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In Vivo Expression of Mammalian BiP ATPase Mutants Causes Disruption of the Endoplasmic Reticulum

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BiP possesses ATP binding/hydrolysis activities that are thought to be essential for its ability to chaperone protein folding and assembly in the endoplasmic reticulum (ER). We have produced a series of point mutations in a hamster BiP clone that inhibit ATPase activity and have generated a species-specific anti-BiP antibody to monitor the effects of mutant hamster BiP expression in COS monkey cells. The enzymatic inactivation of BiP did not interfere with its ability to bind to Ig heavy chains in vivo but did inhibit ATP-mediated release of heavy chains in vitro. Immunofluorescence staining and electron microscopy revealed vesiculation of the ER membranes in COS cells expressing BiP ATPase mutants. ER disruption was not observed when a "44K" fragment of BiP that did not include the protein binding domain was similarly mutated but was observed when the protein binding region of BiP was expressed without an ATP binding domain. This suggests that BiP binding to target proteins as an inactive chaperone is responsible for the ER disruption. This is the first report on the in vivo expression of mammalian BiP mutants and is demonstration that in vitro-identified ATPase mutants behave as dominant negative mutants when expressed in vivo.

INTRODUCTION

The folding and assembly of proteins in a cell appears to depend on the action of a group of proteins termed molecular chaperones (for reviews see Gething and Sambrook, 1992; Hendrick and Hartl, 1993). Perhaps the best characterized are the hsp70 family members, which are approximately 70 kDa and are constitutively expressed in every organisms' cellular organelles (Lindquist and Craig, 1988). Physiological stresses such as increased temperature cause the synthesis of these proteins to be up-regulated. They apparently function to ensure that nascent polypeptides attain, and maintain, the required conformation for folding by binding transiently to the unfolded proteins, possibly masking regions that might otherwise interact and lead to misfolding or aggregation. Hsp70 family members also stably bind to proteins that are unable to fold correctly and to protein subunits produced in stoichiometric excess (Gething and Sambrook, 1992). It remains to be seen if the hsp70 proteins merely serve to prevent errors in folding and assembly, or actually play a more active role in mediating these processes.

BiP/GRP78 is the endoplasmic reticulum (ER) homologue of the hsp70 family, and is transcriptionally regulated by agents that alter the levels of unfolded proteins in the ER (Lee, 1987; Kozutsumi *et al.*, 1988). Although extensive data are available on the regulation of mammalian BiP and its binding to nascent and aberrant ER proteins (Gething and Sambrook, 1992; Haas, 1991), the full extent of its involvement in the maturation of secretory pathway proteins is not known. Kar2, the yeast BiP homologue, is an essential

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protein (Normington *et al.*, 1989; Rose *et al.*, 1989). Kar2 is involved in protein translocation into the ER (Vogel *et al.*, 1990; Sanders *et al.*, 1992) and is required for homotypic fusion of ER membranes during karyogamy (Latterich and Schekman, 1994). ATP binding or hydrolysis may be required for these functions because several of the Kar2 mutations map to the ATP binding domain. However, no biochemical data are available on these mutants.

Like all members of the hsp70 family, BiP has a weak ATPase activity (Kassenbrock and Kelly, 1989) that can result in the in vitro release of BiP from bound Ig heavy chains (Munro and Pelham, 1986). Depletion of cellular ATP causes prolonged association of proteins with BiP, thereby blocking their secretion (Dorner *et al.*, 1990), whereas lowering ER calcium levels leads to premature release of proteins from BiP in vivo, presumably by activating ATP hydrolysis (Suzuki *et al.*, 1991). These results, together with the recent demonstration of ATP transport into the ER (Clairmont *et al.*, 1992), provide evidence that ATP binding/hydrolysis is an important part of BiP's in vivo function.

Based on crystallographic data from another hsp70 family member, hsc70 (Flaherty *et al.*, 1990), we recently mutated codons in the hamster BiP gene that correspond to residues implicated in interactions with the β-phosphate (T37), γ-phosphate (T229), and divalent cation (E201) of ATP. Bacterially produced–recombinant proteins bearing these mutations have severely diminished ATPase activity in vitro (Gaut and Hendershot, 1993). The mutant proteins can still dimerize and associate with Ig heavy chains, suggesting that these mutations do not drastically alter the structure of BiP. However, the mutant proteins are unable to release Ig heavy chains in vitro in the presence of ATP (Gaut and Hendershot, 1993). Reports on mutation of some of the corresponding residues in bovine hsc70 (Wilbanks *et al.*, 1994) and bacterial dnaK (McCarty and Walker, 1991; Wild *et al.*, 1992) confirm the universal importance of these amino acids in hsp70 proteins for in vitro ATP binding and hydrolysis. However, optimal conditions for in vitro ATP hydrolysis probably do not exist in the ER. In fact, ATP hydrolysis assayed in vitro is almost completely inhibited at pH 7.5 and by millimolar calcium concentrations (Kassenbrock and Kelly, 1989; Gaut and Hendershot, 1993). One explanation for this paradox could be the regulation of BiP's ATPase activity by as yet unidentified mammalian ER homologues of dnaJ and grpE, which regulate dnaK activity (Liberek *et al.*, 1991) or by other ER molecular chaperones such as grp94, ERp72, or calnexin (Mazzarella *et al.*, 1990; Ahluwalia *et al.*, 1992; Melnick *et al.*, 1992). Alternatively, the mutants identified by in vitro assays may not behave as mutants in vivo.

To address this paradox, we generated antisera that allowed us to examine the in vivo behavior of transfected BiP mutants against a background of endogenous BiP, and to gain some insight into BiP's in vivo function. Here, we provide the first evidence that in vitro-identified BiP ATPase mutants function as mutants in vivo, and that their expression results in vesiculation of the ER. We show that this phenotype is not observed if the mutations are expressed on a BiP mutant that cannot bind to proteins but is seen if only the protein binding domain of BiP is expressed. We discuss the likely implications of these observations on the role of BiP in the ER.

MATERIALS AND METHODS

Cell Lines

A mouse plasmacytoid line, Ag8.653 (Kearney *et al.*, 1979), a Chinese hamster fibroblast line CHO (Puck *et al.*, 1958), an African green monkey kidney fibroblast line COS (subclone COS-7) (Gluzman, 1981), and a human pre-B cell line Nalm-6 (Nilsson, 1979) were used for these experiments. The mouse and human cell lines were maintained in RPMI 1640 medium (Whittaker M.A. Bioproducts, Walkersville, MD); the CHO and COS cells were grown in Dulbecco's modified Eagle's medium (Whittaker M.A. Bioproducts). Both media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin (Irving Scientific, Santa Ana, CA).

Polyclonal Anti-Peptide Antibody Production

An 11-amino acid peptide (GEEDTSGKDEL) corresponding to the C-terminus of hamster BiP (Ting *et al.*, 1987) was synthesized using standard F-moc chemistry on an Applied Biosystems (Foster City, CA) 431A peptide synthesizer. The purified peptide was conjugated to keyhole limpets hemocyanin for immunization of New Zealand White female rabbits. Immune sera was affinity purified on peptide-conjugated sepharose beads, eluted with Acti-Sep (Sterogene, Arcadia, CA), and desalted on a PD-10 Sephadex column (Pharmacia, Uppsala, Sweden).

