BAP, a Mammalian BiP-associated Protein, Is a Nucleotide Exchange Factor That Regulates the ATPase Activity of BiP*

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We identified a mammalian BiP-associated protein, BAP, using a yeast two-hybrid screen that shared low homology with yeast Sls1p/Sil1p and mammalian HspBP1, both of which regulate the ATPase activity of their Hsp70 partner. BAP encoded an ∼54-kDa protein with an N-terminal endoplasmic reticulum (ER) targeting sequence, two sites of N-linked glycosylation, and a C-terminal ER retention sequence. Immunofluorescence staining demonstrated that BAP co-localized with GRP94 in the endoplasmic reticulum. BAP was ubiquitously expressed but showed the highest levels of expression in secretory organ tissues, a pattern similar to that observed with BiP. BAP binding was affected by the conformation of the ATPase domain of BiP based on in vivo binding studies with BiP mutants. BAP stimulated the ATPase activity of BiP when added alone or together with the ER DnaJ protein, ERdj4, by promoting the release of ADP from BiP. Together, these data demonstrate that BAP serves as a nucleotide exchange factor for BiP and provide insights into the mechanisms that control protein folding in the mammalian ER.

The Hsp70 family of molecular chaperones are highly homologous and consist of two distinct domains: a highly conserved N-terminal ATPase domain and a less conserved C-terminal polypeptide-binding domain (1). The chaperone activity of Hsp70 proteins is controlled by the ATPase domain that undergoes a reaction cycle comprised of ATP binding, hydrolysis, and nucleotide exchange, which is regulated by co-chaperones and co-factors. In bacteria, DnaJ accelerates ATP hydrolysis, whereas GrpE promotes nucleotide exchange of ADP to ATP (2, 3). The mammalian cytosolic Hsc70 is similarly regulated by Hsp40, a homologue of DnaJ (4), and a number of both positive and negative regulators of nucleotide exchange have been identified. Bag-1 can stimulate the ATPase activity of Hsc70, presumably by facilitating nucleotide exchange, although the precise function of Bag-1 is still somewhat controversial (5–7). A negative regulator of the ATPase activity of Hsc70, HspBP1, has been identified (8). Additional Hsc70-interacting proteins such as Hip (9), Hop (10), 11, and CHIP (12) have been identified that further contribute to the regulation of the Hsc70 ATPase cycle and, as such, serve to control the chaperone function of Hsc70.

BiP (also known as GRP78) is a mammalian endoplasmic reticulum (ER) homologue of the Hsp70 family. The ER is the site of synthesis, folding, and assembly of secretory pathway proteins that include resident proteins of the endocytic and exocytic organelles, as well as surface and secreted proteins. 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 eventually targeted for ER-associated degradation through the action of the chaperones (13, 14).

BiP was first identified bound to nonsecreted free heavy chains in pre-B lymphomas (15) and was consequently shown to interact with a number of other secretory pathway proteins to prevent their premature transport from the ER and to promote their proper folding and assembly (16). As such, BiP was the first ER chaperone and component of the ER quality control apparatus to be identified. In addition, BiP plays an essential role in maintaining the permeability barrier of the ER translocon during early stages of protein translocation (17), targeting misfolded proteins for proteasomal degradation (18), serving as a sensor for ER stress (19, 20), and contributing to ER calcium stores. The ATPase activity of BiP is required for at least some of these roles; thus, it is reasonable to speculate that the ATPase activity of BiP should be regulated in a way similar to that of other members of the Hsp70 family.

