Biotechnology and Bioprocess Engineering **2003**, 8: 192-198 ©KSBB

Isolation of Proteins that Specifically Interact with the ATPase Domain of Mammalian ER Chaperone, BiP

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Abstract BiP, immunoglobulin binding protein, is an ER homologue of Hsp70. However, unlike other Hsp70 proteins, regulatory protein(s) for BiP has not been identified. Here, we demonstrated the presence of potential regulatory proteins for BiP using a pull-down assay. Since BiP can bind any unfolded protein, only the ATPase domain of BiP was used for the pull-down assay in order to minimize nonspecific binding. The ATPase domain was cloned to produce recombinant protein, which was then conjugated to CNBr-activated agarose. The structural conformation and ATP hydrolysis activity of the recombinant ATPase domain were similar to those of the native protein. Eight proteins from metabolically labeled mouse plasmacytoma cells specifically bound to the recombinant ATPase protein. The binding of these proteins was inhibited by excess amounts of free ATPase protein, and was dependent on the presence of ATP. These proteins were eluted by ADP. Of these proteins, Grp170 and BiP where identified, while the others were not identified as known ER proteins, from Western blot analyses. The presence of the ATPase-binding proteins for BiP was first demonstrated in this study, and our data suggest similar regulatory machinery for BiP may exist in the ER, as found in prokaryotes and other cellular compartments.

Keywords: BiP, endoplasmic reticulum, regulatory protein, ATPase domain

INTRODUCTION

The endoplasmic reticulum (ER) is the site of synthesis, folding and assembly of secretory pathway proteins, which 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 are eventually targeted for ER-associated degradation (ERAD) through the action of the chaperones [1,2]

BiP (also known as GRP78) is a mammalian endoplasmic reticulum (ER) homologue of the Hsp70 family. It was first identified bound to non-secreted free heavy chains in pre-B lymphomas [3]. BiP has been shown to interact with a number of other secretory pathway proteins, preventing their premature transport from the ER and promoting their proper folding and assembly [4]. 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 the early stages of protein translocation [5], targeting misfolded proteins for proteasomal degradation [6], serving as a sensor for ER stress [7] and contributing to ER calcium stores.

The Hsp70 family of molecular chaperones, including BiP, is highly homologous and consists of two distinct domains: a highly conserved N-terminal ATPase domain, and a less conserved C-terminal polypeptide binding domain [8]. 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 cofactors. In bacteria, DnaJ accelerates ATP hydrolysis, whereas GrpE promotes nucleotide exchange of ADP to ATP [9,10]. The mammalian cytosolic Hsc70 is similarly regulated by Hsp40, a homologue of DnaJ [11], 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 [12, 13]. A negative regulator of Hsc70's ATPase activity, HspBP1, has been identified [14]. Additional Hsc70 interacting proteins, such as Hip [15], Hop [16,17] and CHIP [18], have been identified, which further contribute to the regulation of the Hsc70's ATPase cycle, and as such, serve to control Hsc70's chaperone function.

At least some roles of BiP in the ER are dependent on

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its ATPase activity, thus it is reasonable to speculate that the ATPase activity of BiP should be regulated in a similar way to that of other members of the Hsp70 family. However, unlike other Hsp70s, proteins that modulate mammalian BiP function, with the exception of the J homologue proteins, have not been identified. A total of four mammalian ER DnaJ homologues have been identified [19-23], and it has been proposed that they be referred to as ERdj1-4. *In vitro* biochemical studies have shown that the J domains of ERdj3 and ERdj4 can stimulate the ATPase activity of BiP [21,23], and both ERdj proteins can bind to BiP *in vivo* [21]. Recently, a yeast ER protein has been isolated from two different genera (Sls1p/Sil1p), which interacts with the ATPase domain of Kar2p, the yeast homologue of BiP [24,25]. Defects in the *Sls1* gene are not lethal, but affect protein translocation into the ER. Sls1p is proposed to be a GrpE-like protein, which is 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, S1s1p has not been directly demonstrated to serves as a nucleotide exchanger for Kar2p.

