, and 0.1% NP-40) and was incubated at 30 °C. The enzyme reaction was removed at indicated time points, spotted onto TLC plates (Sigma), and developed in 1 M formic acid, 0.5 M LiCl. ATP and free phosphate signals were quantified by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software. The statistical data were deduced from three independent experiments, and the error bars in Fig. 7, A–D, represented S.D.

Northern Analysis

B16, NIH3T3, HeLa, and HepC2 cells were treated in the absence or presence of 10 μM tunicamycin or 2 μM thapsigargin for 6 h. Total RNA was extracted using the RNeasy mini prep kit (Qiagen), and 20 μg of RNA was loaded on a formaldehyde gel and transferred for Northern blotting as described (37). The probes used for Northern blotting corresponded to the coding sequences of human ERdj4, mouse ERdj4, hamster BIP, mouse GRP94, and human G3PDH (CLONTECH). The ERdj4 probe was a 1.5-kb fragment isolated from XhoI and EcoRI sites of EST clone BE255328 (Incyte Genomics). The ERdj4 probe was a 520-bp fragment removed with EcoRV and PstI from the cDNA. BIP, GRP94, and G3PDH probes were prepared as described (37) using the Prime-it II kit (Stratagene) and purified by NucTrap columns (Stratagene). Human 12-lane MT blot containing 1 μg of poly(A)+ RNA per lane from 12 different human tissues was purchased from CLONTECH (catalog number 7780-1). Probes corresponding to three different regions of the human ERdj4 sequence were hybridized with the blot, as indicated under “Results.”

RESULTS

Sequence Analysis of ERdj4—By searching the DDBJ/EMBL/GenBank™ data base for proteins that contained both a DnaJ domain and a potential ER localization signal sequence, we identified a novel mouse cDNA with GenBank™ accession number AB028857. PSORT analysis of the deduced amino acid sequence of this gene revealed the presence of a type II N-terminal hydrophobic sequence (aa 7–23) (43), immediately followed by the highly conserved 70 amino acid signature J domain (aa 24–93) (Fig. 1A). Type II signal sequences are not normally cleaved and serve to both target and anchor proteins in the ER membrane (44). We named this novel gene ERdj4, because it represents the fourth mammalian ER DnaJ protein to be reported. A full-length cDNA was assembled from overlapping clones in the mouse EST data base and used to blast the entire mouse and human EST data bases. Nearly 100 partial clones were identified in the mouse data base, which had been cloned from a variety of tissues including kidney, lung, testis, and embryo. In addition, 170 partial clones were found in the human data base, again from a variety of tissues including placenta, testis, colon, pancreatic islet, infant brain, and fetal heart, suggesting that ERdj4 was a ubiquitously expressed gene. The majority (33:36) of the mouse sequences that extended to the 3′ region ended at bp 1928, thus producing an ~1.9-kb mRNA (Fig. 1A), although three clones extended another 150 bp. Of the 88 human cDNAs that extended into the 3′-untranslated region, 50 stopped at bp 1923 producing an ~1.9-kb mRNA and the other 38 stopped at bp 2370 encoding an ~2.4-kb message. However, in both species the extension is in the 3′-untranslated region and should not alter the protein product. The largest open reading frame was found in frame 1 beginning at bp 202, which was preceded by an in-frame stop codon at bp 19, suggesting that translation of ERdj4 begins with this methionine to produce a 222-amino acid protein with a predicted molecular mass of ~26 kDa (Fig. 1A). The first methionine in the human cDNA occurs at the same site as in the mouse cDNA and encodes a 223-amino acid protein. Comparison of the amino acid sequences of various ERdj4s from mammals revealed that it is highly conserved gene with 91% identity between mouse and human and 97% identity between mouse and rat (Fig. 1B). In addition, a number of partial cDNA clones from other organisms were found as follows: a pig EST clone (BE014395) encoded the N-terminal 110 amino acids of ERdj4 and showed 94% identity to the corresponding region of mouse ERdj4. Several Xenopus EST clones (BG017796, BG51477, and BG817035) were also identified, which showed 60–67% identity to the corresponding mouse regions. We were unable to identify any homologous genes in Caenorhabditis elegans, yeast, or Drosophila, suggesting that ERdj4 may represent a vertebrate specific DnaJ protein.

