In the present study, we produced single point mutations in the ATP binding site of hamster BiP, isolated recombinant proteins, and characterized them in terms of their affinity for ATP and ADP, their ability to undergo a conformational change upon nucleotide binding, and their rate of ATP hydrolysis. These analyses allowed us to classify the mutants into three groups: ATP hydrolysis (T229G), ATP binding (G226D, G227D), and ATP-induced conformation (T37G) mutants, and to test the role of these activities in the in vitro ATP-mediated release of proteins from BiP. All three classes of mutants were still able to bind peptide demonstrating that nucleotide is not involved in this function. Addition of ATP to either wild-type BiP or the T229G mutant caused the in vitro release of bound peptide, confirming that ATP hydrolysis is not required for protein release. ATP did not dissociate G226D, G227D, or T37G mutant BiP-peptide complexes, suggesting that ATP binding to BiP is not sufficient for the release of bound peptides, but that an ATP-induced conformational change in BiP is necessary. The identification of BiP mutants that are defective in each of these steps of ATP hydrolysis will allow the in vivo dissection of the role of nucleotide in BiP's activity.

The heat shock protein 70 (HSP70) family of chaperones are components of the cellular machinery for folding, assembly, and degradation of proteins (1). These proteins are thought to undergo cycles of nucleotide-mediated binding and release to unfolded polypeptides. The binding of peptides to HSP70 proteins stimulates their ATPase activity (2), and bound peptides or proteins are released with ATP but not with non-hydrotlyzable analogues (2–5). These observations have led to the conclusion that ATP hydrolysis is required for HSP70 activity and that there are functional interactions between the ATP binding and protein binding domains. All HSP70 proteins bind ATP tightly but the ATP hydrolysis rates of purified proteins are so low under physiological conditions, that other co-factors may be required to enhance the rate of ATP hydrolysis. In bacteria two co-factors, dnaJ and grpE, have been identified that together increase the ATPase activity of dnaK (bacterial HSP70 homologue) up to 50-fold (6). Although dnaJ homologues have been identified in several organelles in various organisms (7), the only euakaryotic grpE homologues found thus far are mitochondrial (8, 9). Alternatively, it is possible that ATP binding rather than ATP hydrolysis is essential for HSP70 function. In support of this hypothesis, investigators have recently demonstrated that peptides are released from both dnaK and hsc70 (mammalian cytosolic homologue) after ATP binding but before ATP hydrolysis occurs (10). Thus, it is presently unclear whether ATP binding, ATP hydrolysis, or both are important for HSP70 function in vivo.

In Vitro Dissociation of BiP-Peptide Complexes Requires a Conformational Change in BiP after ATP Binding but Does Not Require ATP Hydrolysis*

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protein. A rigorous biochemical characterization of these mutants has not been done.

Thus, there is a growing amount of data to demonstrate that either the ATP binding or hydrolysis properties of HSP70 proteins are important in their in vivo function, but in most cases, ATP binding site mutants have not been characterized in enough detail to determine the role of nucleotide in regulating HSP70 activity. In this study, we have produced a series of single point mutants in the ATP binding site of hamster BiP and purified these recombinant proteins from bacteria. Mutants that retained the structural integrity of wild-type BiP were selected for further characterization in terms of ATPase activity, nucleotide binding affinity, and the ability of nucleotide to induce a conformational change in the mutant protein. Through these characterizations, we were able to classify the ATP binding site mutants into three separate groups: ATP binding mutants, ATP hydrolysis mutants, and conformational change mutants. The mutants were used to determine the requirements for the in vitro release of bound peptides.

**Materials and Methods**

Producing BiP Single Point Mutants—The single site mutants (G226D, G227D, and E201K) were made in a hamster BiP cDNA done (24) through directed mutagenesis using an overlap extension polymerase chain reaction approach as described (25). The polymerase chain reaction products were digested with MscI and BstEII, and then inserted back into the BiP cDNA done in place of the wild-type coding region. The mutations were verified by dideoxy DNA sequencing through the entire region that was ligated into the BiP cDNA using a Sequenase kit (U.S. Biochemical Corp.). Each of these cDNA clones was inserted into the QE-10 vector (Oligene, Chatsworth, CA) and expressed in M15 bacteria. Recombinant BiP (rBiP) proteins were purified on Ni2+ agarose as described (16, 33). The T37G and T229G mutants were described previously (16, 22).