We attempted to identify potential mammalian regulators of the ATPase activity for BiP using an affinity column of the ATPase domain of BiP. Several BiP interacting proteins were isolated from mouse lymphoma cells. Of these are some known ER chaperones, but others still remain to be identified. Their binding to BiP was specific, and affected by nucleotides associated with the ATPase domain of BiP. This study, therefore, suggests the presence of potential BiP regulator(s).

MATERIALS AND METHODS

DNA Cloning of the ATPase Domain of BiP

The cDNA encoding the ATPase domain was cloned by deleting the substrate binding-encoding part from a full length of hamster BiP cDNA in the pTZ vector (pTZSSBBiP), but lacking the coding region for the BiP signal sequence [26]. A DNA fragment encoding the ATPase region including the 14 amino acid from the C-terminus was produced by PCR with two pairs of primer that generate overlapping parts: 5'-GTGCCGGCTAATG AGCCCACAGCAGC-3' and 5'-TGGGGGAGGACCAAG TGTAAGAGGACA-3' for the ATPase region, and 5'-ACA CTTGGTCCTCCCCCAACTGGTG-3' and 5'-TTCGAG ACTTAAGTTCTCTCAATTT-3' for the 14 amino acids of the C-terminus. The 14 amino acids of the C-terminus serve as an epitope for the anti-rodent BiP antibody. These PCR products were linked together by a second round PCR with 5'-GTGCCGGCTAATGAGCCCACAG CAGC-3' and 5'- TTCGAGACTTAAGTTCTCTCAATTT -3'. The second round PCR product was digested with AccI and AflII, and ligated into AccI and AflII-digested pTZSSBBiP, resulting in pTZ(BiP)44 K. The pQE10 vector (Qaigen) was digested with BamHI and HindIII, and ligated with a DNA fragment of the ATPase containing the 14 amino acids, which was produced by digesting pTZ(BiP)44 K with the same restriction enzymes. The resulting construct, pQE(44 K), tagged 6 histidines to the N-terminus of the ATPase domain, and was transformed into M15 *Escherichia coli* in order to produce the recombinant protein.

Purification of the Recombinant ATPase Domain

The histidine-tagged protein was expressed by incubating bacteria, in the presence of 1 mM isopropyl 1-thio-β-D-galactopyranoside, for 1 h at 37°C. Bacterial cells were sonicated in lysing buffer (50 mM Na_2PO_4, 500 mM NaCl, 10 mM imidazole at pH7.2), and supernatant was obtained by centrifugation for 10 min at 14,000 rpm and 4°C. The histidine-tagged recombinant protein was purified by binding to a Ni^{2+}-agarose column at pH 8.0 and eluting at pH5.0. The purified protein was analyzed for homogeneity by SDS-PAGE.

Proteolysis of the Recombinant ATPase Domain of BiP

The recombinant ATPase domain of BiP (rATPase) was digested with protease K, as described by Kassenbrock and Kelly [27], with minor modifications. 6 μg of the rATPase protein was incubated with 3 μg of protease K in PBS for 10 min at 37°C. After incubation, the reaction was stopped by the addition of 10 μL of phenylmethylsulfonyl fluoride (1 mg/mL) while incubating on ice for 30 min. The digested samples, along with an undigested control, were analyzed by SDS-PAGE.

ATPase Activity Assay of the ATPase Domain of BiP

The ATPase activity of the rATPase protein was assayed, as previously described, with minor modifications [9, 11]. Briefly, 0.5 μM of the rATPase protein was incubated in a total volume of 50 μL of ATPase assay buffer (20 mM HEPES pH7.2, 50 mM KCl, 5 mM $MgCl_2$ and 10 mM DTT), containing 100 μM ATP and 1 μCi [γ-^{32}P]ATP (3,000 Ci/mmol, Amersham-Pharmacia). After incubating for 30 min, 2 μL aliquots were removed and analyzed by thin layer chromatography on polyethyleneimine (PEI) cellulose sheets (Sigma, St. Louis) using 0.5 M formic acid and 0.5 M LiCl as the mobile phase. The PEI sheet was dried, and exposed to Kodak OMAT X-ray film, to visualize hydrolyzed γ–phosphate and ATP. Bovine serum albumin was used as a negative control of the ATPase activity.