Vectors and BiP Mutants

Hamster BiP ATPase mutants 1ADel, T37G, and T229G have been described previously (Gaut and Hendershot, 1993). The 1ADel mutant was created by removing 27 amino acids, Tyr175–Glu201 from the 1A domain of the ATP binding cleft, and the T37G and T229G mutants are single amino acid substitutions. To easily distinguish between endogenous and transfected BiP, an additional deletion mutant, *Kpn*I, was made by removing a 154-bp *Kpn*I fragment from hamster BiP. This allows an in-frame deletion of Pro444–Val494. ATP binding domain mutants 44K and 44K-T37G were constructed by deletion mutagenesis using an overlap extension polymerase chain reaction method (Ho *et al.*, 1989). Oligonucleotides were designed to give a product that joined the sequence encoding Gly425 (which corresponds to the end of the crystallized hsc70 ATPase fragment) with that for Pro640, resulting in a deletion of Iso426–Phe639. The resulting cDNA encoded a protein with a predicted molecular weight of approximately 50K that had the carboxyterminal 15 amino acids of the wild-type protein. We chose to call the mutant 44K to reflect its similarity to the hsc70 44K fragment. The entire PCR-amplified region (*Acc*I–*Afl*II) was cut with the appropriate restriction enzymes, ligated into the pTZ-WTBiP or pTZ-T37GBiP vector in place of the wild-type *Acc*I–*Afl*II fragment, and sequenced in entirety. The truncated cDNAs were excised from the pTZ vector and inserted into QE-10 vector for production of recombinant protein (Qiagen, Chatsworth, CA) and into the pMT vector for expression in eucaryotic cells. A chimeric mouse:human Ig

heavy chain cDNA (Liu *et al.*, 1987) was inserted into pSVL for expression in eucaryotic cells. An *Eco*RI fragment containing the entire human α_1-antitrypsin cDNA (Kurachi *et al.*, 1981) was cloned into the pMT vector for expression in COS cells. The α_1-antitrypsin-BiP chimera was produced by cutting the α_1-antitrypsin cDNA with *Bst*XI and *Hin*dIII (to remove one-fourth of the 3′ end), isolating the *Eco*RI–*Hin*dIII fragment of BiP, and ligating the two together along with a *Bst*XI–*Eco*RI double stranded oligonucleotide linker (5′-actgg-3′ and 5′-aattccagtgaac-3′) to allow in-frame ligation. The chimera comprises the aminoterminus of α_1-antitrypsin (Met1–Phe297) joined directly to the carboxyterminus of BiP (Thr485–Leu654). The construct was sequenced to confirm the integrity of the join.

Production of Recombinant Protein and ATPase Assay

Recombinant proteins were expressed using the Qiagen system (Qiagen) and purified on Ni^{2+}-agarose as previously described (Gaut and Hendershot, 1993). ATPase activity was assayed by two methods: the first was based on the protocol of Kassenbrock and Kelly (1989), which has been used previously by us to identify BiP ATPase mutants (Gaut and Hendershot, 1993); and the second measured the free phosphate liberated during hydrolysis and permits the use of saturating concentrations of ATP (Seals *et al.*, 1978). Two micromolar recombinant BiP was assayed using 1 mM ATP.

COS Cell Transfection and Immune Complex Precipitation

BiP mutant and γ heavy chain cDNAs were cotransfected into COS cells using the DEAE-dextran procedure as previously described (Gaut and Hendershot, 1993). Immune complexes were obtained by lysing the cells in NP-40 lysing buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% NP-40, and 0.5% deoxycholate) containing 10 U apyrase (Sigma Chemical, St. Louis, MO). Clarified lysates were incubated with the indicated antisera, and immune complexes were precipitated by binding to protein A-Sepharose beads. BiP or BiP mutants were examined for their ability to be released from heavy chains by incubating the immune complexes with 1 ml of ATP release buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.0, 75 mM KCl, 1 mM $MgCl_2$, and 1 mM ATP) for 30 min at room temperature.

Western Blotting

Western blotting was performed according to the procedure of Burnette (1981). Transferred proteins were detected by incubation with the indicated antisera followed by horseradish peroxidase-conjugated protein A (EY Laboratories, San Mateo, CA). Nitrocellulose sheets were subsequently developed with ECL reagents (Amersham, Arlington Heights, IL) and exposed directly to XAR film (Kodak, Rochester, NY).

Fluorescence Staining

COS cells (10^5) were seeded into 35-mm tissue culture dishes containing a sterile coverslip. Twenty-four hours later, the cells were transfected as described above. Forty hours after transfection, the coverslips were removed from the dishes, washed once in phosphate-buffered saline (PBS) containing 10% fetal bovine serum, and fixed in acid-alcohol (5% acetic acid:95% ethanol) for 20 min at −20°C. After rehydration of the coverslips in PBS, transfected BiP was detected with a polyclonal anti-GRP78 antibody (Affinity Bioreagents, Neshanic Station, NJ), which is also rodent specific. Transfected α_1-antitrypsin was detected using a goat anti-human α_1-antitrypsin antibody (Organon Teknika-Cappel, Durham, NC), and endogenous monkey BiP was detected with a monoclonal rat anti-BiP antibody (Bole *et al.*, 1986). Each of the primary antibodies was used at a concentration of 0.05 mg/ml and incubated for 20 min at room temperature. Species-specific fluorochrome-conjugated secondary antibodies (Southern Biotechnology Associates, Birmingham, AL) were used at 0.05 mg/ml. Coverslips were washed extensively in PBS between each antibody. Tetramethylrhodamine isothiocyanate (TRITC)-conjugated wheat germ agglutinin (Sigma Chemical) was used at a dilution of 1:50 to identify the Golgi. Fluorescence staining was observed on an Olympus fluorescence microscope.

Electron Microscopy

Forty hours post-transfection, COS cell monolayers were fixed in cacodylate-buffered 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in graded series of alcohols, and embedded in Spurr low-viscosity embedding medium (Ladd Research Industries, Burlington, VT). Ultrathin sections of cells were cut with a diamond knife on a Sorvall MT 6000 ultramicrotome. The sections were stained with uranyl acetate and lead citrate and then examined in a Philips EM 301 electron microscope at 80 kV.

RESULTS

Production of a Rodent Specific Anti-BiP Antisera

Rabbits were immunized with an 11-amino acid peptide, GEEDTSEKDEL, that corresponds to the carboxyterminus of hamster BiP (Ting *et al.*, 1987). Inspection of BiP from other species revealed that this sequence is completely conserved in all known rodent BiP proteins (Chang *et al.*, 1987; Haas and Meo, 1988), whereas the human sequence shows a single amino acid deviation: GEEDT*A*EKDEL (Ting and Lee, 1988). On Western blotting analysis, the control rat monoclonal anti-BiP antibody recognized a 78-kDa protein in mouse, hamster, monkey, and human cell lines, whereas the polyclonal anti-peptide antibody only detected BiP in the mouse and hamster cells (Figure 1A). Immunoprecipitation of BiP from hamster BiP-transfected and mock-transfected COS cells demonstrated that the monoclonal antibody precipitated both endogenous monkey BiP (Figure 1B, panel 1, lanes 1–4) as well as the transfected hamster BiP (Figure 1B, panel 1, lanes 2–4; 1ADel and *Kpn*I mutant proteins migrate faster due to deletions). Conversely, the polyclonal anti-peptide antibody recognized transfected hamster BiP but not the endogenous monkey BiP. The small amount of endogenous BiP observed in the 1ADel and *Kpn*I precipitates must be due to its association with the transfected hamster BiP, because no endogenous BiP is immunoprecipitated in the mock-transfected COS cells (Figure 1B). Thus, our anti-peptide antiserum is able to recognize both native and denatured BiP from rodent cells but is incapable of recognizing either form of BiP from primate cells. Apparently the serine in the sixth position of our peptide contributes to the epitope and the substitution of an alanine at this position in the human BiP abolishes the determinant. The sequence of monkey BiP is not known, but we