Nondenaturing Gel Electrophoresis—2–15% gradient nondenaturing polyacrylamide gels were prepared as described previously (26). 15 μg of purified rBiP was electrophoresed at 4°C for 16 h at 150 V. After electrophoresis, the gel was stained with Coomassie Brilliant Blue to detect proteins.

Proteolysis of Recombinant BiP in the Presence of Nucleotide—Recombinant BiP was digested with proteinase K as described by Kassenbrock and Kelly (11) with some modifications. 10 μg of rBiP was incubated with 2 μg of proteinase K in the presence of 100 μM ATP, 100 μM ADP, or with no added nucleotide in 65 μl of the standard ATPase buffer (20 mM HEPES, 25 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 0.5 mM dithiothreitol, at pH 7.0). After incubation at 37°C for 25 min, the reactions were stopped by adding 10 μl of 1 M phenylmethylsulfonyl fluoride and incubating on ice for 30 min. The digested samples along with an undigested control were analyzed by SDS-PAGE.

ATPase Assay—ATPase assays were performed on purified recombinant protein containing the 6X-His tag as described in the accompanying article (33). The standard assay contains 20 mM HEPES (pH 7.0), 25 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 0.5 mM dithiothreitol, 1 mM Kα3PjATP, and 2 μM rBiP. Reactions were performed at 37°C in a volume of 100 μl and at appropriate times 20-μl aliquots were removed for P i extraction and counted. For characterization of the BiP mutants, 5–6 different recombinant preparations were assayed.

Nucleotide Binding Measurement—The binding affinities of wild-type and mutant rBiP proteins for ATP and ADP were determined as described in the accompanying article (33) using equilibrium dialysis. Briefly, site-directed mutagenesis using an overlap extension polymerase chain reaction approach was used. The standard dialysis buffer contained 100 μM ATP, 100 μM ADP, or no added nucleotide in 65 μl of the standard ATPase buffer (20 mM HEPES, 25 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 0.5 mM dithiothreitol, at pH 7.0). After incubation at 37°C for 25 min, the reactions were stopped by adding 10 μl of 1 M phenylmethylsulfonyl fluoride and incubating on ice for 30 min. The digested samples along with an undigested control were analyzed by SDS-PAGE.

RESULTS

**BiP Binding to Peptide and Release by ATP—Peptide C (2) was coupled to cyanogen bromide-activated Sepharose beads (Pharmacia, Sweden) according to the manufacturer’s suggestions. 50 μg of rBiP was incubated with 100 μl of Peptide C beads (1:1 beads:buffer) in 1 ml of ATPase buffer. After rotating at 4°C for 1 h, the beads were washed three times with ATPase buffer containing 0.2 M NaCl. The beads were then divided equally into two halves and incubated in 1 ml of ATPase buffer either with or without 100 μM ATP for 30 min at room temperature. The beads were re-washed in ATPase buffer containing 0.2 M NaCl, resuspended in SDS sample buffer, heated to 100°C for 5 min, and analyzed by SDS-PAGE. Proteins were visualized by Coomassie Blue staining and quantitated by scanning densitometry (BioImage, Ann Arbor, MI).

**Mutations of Residues in the ATP Binding Site—** In order to produce and isolate BiP mutants that were either defective in ATP binding or ATP hydrolysis, our first step was to identify candidate residues to be mutated. Because the ATP binding domains of HSP70 members are highly homologous (1), we previously took advantage of structural data obtained for the hsc70 ATPase domain and mutated several residues in hamster BiP that could potentially participate in either ATP binding or hydrolysis (16). These included Thr37 (Thr41 in hsc70) and Thr229 (Thr204 in hsc70) which lie in close proximity to the γ-phosphate of ATP in the hsp70 structure (Fig. 1), and Glu202 (Glu175 in hsc70) which interacts with the divalent cation required for nucleotide binding. Mutation of these residues to glycine inhibited the ATPase activity of rBiP (16).

