HEDJ, an Hsp40 Co-chaperone Localized to the Endoplasmic Reticulum of Human Cells*

Min Yu, Robert H. A. Haslam, and David B. Haslam‡

From the Departments of Pediatrics and Molecular Microbiology, Washington University School of Medicine and St. Louis Children’s Hospital, St. Louis, Missouri 63110

Hsp40 co-chaperones, characterized by the presence of a highly conserved J domain, are involved in nearly all aspects of protein synthesis, folding, and secretion. Within the lumen of the endoplasmic reticulum, these chaperones are also involved in reverse translocation and degradation of misfolded proteins. We describe here the cloning and characterization of a novel Hsp40 chaperone, which we named HEDJ. Epitope-tagged HEDJ was demonstrated by confocal microscopy to be localized to the endoplasmic reticulum. Protease susceptibility, glycosidase treatment, and detergent solubility assays demonstrated that the molecule was luminally oriented and membrane-associated. In vitro experiments demonstrated that the J domain interacted with the endoplasmic reticulum-associated Hsp70, Bip, in an ATP-dependent manner and was capable of stimulating its ATPase activity. HEDJ mRNA expression was detected in all human tissues examined. Highly homologous sequences were found in mouse, Drosophila, and Caenorhabditis elegans data bases. These results suggest potential roles for HEDJ in protein import, folding, or translocation within the endoplasmic reticulum.

The endoplasmic reticulum (ER) plays several vital roles in protein processing and secretion. For example, the ER is a site not only of protein modification and sorting but is also an important site of quality control. Intramolecular folding, a requisite for normal protein function, must occur upon import into the ER lumen, where molecular chaperones such as calnexin and calreticulin interact with nascent proteins and retain them in the ER until folding has been correctly achieved (1–3). In an incompletely understood manner, proteins that remain misfolded are exported from the endoplasmic reticulum to the cytoplasm where they are commonly ubiquitinated and degraded by the proteosome (4, 5).

Central to each of these activities are molecular chaperones belonging to the Hsp70 and Hsp40 families. Within the ER lumen, Bip (also known as Grp78 in mammalian cells or Kar2 in yeast), a member of the Hsp70 chaperone family, is involved in essentially all aspects of protein synthesis and secretion. For example, in the yeast Saccharomyces cerevisiae, mutations in Bip result in defects in protein translocation, folding, and reverse translocation (6, 7). However, Bip, like other Hsp70 chaperones, cannot work in isolation. The intrinsic ATPase and folding activity of Bip is stimulated by members of the Hsp40 co-chaperones, also known as DNAJ-related proteins, after Escherichia coli DNAJ, the progenitor family member. These co-chaperones are characterized by the presence of a highly conserved 75-amino acid region termed the J domain, which is the predominant site of interaction with Hsp70 partners (8).

The main function of Hsp40 co-chaperones appears to be stimulation of Hsp 70 chaperones (9–12). Given their importance for Hsp70 function, Hsp40 molecules are involved in nearly all aspects of protein synthesis and secretion (13, 14). In addition to their role in stimulating ATP hydrolysis by Hsp70, some Hsp40 chaperones are felt to have an intrinsic ability to bind and fold at least some misfolded proteins (15, 16).

During post-translational protein translocation into the yeast ER, Bip interacts with Sec63, a membrane-anchored protein bearing a J domain (17–21). It is assumed that Sec63, which forms part of the translocation apparatus, attaches Bip to the site of protein import via its J domain. Upon activation by Sec63, Bip binds nascent peptides and assists in protein import either by serving as an anchor during passive forward movement (a “Brownian ratchet”) (22), by actively pulling nascent molecules into the ER lumen (23), or perhaps by both mechanisms. Mutations in either Bip or Sec63 result in defects in protein translocation into the ER (24). As mentioned previously, it is known that Bip also participates in refolding misfolded proteins once they reach the ER lumen and assists in reverse translocation of those that remain misfolded (25, 26).

Recently it has become evident that the functions of Bip in import and export are distinct (25), perhaps suggesting that Bip interacts with Hsp40 co-chaperones other than Sec63 in reverse translocation. Similarly, Bip likely interacts with another (or several other) Hsp40 molecules to enable protein folding within the lumen. Indeed, in S. cerevisiae, two additional Hsp40 co-chaperones have thus far been identified in the ER and found to interact with Bip in protein folding (27–30). In contrast, no Hsp40 molecules have yet been identified in the ER of higher eucaryotes, including mammalian cells (28, 29).