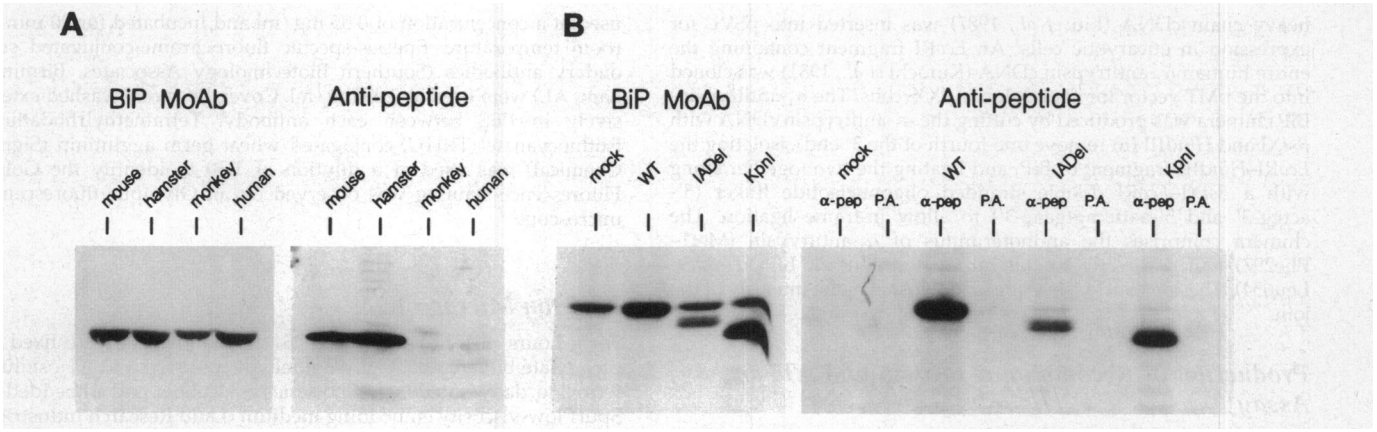

Figure 1. Characterization of species-specific anti-BiP antisera. (A) 2×10^6 cells from mouse (Ag8.653), hamster (CHO), monkey (COS-7), and human (Nalm-6) were lysed and directly applied to 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose and blotted with either a rat monoclonal anti-BiP antibody (BiP monoclonal antibody) or a rabbit polyclonal anti-peptide antisera. (B) Forty hours post-transfection, three dishes of COS cells expressing each hamster BiP construct were labeled with [^{35}S]methionine for 3 h, lysed, and immunoprecipitated with either BiP monoclonal antibody, the polyclonal anti-peptide sera (α-pep), or protein A-Sepharose alone (P.A.). Precipitated proteins were analyzed on 10% SDS-polyacrylamide gels and visualized by fluorography.

assume that it either does not have a serine at position six or has another substitution in this region.

Binding of Hamster BiP ATPase Mutants to γ Heavy Chains Co-expressed in COS Cells

We previously showed that the ATPase inactive BiP 1ADel mutant binds to Ig heavy chains that are co-expressed in COS cells, demonstrating that the deletion did not result in a badly misfolded protein and that ATPase activity is not required for protein binding (Gaut and Hendershot, 1993). Further, the 1ADel protein could not be released from heavy chains in vitro with exogenous ATP. The ATPase point mutants could not be analyzed by the same method because they co-migrate with endogenous BiP and the monoclonal antibody used in that study does not distinguish transfected BiP from endogenous BiP. However, our species-specific polyclonal sera allowed us to examine the association and dissociation of point mutants with Ig heavy chains. COS cells were cotransfected with cDNAs for γ heavy chains together with either wild-type (WT), 1ADel, T37G, or T229G BiP. The latter three were all shown previously to have essentially no ATPase activity when analyzed at 25 μM ATP (Gaut and Hendershot, 1993). Immunoprecipitation of labeled cell lysates with anti-γ demonstrated that all four BiP proteins bound to γ heavy chains (Figure 2). Because these heavy chains bind directly to protein A, their presence in the polyclonal anti-BiP immunoprecipitates is not meaningful. Additionally, the amount of endogenous BiP found in the polyclonal anti-BiP precipitated material is due to its association with the γ heavy chains. This lane serves only to demonstrate the size and amount of transfected hamster BiP expressed. ATP addition to the anti-γ precipitates released both transfected WT BiP and endogenous BiP from the γ heavy chains. Conversely, the 1ADel mutant and the point mutants bound to heavy chains but were not released upon ATP addition (Figure 2). The T37G mutant migrates slightly slower than endogenous BiP on this gel (Figure 2) and remains bound after ATP addition,

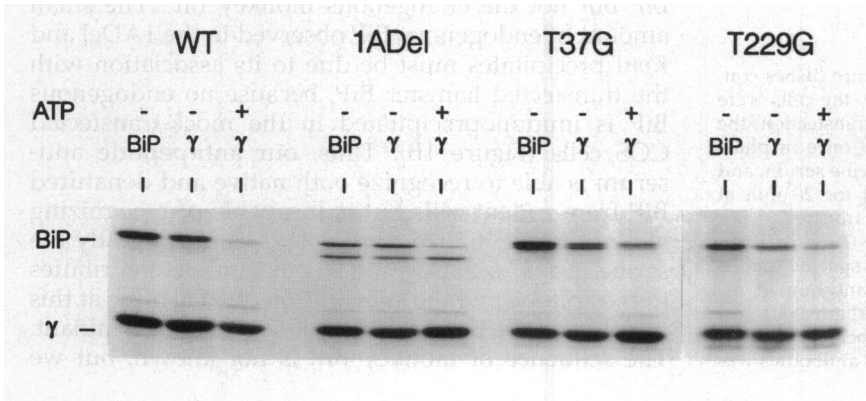

Figure 2. ATPase inactive BiP mutants bind to Ig heavy chains in vivo. Transfected COS cells were metabolically labeled as described in Figure 1. BiP was precipitated from cell lysates using polyclonal anti-peptide sera (BiP) or anti-γ sera. One of the anti-γ precipitates was left untreated (−) and one was incubated with 1 mM Mg-ATP (+) to release the bound BiP. Samples were electrophoretically separated on 10% SDS-polyacrylamide gels and visualized by fluorography.

whereas the endogenous BiP is almost completely released. The presence of hamster BiP in these coprecipitating bands was confirmed by eluting the proteins from this portion of the gel and immunoprecipitating with the polyclonal anti-peptide antisera (our unpublished data).

In Vivo Effects of Expression of BiP ATPase Mutants

The in vivo effects of mutant BiP expression were monitored by immunofluorescence staining of COS cells transiently expressing either WT hamster BiP or one of the ATPase mutants. Microscopic inspection of dual labeled BiP-transfected COS cells revealed a staining pattern for transfected WT BiP identical to that of endogenous BiP in these cells (Figure 3), which looked very similar to the staining pattern seen in nontransfected cells in the same field. This pattern was consistent with an ER localization of hamster BiP. When cells expressing the T37G ATPase mutant were examined in the same way, the mutant BiP appeared to be confined to vesicle-like structures (Figure 3). Some cells displayed a small amount of normal ER staining, but these regions did not contain the ATPase mutant BiP. This organelle disruption apparently did not include the Golgi apparatus because normal Golgi was observed in both WT and mutant BiP-expressing cells, when they were stained with wheat germ agglutinin (Figure 3). Normal Golgi was also observed in EM micrographs of these cells (our unpublished data).

To conclusively identify the structure disrupted by mutant BiP expression, we examined the transfected COS cells by electron microscopy. Routinely, about 10% of the COS cells expressed the transfected BiP as determined by immunofluorescence staining. COS cells expressing the T37G mutant were easily identified by electron microscopy. We found cells full of small vesicles at a frequency of approximately 1 in 10. The membranes of these vesicles were studded with ribosomes, suggesting that they were ER derived and still active in the translation and translocation of proteins (Figure 4, B and C). In most cells, the rough endoplasmic reticulum (RER) appeared rounded and stubby (Figure 4B), however, in some cells the RER vesicles were quite large and dilated (Figure 4C). The cisternae of the vesicles contained electron dense material, which is consistent with the accumulation of protein in them. Cells expressing WT hamster BiP protein were also readily identified by the presence of a much more clearly defined RER. Aside from the increased amount of RER, these cells looked quite normal (Figure 4A). Even after scanning many fields on the grid, we did not detect any cells with RER resembling that seen in T37G BiP mutant-expressing cells.