Two dominant negative mutations have been identified in Kar2 (yeast BiP homolog) that occur at residues corresponding to Gly226 and Gly227 in hamster BiP. In both cases, the mutation involved a glycine to aspartic acid change. The corresponding residues were identified on the three-dimensional structure of the hsc70 ATPase domain (Fig. 1), and the distances between the α carbon of these glycines and the oxygen group of the γ-phosphate of ADP were measured. Gly226 was 3.66 Å from ADP and Gly227 was 4.09 Å away, suggesting that the substitution of an amino acid with a large charged side chain at either position might have profound effects on nucleotide binding or hydrolysis. All of these structurally and genetically identified residues (Thr37, Thr229, Glu202, Gly226, and Gly227 in BiP) are completely conserved in Escherichia coli, yeast, and mammalian HSP70 family members.

**Mutant BiP Proteins Retain Overall Structural Integrity—** Endogenous BiP (26) and wild-type BiP (27, 33) exist as mono-
The proteins were then visualized by Coomassie Blue stain. The positions of monomers, dimers, and higher order oligomers are marked on the left.

The effect of the various mutations on the ATPase activity of BiP was determined at a concentration of 1.0 mM ATP, the T229G mutant had no detectable ATPase activity, while the other three mutants (G226D, G227D, and T37G) had ATPase activities that were reduced relative to WT BiP (Fig. 3). The actual rates of hydrolysis for the individual mutants varied between different preparations but were reproducible for a single preparation. The variations between preparations are provided for the 30-min time point (Fig. 3). As shown in Table I, the G226D and G227D BiP mutants had decreased $V_{\text{max}} (1.7 \pm 0.2$ and $1.5 \pm 0.2$ pmole/min/mg, respectively) compared to wild-type BiP ($V_{\text{max}} = 5.2$ pmole/min/mg), and their $K_m$ values ($97 \pm 14$ and $93 \pm 17$ M), were significantly increased compared to that of WT BiP ($K_m = 1.5$ M). The finding that the G226D and G227D mutants had elevated $K_m$ values was further supported by the observation that the ATPase activity of these two mutants relative to WT BiP increased as the concentration of ATP was increased from 5 M to 1 mM (data not shown).

Nucleotide Binding Properties of BiP Mutants—The reduced ATPase activity of the various BiP mutants could result from a defect in ATP binding or ATP hydrolysis. To distinguish these two possibilities, we measured the binding affinity of our mutants for ATP and ADP using equilibrium dialysis and the results are summarized in Table I. The T229G and T37G mutants had ATP and ADP binding affinities that were essentially the same as those calculated for wild-type BiP (see Ref. 33). This demonstrated that the impaired ATPase activity of these two mutants was due to their inability to bind ATP but to an inability to hydrolyze it. Perhaps this is not surprising since both of the mutations occur at residues that are close to the $\gamma$-phosphate of ATP and involve the substitution of a glycine which should not hinder the binding of nucleotide in this cleft.

Similar studies on the G226D and G227D mutants revealed that, although both mutants retained some ATPase activity (K$_m$ ~ 100 M), we were unable to measure nucleotide binding by equilibrium dialysis using nucleotide concentrations ranging from 50 to 400 M with 50 M of mutant protein. These binding data were further supported by HPLC analyses of the nucleotide content of these mutants after they were purified from bacteria. Unlike WT BiP and the T229G mutant, which both contained bound ADP that was resistant to dialysis, the G226D and G227D mutants had no detectable nucleotide bound to them (data not shown).

Thus, we were concerned that the ATPase activity observed with G226D and G227D could be due to a trace contamination by a stronger ATP hydrolyzing protein. To rule out the possibility of contamination, we first rebound the recombinant protein to Ni$^2+$-agarose and washed the column with 1 M salt to remove any BiP-associated proteins, and second, we chose several new bacteria colonies expressing each of the mutants. Both the salt-extracted and the new isolates were analyzed for ATPase activity and nucleotide binding. Identical results were obtained: the G226D and G227D mutants still had some ATPase activity but no detectable nucleotide binding capability. Photoaffinity labeling of the G227D mutant with 100 M radioactive azido-ATP demonstrated that this mutant could indeed bind ATP (data not shown), further ruling out the possibility that the ATPase activity came from a contaminating protein. Thus, these two mutants evidently bind ATP, but their affinity for ATP is decreased to such an extent that the binding assay used here is unable to detect this binding.