We describe here the expression pattern and intracellular localization of a novel Hsp40 chaperone, which we have termed HEDJ (for human ER-associated DNAJ; the nomenclature follows that of yeast and human cytoplasmic Hsp40 molecules). HEDJ was found to be expressed in all human tissues examined. It was localized to the endoplasmic reticulum with a luminal orientation, was membrane-associated, and was able to bind and stimulate the activity of Bip. Highly homologous

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1 The abbreviations used are: ER, endoplasmic reticulum; HA, hemagglutinin; GST, glutathione S-transferase; Endo H, endoglycosidase H; PNGase F, protein N-glycosidase F; DOC, deoxycholate; EST, expressed sequence tag; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

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genes were found in mouse, Drosophila, and Caenorhabditis elegans data bases.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Antibodies, and Reagents—**Vero cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Transfections were performed as described (31). Approximately 5 × 10^6 cells were transfected with a mixture of plasmid DNA (3 μg) and LipofectAMINE (50 μl; Life Technologies, Inc.) in serum-free medium. 24 h later, medium was added. Experiments on transiently transfected cells were performed 48 h after transfection. For stable transfection, gene-ticin (1000 μg/ml; G418, Life Technologies, Inc.) was added to culture medium 48 h after transfection, and the cells were continually passaged in the presence of genetin at 1000 μg/ml. Monoclonal anti-V5 antibody was obtained from Invitrogen. Monoclonal anti-HA antibody was obtained from Babco. Affinity purified polyclonal anti-calreulin and anti-Bip were obtained from StressGen. Human multiple tissue RNA blots were purchased from CLONTECH. Double-stranded DNA probes were radiolabeled with [32P]dCTP (3000 Ci/mmol) by random priming using reagents from Roche Molecular Biochemicals. Taq DNA polymerase and dNTPs were from Life Technologies, Inc.

**cDNA cloning—**A portion of the HEDJ cDNA was isolated in a genetic screen for molecules involved in intracellular trafficking of shiga toxin, which will be described in detail elsewhere. In brief, Vero cells were transiently transfected with a cDNA expression library and exposed to shiga toxin. Resistant cells were enriched, and plasmid DNA was harvested by Hirt extraction and then used in a further two rounds of transfection and enrichment. After the third round of transient transfection and enrichment, cells were stably transfected with the enriched cDNA clones and selected in the presence of shiga toxin for two weeks. The cDNA inserts in toxin-resistant cells was amplified by polymerase chain reaction from cell lysates using primers flanking cDNA inserts. The resulting polymerase chain reaction products were directly cloned into plasmid pCR3.1Topo/V5/His (Invitrogen). Two transformants characterized contained identical inserts of approxi-mately 1.1 kb and demonstrated homology to the Hsp40 family of co-chaperones. Although no matching sequences were identified in the GenBank™ data base, several identical sequences were identified in the human EST data base. Successive searches using BLAST against the human EST data base allowed identification of the entire coding region.

**Northern Blotting of Human RNA to Detect HEDJ Expression—**The insert from plasmid pHED3 was released by digestion with HindIII and XhoI, gel purified, labeled with [32P]dCTP, and used as probe for Northern blots. 50 ng of purified β-actin DNA (CLONTECH) was similarly labeled. Human multiple tissue RNA blots were prehybridized for 30 min at 68 °C in RapidHyb solution (CLONTECH) and then hybridized for 1 h at 68 °C in the same solution containing 1.5 million cpm/ml of probe. Blots were washed at 50 °C in 2× SSC, 0.1% SDS and exposed to Kodak Biomax MS film at −70 °C.

**Generation of Epitope-tagged HEDJ cDNA—**A full-length, V5 epitope-tagged molecule was generated by amplifying the HEDJ cDNA from a human skeletal muscle library (CLONTECH) using primers Hsp-1 (5′-GGCTCTACAGGGCGGTGGGCTG-3′) and Hsp-3 (5′-ATAATTTTCTTGGTTTGAACTTCTC-3′), which deleted the stop codon. The resulting product was ligated into plasmid pCR3.1Topo/V5/His to create plasmid pHED5, which contains the full-length HEDJ, an in-frame 3′ fusion with the V5 epitope and a C-terminal histidine tag. The insert was sequenced to ensure fidelity. A truncated, HA epitope-tagged HEDJ was constructed by amplifying the HEDJ cDNA with primers Hsp-1 and Hsp-5′ (5′-CTAGCTTGAGGATGCTCGAAGCAGTCAGACCTTACACAGAAGTCTC-3′). The resulting product, which encodes amino acid residues 1–81 of the HEDJ cDNA, a C-terminal HA epitope (GGYPYDVPDYASG), and a stop codon, was ligated into vector pCR3.1Topo/V5/His to create plasmid pHED-HA. The insert was sequenced to verify that errors had not been introduced by polymerase chain reaction.