The T37G and T229G BiP mutants were defective in ATP hydrolysis and in the release of target proteins. Bacterially expressed T37G and T229G mutants appeared identical to WT BiP in terms of their sedimentation on sucrose gradients, their ability to form dimers and monomers, their proteolytic patterns in the presence of ADP, and their binding to peptide-sepharose beads (our unpublished data). However, it was still possible that the point mutations subtly change the conformation of the BiP mutants causing them to aggregate in the ER. Alternatively, the binding of enzymatically inactive BiP to nascent proteins in the ER could be responsible for the ER dilation. To resolve this, we constructed these point mutations on the background of a BiP deletion mutant that contained only the ATP binding domain so binding of the mutant BiP to target proteins could not occur. We also made a hybrid protein expressing the ER-targeting sequence of $\alpha_1$antitrypsin and a protein binding region of BiP. In theory, this hybrid protein should be translocated into the ER and bind to BiP target proteins. However, because it lacks an ATP binding domain, this BiP hybrid should remain bound to target proteins.

Expression of the BiP ATP Binding Domain Alone Does Not Cause ER Disruption

An aminoterminal 44-kDa proteolytic fragment of hsc70 can be generated with chymotrypsin in the presence of ADP. This fragment still has full ATPase activity but no longer binds to clathrin (Chappell *et al.*, 1987). We constructed a BiP ATP binding domain mutant (termed 44K) that included the region of hamster BiP corresponding to the hsc70 proteolytic fragment (amino acids 1–425) followed by the last 15 amino acids of BiP (amino acids 640–654). The C-terminal amino acids should allow the mutant protein to be retained in the ER and to be recognized by our polyclonal antisera. The T37G substitution was engineered onto this ATP binding domain mutant generating a 44K-T37G mutant. Recombinant protein was isolated from the 44K and 44K-T37G constructs and assayed for ATPase activity at saturating ATP concentrations. The 44K mutant had approximately three times the ATPase activity of wild-type protein (318 ± 2.5%) (Figure 5). A similar increase was seen when the ATPase activity of the purified 44-kDa proteolytic fragment of hsc70 was compared with that of full-length hsc70 (Chappell *et al.*, 1987). The recombinant 44K-T37G protein had only 7.5 ± 0.7% of WT BiP ATPase activity or approximately 2.5% of 44K recombinant BiP's ATPase activity (Figure 5), suggesting that this protein should behave as an ATPase mutant in vivo regardless of ER concentrations of ATP.

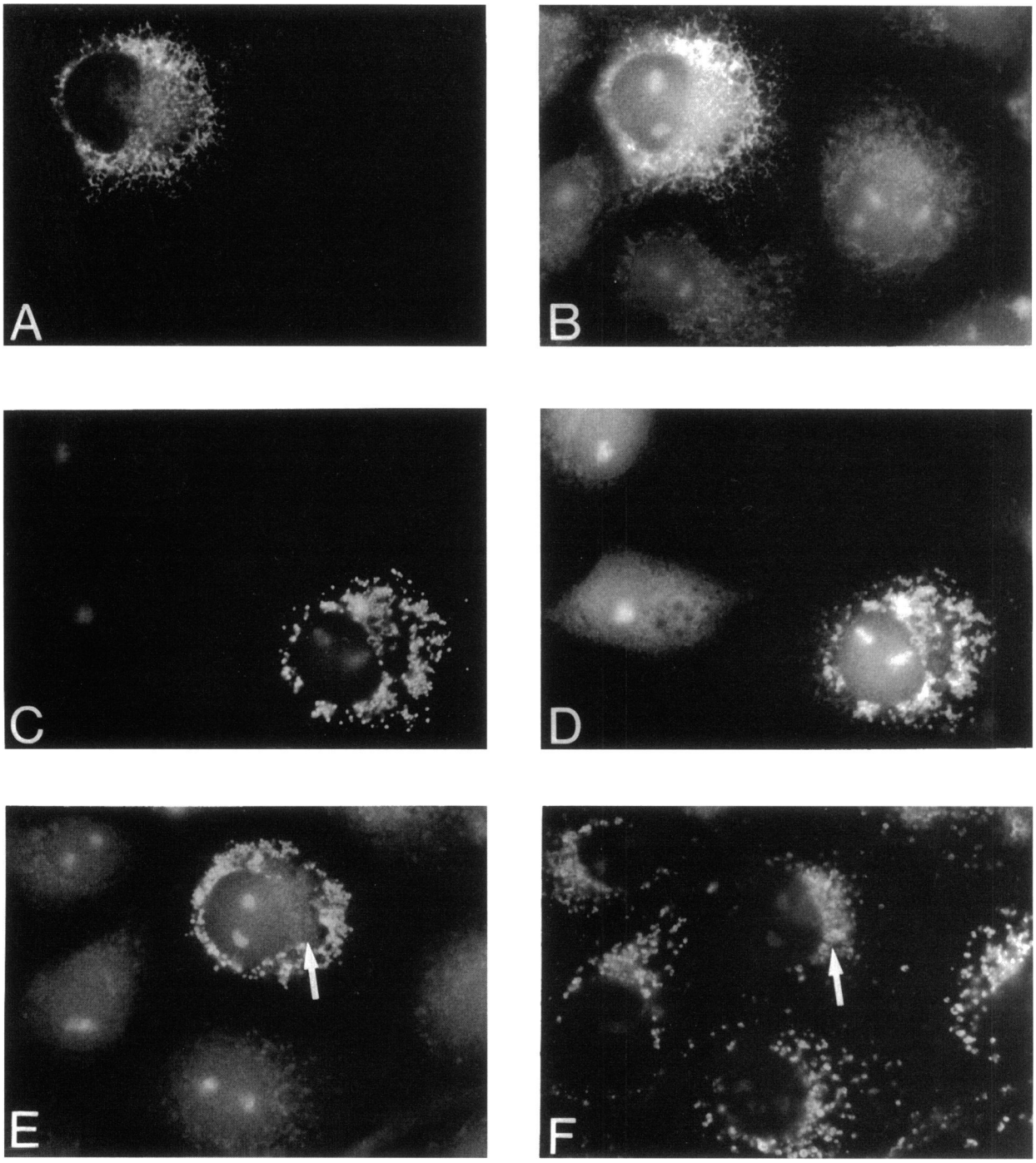

Figure 3. ATPase mutants are localized to vesicles that contain endogenous BiP. COS cells were grown on coverslips and then transfected with wild-type hamster BiP (A and B) or T37G mutant BiP (C, D, E, and F). Transfected cells were fixed and then stained with polyclonal anti-BiP and TRITC-conjugated anti-rabbit sera (A and C) followed by monoclonal anti-BiP and FITC-conjugated anti-rat sera (B and D). T37GBiP-transfected COS cells were stained with anti-grp78 (Affinity Bioreagents) and FITC-conjugated anti-rabbit sera (E) followed by TRITC-conjugated wheat germ agglutinin to detect the Golgi (F). The arrows in A and C denote the Golgi.