Protease Digestion of BiP Mutants in the Presence of Nucleotide—BiP and other HSP70 members produce characteristic proteolytic patterns when digested with protease in the presence of ATP versus ADP (11, 12). An NH$_2$-terminal 44-kDa fragment that comprises the nucleotide binding domain of HSP70 proteins is protected from proteolysis when ADP is present in this cleft. Upon ATP binding, a conformational change occurs in the HSP70 protein resulting in the protection of a 60-kDa fragment that includes both the protein binding domain and the ATP binding domain (11). We wished to use this assay to determine if the two BiP mutants with wild-type ATP binding affinity underwent a proper conformational change upon ATP binding and to verify that the ATP binding mutants were unable to protect the 60-kDa fragment. WT and mutant BiP proteins were digested with proteinase K, a non-
specific serine protease, in the presence of ADP, ATP, or no added nucleotide (Fig. 4). In the accompanying paper (33), we demonstrated that WT rBiP exhibited the same proteolytic patterns as native BiP isolated from dog pancreas (i.e. ATP protected both a 60- and 44-kDa fragment, while ADP protected a 44-kDa fragment). Proteolytic digestion of the T229G mutant generated a pattern identical to that observed with WT BiP (Fig. 4). This is consistent with the observation that the T229G mutant binds ATP with the same affinity as WT BiP. Furthermore, it suggests that ATP induced a similar conformational change in this mutant.

ATP did not protect a 60-kDa fragment in either the G226D or G227D mutant, which is in agreement with our inability to demonstrate stable binding of ATP to these mutants. However, the 44-kDa fragment was still protected in the absence of any added nucleotide. HPLC analyses revealed that these mutants did not contain ATP or ADP (data not shown), suggesting that the NH2-terminal ATP binding domain folds compactly even in the absence of nucleotide and is in keeping with data obtained for nucleotide-free WT BiP that was also analyzed (33). Surprisingly, ATP did not protect a 60-kDa fragment in the T37G mutant, even though its ATP binding affinity was normal. This suggests that T37G is unable to undergo the appropriate conformational change upon binding to ATP that leads to changes in the protein binding domain.

Conformational Mutant Is Defective in Peptide Stimulated ATPase—We tested the possibility that the T37G mutant was impaired in its ability to transduce a signal from the ATP binding domain to the protein binding domain by examining the effect of peptide on the ATPase activity of both WT BiP and the T37G mutant (Fig. 5). As expected, we found that the ATPase activity of WT BiP could be stimulated about 2–3-fold by peptide. However, the ATPase activity of T37G was not significantly lowered as estimated from their Km values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained, their binding affinities for ATP were significantly lowered as estimated from their Kd values for these two mutants could not be directly obtained. However, our inability to detect nucleotide binding for the G226D and G227D mutants remains a concern. We checked to see if this could be a result of aggregate formation at the high protein concentrations used for this assay. However, we found that the proteins were completely soluble after equilibrium dialysis and existed as both dimers (~60%) and monomers (~40%). Interestingly, the mutant dimers could not be shifted to monomers by the addition of 200 μM ATP, whereas the WT BiP dimers were readily converted to monomers. Therefore, under the nucleotide binding conditions, the WT BiP was monomeric while these two mutants were largely dimeric (data...
not shown). This difference in molecular forms could further contribute to our inability to measure stable nucleotide binding for the G226D and G227D mutants since BiP dimers may have a lower affinity for ATP (29). In summary, we have been as rigorous as possible in an attempt to resolve the discrepancy between the \( K_m \) measured for these mutants and our inability to detect stable nucleotide binding and can find no obvious technical reason for it. Despite this, all of our assays demonstrate that both mutants are impaired in their ability to bind to nucleotide.

The T229G mutant was described previously as a BiP ATPase mutant (16). The characterization presented here demonstrates that the defect leading to reduced ATPase activity is not due to an inability of this mutant to bind ATP or to undergo a conformational change after binding ATP, but is due to its inability to hydrolyze ATP. The importance of T229 in ATP hydrolysis was anticipated by structural predictions (13, 14). This threonine has been mutated in both dnaK (Thr199) and hsc70 (Thr204). Characterization of the dnaK Thr199 mutants revealed that substitution of an alanine, valine, or aspartic acid at this site greatly reduced the ATPase activity of the mutants (0.6, 3, and 7%, respectively) compared to that measured for WT dnaK (19). These mutants still bind ATP-agarose (19), and the T199A mutant undergoes a conformational change upon ATP binding resulting in the release of substrate protein (10), which is entirely consistent with our characterization of the BiP T229G mutant. Unlike the data obtained for dnaK and BiP, mutation of the corresponding threonine (Thr204) in hsc70 results in an increase of both \( k_{cat} \) and \( K_m \) (21).