A total of four mammalian ER DnaJ homologues have been identified (21–25), and it has been proposed that they be referred to as ERdj1–4. In vitro biochemical studies show that the J domains of ERdj1, ERdj3, and ERdj4 can stimulate the ATPase activity of BiP (24–26), and both ERdj3 and 4 can bind to BiP in vivo (25).2 Recently, a yeast ER protein (Sls1p/Sil1) was isolated from two different genera that interacts with the ATPase domain of Kar2p, the yeast homologue of BiP (27, 28). Defects in the SLS1 gene are not lethal but affect protein translocation into the ER, especially associated with LH51 mutations. Sls1p is proposed to be a GrpE-like protein based on its preference for the ADP-bound conformation of Kar2p and its ability to enhance the ATPase activity of Kar2p in the presence of the J domain of Sec63p, a yeast ER transmembrane DnaJ homologue. However, nucleotide exchange activity for Sls1p has not been directly demonstrated. The existence of potential mammalian and invertebrate homologues of Sls1p was reported (28), but no data are available on their activity.

We attempted to identify potential mammalian regulators of the ATPase activity of BiP using the ATPase domain of a BiP

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2 The abbreviations used are: ER, endoplasmic reticulum; EST, expressed sequence tag; HA, hemagglutinin; TRITC, tetramethylrhodamine isothiocyanate; Endo H, endoglycosidase H; DSP, dithiothreitol/succinimidyl propionate.

3 K. T. Chung, Y. Shen, and L. M. Hendershot, unpublished data.
mutant as the bait protein in a yeast two-hybrid screen. A BAP-interactive protein was identified by screening a human liver cDNA library. We designated the protein, which bears low sequence homology with both yeast ER Sis1p, a positive regulator of the ATPase activity of Kar2p, and mammalian cytosolic HspBP1, a negative regulator of the ATPase activity of Hsc70, as BAP for BIP-associated protein. In this study, we demonstrate that BAP is a resident ER luminal protein that interacts with the ATPase domain of BIP and that BAP binding is affected by nucleotide-induced conformational changes in the ATPase domain of BIP. In vitro assays demonstrated that BAP functioned as a nucleotide exchanger for BiP and consequently enhanced the positive effect of ERdj4 on the ATPase activity of BiP. Thus, BAP represents the first mammalian ER nucleotide exchange factor for BiP and reveals a conservation in the regulatory machinery for the ATPase activity of the Hsp70 chaperones found in other eukaryotic cellular compartments and in bacteria.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screen and cDNA Cloning**—The procedure for yeast two-hybrid screening was performed as published (29). DNA encoding the ATPase domain of the human BiP, which contains the ER targeting signal sequence (30, 31), was cloned into the Ndel and BumHI sites of the pSA1 vector. The resulting construct, pAS1(T229G) was transformed into the yeast HF7c strain. Trp' transformants were isolated and subsequently transformed with a human liver cDNA library that contained ~5.5 × 10^6 independent clones in the LEU2 marker plasmid pCT2 (Clontech Laboratory Inc., Palo Alto, CA). Transformants were plated on SD-Trp-Leu-His medium containing 20 mM 3-aminoantizole, and colonies that grew on this medium were assayed for β-galactosidase activity. Target plasmids from the positive colonies were isolated, sequenced, and used to search for homologous proteins in the data base. Four independent clones of varying lengths of mouse full-length cDNA were obtained by searching the EST database that contained 3.5 × 10^4 ESTs. The sequence of the inserted cDNA of the EST was verified by DNA sequencing. An EST clone encoding the complete cDNA of the positive target gene (identification number 5102998) from Incyte Genomics Systems Inc. was transformed into the yeast HF7c strain. Trp' and His' colonies were isolated, sequenced, and used to search for homologous proteins in the data base. Four independent clones of varying lengths of mouse full-length cDNA were obtained by searching the EST database that contained 3.5 × 10^4 ESTs. The sequence of the inserted cDNA of the EST was verified by DNA sequencing.