Conjugation of the ATPase Domain of BiP to CNBr-activated Sepharose

Approximately 20 mg of the rATPase protein were conjugated to 0.5 g of dried cyanogen bromide-activated Sepharose beads (Pharmacia), according to the manufacturer's instruction. Beads alone, without protein, were also prepared as a negative bead control.

Affinity Pull-down Assay for ATPase Interacting Proteins

Ag8.653, mouse plasmacytoma cells, were maintained in complete RPMI1640 medium, and approximately 1 × 10^6 cells metabolically labeled incubating in RPMI1640 containing [^{35}S]-Translabel (ICN) for 18 h. Labeled-cells were then disrupted in 1 mL of lysing buffer (20 mM HEPES, 50 mM KCl, 2 mM MgCl2, 20 mM Imidazole, 1% Triton X-100, 1 mM PMSF, 2 mg/mL aprotinin and 0.5 mg/mL leupeptin, at pH 7.2) and centrifuged at 14,000 rpm and 4°C for 20 min in an Eppendorf centrifuge. The supernatant was incubated with 50 μL of 50% rATPase-coupled beads in the lysing buffer containing 1 mM ATP at 4°C for 30 min. After incubation, the beads were washed three times with the same buffer, and analyzed by SDS-PAGE. The SDS gel was dried, and exposed to X-ray film. For the elution of bound proteins, precipitated beads were washed with the same lysing buffer without ATP, and then further incubated in 100 μL of the same buffer, containing 1mM ADP, at room temperature for 20 min. The eluted proteins were recovered by centrifugation at 3,000 rpm for 3 min, and analyzed by SDS-PAGE. Bound proteins were identified by western blot analyses using an ECL kit (Pharmacia). Rabbit anti-Grp170 and anti-BiP antibodies were produced in our lab, while the other antibodies were purchased from Stressgen (Vancouver, Canada).

Endoglycosidase H digestion

The ATPase interacting proteins were obtained as described above, and resuspended in 0.1 M sodium acetate buffer at pH 5.5. The digestion was carried out with 0.5 U Endoglycosidase H (Endo H) (Boehringer Mannheim, Indianapolis, IN) for 18 h at 37°C, and the digested proteins analyzed by SDS-PAGE.

RESULTS

We used the ATPase domain of BiP to isolate any potential regulatory protein for BiP. The recombinant ATPase protein was designed to contain 6 histidines at the N-terminus and to keep the C-terminal 14 amino acids, deleting the substrate-binding domain. Six histidines are utilized to purify the recombinant protein, and the C-terminal 14 amino acids provide an epitope for the rabbit anti-BiP polyclonal antibody (Fig. 1). The rATPase protein was expressed in M15 *E. coli,* and purified using a Ni^{2+}-agarose column. The purified rATPase protein was found to be homogenous from SDS-PAGE (Fig. 2). Since Kassenbrock and Kelly [27] demonstrated that ADP-associated BiP generates an approximately 50 kDa proteolysis resistant protein, the rATPase protein was digested by protease K. The digested product was separated mainly as 50 kDa and 33 kDa protein bands (Fig. 2). This result indicated that the rATPase protein is properly folded and dominantly ADP-associated. This data was consistent with the previous data of full length recombinant BiP [28]. We also attempted whether or not