**Confocal Microscopy—**Vero cells were transiently transfected with pPHED3 (HA epitope-tagged HEDJ) or pHED-HA (truncated, HA epitope-tagged HEDJ). Transfected cells were seeded in multwell chambers on a glass slide, fixed with 2% paraformaldehyde at 4 °C for 1 h, permeabilized by the addition of 0.1% Triton X-100 in PBS for 5 min at room temperature, and then incubated for 1 h at room temperature with Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum plus 2% BSA (blocking buffer). Primary antibodies used were mouse anti-V5 or anti-HA at 1:200 dilution in blocking buffer. Double labeling experiments included goat anti-calnexin (DPP-23) at 1:200 dilution for 1 h at room temperature. After washing, Oregon yellow-conjugated anti-mouse (Molecular Probes, Eugene, OR) or Texas Red-conjugated anti-goat (Sigma) secondary antibody was added at 1:200 dilution in blocking buffer and incubated a further 1 h at room tempera-ture. Slides were washed with PBS, mounted with Aqui PolyMount (Polysciences, Warrington, PA), and visualized by epifluoresence confocal microscopy (Bio-Rad). Image processing was performed with the LaserSharp 1024 software package.

**Proteinase K Digestion, Endoglycosidase Treatment, and Solubiliza-tion of HEDJ—**To isolate crude microsomal extracts pHED3 transfectants were homogenized in 0.5 ml of PBS, centrifuged, resuspended in 100 μl MES buffer, pH 6.7, and then disrupted by 10 strokes in a tightly fitting glass Dounce homogenizer. Nuclei and cellular debris were removed by centrifugation at 4000 × g. Microsomes were precipitated from the supernatant by the addition of MnCl2 to 20 mM and incubation on ice for 30 min. Microsomes were sedimented for 5 min at 4000 × g and resuspended in the appropriate buffer for later experiments.

**Protease treatment—**was performed by adding proteinase K to a concentration of 100 μg/ml to microsomes resuspended in PBS either in the presence or absence of Triton X-100 at 1% final concentration. After incubating at 4 °C for 1 h, SDS-PAGE buffer was added, and the samples were boiled, electrophoresed on a 4–20% polyacrylamide gradient gel, and transferred to nitrocellulose. After blocking in 5% skim milk in PBS, blots were incubated in the presence of antibody against V5 (1:2500) or calreulin (1:500), respectively, and incubating for 2 h at room temperature. After washing and adding secondary antibody (horseradish peroxidase-conjugated anti-mouse or anti-goat; 1:5000) for 1 h, filters were washed, immersed in ECL reagents (Amersham Pharmacia Biotech), and exposed to film.

**Glycosidase treatment was performed by resuspending microsomes to a final volume of 40 μl in water containing protease inhibitors (complete mixture; Roche Molecular Biochemicals). 40 μl of denaturation buffer (0.5% SDS, 1% β-mercaptoethanol) was added, and the samples were placed in a boiling water bath for 10 min and then divided into three aliquots. To these were added either Endo H (20 milliunits; Roche Molecular Biochemicals), PNGase F (100 units; New England Biolabs), or no glycosidase. The appropriate buffer was added, the sample volume was brought to 80 μl with water, and the samples were in-cubated at 37 °C. Aliquots were removed after 1 or 2 h and subjected to electrophoresis on a 10% polyacrylamide gel followed by Western blotting as described above.

**Solubilization of HEDJ was performed by preparing microsomes from pHED3 transfected cells. After freezing at −20 °C, the microsomes were resuspended in either water or PBS containing Triton X-100, 1% Triton X-100, or 1% deoxycholate (DOC). After rocking for 4 °C for 1 h the samples were centrifuged at 4000 × g for 5 min to sediment residual membranes and microsomes. The supernatant was removed to a new tube, and the pellet was resuspended in an equal volume of water. SDS-PAGE sample buffer was added, and the products were subjected to electrophoresis and Western blotting as described above.