**Vectors**—The BAP-HA tagged construct was produced by inserting full-length BAP without a stop codon into the EcoRI and Xhol sites of the 3HA DSL vector (a kind gift from Dr. Daesik Lim of St. Jude Children's Research Hospital). The resulting construct, pBSLBP-HA-3HA, was transformed into the yeast HF7c strain. Trp' transformants were isolated and subsequently transformed with a human liver cDNA library that contained ~5.5 × 10^6 independent clones in the LEU2 marker plasmid pCT2 (Clontech Laboratory Inc., Palo Alto, CA). Transformants were plated on SD-Trp-Leu-His medium containing 20 mM 3-aminoantizole, and colonies that grew on this medium were assayed for β-galactosidase activity. Target plasmids from the positive colonies were isolated, sequenced, and used to search for homologous proteins in the data base. Four independent clones of varying lengths of mouse full-length cDNA were obtained by searching the EST database that contained 3.5 × 10^4 ESTs. The sequence of the inserted cDNA of the EST was verified by DNA sequencing.

**Expression of Recombinant BAP and Antibody Production**—The sequence encoding BAP without the ER targeting signal was cloned into the pcDNA3.0 vector and the ATPase domain of BIP was confirmed by back-transforming yeast with target and bait DNAs. We obtained an EST clone forming yeast with target and bait DNAs. We obtained four independent clones of varying lengths of mouse ESTs. Four independent clones of varying lengths of mouse ESTs were isolated, sequenced, and used to search for homologous proteins in the data base. Four independent clones of varying lengths of mouse ESTs were isolated, sequenced, and used to search for homologous proteins in the data base. Four independent clones of varying lengths of mouse ESTs were isolated, sequenced, and used to search for homologous proteins in the data base.

**ATPase Activity of BiP**—The ATPase activity of BAP was assayed as described previously with minor modifications (2, 4). Briefly, 0.5 μM recombinant BAP was incubated with or without 1 μM recombinant BAP and/or 2 μM recombinant J domain from ERdj4 at ATPase assay buffer (20 mM HEPES, pH 7.2, 50 mM KCl, 5 mM MgCl2, and 10 mM dithiothreitol) at a total volume of 50 μl. Each reaction mixture contained 100 μM ATP and 1 μCi of [γ-32P]ATP (3000 Ci/mmol; Amersham Biosciences). At various time points, 2-μl aliquots were removed and analyzed by thin layer chromatography on polyethyleneimine cellulose sheets (Sigma) using 0.5 mM formic acid, 0.5 mM LiCl. ATP was not detected by the ATP assay activity by itself or bind to nucleotides. Natural hydrolysis of ATP in the presence of bovine serum albumin was routinely subtracted to determine the ATPase activity. The reaction was terminated by passing through a PD-10 column (Amersham Biosciences) that had been equilibrated with PBS containing 10% glycerol. The isolated His6-BAP was fairly pure as judged by Coomassie staining of SDS-PAGE gels and was used for in vitro assays and to produce a polyclonal antisera in rabbits. The resulting antisera was affinity-purified using His6-BAP immobilized on CNBr-activated agarose. BAP and the J domain of ERdj4 were purified as described below (25, 30).

**Northern Blot Analysis**—A Northern blot of multiple human tissue mRNAs was obtained (Clontech Laboratory Inc., Palo Alto, CA) and probed as previously described (32). The human BAP probe was prepared from the coding sequence (1,386 bp) of the BAP gene using a Promega P3 kit and a Northtrap column (Stratagene, La Jolla, CA). The 1,386-bp coding segment of mouse BAP homologue was produced by PCR from a mouse B cell cDNA library. The forward primer was 5'-CAACCTCGACATCTCAAGCTTCAAGACTCACAT-3' and the reverse primer was 5'-CTGGGAAACTTCTGTAGTTCTCCTGACATC-3'. This PCR product was used to prepare the mouse BAP probe as above. BAP and β-actin probes were prepared as described (32).