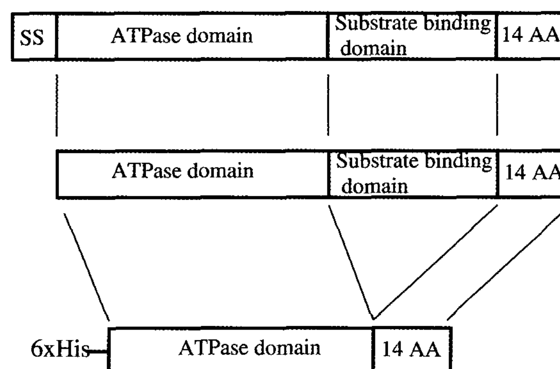

Fig. 1. Schematic diagram of BiP and its ATPase domain. BiP is composed of an endoplasmic reticulum targeting signal sequence (SS), an ATPase domain, a substrate binding domain and the ER retention tetrapeptide (KDEL). The recombinant ATPase protein was produced to tag six histidines at the N-terminus and to include the C terminal 14 amino acids from a full length of BiP, as described in Material and Methods.

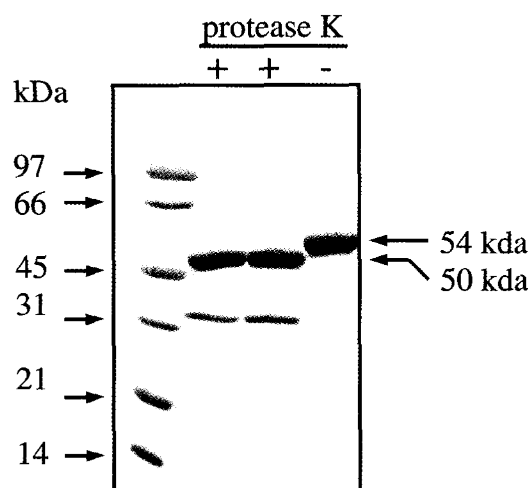

Fig. 2. Purified recombinant ATPase protein and protease K digestion. Six histidines tagged-ATPase protein was purified to almost homogeneity using a Ni^{2+}-agarose column, and the molecular weight of the recombinant protein was approximately 54 kDa (Lane 4). The 50 kDa fragment was protected from protease K digestion for 10 min at 37°C (duplicated samples in lane 2 and 3).

the rATPase protein is functional for ATP hydrolysis activity by an ATPase assay. As shown in Fig. 3, the rATPase protein was able to hydrolyze ATP to ADP *in vitro.* Both the protease K protection and ATP hydrolysis results strongly indicated that the structure and function of the recombinant ATPase protein was very similar to those of the native ATPase domain of BiP. Therefore, the rATPase protein should be recognized by its potential interacting protein(s) from an *in vitro* assay. The rATPase protein was coupled to CNBr-activated Sepharose with a 98%

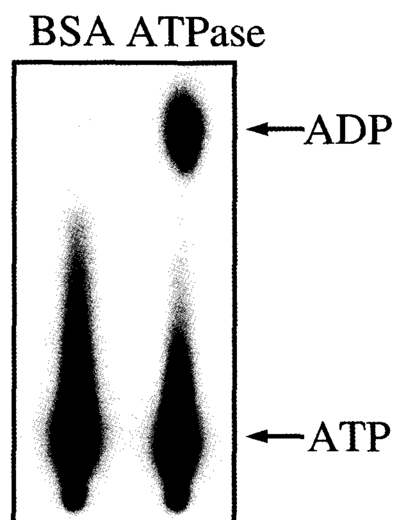

Fig. 3. The ATPase activity assay of the recombinant ATPase domain. The ATPase activity of the recombinant ATPase domain of BiP was assayed as described [9,11]. The recombinant ATPase protein (0.5 μM) was incubated in a total volume of 50 μL of the ATPase assay buffer (20 mM HEPES pH7.2, 50 mM KCl, 5 mM $MgCl_2$ and 10 mM DTT), containing: 100 μM ATP and 1 μCi [γ-^{32}P]ATP (3,000 Ci/mmol, Amersham-Pharmacia). After a 30 min incubation, 2 μL aliquots were removed and analyzed on polyethyleneimine (PEI) cellulose sheets using 0.5 M formic acid and 0.5 M LiCl. The PEI sheet was dried and exposed to Kodak OMAT X-ray film to visualize hydrolyzed γ-phosphate and ATP. Bovine serum albumin was used as a negative control.