**J Domain-GST Fusion and Purification of GST Fusion Protein—**The J domain and a segment of the G/F linker region (residues 18–120) were amplified from plasmid pHED3 transfected cells. After freezing at −20 °C, the microsomes were resuspended in either water or PBS containing Triton X-100, 1% Triton X-100, or 1% deoxycholate (DOC). After rocking for 4 °C for 1 h the samples were centrifuged at 4000 × g for 5 min to sediment residual membranes and microsomes. The supernatant was removed to a new tube, and the pellet was resuspended in an equal volume of water. SDS-PAGE sample buffer was added, and the products were subjected to electrophoresis and Western blotting as described above.

**Generation and Purification of His-tagged Bip—**The Bip cDNA, excluding the signal peptide, was amplified from a Daudi (human Burkitt lymphoma) cell line by polymerase chain reaction using primers HED-10 (5′-CCAGGACTCCTGAGGGCGGCAGGAGGAAGC-3′) and HED-11 (5′-GGACCACCTCGAGCTCACTAATGTTTPCTGC-3′). The product was

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ligated into vector pCR3.1-Topo/V5/His, released by XhoI digestion and subcloned into XhoI-digested plasmid pET-14b (Novagene), which placed Bip in-frame with an N-terminal His 6 tag to create plasmid pET-Bip. Subsequently, nucleotide sequencing revealed a nucleotide substitution in the Bip cDNA, which resulted in substitution of proline for leucine at amino acid position 604. Nevertheless, we found that purified Bip bound the HEDJ J domain in an ATP-dependant fashion (see “Results”), indicating that the amino acid substitution did not interfere with the Bip-J domain interaction.

His-tagged Bip was purified from a 1-liter culture of E. coli BL21 (DE3) harboring pET-Bip by growing to an A600 value of 0.8 and then inducing protein expression by the addition of isopropyl-1-thio-b-D-galactopyranoside to 1 mM and incubating a further 2 h. Bacteria were sedimented, washed with PBS, frozen at -220 °C, and then resuspended in PBS containing 1% Triton X-100. The cells were sonicated, debris was removed by centrifugation, and the supernatant was applied to a 1-ml Hi-Trap column (Amersham Pharmacia Biotech). After washing with 10 ml of PBS, proteins were eluted with a linear gradient of imidazole in PBS. Fractions containing Bip were identified by slot-blotting, pooled, and dialyzed against 20 mM Tris, pH 7.0.

HEDJ-Bip Binding Assays—Binding assays were performed as described by Corsi and Schekman (17) with modifications as follows. 50 µg of purified GST-HEDJ or GST were added to 50 µl of glutathione agarose (Amersham Pharmacia Biotech) in a final volume of 500 µl of binding buffer. After 1 h at 4 °C the beads were sedimented, washed several times with ice-cold binding buffer, and then resuspended in 500 µl of binding buffer. Purified Bip (50 µg) was added to each sample in the presence or absence of 1 mM ATP. Following a 2-h incubation while rotating at 4 °C, the beads were sedimented and washed five times with 1 ml of binding buffer. GST-HEDJ, GST, and associated Bip were released by the addition of 100 µl of binding buffer containing 20 mM reduced glutathione. Laemmli buffer was added to the supernatant, and the samples were boiled and subjected to SDS-PAGE. Proteins were detected by Coomassie staining.

ATPase Assays—to determine whether the HEDJ J domain was capable of stimulating the ATPase activity of Bip, in vitro assays were performed using GST fusion proteins and purified recombinant Bip. GST-HEDJ, GST, and recombinant Bip were purified as described above. Each protein was dialyzed overnight into ATPase assay buffer (50 mM HEPES, pH 6.8, 50 mM NaCl, 2 mM MgCl2). Assays were performed essentially as described (17), in a final volume of 100 µl consisting of ATPase buffer, to which was added KCl to 20 mM, 0.5 mCi of [γ-32P]ATP, and 100 µM ATP. Reactions contained 1.0 µg of Bip (140 nM), and either no addition or 2.5 µg of GST or GST-HEDJ. Comparison was made with a reaction containing neither Bip or a GST containing protein. At each time point between 0 and 75 min, which was in the linear range, 5 µl was removed and spotted on a plastic backed polyethyleneimine-cellulose thin layer plate (Sigma). Radioactive ADP was visualized by autoradiography.
was separated from ATP by chromatography in 0.5 M formic acid in 0.5 M LiCl. The plates were exposed for 1 h to x-ray film to identify the Rf and location of ADP. Individual spots were cut from the TLC plate, and the amount of radioactive ADP was determined in a scintillation counter.