As a DnaJ family member, ERdj4 contained the highly conserved 70-amino acid (aa 24–93) region known as the J domain, which interacts with Hsp70 partners to stimulate their ATPase activity.
activity. Unlike ERdj1/Mtj-1 and ERdj2/hSec63, which have only a J domain and therefore belong to the type III subgroup of DnaJ proteins, ERdj4 also contains a glycine/phenylalanine-rich region following its J domain (Fig. 1C). However, it lacks the cysteine-rich Zinc finger binding domain, which exists in all type I J proteins like ERdj3/HEDJ and E. coli DnaJ (Fig. 1C). Thus, ERdj4 is a type II DnaJ protein and, as such, represents the first member of this subgroup to be found in the ER of eucaryotic cells. In the yeast ER, there are three ER DnaJ homologues as follows: Sec63 and Jem1 are type III J proteins and Scj1 is a type I J protein.

**Tissue Distribution of ERdj4**—In order to determine the tissue distribution of ERdj4 expression, we used the entire coding region (bp 194–870) of human ERdj4 as a probe to hybridize to a blot containing poly(A) RNA isolated from various human tissues. Two transcripts of ~2.4 and ~1.9 kb were detected in all human tissues examined (Fig. 2). The highest level of ERdj4 expression was found in the liver, placenta, and kidney, which also showed the highest level of BiP expression. All three tissues contain cells with well developed ER that produce large quantities of secretory proteins. The presence of two distinct transcripts may be because of the different lengths of the 3'-untranslated region that were reflected in the EST clones and that were predicted to produce messages of ~1.9 and ~2.4 kb. A probe corresponding to the C-terminal coding

**Fig. 1. Sequence analysis of ERdj4 cDNA.** A, complete nucleotide sequence of mouse ERdj4, and its deduced amino acid sequence. *Italic* letters (aa 7–23) indicate the predicted signal peptide. The J domain is *underlined* (aa 24–93), and the HPD motif is *shaded* and indicated with *boldface* type. In the 5'-untranslated region, an in-frame stop codon TAG is *underlined*. B, comparison of ERdj4 amino acid sequences from human (h), mouse (m), and rat (r). The peptide sequences were aligned using BioEdit Software (North Carolina State University). To optimize the homology, a gap was inserted as denoted by a dot. *Black boxes* and *gray boxes* indicate identities or similarities, respectively. C, classification of ERdjs. ERdj1 and ERdj2 are type III J proteins and have only a J domain; ERdj3 belongs to type I J proteins and has a high degree of domain conservation with E. coli DnaJ, containing J, G/F-rich, and Cys-rich Zinc finger domains; ERdj4 has J and G/F domains and belongs to type II J proteins. All of ERdjs have the signal peptide that allows them to translocate into ER.

**Fig. 2. Tissue distribution of ERdj4.** Human 12-lane multi-tissue Northern blot was purchased from CLONTECH (catalog number 7780-1) and probed with ERdj4, BiP, and ß-actin probes, sequentially. The transcripts were detected by autoradiography. ERdj4 and BiP showed similar expression patterns in the various tissues. ß-Actin served as a loading control. Lk., peripheral blood leukocyte; Lu., lung; Pl., placenta; In., small intestine; Li., liver; Ki., kidney; Sp., spleen; Th., thymus; Co., colon (no mucosa); Sh., skeletal muscle; Ht., heart; Br., brain.
antiserum. After mounting, cells were visualized by immunofluorescence microscopy. A and D, endogenous GRP94; B and E, HA-tagged ERdj4; and C and F, merged images of A and B and D and E. The white arrows indicate the Golgi apparatus, which excludes resident ER proteins.