**Cellular Localization of BAP**—pDSLBP-HA-BAP was used to transfect COS-1 cells using the FuGENE 6 reagent (Roche Molecular Biochemicals). 18 h after transfection, the cells were fixed with 5% glutaraldehyde, 95% ethanol and incubated overnight with a mouse anti-HA tag monoclonal antibody (kindly provided by Dr. Al Reynolds, Vanderbilt University) and with rabbit anti-GFP polyclonal antibody produced in our lab. The cells were then washed with PBS and incubated with TRITC-labeled goat anti-mouse antibody and fluorescein isothiocyanate-labeled goat anti-rabbit antibody for 2 h. The slides were examined on a confocal microscope (Leica TCS NT with Leica DMRBE). The images were processed by Adobe Photoshop 5.0.

**Yeast Two-hybrid Screen and cDNA Cloning**—The procedure for yeast two-hybrid screening was performed as published (29). DNA encoding the ATPase domain of the human BiP, which contains the ER targeting signal sequence (30, 31), was cloned into the Ndel and BumHI sites of the pSA1 vector. The resulting construct, pAS1(T229G) was transformed into the yeast HF7c strain. Trp' transformants were isolated and subsequently transformed with a human liver cDNA library that contained ~5.5 × 10^6 independent clones in the LEU2 marker plasmid pCT2 (Clontech Laboratory Inc., Palo Alto, CA). Transformants were plated on SD-Trp-Leu-His medium containing 20 mM 3-aminoantizole, and colonies that grew on this medium were assayed for β-galactosidase activity. Target plasmids from the positive colonies were isolated, sequenced, and used to search for homologous proteins in the data base. Four independent clones of varying lengths of mouse full-length cDNA were obtained by searching the EST database that contained 3.5 × 10^4 ESTs. The sequence of the inserted cDNA of the EST was verified by DNA sequencing. An EST clone encoding the complete cDNA of the positive target gene (identification number 5102998) from Incyte Genomics Systems Inc. was transformed into the yeast HF7c strain. Trp' and His' colonies were isolated, sequenced, and used to search for homologous proteins in the data base. Four independent clones of varying lengths of mouse full-length cDNA were obtained by searching the EST database that contained 3.5 × 10^4 ESTs. The sequence of the inserted cDNA of the EST was verified by DNA sequencing.
BiP from free nucleotides. For nucleotide release under limited conditions, 0.5 \mu M BiP was incubated with 1 \mu C of \[\text{ATP}\] for 30 min at 30\degree C in the presence of 1 \mu M BAP alone, 1 \mu M J domain alone, or 1 \mu M J domain and 1 \mu M BAP. The reaction mixtures were applied to MicroSpin G-50 columns and analyzed as above.

RESULTS

Identification of BAP—To identify BiP interacting proteins, a yeast two-hybrid screen was performed using the ATPase domain of a BiP mutant, BiPT229G, as the bait protein and a human liver cDNA library as the target. The BiPT229G binds ATP but cannot hydrolyze it (30, 31). We isolated four independent positive clones that carried target genes encoding putative BiP-interacting proteins. DNA sequencing data revealed that all four clones represented the same gene but had various amounts of the 5' coding region missing. We obtained an EST clone (identification number 5102998) that contained the complete open reading frame of the BiP-associated protein, which we named BAP. The BAP cDNA encoded a 461-amino acid protein with a potential N-terminal ER targeting sequence, two possible N-linked glycosylation sites, and an ER retention tetrapeptide (KELR) at the C terminus (Fig. 1A). Interestingly, two polyadenylation signal sequences were identified in the human BAP mRNA. Both may be used, because EST sequences corresponding to both were found in the data base. However, our Northern blot analyses of primary human tissues revealed only a single BAP transcript (Fig. 2A). BAP shared low sequence homology with a recently identified yeast ER protein Sls1p (Fig. 1B), which interacts with the ATPase domain of yeast BiP and enhances the Sec63p-mediated increase in the ATPase activity of BiP (27) and was identical to an EST-derived sequence described as a potential human homologue of Sls1p (28). BAP also shared 29% homology with a cytosolic Hsp70-binding protein, HspBP1, which in contrast to Sls1p, appears to inhibit the ATPase activity of Hsp70 (8).