The 44K and the 44K-T37G mutants were transiently expressed in COS cells together with γ heavy chains to determine their protein binding capability. Transfected γ heavy chains bind directly to protein A-Sepharose so their presence in the anti-BiP precipitates is not meaningful. In cells expressing wild-type

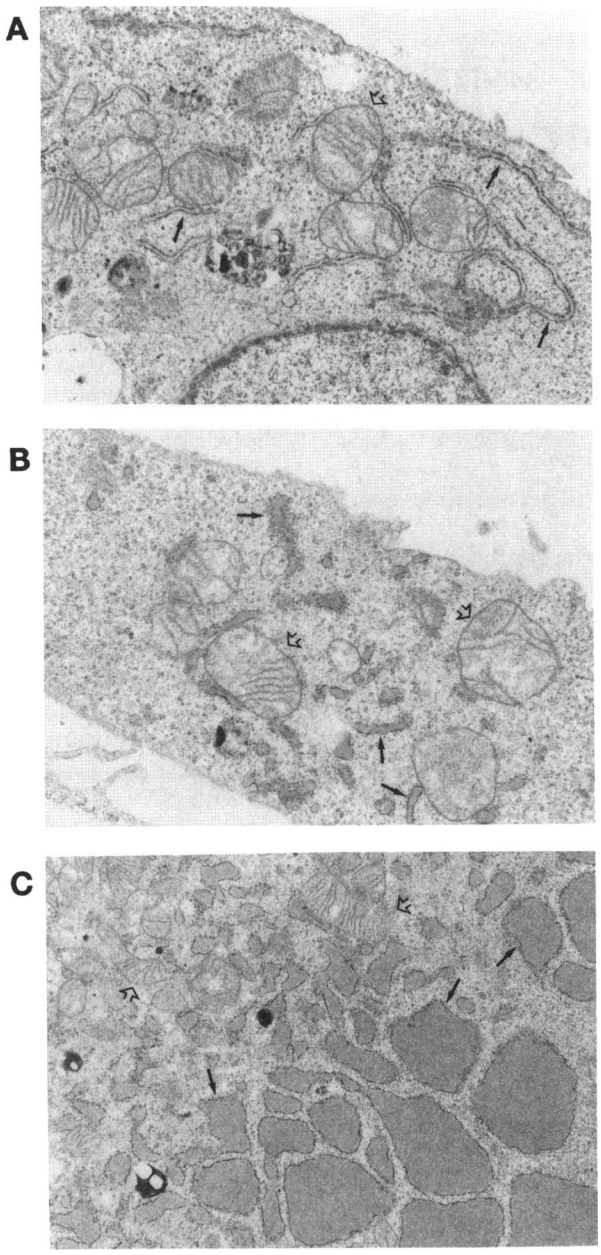

Figure 4. In vivo expression of ATPase mutant results in ER vesiculation. COS-transfected cells were fixed and embedded in Spurr low-viscosity embedding medium. Ultrathin sections were stained in uranyl acetate and lead citrate. Single arrows identify RER and double arrows indicate mitochondria. (A) WT hamster BiP-transfected; (B and C) T37G BiP-transfected.

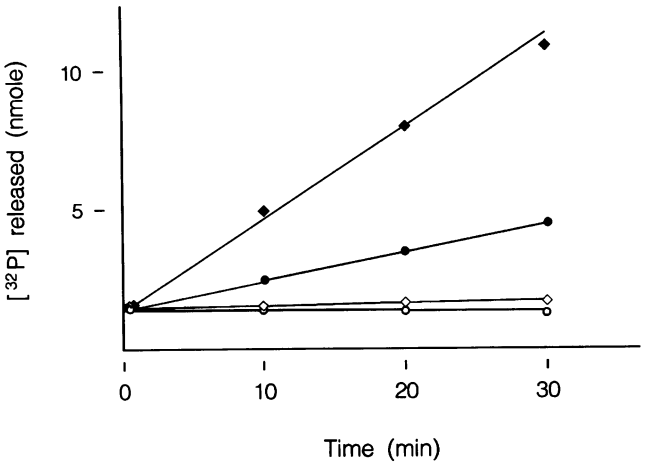

Figure 5. 44K aminoterminal fragment of BiP possesses increased ATPase activity, which is abolished with T37G substitution. Two micromolar recombinant BiP or BiP mutants were added to the ATPase reaction mixture containing 1 mM ATP, and at 0, 10, 20, and 30 min a 20 μl aliquot was removed, liberated [^{32}P]phosphate was extracted, and nanomoles of released phosphate were calculated. Wild-type (●), 44K (◆), 44K-T37G (◇), and BSA (○). Data from three or more assays were averaged.

hamster BiP and γ heavy chains, the anti-γ antibodies co-precipitated BiP as expected (Figure 6A). Both mutant proteins (apparent molecular weight ~50K) and the γ heavy chains migrate with similar mobilities on denaturing gels making it very difficult to determine whether or not the mutants bound to γ heavy chains (Figure 6A). The 78-kDa protein co-precipitated with the γ chains in both cases represents endogenous monkey BiP. To circumvent the co-migration problem, we repeated this experiment with unlabeled cells. After electrophoresis, the proteins were transferred to nitrocellulose and blotted with the polyclonal anti-BiP antisera. The antibody recognized BiP in both the anti-BiP and the anti-γ immunoprecipitates from cells transfected with wild-type BiP (Figure 6B). However, in the 44K– and 44K-T37G–expressing cells, the antibody only detected BiP in the anti-BiP–precipitated material. No 44K or 44K-T37G proteins were co-precipitated with γ heavy chains, demonstrating their inability to bind to this target protein (Figure 6B). Clearly the protein binding domain of BiP is not included within the first 425 amino acids or the last 15 amino acids of BiP. Additionally, these data verify that the functional effects of the ATPase-inactivating point mutations can be assayed independently of protein binding effects.

The wild-type, 44K, and 44K-T37G mutants were transiently expressed in COS cells and examined for their ability to distort the ER. Immunofluorescence staining of the cells expressing the 44K mutant was indistinguishable from that of cells expressing WT BiP (Figure 7). The staining pattern completely overlapped with that for endogenous BiP demonstrating that the 44K protein was expressed in the ER. Furthermore, expressing the ATPase domain alone apparently had no deleterious effects on the cell. The immunofluorescence staining pattern of the 44K-T37G mutant showed the same "normal" ER staining. Evidently the point mutation itself did not

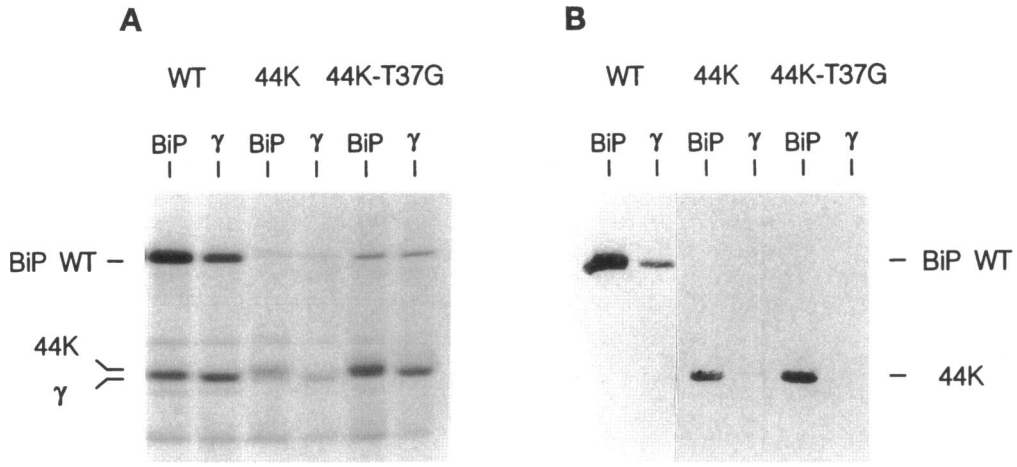

Figure 6. The BiP ATPase domain does not bind to substrate proteins. (A) Forty hours post-transfection, COS transfectants were ^{35}S-labeled and immunoprecipitated with either polyclonal anti-BiP or anti-γ to precipitate heavy chains, and then analyzed on 10% SDS gels. (B) Forty hours post-transfection, unlabeled COS transfectants were immunoprecipitated and analyzed as in A. After electrophoresis, proteins were transferred to nitrocellulose and blotted with polyclonal anti-BiP peptide antisera.

cause aggregation or instability of BiP leading to ER distortion. Additionally, these data suggest that the ER-modified phenotype is caused not by some alteration in the ER ADP/ATP ratio by the ATPase mutants but by the binding of BiP to target proteins as an inactive chaperone.