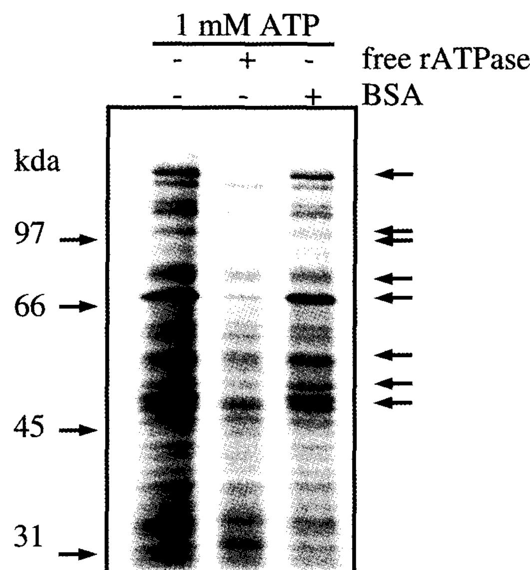

Fig. 4. ER proteins bind to the ATPase domain of BiP. Ag8.653 cells were metabolically labeled, and 1% Triton X-100 solubilized proteins were incubated with the recombinant ATPase protein-conjugated agarose beads in the presence of ATP. Excess amount of free recombinant ATPase protein was added to compete for binding. Bovine serum albumin was used as a negative control for the binding competition.

coupling efficiency, and the protein-coupled beads were used to pull down interacting proteins from [S^{35}]-labeled Ag8.653 cells. Ag8.653 cells are mouse plasmacytoma cells that originate from B cells, but lack the ability to produce antibody that is a major substrate of BiP. The preliminary experiments with the whole cell lysate and endoplasmic reticulum vesicles showed almost identical binding patterns, indicating that binding to the beads is specific. Therefore, whole cell lysate was used for most experiments.

A pull down experiment was carried out in the presence of ATP; this was because the conformation of the ATPase domain should be more favorable with positive regulator(s), which prefer an ATP-bound conformation of the ATPase domain to hydrolyze the ATP. Although the rATPase protein was very similar to the native ATPase domain of BiP, nonspecific binding was observed (Fig. 4. lane1). Therefore, competition binding was employed, in the presence of an excess amount of free rATPase and bovine serum albumin, to subtract the background, and distinguish specific binding. Several dominant proteins competed with free rATPase protein for binding to the beads, but not with bovine serum albumin. Those proteins were about 150, 110, 100, 70, 54, 48 and 46 kDa. These results suggested that binding of these proteins appears to be specific to the rATPase-coupled beads. Since we used whole cell lysate for the pull down technique, although similar results were obtained with ER vesicles, we wanted to know if ER proteins are included in the bound proteins. ER resident glycoproteins are susceptible to endoglycosylase H (Endo H) digestion because N-linked glycosylation occurs in the ER. After Endo H digestion of the bound proteins, the migration of a 150 kDa protein band was greatly shifted down, indicating digestion of the glycosyl moieties (Fig. 5). No other protein bands were obviously changed. Together with the results of the binding competition assay, the presence of Endo H susceptible protein among the bound proteins suggested that the bound proteins are specifically interacting with the recombinant ATPase protein, and at least some of the bound proteins are ER resident. We then attempted to identify some of the bound proteins with a series of western blot analyses using available ER protein specific antibodies. Two ER proteins, Grp170 and BiP, were identified, while the identification of the others using Western blot analyses failed (Fig. 6). Indeed, the Endo H-sensitive 150 kDa protein turned out to be Grp170, which is another Hsc70 homologue in the ER [29]. The precise function of Grp170 has not been elucidated. Binding of Grp170 to the ATPase domain of BiP had not been demonstrated before. However, it was not clear whether binding of Grp170 to the rATPase protein was a direct or indirect interaction in this pull-down assay.