HSP70 proteins bind nucleotide in the NH2-terminal domain and polypeptide in the C-terminal domain. ATP causes the release of bound polypeptides and peptide binding can activate the ATPase activity, suggesting that communication between the two domains is important for the functions of HSP70 proteins. The protection of a 60-kDa fragment with ATP versus a 44-kDa fragment with ADP in the presence of proteases (11,12) implies that a conformational change takes place in HSP70 proteins upon ATP binding that affects the protein binding region residing between the 44- and 60-kDa cleavage sites (30). The T37G mutant binds ATP and ADP with normal affinity, but a 60-kDa fragment was not protected during proteolysis in the presence of ATP, suggesting that the ATP-induced conformational change does not occur in the T37G mutant. This uncoupling was further confirmed by the finding that its ATPase activity was not stimulated by peptide. The fact that the \( K_m \) for ATP and ADP were not altered in this mutant suggests that the conformational change in the protein binding domain does not act to stabilize ATP in the pocket, but to transduce a signal from the ATPase domain to the protein binding domain for substrate release. A dnaK transducing mutant was recently described that resulted from the mutation of Glu171 (Glu201 in BiP) to alanine, leucine, or lysine (31) suggesting that there may be several ways to uncouple the ATP-mediated release of proteins from HSP70 members.

A recent report demonstrated that the rate of peptide release from dnaK and hsc70 after ATP binding was faster than the rate of ATP hydrolysis (10), implying that ATP binding, not hydrolysis, triggered the release of peptide. The dnaK T199A mutant is impaired in its ability to hydrolyze ATP, but it is able...
Identification of Three Classes of BiP ATPase Mutants

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REFERENCES

1. Gething, M. J., and Sambrook, J. (1992) Nature 355, 33–45
2. Flynn, G. C., Chappell, T. G., and Rothman, J. E. (1989) J. Biol. Chem. 264, 385–390
3. Munro, S., and Pelham, H. R. (1986) Cell 61, 291–300
4. Liberek, K., Skowrya, Z., Zylicz, M., and Kohn, C., and Georgopoulos, C. (1991) J. Biol. Chem. 266, 14491–14496
5. Pallari, D. R., Welch, W., J., and Fink, A. L. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 5719–5723
6. Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C., and Zylicz, M. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 2874–2878
7. Caplan, A. J., Cyr, D. M., and Douglas, M. G. (1993) Mol. Biol. Cell 4, 555–563
8. Naylor, D. J., Ryan, M. T., Condon, R., Hoogenraad, N. J., and Hqi, P. B. (1996) Biochim. Biophys. Acta 1296, 75–79
9. Ikeda, E., Yoshida, S., Mitsuzawa, H., Uno, I., and Toh-e, A. (1994) FEBS Lett. 339, 265–268
10. Pallari, D. R., Reid, K. L., Shi, L., Welch, W. J., and Fink, A. L. (1993) Nature 365, 664–666
11. Kassenbrock, C. K., and Kelly, R. B. (1989) EMBO J. 8, 1461–1467
12. Chappell, T. G., Konforti, B. B., Schmid, S. L., and Rothman, J. E. (1987) J. Biol. Chem. 262, 746–752
13. Flaherty, K. M., Deluca Flaherty, C., and McKay, D. B. (1990) Nature 346, 623–628
14. Flaherty, K. M., McKay, D. B., Kabsch, W., and Holmes, K. C. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 5041–5045
15. Willbanks, S. M., Deluca Flaherty, C., and McKay, D. B. (1994) J. Biol. Chem. 269, 12893–12898
16. Gaut, J. R., and Hendershot, L. M. (1993) J. Biol. Chem. 268, 7248–7255
17. Wild, J., Kamath Loeb, A., Ziegelhoffer, E., Lonetto, M., Kawasaki, Y., and Gross, C. A. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 7139–7143
18. Vigny, J. P., Misra, L. M., and Rose, M. D. (1991) J. Cell Biol. 110, 1885–1895
19. McCarty, J. S., and Walker, G. C. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 9513–9517
20. McCarty, J. S., and Walker, G. C. (1994) J. Bacteriol. 176, 764–780
21. O'Brien, M. C., and McKay, D. B. (1993) J. Biol. Chem. 268, 24323–24329
22. Gaut, J. R., and Hendershot, L. M. (1993) J. Biol. Chem. 268, 12691–12698
23. Hendershot, L. M., Wei, J.-Y., Gaut, J. R., Lawson, B., Frieden, P. J., and Murti, K. G. (1995) Mol. Biol. Cell 6, 283–296
24. Ting, J., Wooden, S. K., Kriz, R., Kelleher, K., Kaufman, R. J., and Lee, A. S. (1987) Gene (Amst.) 55, 147–152
25. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 51–59
26. Frieden, P. J., Gaut, J. R., and Hendershot, L. M. (1992) EMBO J. 11, 63–70
27. Carlino, A., Toledo, H., Skaleris, D., Delisio, R., Weissbach, H., and Brot, N. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 2081–2085
28. Landry, S. J., Jordan, R., McMacken, R., and Gierasch, L. M. (1992) J. Cell Biol. 119, 63–70
29. Toledo, H., Carlino, A., Vidal, V., Redfield, B., Nettleton, M. Y., Kochan, J. P., Brot, N., and Weissbach, H. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 2505–2508
30. Wang, T. F., Chang, J. H., and Wang, C. (1993) J. Biol. Chem. 268, 26049–26051
31. Buchberger, A., Valencia, A., McMacken, R., Sander, C., and Bukau, B. (1994) EMBO J. 13, 1687–1695
32. Ladjimi, M. M., Benaroudj, N., Fouchaq, B., Batelier, G., and Trinolides, F. (1995) J. Cell. Biochem. 61, 198
33. Wei, J., and Hendershot, L. M. (1995) J. Biol. Chem. 270, 26670–26676