To demonstrate saturability of the ATPase stimulation of Bip, ATPase assays were repeated with varying concentrations of either GST-tHEDJ or GST in the presence or absence of Bip (0.5 μg; 70 nM). Reactions were incubated for 60 min at room temperature, and the quantity of ADP released was determined by TLC and scintillation counting as described above. The V_max was determined by correcting for the specific activity of [32P]ATP (220 cpm/pmol) and the final concentration of GST-tHEDJ in each reaction and then performing nonlinear regression analysis of the data using GraphPad Prism (version 2.0).

RESULTS

A partial Vero cell cDNA was isolated in the process of a phenotypic cloning approach to identify genes involved in shiga toxin trafficking. When compared by BLAST with the GenBank data base there were no sequences that matched with high probability. However, when compared with the human EST data base, several matching sequences were identified. When the nucleotide sequence was translated into predicted peptide sequence using the first ATG as the start codon and compared with the SwissProt data base, at least 50 matching sequences were identified, all belonging to the DNAJ or Hsp40 family of co-chaperones. The role of this chaperone in shiga toxin pathogenesis is currently under study.

Iterative BLAST searches were performed against the human EST data base to identify the entire HEDJ coding region and predicted stop codon. Based on the deduced cDNA sequence, primers were designed to allow amplification of approximately 150 base pairs of 5' untranslated DNA and the entire coding region of HEDJ from human skeletal muscle cDNA. Nucleotide sequencing of the insert demonstrated that the HEDJ sequence matched that predicted from EST sequences (Fig. 1A). The cDNA encodes a peptide consisting of 358 amino acids, which demonstrate high homology to the Hsp40 (DNAJ) co-chaperones. Blast and tblastx searches were performed against the mouse EST, C. elegans, and Drosophila data bases. Highly homologous sequences were found in each (Fig. 1B). No Saccharomyces sequences matched with high
probability. Like other Hsp40 chaperones, HEDJ contained a highly conserved region of 75 amino acids known as the J domain, which interact with Hsp70 partners to stimulate ATPase and folding activity. Additionally, HEDJ contained domains shared by many other Hsp40 molecules such as a glycine/phenylalanine-rich putative linker region, a cystine-rich domain, and a C-terminal domain, which in other Hsp40 molecules has been postulated to directly bind misfolded peptides (Fig. 1C). Unlike essentially all other known Hsp40 genes in higher eucaryotes, HEDJ contained a predicted signal peptide at the N terminus, suggesting that it is likely translocated into the endoplasmic reticulum.

To determine the pattern of HEDJ expression in human tissues, RNA hybridization experiments were performed using a radiolabeled full-length HEDJ as probe against poly(A) RNA isolated from various tissues. Hybridizing transcripts of 2.2 and 2.0 kilobases were found in all tissues examined, with relatively highest HEDJ expression found in the pancreas and testis and the least hybridization to RNA from thymus and small intestine (Fig. 2). The two bands likely represent alternately spliced HEDJ message, although the possibility of a highly related, cross-hybridizing message cannot be excluded. In all cases, hybridization with a β-actin probe confirmed that HEDJ was localized to the endoplasmic reticulum.

As described previously, Hsp40 chaperones are involved in nearly all aspects of protein folding, secretion, and degradation. These activities have been intensively studied in yeast, where three Hsp40-like proteins have been identified within the ER; each apparently with overlapping but distinct activities. In contrast, in higher eucaryotes, HEDJ contained a predicted signal peptide protruded into the cytoplasm, where it might interact with Bip (or an as yet uncharacterized Hsp70), and then fluorescein isothiocyanate-conjugated secondary antibody. In each case, cells were also labeled with antibody against calnexin and Texas Red-conjugated secondary antibody. After mounting, cells were visualized by confocal microscopy, as described in the text.