Proteins bound to the rATPase-coupled beads were eluted with 1 mM ADP. The eluted proteins were the

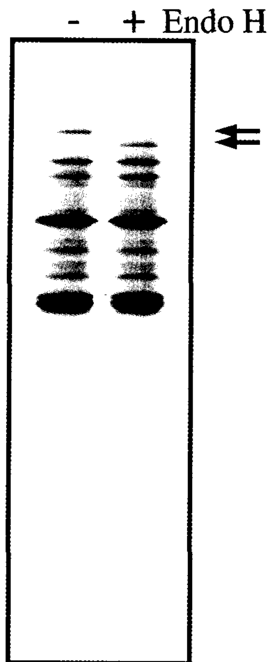

Fig. 5. Endoglycosidase H digestion of the ATPase interacting proteins. The ATPase interacting proteins were digested with 0.5 U Endo H (Boehringer Mannheim, Indianapolis, USA) for 18 h at 37°C in 0.1 M sodium acetate buffer at pH 5.5. Digested proteins were analyzed by SDS-PAGE.

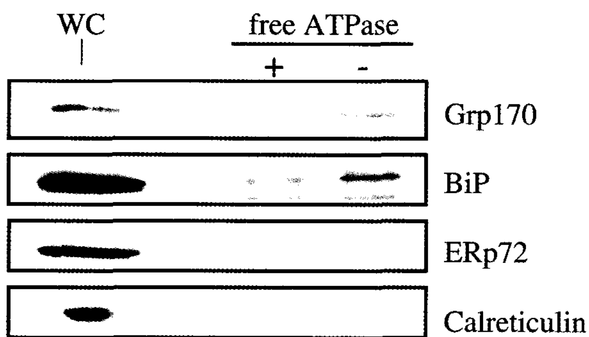

Fig. 6. Western blot analysis of the ATPase interacting proteins. Western blot analyses were applied to identify ATPase interacting proteins. Representative data are shown here. Grp170 and BiP were identified among the ATPase interacting proteins and competed with the free ATPase protein. Erp72 and calreticulin were not detected among the ATPase interacting proteins. Whole cell lysate (WC) was used as a positive control for western blots.

same as those that competed with the free rATPase protein in the competition binding assay in the presence of ATP (Fig. 7). Therefore, these proteins apparently prefer the ATP-associated conformation of the ATPase for binding, and the ADP-associated conformation of the ATPase for the release, suggesting that at least one of these proteins is potentially a regulatory protein for BiP.

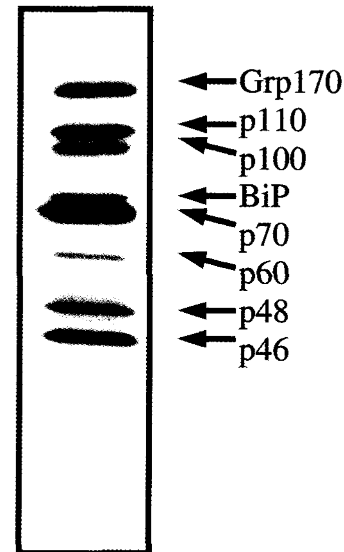

Fig. 7. Elution of the ATPase interacting proteins by ADP. Proteins were pull-downed with the rATPase-coupled beads in the presence of 1 mM ATP, and washed three times with lysing buffer omitting ATP. The beads were resuspended with 100 μL of the same buffer, containing 1 mM ADP, and incubated for 20 min at room temperature. Eluted proteins were analyzed by SDS-PAGE.

DISCUSSION

The family of Hsp70 proteins is highly conserved from bacteria to mammals. The ATPase domain (N-terminal domain) shares more amino acid sequence homology than the substrate binding domain (C-terminal domain). In bacteria, the chaperonic function of DnaK, a bacterial homologue of Hsp70, is controlled by two regulatory proteins, GrpE and DnaJ. Mitochondria contain all the homologues of Hsp70, GrpE and DnaJ. A few years ago, a similar homologue system was also found in mammalian cytosol, with even more diverse regulatory proteins for Hsp70. BAG-1 was first identified as an anti-apoptotic protein [30], and was later found to regulate the ATPase activity of Hsp70, serving as a nucleotide exchanger [31]. Another cytosolic protein, HspBP1 was identified as a negative regulator of the ATPase and protein refolding activities of Hsp70 by blocking the binding of nucleotides [14]. However, both BAG-1 and HspBP1 do not share amino acid sequence homology with each other, or with bacterial GrpE, suggesting that the mammalian homologue, Hsp70, also requires the functional homologue of GrpE to exert its chaperonic function, perhaps in a more tightly regulated manner.