Subcellular Localization of ERdj4—To verify further its cellular localization and membrane orientation, we produced a C-terminal HA epitope-tagged version of ERdj4 and transfected COS-1 cells with it. Cells were fixed 24 h after transfection and co-stained with an anti-HA monoclonal antibody and a rabbit polyclonal anti-GRP94 antisera (Fig. 3). Approximately 10% of cells expressed the epitope-tagged ERdj4 (Fig. 3, B and E), which was localized to the perinuclear area and showed a lacy reticular staining pattern. Co-staining with the resident ER luminal protein GRP94 (Fig. 3, A and D) revealed completely overlapping patterns (Fig. 3, C and F), which strongly suggests that ERdj4 is a resident ER protein. Like GRP94, ERdj4 appeared to be excluded from the Golgi region (as shown in Fig. 3 with white arrows), suggesting that it is not transported further along the secretory pathway. Control experiments were performed in the absence of primary antibodies or by using cells transfected with an empty 3HA DSL vector and demonstrated negligible background staining (data not shown).

Orientation and Glycosylation Status of ERdj4—To eliminate the possibility that ERdj4 was associated with the cytosolic side of ER membranes, we translated ERdj4 in vitro in the presence of microsomes and examined its sensitivity to proteinase K digestion (Fig. 4A). Adding detergent to the microsomes rendered ERdj4 resistant to proteinase K digestion. These results indicated that ERdj4 encodes an ER targeted protein that is translocated into the ER lumen.

As shown in Fig. 4A, in vitro translated ERdj4 migrated with
an apparent molecular mass of ~27 kDa both in the presence and absence of microsomes. Inspection of the sequence revealed that mouse ERdj4 has two potential N-linked glycosylation sites, which could allow addition of high mannose sugars to the protein in ER. A combination of glycosylation at a single site and signal sequence processing could produce a protein without an obvious change in molecular weight. In this case, ERdj4 would be a soluble ER luminal protein, because the signal peptide represents the only hydrophobic stretch in the protein sequence. Endo-H treatment of the in vitro translocated protein did not result in a mobility increase and suggested that ERdj4 was not glycosylated (data not shown). As an independent way to examine this question, we investigated the glycosylation status of HA-tagged ERdj4 that was transiently expressed in COS-1 cells. Cells were labeled with or without tunicamycin, which inhibits N-linked glycosylation, and a portion of the protein synthesized in the absence of tunicamycin was treated with Endo-H to remove N-linked glycans. As shown in Fig. 4B, neither Endo-H digestion (lane 5) nor tunicamycin treatment (lane 6) altered the mobility of in vivo synthesized ERdj4 (lane 4). The \(\alpha\)CH1 light chain, which has a single N-linked glycosylation site, was used as a positive control, because it has a molecular weight similar to that of ERdj4. Both Endo-H digestion and tunicamycin treatment caused an easily detectable change in the mobility of the light chain (the glycosylated one migrated at ~32 kDa, as indicated with a solid arrow in lanes 8 and 9; the non-glycosylated one migrated at ~30 kDa as indicated with a white arrow in lanes 9 and 10). Because ERdj4 did not appear to be either glycosylated or possess a KDEL retention sequence, features that are commonly found on ER resident proteins, we made a HA-tagged chimeric protein, which is composed of ERdj4 at its N terminus ligated in-frame with the first amino acid of the mature \(\alpha\)-antitrypsin (lacking its signal sequence) at its C terminus, to further investigate the orientation of ERdj4 in the ER. With three N-linked glycosylation sites in its sequence, \(\alpha\)-antitrypsin should be glycosylated if ERdj4 translocates it into the ER lumen. As shown in Fig. 4B, the chimeric protein was glycosylated (lanes 12 and 13), as demonstrated by sensitivity to both Endo-H digestion (lane 14) and tunicamycin treatment (lane 15). These data further confirmed that ERdj4 was an ER resident protein with its J domain located in the ER lumen. Coupled with the fact that in vitro translocated ERdj4 did not migrate more rapidly, our data suggest that the signal sequence of ERdj4 might not be cleaved and that it was likely to be an integral membrane protein.