BAP Was Ubiquitously Expressed and Co-localized with BiP in the ER—A cDNA probe corresponding to the mature form of BAP was hybridized to a human multiple-tissue blot to determine the expression pattern of BAP. An 1.8-kb transcript was detected in all of the tissues examined, but BAP was expressed most highly in secretory tissues such as liver, placenta, and kidney (Fig. 2A). The expression pattern of BAP on the multiple-tissue blot was almost identical to that of BiP and two recently identified ER DnaJ proteins, ERdj4 and ERdj3 (not shown), suggesting that they may function together. To determine the subcellular localization of BAP, COS-1 cells were transfected with a cDNA encoding a HA-tagged version of BAP.
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Immunofluorescence staining revealed that BAP co-localized with endogenous GRP94 in the ER of COS-1 cells (Fig. 2B).

**Fig. 2. Tissue distribution and cellular localization of human BAP.** A, a human multiple tissue Northern blot was hybridized with probes corresponding to the coding regions of the human BAP, hamster BAP, and human β-actin genes. Lane 1, peripheral blood leukocytes; lane 2, lung; lane 3, placenta; lane 4, small intestine; lane 5, liver; lane 6, kidney; lane 7, spleen; lane 8, thymus; lane 9, colon; lane 10, skeletal muscle; lane 11, heart; lane 12, brain. B, COS-1 cells were transfected with pDStk/BAP-HA-A. At 18 h post-transfection, the cells were immunostained with a monoclonal anti-HA tag antibody and a polyclonal anti-GRP94 antibody followed by TRITC- and fluorescein isothiocyanate-conjugated secondary antibodies, respectively. The images were visualized on a confocal microscope and processed with Adobe Photoshop 5.0. The merged image reveals co-localization of these two proteins (yellow).

**Fig. 3. Endogenous BAP is an ~54-kDa glycoprotein.** A, HepG2 cells were metabolically labeled in the presence of Me2SO or tunicamycin (2.5 μg/ml). The cells lysates were prepared and immunoprecipitated with the anti-BAP antibody or protein A-purified preimmune IgG (PI). For Endo H digestion, immunoprecipitated BAP was digested with Endo H for 18 h at 37 °C. The proteins were analyzed by SDS-PAGE. In addition, 293T cells were transfected with pCDNA(BAP) and analyzed in a manner similar to that of HepG2 cells. Glycosylated (+CHO) and nonglycosylated (−CHO) BAP are indicated. B, aliquots of the same cell lysates of HepG2 cells prepared in A were analyzed by Western blot with antisera specific for rodent BiP and BAP.

The binding of BAP to BiP was examined in *vivo* by transiently co-expressing BAP with various BiP mutant proteins in COS-1 cells. We have identified a number of BiP mutants that are impaired in nucleotide binding, in the ATP-dependent conformational change, or in ATP hydrolysis (30, 31). Transfected cells were metabolically labeled with [35S]methionine and [35S]cysteine and then treated with the membrane-permeable cross-linking agent DSP to stabilize BAP-BiP complexes within the cell. The complexes were isolated with rodent-specific anti-BiP antisera and separated on a SDS gel. An aliquot of the total cell lysate was analyzed by Western blotting, which revealed that each of the co-transfected set of cells expressed very similar levels of the two proteins (Fig. 4A). However, the co-precipitation experiments demonstrated that much more BAP was co-precipitated with the various BiP mutants than with wild type BiP. This indicated that the binding of BAP to BiP

Antiserum for BiP did not react with the antiserum. Labeling cells in the presence of tunicamycin or Endo H produced a protein that migrated more rapidly on SDS gels, demonstrating that BAP is a N-linked glycoprotein and therefore must enter the ER (Fig. 3A, left panel). cDNAs encoding BAP with and without an HA tag were transfected into 293T cells, and the 293T cells were examined by similar methods. When full-length BAP without an HA tag was expressed, a 54-kDa protein was precipitated with the anti-BAP antiserum, which migrated more rapidly after labeling in the presence of tunicamycin or Endo H digestion (Fig. 3A, right panel). Detection of the HA-tagged form with a HA-specific monoclonal antibody showed similar results (data not shown).