Transfection efficiency of approximately 15% and demonstrate that V5-tagged HEDJ is expressed in a similar distribution as calregulin. Control experiments were performed by omitting primary antibody or by substituting cells transfected with vector alone and demonstrated minimal background labeling (data not shown). We have been unable to raise antiserum in rabbits either to full-length recombinant HEDJ or to peptide antigens, presumably because of the extremely high conservation of the HEDJ protein (Fig. 1C). Thus, we were unable to examine the intracellular localization of HEDJ that did not bear an epitope tag. To confirm that the ER localization seen with the V5 epitope-tagged protein was not an artifact of the V5 epitope and to determine whether the signal peptide and J domain alone would be sufficient to target the protein to the endoplasmic reticulum, confocal microscopy was repeated with a truncated, HA-tagged HEDJ cDNA. Transfected cells were subjected to confocal microscopy as described above, except that mouse anti-HA antibody was substituted for anti-V5. As was seen with the full-length V5-tagged molecule, HA-tagged truncated HEDJ was found to co-localize with calregulin. These results confirmed that HEDJ was localized to the endoplasmic reticulum irrespective of the epitope tag.

We next sought to determine the orientation of HEDJ with respect to the ER lumen. Although we anticipated that HEDJ would be localized within the lumen where it would be available to interact with Bip (or an as yet uncharacterized Hsp70), it was conceivable that HEDJ was oriented such that the peptide protruded into the cytoplasm, where it might interact with
cytosolic Hsp70 molecules such as Hsc70. For example, the yeast DNAJ homologue, Ydj1p, in addition to having a cytoplasmic localization, is associated with the outer surface of the ER membrane (32).

As one means of examining orientation of HEDJ within the ER, protease susceptibility assays were performed. Microsomes were prepared from cells transfected with full-length, epitope-tagged HEDJ and exposed to proteinase K either in the presence or absence of Triton X-100. Luminally oriented proteins were expected to be protected from proteinase K, because this protease cannot cross the microsomal membrane. Addition of Triton X-100 was expected to disrupt the membrane and allow access of the protease to intraluminal proteins. We found that in the absence of Triton X-100, HEDJ was protected from proteinase K, as demonstrated by no decrease in the amount of full-length protein after a 1-h incubation with proteinase K, as compared with untreated microsomes (Fig. 4, first and third lanes). In contrast, addition of 1% Triton X-100 allowed complete degradation of HEDJ. Control experiments demonstrated similar results with other luminal ER proteins such as calregulin (Fig. 4), Bip, and calnexin (not shown). These results indicate that HEDJ is localized within the lumen of the endoplasmic reticulum.

Another means of determining the orientation and localization of proteins within the secretory pathway is to examine patterns of glycosylation. The presence of asparagine-linked carbohydrate indicates transit to the endoplasmic reticulum lumen, where high mannose-type sugars are transferred from dolichol-phosphate to nascent proteins, provided they bear at least one potential glycosylation site. High mannose carbohydrates are characterized by susceptibility to cleavage by Endo H. During transit from the ER through the Golgi, N-linked carbohydrates undergo trimming of glucose residues and further modification, which result in resistance to cleavage by Endo H. Notably, these N-linked sugars, like high mannose carbohydrate, remain susceptible to cleavage by PNGase F. HEDJ bears two potential N-linked glycosylation sites at residues 5 and 261. However, the first site is located within the putative signal peptide and transmembrane domain, likely indicating that only the second site is accessible for modification.

The glycosylation pattern of HEDJ was examined in transfected cells by subjecting microsomes from pHED3-transfected cells to digestion either with Endo H, PNGase F, or no glycosidase and examining the migration of HEDJ on SDS-PAGE gels by Western blotting. After 1 h of incubation with either glycanase, the molecular mass was reduced by approximately 2 kDa in approximately half of the HEDJ protein. After 2 h of incubation, all of the HEDJ was reduced in apparent molecular mass by Endo H and PNGase F, as compared with sample incubated in the absence of glycanase (Fig. 5). Glycosylation of HEDJ confirmed that the protein was luminally oriented. Moreover, demonstration that essentially all of the N-linked sugar is Endo H-susceptible indicated that HEDJ remained resident within the ER.