**Membrane Integration of ERdj4**—To determine more accurately whether ERdj4 was a membrane-anchored ER protein, we examined the endogenous protein in the mouse plasmacytoma cell line, Ag8.653 cell, which has a well developed ER membrane system, by using a rabbit polyclonal anti-ERdj4 antiserum that we developed. ER vesicles were isolated, divided evenly into 4 aliquots, and then incubated with PBS alone or PBS containing 0.1% digitonin, 0.2% digitonin, or 1% deoxycholate. The samples were centrifuged to pellet ER membranes, and both the pellets and the supernatants were subjected to the electrophoresis and Western blotting. Several ER resident proteins (calnexin, BiP, and ERdj3/HEDJ) were used as controls for the method. As a membrane-bound protein, calnexin remained associated with ER vesicles in 0.1 and 0.2% digitonin, whereas BiP, an ER luminal protein, was partially released into supernatant by these conditions (Fig. 5). Because BiP is part of a large chaperone complex in the ER (3, 31) and also binds to the translocon (5) and the Ire1 and PERK transmembrane kinases (8), it is perhaps not surprising that part of BiP remained associated with ER membranes in 0.2% digitonin. However, another ER luminal protein, ERdj3/HEDJ, was partially released with 0.1% digitonin and almost completely in 0.2% digitonin. In keeping with the in vitro translocation data suggesting that ERdj4 might be a membrane-anchored protein, the endogenous ERdj4 protein was still largely associated with membrane fraction even in 0.2% digitonin. When the microsomes were disrupted with 1% deoxycholate, all four ER resident proteins were released to supernatant.

**In Vivo Interaction of ERdj4 with BiP**—The data presented above indicated that ERdj4 remained associated with the ER membrane, presumably via its signal sequence. Therefore the majority of the protein, including the J domain, would reside inside the ER lumen where it could interact with an Hsp70 partner. Because BiP is the major Hsp70 protein in the ER, we determined if ERdj4 could associate with BiP in vivo. COS-1 cells were transfected with either empty vector, ERdj4, BiP, or ERdj4 and BiP. Cells were metabolically labeled and then treated with DSP, a membrane-permeable cross-linker, to stabilize the naturally existing complexes. Cell lysates were prepared and immunoprecipitated with the indicated antisera (Fig. 6). The precipitated proteins were treated with a reducing reagent to disrupt the cross-links before analyzing the sample by SDS-PAGE. Exogenously expressed ERdj4 was specifically immunoprecipitated with anti-ERdj4 antisera (lane 5) but not with either protein A (lane 4) or the anti-rodent BiP antisera (lane 6). In addition, an ~100-kDa protein (as indicated with *) was immunoprecipitated with our immune serum from both transfected and non-transfected COS cells. It is possible that this band represents an unidentified COS cell protein that contains a J domain. When hamster BiP was co-expressed with ERdj4, a small amount of BiP co-precipitated with ERdj4 (lane 11), and more easily detected, ERdj4 was co-precipitated with transfected hamster BiP when the anti-rodent BiP antisera was used (lane 12). Interactions between BiP and ERdj4 could also be detected without using the covalent cross-linker; however, they were somewhat sensitive to the lysing conditions used (data not shown). These data indicated that ERdj4 could interact with BiP in vivo and might regulate the activity of BiP.