An aliquot of whole cell lysates from control and tunicamycin-treated HepG2 cells was analyzed by Western blotting with antisera specific for both BAP and BiP. In the control cells, a major band migrating at 54 kDa was observed, which migrated faster after tunicamycin treatment (Fig. 3B). After 16 h of tunicamycin treatment, none of the glycosylated protein could be detected, suggesting that the half-life of BAP, at least during ER stress, is relatively short. In addition, unlike BiP, the amount of BAP protein present in the tunicamycin-treated cell lysates was actually less than in the untreated cell lysates. This suggested that unlike other ER chaperones, BAP might not be a target of the unfolded protein response.
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**Fig. 4.** BAP binding to BiP in mammalian and yeast system. A, COS-1 cells were co-transfected with a combination of BAP-HA and either BiP, BiPT229G, BiPG227D, or BiPT37D. 30 h after transfection, the cells were metabolically labeled for 18 h and subjected for cross-linking with DSP. Immunoprecipitation (IP) was performed with the anti-BiP antiserum, and the precipitated proteins were separated by SDS-PAGE (bottom panel). An aliquot of each cell lysate was blotted separately to determine expression levels of BAP and BiP in each sample using an anti-HA tag or anti-BiP antibody (top panel). WB, Western blot. B, BAP was co-transformed with either the wild type (WT) or T229G ATPase domain of BiP into the HF7c yeast strain. Transformants were grown on SD-Trp/-Leu/-His in the presence of 20 mM 3-aminotriazole. The left panel shows combinations of vectors co-transformed.

was significantly affected by the conformation of the ATPase domain of BiP (Fig. 4A). Similar results were observed when we expressed BAP in yeast. The BAP-Gal4 activation domain fusion protein was transformed into yeast together with the Gal4 DNA-binding domain fused to either the wild type or T229G BiP ATPase domains and used to drive Gal4-mediated His3 gene expression. Only the combination of BAP and T229G was able to grow on His- selective medium. Yeast cells expressing BAP with the wild type ATPase domain of BiP failed to do so (Fig. 4B). Together, these data showed that BAP binds better or more stably to the BiP mutants than to wild type BiP, possibly because of conformational differences in the ATPase domains or in the nucleotide bound state of the domains.

**BAP Stimulates the ATPase Activity of BiP**—Because BAP specifically interacted with the ATPase domain of BiP, we determined whether BAP could modulate the ATPase activity of BiP under conditions of nucleotide excess. In this experiment, aliquots of the ATP hydrolysis assay mixture were removed at 10-min intervals and analyzed on TLC plates. Our recombinant BAP preparation was first tested for nucleotide binding and intrinsic ATPase activity and found to be negative for both (data not shown). When BAP was added to BiP (2:1 molar ratio of BAP:BiP), it increased the ATPase activity of BiP by about 2-fold, which was similar to the increase observed when the J domain of ERdj4 was added to BiP (Fig. 5). When both BAP and the J domain were added to BiP, the rate of ATP hydrolysis by BiP was stimulated by about 4-fold over basal levels, indicating that both BAP and ERdj4 positively regulate the ATPase activity of BiP (Fig. 5).