In contrast to bacterial DnaK and cytosolic Hsp70, nucleotide exchanging regulators of the ER BiP have not been identified. It is plausible to speculate that BiP would also have a nucleotide exchanging regulator based on

functional homology with Hsp70s and the presence of DnaJ homologues for BiP. With this in mind, we attempted to identify proteins whose interactions with the ATPase domain of BiP were dependent on nucleotides. The six histidines tagged-recombinant ATPase domain of BiP (rATPase) was expressed in *E. coli,* and purified to homogeneity by a Ni^{2+}-agarose column. The rATPase protein was protease digestion resistant and able to hydrolyze ATP to ADP, indicating that the recombinant protein is structurally and functionally similar to the native protein. We used only the ATPase domain of BiP, not including the substrate binding domain, to increase the ATPase-specific binding and to eliminate the chaperone-substrate interaction. We used a pull-down technique, with the rATPase-coupled beads, and isolated several proteins that appeared to be specifically binding to the rATPase protein in a nucleotide dependent manner. These proteins were eluted by ADP, with two of these being identified as Grp170 and BiP, from Western blot analyses. BiP exist as monomers, dimers and some higher order oligomers [32,33]. Therefore, it was not surprising to find BiP among the interacting proteins. However, the presence of Grp170 among the bound proteins is interesting. Grp170 is another ER homologue of hsp70, with no known precise function in the ER [29]. In this study we could not define if the interaction between Grp170 and the rATPase protein was direct or indirect. Since the ATPase domains of the two proteins share 29% amino acid sequence homology it is plausible that the ATPase domain of Grp170 is dimerized directly to the ATPase of BiP as BiP is dimerized through its ATPase domain. We do not rule out the possibility that other proteins are involved in linking these two proteins, similarly to the association of Hsp70 with Hsp90 through Hop in cytosol [16, 17].

Six other proteins could not be identified by the Western blot analyses, which was employed to distinguish the unknown proteins from the known ER proteins. Although we did not use all the antibodies available against ER proteins they are possibly, based on their binding specificity, ER proteins. It was demonstrated that cytosolic Hsp70 does not interact with an ER J protein [34], indicating the importance of the binding specificity between a chaperon and its regulators in different cellular compartments. As an alternative way of verifying the isolated proteins as being ER proteins, we are currently conducting cross-linking experiment using ER vesicles treated with a membrane permeable reagent, such as DSP (Dithiobis (succinimidylpropionate)), which can be cleaved by reducing agents. From our preliminary data a few proteins from the pull-down assay were also found in a cross-linked protein complex, supporting the fact that proteins isolated by the pull-down assay are ER proteins. As the Western blot analyses failed to identify some proteins, we attempted to sequence the 70 kDa protein by automatic the Edman degradation method, but sequence data were not obtained due to possible N-terminal block. We are currently scaling up to obtain enough proteins for amino acid sequence analyses. Once we have protein sequence data we will take advantage of EST clones. We are very anxious to identify the six other proteins, which will be utilized to dissect the multiple functions of BiP.

In summary, we isolated a group of proteins that specifically interact with the ATPase domain of BiP. Two of the interacting proteins are the ER chaperones, Grp170 and BiP. The other interacting proteins were not identified, but strongly suggest the presence of potential regulator(s) for BiP. Data from this study indicate that the ER chaperone, BiP, has possibly similar regulatory machinery as found in other Hsp70 chaperones members.

Acknowledgement This work was supported by the Brain Busan 21 Project in 2002.

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[Received October 16, 2002; accepted April 9, 2003]