**Activation of ATPase Activity of BiP by J Domain of ERdj4**—To determine whether the J domain of ERdj4 could stimulate the ATPase activity of BiP, we purified recombinant BiP and both wild type and mutant ERdj4 J domains and assayed their effects on the ATPase activity of BiP. The rate of ATP hydrolysis by BiP was in the linear range during course of the experiment under all conditions examined (Fig. 7A). In the absence of ERdj4, the ATP hydrolysis rate of BiP was about

\[ Y. Shen, L. Meunier, and L. M. Hendershot, unpublished data. \]
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ERdj4 interacts with BiP in vivo. COS-1 cells were transiently transfected with empty vector, full-length ERdj4, rodent BiP, or ERdj4 + rodent BiP. 42 h after transfection, cells were labeled with [35S]methionine for 2.5 h and treated with 150 µg/ml DSP. Cell lysates were immunoprecipitated with (+) or without (−) antisera against ERdj4 or rodent BiP as indicated. The rodent-specific anti-BiP antiserum does not recognize endogenous monkey BiP in COS-1 cells. * indicated an −100-kDa protein that was recognized by the anti-ERdj4.

To determine the concentration at which the J domain of ERdj4 provided optimal stimulation of the ATPase activity of BiP, we performed the ATPase assay with a fixed amount of BiP and increasing amounts of either wild type or mutant J domains (Fig. 7C). The mutant J domain was made by changing His54 → Gln in the HPD motif. BiP (final concentration 0.5 µM) was incubated with either wild type (WT J-ERdj4) or mutant (Mu J-ERdj4) J domain (final concentration 0.25–4 µM) for 30 min. We found that the stimulation of the ATPase activity of BiP by WT J-ERdj4 was concentration-dependent, with maximal stimulation occurring at a 4:1 ratio of WT J-ERdj4 to BiP. Mutation of the HPD motif in the J domain totally abolished its ability to activate the ATPase activity of BiP at any concentration (Fig. 7C and D).

Induction of ERdj4 mRNA and Protein during ER Stress—Most of the ER molecular chaperones are transcriptionally up-regulated during conditions of ER stress, via a signaling cascade termed the unfolded protein response (UPR) pathway. This serves to prevent the aggregation of misfolded proteins in the ER and to aid in protein refolding when the stress subsides. The data above indicated that ERdj4 may work as a partner of BiP in the ER and to aid in protein refolding when the stress subsides. Third, a chimeric protein composed of ERdj4 at its N terminus and 1-antitrypsin at its C terminus was glycosylated (Fig. 8B, lane 2) from transfected COS-1 cells. This serves to prevent the aggregation of misfolded proteins in the ER lumen.

ERdj4 was expressed in all tissues and showed a similar distribution pattern as BiP, with the highest level of expression in liver, placenta, and kidney. These three tissues contain cells...
with well-developed ER and produce large quantities of secretory proteins. Moreover, ERdj4 interacted with BiP in vivo when they were co-expressed in COS-1 cells, and the J domain of ERdj4 activated the ATPase activity of BiP in vitro. These data suggest that ERdj4 may be one of the natural partners of BiP in vivo and therefore must regulate one of the functions of BiP. BiP plays a role in maintaining the permeability barrier of the ER translocon during early stages of protein translocation (5), aiding the folding and assembly of nascent proteins (3, 4), targeting misfolded proteins for retro-translocation and ERAD (6, 7), serving as a sensor of ER stress by binding or releasing ER kinases (8), and maintaining ER calcium stores (9). Based on other hsp70 systems, it is reasonable to assume that different DnaJ partners may assist BiP in these various functions.