**BAP Promotes Nucleotide Exchange from BiP**—DnaJ homologues, including ERdj4, bind to the ATP-bound form of Hsp70 family members and accelerate their intrinsic ATPase activity (33). In contrast, binding studies with our BiP mutants suggested that BAP might preferentially interact with the ADP-bound state of BiP. For BAP to stimulate the ATPase activity of BiP, it was conceivable that BAP served as a nucleotide exchange factor for BiP. We examined the ability of BAP to affect nucleotide binding under steady state conditions. BiP alone or BiP with the indicated combinations of BAP and ERdj4 was incubated in the presence of limited quantities of [\(^32\)P]ATP for 30 min. Under these conditions, in the reaction mixture containing BiP alone, BiP remained associated with both ADP and ATP (Fig. 6A). Addition of the J domain to BiP caused increased hydrolysis of labeled ATP to ADP, which remained bound. In contrast, the addition of BAP either alone or together with ERdj4 resulted in the almost complete absence of a signal for labeled nucleotide with BiP (Fig. 6A). This finding is compatible with BAP inhibiting nucleotide binding, as has been suggested for the function of HspBP1 (8). Conversely, these data could suggest that BAP is serving to accelerate the exchange of labeled nucleotide with unlabeled nucleotide, which is in molar excess in this experiment.

Thus, we directly examined the nucleotide exchange function of BAP. BiP was loaded with [\(^32\)P]ATP and separated from unbound nucleotide (Fig. 6B, first lane). The labeled BiP was then incubated alone, with the J domain from ERdj4, or with the J domain and recombinant BAP for the indicated times and then separated on a second column. In the absence of additional proteins, approximately half of the labeled ADP was exchanged for cold ATP within 5 min. Unlike other members of Hsp70 family, however, BiP hydrolyzed the remaining labeled ATP to ADP during the second spin column process, even though it was carried out at 4 °C (Fig. 6B, compare first and
second lanes). The amount of ADP that remained bound to BiP in the presence of the J domain was 20% greater than BiP alone, but the rates of exchange were very similar in both cases. Thus, the J protein accelerated ATP hydrolysis but did not affect the rate of nucleotide exchange, which is in keeping with studies on the bacterial and cytosolic DnaJ homologues. When both ERdj4 and BAP were added to BiP, the [α-32P]ADP was quickly replaced with cold ATP. Even after a 1-min incubation, almost all of the labeled ADP was released (Fig. 6B). Thus, BAP accelerates the rate of ATP hydrolysis of BiP by increasing the rate of nucleotide exchange.

DISCUSSION

All of the Hsp70 family members bind and hydrolyze ATP, and their functions are regulated by the nucleotide-bound state. Hsp70s bind to unfolded substrate proteins when they are in the ATP-bound state. ATP hydrolysis, catalyzed by DnaJ family members, serves to stabilize their binding to these substrates, and exchange of ATP back into the nucleotide-binding cleft allows the Hsp70 protein to release the substrate. For most Hsp70s, co-factors that regulate ATP hydrolysis and nucleotide exchange have been identified. However, until now no regulators of nucleotide exchange for the mammalian ER Hsp70 family orthologue had been identified. In this study, we provide the first description of a resident ER protein that serves as a nucleotide exchange factor for BiP. BAP was isolated as a protein that bound to the ATPase domain of a hamster BiP mutant in a yeast two-hybrid screen. Our characterization of BAP revealed that it possessed an N-terminal ER targeting signal sequence, a C-terminal ER retention motif, and N-linked glycans that remained Endo H-sensitive, implying that BAP is not transported beyond the ER. In addition, BAP was associated with BiP in mammalian cells and interacted with BiP functionally in vitro.

The amino acid sequence of BAP shows homology with two groups of proteins that have been implicated in regulating the ATPase cycle of different Hsp70 proteins. The region between amino acids 70 and 108 of BAP is highly homologous to a similar region of Sls1p, which was recently identified as a resident ER protein in both Yarrowia lipolytica and Saccharomyces cerevisiae (27, 28). Sls1p binds to the ATPase domain of Kar2p, the yeast homologue of BiP, and functionally interacts with the yeast DnaJ protein Ser63p to increase the ATPase activity of Kar2p (27). SLS1 also interacts genetically with LHIS1, another Hsp70 homologue in the yeast ER (28). BAP also shares some sequence homology with the cytosolic protein HspBP1 in its more central region, which includes the armadillo repeat domain. HspBP1 interacts with Hsp70 and inhibits the Hsp40-mediated activation of Hsp70s (8), perhaps by preventing nucleotide rebinding (34). Homologues of HspBP1 can be found in databases for a vast variety of species. Thus, BAP shares homology with two functionally different families of nucleotide exchange regulators for Hsp70 proteins.