Expression of the Protein Binding Domain of BiP on a Heterologous Protein Produces ER Phenotype

If this hypothesis is correct, expression of the BiP protein binding domain in the absence of an ATP binding domain should cause the same phenotype, because this type of mutant BiP should still bind to target proteins but would be unable to release them. The actual protein binding domain of hsp70 family members is poorly defined. However, guided by deletional analyses of various hsp70 proteins (Munro and Pelham, 1984; Chappell *et al.*, 1987; Milarski and Morimoto, 1989), secondary structural predictions (Chou and Fasman, 1974), and convenient restrictions sites in the hamster BiP cDNA clone (Ting *et al.*, 1987), we identified a potential protein binding region in BiP. To target this protein to the ER lumen, we constructed an $\alpha_1$antitrypsin-BiP hybrid protein, comprised of the aminoterminus of $\alpha_1$antitrypsin (amino acids 1–296) tethered to the carboxyterminus of BiP (amino acids 485–654). This hybrid protein should be targeted to the ER lumen, be retained there, and be immunoprecipitated with antibodies specific for both $\alpha_1$antitrypsin and BiP.

COS cells were cotransfected with cDNAs for wild-type BiP, $\alpha_1$antitrypsin, or $\alpha_1$antitrypsin-BiP together with the cDNA for γ heavy chains. Labeled lysates were divided and immunoprecipitated with antibodies specific for BiP, $\alpha_1$antitrypsin, or γ heavy chains. In cells co-expressing BiP and heavy chains, the anti-γ antibody co-precipitated BiP as expected (Figure 8B). The anti-$\alpha_1$antitrypsin antibody appears to have precipitated BiP and γ because of the direct binding of the γ heavy chains to protein A and does not imply any cross-reactivity of $\alpha_1$antitrypsin antisera with either BiP or γ heavy chains. When $\alpha_1$antitrypsin was co-expressed with γ heavy chains, there was no detectable association of the two proteins even when $\alpha_1$an-

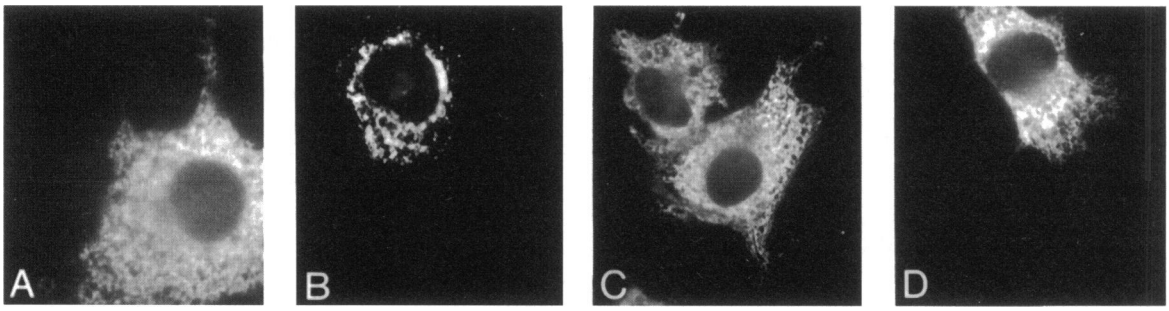

Figure 7. ATPase mutant that cannot bind to proteins in the ER does not cause ER disruption. Fixed cells were stained with anti-grp78 sera followed by TRITC-conjugated anti-rabbit sera. (A) Wild-type hamster BiP transfectants; (B) T37G transfectants; (C) 44K-transfected cells; and (D) 44K-T37G-transfected cells.

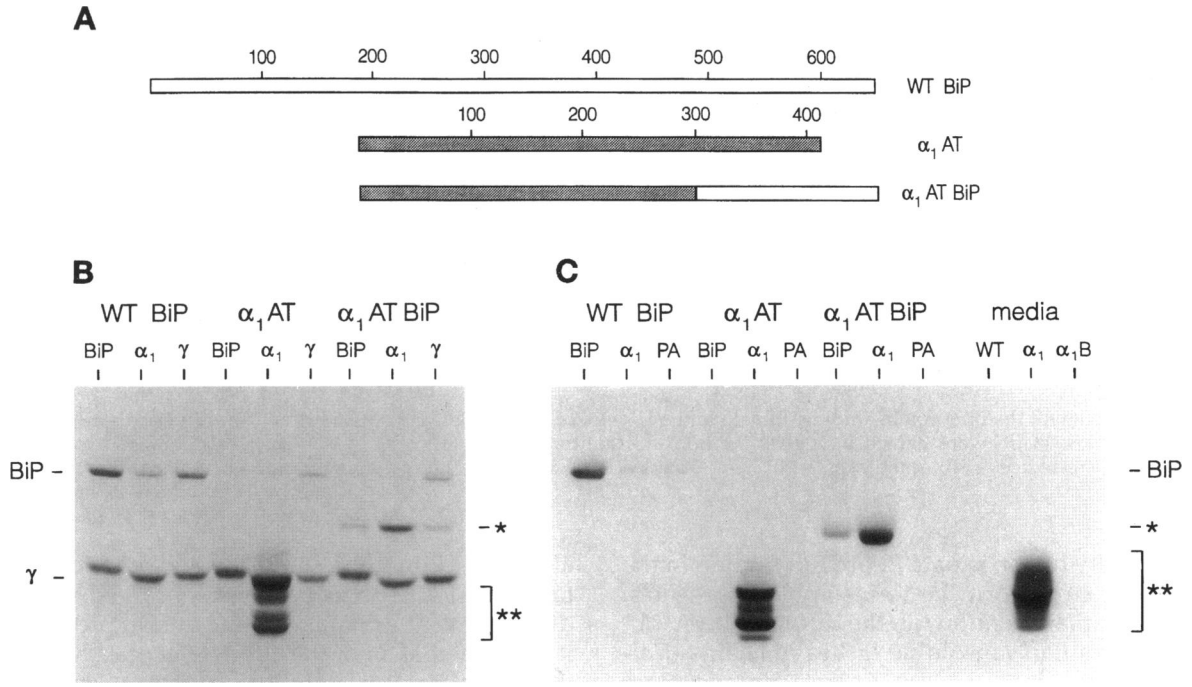

Figure 8. Transfer of BiP protein binding activity to heterologous protein. (A) Stick diagrams representing BiP, $\alpha_1$antitrypsin, and an $\alpha_1$antitrypsin-BiP hybrid protein. The hybrid was constructed using the aminoterminal three-fourths of $\alpha_1$antitrypsin and the carboxyterminal one-fourth of BiP. (B) Three dishes of COS cells transfected with cDNAs encoding hamster BiP, $\alpha_1$antitrypsin, or $\alpha_1$antitrypsin-BiP in addition to γ heavy chain were labeled, lysed, and immunoprecipitated with polyclonal anti-BiP, anti-$\alpha_1$antitrypsin, or antiγ antisera. Precipitated proteins were analyzed and visualized as described in the legend to Figure 2. (C) COS cells were transfected, labeled, and analyzed as described in B except that the cDNA for γ heavy chain was not included in the transfection. Media from the labeling was saved and precipitated with a combination of polyclonal anti-BiP and anti-$\alpha_1$antitrypsin sera. Lettering at the top of the panel for the media refers to the transfected cDNA; hamster BiP (WT), $\alpha_1$antitrypsin (α_1), and $\alpha_1$antitrypsin-BiP (α_1B). Small lettering for B and the rest of C refers to the antibody used for immunoprecipitation; the large lettering denotes the transfected cDNA. The single asterisk denotes the location of $\alpha_1$antitrypsin-BiP, and the double asterisk indicates $\alpha_1$antitrypsin.

titrypsin (**) was over-expressed compared with the other proteins. The anti-γ antisera co-precipitated some endogenous monkey BiP from the cells (Figure 8B). In cells co-expressing the $\alpha_1$antitrypsin-BiP hybrid protein (*) and γ chains, we found that the γ chains were associated with both endogenous BiP and the hybrid protein. The exclusion of γ heavy chains from the transfections demonstrated that the precipitation of $\alpha_1$antitrypsin-BiP with the anti-γ antibody was dependent upon the presence of heavy chains, thus showing that the association was specific (Figure 8C). These data demonstrate that the carboxyterminal 169 amino acids of BiP contain a sufficient portion of the protein binding domain to allow transfer of this activity to another protein, and that the ER retention signal expressed on this hybrid protein is adequate to block secretion of the hybrid (Figure 8C).