To date, a total of 4 ERdjS have been identified in the mammalian ER as follows: ERdj1/Mtj1, ERdj2/hSec63, ERdj3/HEDJ, and now ERdj4. ERdj2/hSec63 is a transmembrane protein, which displays similar topology in the ER membrane as Sec63p in yeast. It is expressed at relatively high levels in dog pancreas and associates with mammalian Sec61 and Sec62 in the translocon (31, 47). The J domain of ERdj2 interacts with BiP and stimulates its ATPase activity in vitro (31). Together these data suggest that ERdj2 is a mammalian homologue of yeast Sec63p and may act with BiP to translocate nascent polypeptides into the ER. When mammalian cells encounter ER stress, protein translation is inhibited, and consequently the translocation of newly synthesized peptides slows down. Thus, we would not expect ERdj2 to be up-regulated by ER stress as a component of translocon. Consistent with this, our Northern data showed that the mRNA level of ERdj2 was not elevated under ER stress induced by either tunicamycin or thapsigargin. Although ERdj4 is associated with ER membranes, it is unlikely that it also plays a role in translocation into the ER, because its expression is highest during ER stress when little translocation is occurring. ERdj3 is most closely related to Scj1p in yeast and shows some sequence homology with Scj1p (32). Both are soluble luminal proteins of the type I subgroup of DnaJ proteins. Scj1p cooperates with yeast BiP to fold and assemble proteins in the ER lumen (25). ERdj3 may play a similar role in protein folding and assembly in mammalian cells, because we have found that it interacts with BiP-bound, unassembled immunoglobulin heavy chains in vitro (32). ERdj1/Mtj1 is a member of the type III subgroup of DnaJ proteins and is a transmembrane protein with its J domain facing the ER lumen. Although its J domain interacts with BiP in vitro, the function of ERdj1 is not clear yet. In yeast, there is another type III DnaJ protein, Jem1p, which interacts with Kar2p to mediate nuclear membrane fusion during yeast mating (26). However, Jem1p is a soluble luminal protein and

![Graph A](image_url)

**Fig. 7. J domain of ERdj4 activates ATPase activity of BiP, but mutant J domain cannot.** A, recombinant BiP (0.5 μM) was incubated with (●) or without (○) the recombinant wild type J domain of ERdj4 (4 μM) in ATPase assay buffer containing 100 μM ATP at 30 °C. 2 μl of each sample was removed at 0, 5, 10, 20, 30, 40, 50, and 60 min and spotted onto a thin layer chromatography plate for separation. The hydrolysis of ATP was quantified by PhosphorImager. B, turnover rate calculated from A. C, recombinant BiP (0.5 μM) was incubated with various concentrations (0.25–4 μM) of WT J-ERdj4, the wild type J domain of ERdj4 (●) or Mu J-ERdj4, the His → Gln mutant J domain (○) as indicated, in ATPase assay buffer containing 100 μM ATP at 30 °C for 30 min. 2 μl of each sample was removed and separated by thin layer chromatography. The hydrolysis of ATP was quantified by PhosphorImager. D, turnover rate calculated from C.

3 L. Meunier, Y. K. Usherwood, K. T. Chung, and L. M. Hendershot, submitted for publication.
ERdj4 is induced by ER stress. A, ERdj4 is an ER stress-inducible gene. Total RNA extracted from non-treated (−), tunicamycin-treated (Tu), and thapsigargin-treated (Th) HepG2 cells was probed by Northern blotting using the coding region of ERdj2, ERdj4, GRP94, and BiP. G3PDH served as a loading control. The transcripts were detected by autoradiography. B, protein level of ERdj4 was induced by tunicamycin. Ag8.653 cells and B16 cells were left untreated (−) or treated with (+) tunicamycin for 16 or 8 h, respectively. Microsomes were produced from both cell lines and electrophoresed with cell lysate from COS-1 cells that were transiently transfected with pSG5 vector (Mock) or pSG5-ERdj4 (ERdj4). Western blotting was performed by using antisera against ERdj4 and calnexin. The calnexin antiserum is specific for mouse cell lines and does not recognize COS-1 monkey calnexin. A nonspecific band A, which could be recognized by ERdj4 antisera, shifted to A' indicating that tunicamycin treatment worked properly.

shows no sequence homology to the four known mammalian ERdj proteins. In yeast, both Sj1 and Jem1 are required for the retro-translocation of misfolded proteins for degradation by the proteasome (10).