Recombinant BAP protein stimulated the ATPase activity of BiP in vitro and caused a further increase in the presence of recombinant J domain from ERdj4, a recently identified mammalian ER DnaJ homologue (25), suggesting that BAP was more likely to be a functional homologue of Sls1p than of HspBP1. When nucleotide exchange assays were performed under conditions of excess cold ATP, BAP caused the rapid release of labeled ADP from BiP. When the exchange assays were performed under conditions where both ADP and ATP were associated with BiP, it appeared that both nucleotides were readily released by BAP. This characteristic is more similar to results obtained with GrpE, the nucleotide exchange factor present in bacteria (2) and in organelles like chloroplasts and mitochondria (35), which are thought to be of bacterial origin.

Co-expression of BAP with either wild type or mutant BiP revealed that more BAP was associated with the ATPase mutants than with wild type BiP. In addition the ATPase domain of BiP mutant interacted with BAP in the two-hybrid screen, whereas the wild type domain did not. These data suggest that BAP may prefer the ADP bound form of BiP, which is in keeping with binding data for both Sls1p (27) and Fes1p, a recently identified yeast homologue of HspBP1 (34), both of which bind preferentially to their Hsp70 partners when in the ADP-bound state. In the case of BAP and Sls1p, this should result in the preferential exchange of ADP out of the nucleotide-binding cleft, which explains their positive effects on the ATPase activity of their respective Hsp70 proteins. Our nucleotide binding experiments demonstrated that BAP does not directly bind either ATP or ADP, so its ability to act as an exchanger for BiP must occur as a result of conformational changes that occur in the ATPase domain of BiP when BAP binds.

Like other Hsp70 proteins, BiP binds to unfolded regions in substrate proteins and prevents them from folding or aggregating (16, 36). In vitro, it is assumed that ATP must rebind to the nucleotide-binding cleft to allow release of bound proteins at the appropriate time so they can fold. This hypothesis is supported by data obtained with BiP ATP-binding mutants showing that the mutants prevent the folding of bound substrates but keep them in a soluble form (37). The in vitro addition of ATP to complexes induces the release of BiP, which can provide an opportunity for the substrate to fold or in some cases leads

3 J. L. Brodsky, personal communication.
to aggregation of the unfolded substrate (38, 39). Thus, it is reasonable to assume that the timing or conditions of release might be important and that overexpression of a nucleotide exchange factor could have either positive or negative effects on protein folding. In keeping with the idea of negative effects on protein folding, the overexpression of the cytosolic nucleotide exchange factor BAG-1 inhibited the ability of Hsp70 to refold luciferase (40) and suppressed the positive effect of Hip on Hsp70 chaperone activity (41).

In support of the idea that BAP positively regulates protein folding in the mammalian ER, we found that BAP is expressed most highly in tissues like the liver, kidney, and placenta, which produce large amounts of secreted proteins. These tissues also show high levels of BiP, ERdj3, and ERdj4 (25), which produce large amounts of secreted proteins. These tissues also show high levels of BiP, ERdj3, and ERdj4 (25), which were shown here to interact with BAP. The role of BAP in regulating protein folding by BiP is currently under investigation using in vitro and in vivo experimental models.

In conclusion, we have identified the first mammalian ER nucleotide exchange factor for BiP, which appears to be a homologue of yeast Sls1p. However, although BAP is highly expressed in secretory tissues, unlike Sls1p, BAP is regulated independently of ER chaperones during ER stress. This suggests that mammalian cells have the ability to inhibit the release of BiP from substrate proteins under conditions that are not conducive to proper folding or assembly.

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