Because the $\alpha_1$antitrypsin-BiP protein could bind target proteins, but possessed no ATPase activity, it should remain bound to target proteins and unable to catalyze their folding or assembly. Immunofluorescence staining of cells expressing $\alpha_1$antitrypsin with the anti-$\alpha_1$antitrypsin antibody resulted in a diffuse staining pattern that filled most of the cytoplasm (Figure 9A). This suggested localization to both the ER and Golgi, an anticipated result because $\alpha_1$antitrypsin protein is secreted from these cells (Figure 8C). When COS cells expressing $\alpha_1$antitrypsin-BiP were examined in the same way, we found that the hybrid protein was localized to vesicles very similar to those observed with the BiP ATPase mutants and was excluded from the perinuclear area that contains the Golgi (Figure 9B). This is consistent with ER retention of the hybrid protein and is further supported by the lack of secretion of the hybrid (Figure 8C). These data, coupled with the ATPase domain mutants, confirm that the phenotype we observed with the BiP ATPase mutants was due to BiP binding to target proteins as an inactive chaperone.

ER Disruption Is Not Due to Insolubility of ATPase Defective BiP Molecules

In an initial attempt to understand what may be causing the ER breakdown, we examined whether either the individual point mutations in the ATPase mutants

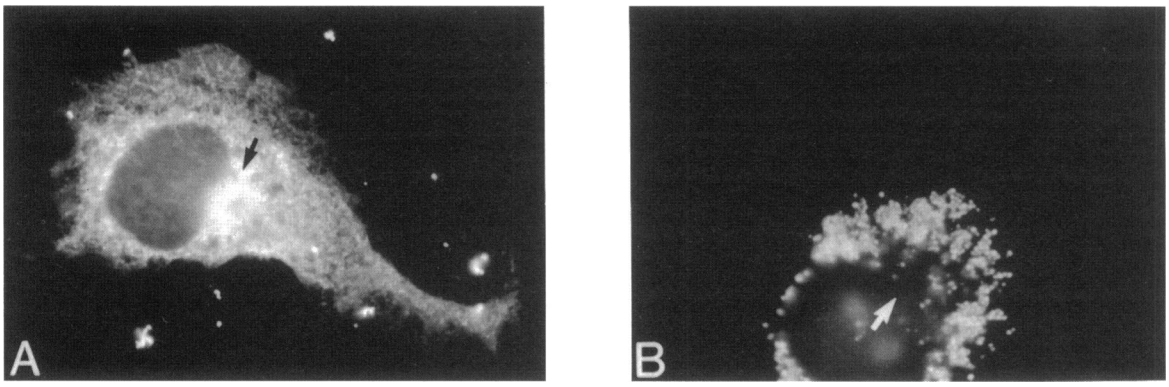

Figure 9. Expression of the BiP protein binding domain in the absence of an ATP binding domain results in the vesiculated ER phenotype. Fixed COS cell transfectants were stained with goat anti-human $\alpha_1$antitrypsin sera followed by FITC-conjugated rabbit anti-goat sera. (A) $\alpha_1$antitrypsin-transfected cells; (B) $\alpha_1$antitrypsin-BiP hybrid-transfected cells. The Golgi region is indicated by an arrow.

or their inability to participate in protein folding in the ER caused them to form large insoluble aggregates. Somewhat similar dilation of the ER has been observed when mutant, insoluble Ig heavy chains (Valetti *et al.*, 1991), low density lipoprotein receptors (Pathak *et al.*, 1988), or $\alpha_1$antitrypsin molecules (Dycaico *et al.*, 1988; Graham *et al.*, 1990) are expressed. COS cells transiently expressing the WT, T37G, T229G, 44K, 44K-T37G, and $\alpha_1$antitrypsin-BiP proteins were lysed in NP-40 buffer and pelleted at 12,000 $\times$ *g*. The supernatants were retained on ice while the pellets were solubilized with sodium dodecyl sulfate (SDS) and then diluted in NP-40 buffer. Both supernatants and solubilized pellets were then immunoprecipitated. All transfected proteins were found only in the NP-40 soluble fraction and not in the SDS-soluble fraction (Figure 10B). This is not because the antibody is incapable of recognizing SDS-solubilized proteins. WT hamster BiP could be immunoprecipitated after SDS denaturation (Figure 10A). In fact, after screening many other point mutants and larger deletion mutants, we have only found one mutant BiP protein that tends to form insoluble aggregates. This demonstrates that the mutation itself is not grossly destabilizing to any of these proteins.

DISCUSSION

Here we describe the first evidence that mammalian BiP mutants, identified in vitro as ATP binding/hydrolysis mutants, have profound effects on the integrity of the ER when expressed in mammalian cells. Given the nonphysiological conditions that are required for optimal in vitro ATPase activity, it was unclear whether in vitro-identified mutants would behave as mutants in vivo. Several lines of evidence suggest that the observed phenotype results from the altered ATP binding/hydrolysis activity of these mutants. First, the phenotype was observed with several different point mutants and the hybrid protein making it unlikely that all of the altered residues had the same effects on protein stability. Structural data have recently been reported for eight different hsc70 ATPase mutants (two of which have mutated residues that correspond to residues altered in BiP) and indicate that none of these eight mutations significantly altered the structure of the ATP binding domain (O'Brien and McKay, 1993; Flaherty *et al.*, 1994). Second, expressing the T37G mutation on the background of the ATP binding domain alone did not cause the phenotype, suggesting that ER vesiculation is coupled to both the disruption of enzymatic activity and protein binding. Finally, the observation that the BiP "protein binding domain" tethered to the aminoterminus of a secretory pathway protein lacking ATP binding capabilities also caused ER vesiculation argues against the amino acid substitutions alone being responsible for the phenotype. Taken together, these results strongly suggest that the phenotype is a result of BiP binding to proteins as an inactive chaperone.