The up-regulation of ERdj4 in the presence of stress may suggest that ERdj4 plays a role in either the folding of unfolded proteins or the retro-translocation of misfolded proteins, both of which diminish the accumulation of unfolded proteins in the ER that occurs during ER stress conditions. Its localization to the membrane of the ER might allow it either to direct unfolded proteins to the translocon for retro-translocation or to aid in the folding of other membrane-anchored proteins. We feel that it is unlikely that ERdj4 plays a role in the activation or silencing of the ER kinases, Ire1 and PERK, because its kinetics of induction suggest it is downstream of UPR induction and not upstream. Finally, it is possible that it plays a role in sealing the translocon during stress. However, because the number of translocons does not increase during ER stress, there is no reason to believe that additional ERdj4 would be required during stress to perform this function. It is possible that the diminished translation that occurs during stress would require more translocons to be sealed; however, this is a very early feature of the UPR and is fairly transient in the case of tunicamycin- and thapsigargin-induced stress. Clearly further studies will be needed to understand which of these roles ERdj4 might perform. Whatever ER functions ERdj4 participates in, it seems to be restricted to vertebrates, because no ERdj4 homologue was found in the Drosophila, C. elegans, or yeast genomic data bases.

Sequence analysis shows that ERdj4 is a member of the type II subgroup of DnaJ proteins, which contain a hallmark J domain (aa 24–93) followed by a glycin/phenylalanine-rich region. As such, ERdj4 is the first type II DnaJ protein to be identified in the ER of any organism. Several type II DnaJ proteins have been characterized in other organelles of eukaryotic cells, including Sis1p, Hdj1/Hsp40, haj1, and haj2. Yeast Sis1p is localized in the cytosol and nucleus and is essential for viability (48). It associates with ribosomes and promotes the initiation of translation (49). Mammalian Hdj1/Hsp40 has a similar cellular localization pattern as Sis1p and also binds to ribosomes, where it aids in the folding of polypeptide chains that are still in the process of elongating (50, 51). HsJ1 is expressed primarily in neuronal tissues (52) and inhibits clathrin uncoating reactions mediated by Hsc70 (53). HsJ2 has some sequence homology with HsJ1 but is expressed mainly in testis where it may play a role in protein translation initiation. Type II DnaJ proteins are usually less efficient in suppressing the aggregation of unfolded proteins than some type I DnaJ proteins, like DnaJ, Ydj1p, Mdj1 and Hdj2, which is probably due to the lack of a C-terminal substrate binding domain in type II DnaJ proteins. However, type II J proteins appear to be as effective as type I J proteins in promoting Hsp70-dependent folding of unfolded substrates. In fact, Hdj1 is even more effective than Hdj2 in refolding luciferase in vitro (54). In general, type I and type II J proteins have a more highly conserved J domain within their own groups and tend to interact with a broader range of substrates, whereas type III J proteins, have a lower level of conservation and a more restricted substrate specificity (55). A broad substrate specificity would be expected if ERdj4 played a role in either the refolding of proteins or the retro-translocation of unfolded proteins during ER stress.

In summary, we have identified a novel mammalian ER DnaJ family member, ERdj4. ERdj4 is a type II DnaJ homologue with its signal sequence anchored to the ER membrane and its J domain and Gly/Phe-rich domain located inside ER. ERdj4 co-localizes and interacts with BiP in the ER lumen and may play a role in one of the functions of BiP. Because ERdj4 is up-regulated during ER stress, it might be involved in refolding of unfolded and misfolded proteins or some aspect of ERAD. It is important to understand which of BiP’s functions ERdj4 regulates and how it works in vivo in order to increase our understanding of how mammalian cells respond to ER stress.

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Identification and Characterization of a Novel Endoplasmic Reticulum (ER) DnaJ Homologue, Which Stimulates ATPase Activity of BiP \textit{in Vitro} and Is Induced by ER Stress

Ying Shen, Laurent Meunier and Linda M. Hendershot

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