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to release denatured proteins with ATP in the presence of KCI (10). The ability of our ATP hydrolysis mutant, T229G, to be released from peptide with ATP supports this data. The ATP binding-mediated release they described requires Ki and does not occur with NaCl (10). We previously reported that the T229G mutant was defective in peptide release using a NaCl containing buffer (16), but this mutant is released as readily as wild-type BiP when Ki is present in the buffer. This suggests that Ki is required for the ATP-induced conformational change in HSP70 proteins. The same group (10) found that ATP analogues were unable to release bound polypeptides from dnaK or hsc70 which is in agreement with earlier reports (2–5). However, they showed that this was not because ATP hydrolysis is necessary for the release but was because the ATP analogues do not induce a conformational change in the HSP70 proteins (10). Our characterization of the three classes of ATPase mutants allowed us to re-examine this question and to confirm their findings in several different ways. First, the ATP hydrolysis mutant (T229G), that binds ATP with wild-type affinity but does not hydrolyze it, released peptide upon ATP addition with the same apparent kinetics as wild-type protein. Second, the ATP binding mutants (G226D and G227D) were not capable of releasing bound peptide. Finally, we were able to determine that ATP binding per se is not sufficient for peptide release, but that a concurrent conformational change induced by ATP binding is also required.

Most current models on HSP70 protein binding and release portray the ADP-HSP70 complex as peptide binding competent and ATP-HSP70 as incompetent for peptide binding. In vivo these may be the only two forms of HSP70, since the binding affinity of both ATP and ADP are very high. However, our in vitro data on the two nucleotide binding mutants demonstrate that ADP binding is not a requirement for making BiP receptive to peptides. This is further supported by the fact that the protein binding domain of hsc70 (32) and BiP (23), expressed without the ATP binding domain, are able to bind polypeptides.

In vitro systems have provided sophisticated and essential information on nucleotide binding, nucleotide-induced changes, and nucleotide hydrolysis by HSP70 proteins and delineated the requirements for in vitro release of bound proteins. These data have been instrumental in allowing investigators to produce models of how the ATP binding/hydrolysis activities of HSP70 proteins regulate their function in vivo. However, it has not been possible to directly test these models because separating these events in vivo is not feasible. Our identification of BiP ATPase mutants that are defective in the various steps in ATP hydrolysis, coupled with an in vivo expression system and a species-specific antisera to recognize the transfected mutants (23) will allow for the first time the direct testing of the various HSP70 functional models in vivo.