We next sought to determine whether HEDJ was free within the ER lumen or was membrane-associated. To determine whether HEDJ was membrane-associated, microsomes were isolated from cells transfected with pHED3 and were subjected to extraction with PBS alone, 0.1% Triton X-100, 1% Triton X-100, or 1% DOC. As expected, calreticulin, a soluble ER luminal protein, was released into the supernatant by 0.1% and 1% Triton X-100 as well as 1% DOC. In fact, freezing and thawing the microsomes without addition of detergent resulted in release of approximately half the calregulin. In contrast, essentially all HEDJ remained in the pellet following 0.1% Triton X-100 extraction. Even exposure to 1% Triton X-100, which was expected to solubilize most of the microsomal membrane, solubilized only a minimal amount of HEDJ. Only the stronger detergent, 1% DOC, was able to solubilize HEDJ, suggesting a strong association with the ER membrane (Fig. 6). Having demonstrated that HEDJ was localized to the ER and luminally oriented, we examined the possibility that this novel Hsp40 interacts with Bip, the only Hsp70 co-chaperone thus far known to be localized to the ER of higher eucaryotes. To determine whether the J domain of HEDJ interacts with Bip, in vitro binding assays were performed using GST fusion proteins, as described by Corsi and Schekman (17), who showed
that the J domain of Sec63 interacted with Bip in an ATP-dependent manner. We found, in the presence of 1 mM ATP, Bip bound to a GST-HEDJ fusion protein containing the HEDJ J domain. In contrast, the amount of Bip bound to GST-HEDJ in the absence of ATP was undetectable by Coomassie staining (Fig. 7). As expected, Bip did not bind the GST control, either in the presence or absence of ATP.

Finally, to determine whether the HEDJ J domain was capable of stimulating the ATPase activity of Bip, in vitro ATPase assays were performed. Reactions contained either no additions or Bip plus GST, GST-tHEDJ, or no GST protein. In the absence of any additions there was minimal ATP hydrolysis. In these experiments, 1 μg of Bip was added to a final concentration of approximately 140 nM. Reactions containing Bip alone demonstrated ATP hydrolysis at a rate of approximately 1.6 mol ADP/mol Bip/min (Fig. 8). Addition of GST had no effect on the rate of ATP hydrolysis, whereas addition of GST-HEDJ resulted in a specific activity of approximately 1.6 mol ADP/mol Bip/min (Fig. 8). GST addition had minimal ability to stimulate ATP hydrolysis, whereas addition of GST-tHEDJ demonstrated that, unlike all other Hsp40 proteins identified among higher eucaryotes, HEDJ possesses a novel human Hsp40 chaperone, HEDJ. Sequence analysis of the cloned HEDJ demonstrated that, unlike all other Hsp40 molecules identified among higher eucaryotes, HEDJ possesses a predicted signal peptide, suggesting secretion into the endoplasmic reticulum. Confocal microscopy demonstrated that recombinant HEDJ bearing either a V5 or HA epitope tag, was indeed co-localized with calnexin to the endoplasmic reticulum. Moreover, essentially all the HEDJ isolated from microsomal extracts was found to be glycosylated, confirming transit to the endoplasmic reticulum. Protease resistance and asparagine-linked glycosylation indicated that the molecule was oriented toward the ER lumen. Finally, extraction of microsomes under various conditions revealed that, unlike calregulin, HEDJ remained insoluble even in the presence of Triton X-100, suggesting a strong association with the ER membrane.

Query of nucleotide and protein data bases revealed highly homologous sequences in mouse, Drosophila, and C. elegans data bases. Interestingly, the yeast S. cerevisiae genome does not contain an HEDJ homologue, perhaps suggesting that HEDJ performs the same function as either Sj1p or Jem1p, two luminal J domain containing molecules found in the yeast ER. Of these two yeast proteins, Sj1 is most closely related to DNAJ and HEDJ. Thus, Bip and Bip may have related functions in the mammalian and yeast endoplasmic reticulum, respectively. Interestingly, Sj1p is known to interact with Bip and is suggested to be the primary co-chaperone for Bip under normal physiological conditions (28).