Although in vivo expression of mammalian BiP mutants has not been previously described, analogous mutants in bacteria (dnaK) and yeast (Kar2) homologues have been reported. The dnaK mutants affect ATP hydrolysis or ATP-mediated release of bound peptides and act as dominant negative mutants when expressed with wild-type dnaK (Wild *et al.*, 1992; Buchberger *et al.*, 1994). Some yeast BiP mutants map to the ATP binding domain, although their effect on ATP binding or hydrolysis has not yet been determined (Vogel *et al.*, 1990). In yeast, BiP has been shown to be an essential protein and the BiP mutants are often lethal (Rose *et al.*, 1989). Similarly, we have been unable to obtain stable cell lines producing these BiP ATPase mutants. Given the profound effects of

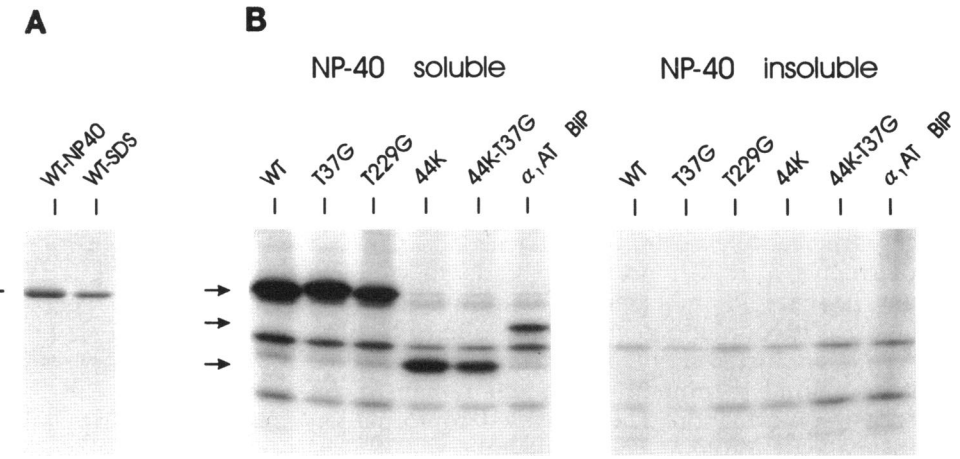

Figure 10. ER breakdown is not due to gross insolubility of ATPase mutants. (A) ^{35}S-labeled COS cells expressing WT hamster BiP were either lysed in NP40 (lane 1) or in SDS (lane 2) buffer, diluted, and then immunoprecipitated with the polyclonal anti-peptide antisera. (B) COS cells were transfected with cDNAs for wild-type hamster BiP, T37G, T229G, 44K, 44K-T37G, or $\alpha_1$antitrypsin-BiP mutants, labeled, lysed, and analyzed as above (NP-40 soluble). Additionally, the 12,000 × *g* pellet obtained during lysate clarification was solubilized in 1% SDS, diluted, and immunoprecipitated with polyclonal anti-BiP antisera (NP-40 insoluble).

mammalian BiP mutant expression on the ER, this may not be too surprising.

Recent data on dnaK and human hsp70 suggest that the ATP binding capability of hsp70 family members may be functionally more important than ATP hydrolysis (Palleros *et al.*, 1993). Although our previous characterization of the T229G and T37G mutants showed that both were able to bind to ATP-agarose, our initial protease protection studies performed in the presence of ATP suggest that the T37G mutant is either impaired in ATP binding or unable to undergo a conformational change in response to nucleotide binding (our unpublished data). Studies are currently underway to directly measure the nucleotide binding capabilities of our ATPase mutants in an attempt to distinguish these two events; however, given the fact that none of our ATPase mutants could be dissociated from heavy chains with 1 mM ATP, we must conclude that either nucleotide binding is not sufficient for BiP function or these mutants are defective in coupling nucleotide binding to protein release.

Understanding why BiP ATPase mutants cause a distortion of the ER is a more interesting and complicated question. Its answer should ultimately provide the most information regarding mammalian BiP function in the ER. Yeast BiP is essential for protein translocation into the ER through its interactions with sec63p, a dnaJ-like protein (Vogel *et al.*, 1990; Sanders *et al.*, 1992). Conceivably, a mammalian BiP ATPase mutant that binds to nascent proteins but fails to release them could block translocation pores. The cell might "pinch off" these blocked regions in an effort to maintain the integrity of the ER. In our experiments, cotransfected heavy chains were glycosylated normally, as was the α_1 antitrypsin-BiP protein, which contains three glycosylation sites (Figures 2 and 8). These data suggest that either mammalian BiP is not involved in translocation or sufficient amounts of endogenous BiP are present to allow translocation to proceed normally; however, we cannot rule out the possibility that translocation is partially impaired in the BiP mutant-producing cells and that the nontranslocated products are degraded too rapidly to be detected.

If BiP's primary function is to bind nascent proteins, keeping them soluble and preventing incorrect interactions, an ATPase mutant should retain this function. Our data suggest that the ATPase mutants bind to target proteins at levels similar to those observed for wild-type BiP. Additionally, both the ATPase mutants themselves (Figure 10), and the γ heavy chains that are associated with them (our unpublished data) are soluble. If it is not the binding of BiP but its failure to release target proteins that causes the defect, then the stable binding of substrate proteins to BiP should result in their blocked or inefficient secretion. Depending on the cell's secretory capacity and the magnitude of nascent proteins that bind to BiP, this block could be sizable. An accumulation of such proteins could result in distortion of the ER architecture. COS cells are not highly secretory cells, making studies on the secretion of endogenous proteins limited. However, when proteins known to associate transiently with BiP are coexpressed with ATPase mutants, their secretion is reduced (our unpublished data).

Because the proteins we expressed (e.g. BiP and γ heavy chains) are not secreted, the observed phenotype might be caused by the transfected cell's inability to deal with the mutant protein. If BiP functions in the ER degradation of associated proteins, an inactive BiP protein would affect ER degradation processes. Some mutant proteins that accumulate in the ER can form insoluble aggregates that are not efficiently degraded (Pathak *et al.*, 1988; Graham *et al.*, 1990; Valetti *et al.*, 1991). These insoluble aggregates cause extreme dilation of the ER. Stable cell lines can be made from

plasmacytoma cells with this phenotype (Alanen *et al.*, 1985), wherein the mutant Ig molecules are not secreted but other nonmutant proteins are (Valetti *et al.*, 1991). This suggests that mechanisms exist to sequester regions of the ER that harbor insoluble aggregates yet retain a secretory pathway. Our data show that the BiP mutants do not form this type of insoluble aggregate; however, if proteins entering the ER contain multiple BiP binding sites, it is possible that some rather large complexes are being formed.

Alternatively, if BiP normally binds to proteins when they are in an extended conformation and, through cycles of binding and release, allows them to fold into an increasingly compacted conformation, proteins bound to mutant BiP might remain in an extended conformation. This extended conformation might make the proteins better substrates for ER proteases, and the dilated areas may represent regions of increased degradative activity. Preliminary data suggest that both the mutants causing the phenotype and associated γ heavy chains are turned over more rapidly than wild-type BiP and associated heavy chains. More extensive studies on turnover of mutant BiP and associated proteins are underway.

Very recently, Latterich and Schekman demonstrated that yeast BiP is required for homotypic ER membrane fusion during karyogamy; depletion of BiP or expression of mutant BiP resulted in membranes that could not participate in fusion reactions (Latterich and Schekman, 1994). The authors of this report hypothesize that yeast BiP is acting either directly or indirectly on an ER membrane protein that is required for membrane fusion. If mammalian ER membranes contain similar proteins required for ER fusion after cell division, one can imagine how failure to fold or assemble these proteins in cells expressing ATPase-defective BiP could cause a similar defect that would result in a vesiculated ER. The time frame of transient expression in our experiments allows for at least one cell division.

In conclusion, a unique epitope in the C-terminus of hamster BiP enabled us to generate an antisera that specifically recognizes transfected hamster BiP in COS monkey cells. Our studies demonstrate that in vitro-identified BiP ATPase mutants behave as mutants when expressed in cells. This was not entirely anticipated because the conditions for optimal ATPase activity in vitro do not exist in the ER. The in vivo synthesis of mutant BiP results in a phenotype of ER vesiculation. This phenotype is not due to some alteration of ER ATP:ADP ratios, nor is it due to gross instability of the mutants. Rather, it is dependent on BiP binding to target proteins and evidently failing to release them. The enzymatically inactive BiP can still bind to target proteins, keeping them soluble and retaining them in the ER. Determining the cause of ER distortion in these cells should provide us with a much clearer understanding of BiP function in mammalian cells.

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