Chaperone-assisted protein folding occurs in the cytosol and in intracellular organelles such as endoplasmic reticulum, mitochondria, and nucleus. At least four Hsp70 chaperones, localized to specific intracellular compartments, are described in S. cerevisiae. Each Hsp70 interacts with a specific Hsp40 partner. Interestingly, an Hsp70 from one intracellular compartment may interact with more than one Hsp40 in the same compartment (such as endoplasmic reticulum) and yet cannot be stimulated by DNAJ co-chaperones from other cellular compartments, indicating a high degree of specificity in the interaction between Hsp70 molecules and their Hsp40 partners (10, 27). Because Bip is the only Hsp70 identified thus far in mammalian cells, it is likely that HEDJ serves to activate the ATPase activity of Bip during protein translocation, intraluminal folding, or reverse translocation. However, it is possible that another as yet unidentified Hsp70 exists in the endoplasmic reticulum from microsomal extracts. Microsomes were prepared from 1 × 10⁶ HEDJ-3 transfected cells and resuspended in 400 μl of PBS. After freezing and thawing, four samples were aliquoted, to which were added either no detergent (None), Triton X-100 to 0.1% (0.1% TX-100) or 1% (1% TX-100), or deoxycholate to 1% (DOC), and the final volume was brought to 100 μl with PBS. After incubating 30 min at 4 °C, the samples were centrifuged at 10,000 × g, the supernatant was removed, 100 μl of PBS was added to the pellet, and 20 ml of each sample was subjected to SDS-PAGE and Western blotting with antibodies against either V5 (HEDJ) or calregulin. P, pellet; S, supernatant.

**Fig. 6.** Detergent solubilization of HEDJ from microsomal extracts. Microsomes were prepared from 1 × 10⁶ HEDJ-3 transfected cells and resuspended in 400 μl of PBS. After freezing and thawing, four samples were aliquoted, to which were added either no detergent (None), Triton X-100 to 0.1% (0.1% TX-100) or 1% (1% TX-100), or deoxycholate to 1% (DOC), and the final volume was brought to 100 μl with PBS. After incubating 30 min at 4 °C, the samples were centrifuged at 10,000 × g, the supernatant was removed, 100 μl of PBS was added to the pellet, and 20 ml of each sample was subjected to SDS-PAGE and Western blotting with antibodies against either V5 (HEDJ) or calregulin. P, pellet; S, supernatant.

**Fig. 7.** In vitro interaction of GST-HEDJ J domain with Bip. 50 μg of GST-HEDJ or GST were immobilized on glutathione agarose in binding buffer. After washing, 50 μg of purified Bip was added to each sample in the presence (+) or absence (−) of 1 mM ATP. After incubating at 4 °C for 2 h, the beads were washed several times with binding buffer. Bound proteins were released by the addition of 20 mM glutathione in binding buffer, and the supernatant was removed and subjected to SDS-PAGE. Proteins in the eluted samples were detected by Coomassie staining.
mic reticulum of mammalian cells. The in vitro assays performed here demonstrate that the HEDJ J domain interacts with Bip in an ATP-dependent manner, suggesting that Bip might be the natural in vivo partner for HEDJ. We cannot exclude the possibility, however, that other Hsp70 chaperones exist in the ER lumen that have higher affinity for HEDJ than does Bip.

Localization of HEDJ to the luminal surface of the ER membrane suggests that this novel Hsp40 co-chaperone is well situated to interact with Bip (or another Hsp70) in the retrograde transport of host proteins across the Sec61 apparatus. The components of this retrograde pathway are largely unknown in mammalian cells. However, reverse translocation has been investigated intensely in yeast, where it is known that the yeast Bip homologue and other chaperones are involved in ER to cytosol transport (20). Much of the mechanism of retrograde transport, however, has yet to be elucidated. The process is under intense scrutiny because the pathogenesis of many diseases such as cystic fibrosis, α1-antitrypsin deficiency, prion-associated disorders, and Alzheimer’s disease all involve misfolding of mutated proteins and abnormalities of their processing in the endoplasmic reticulum (5). These misfolded proteins may accumulate in the endoplasmic reticulum or be transported to the cytosol for proteosomal degradation. Moreover, trafficking of some bacterial toxins within eucaryotic cells may utilize the reverse translocation apparatus (33, 34). It has therefore been suggested that the ability to therapeutically modulate ER to cytosol transport may influence the severity of some of these diseases. Investigations on the role of HEDJ in folding and translocation of misfolded proteins and bacterial toxins may provide insight into normal cellular biology and the pathogenesis of diverse human diseases.

Addendum—Bies et al. (35) recently described the purification of an Hsp40 co-chaperone from dog pancreas microsomes. Peptide sequence analysis indicated that this protein, which Bies et al. have named ERj3p, is a homologue of the yeast Hsp40 Scj1p and is the canine homologue of the chaperone we describe here, HEDJ.
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