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Specificity of Peptide-Induced Depolymerization of the Recombinant Carboxy-Terminal Fragment of BiP/GRP78

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In the present study, we have used a non-denaturing gel electrophoresis assay to characterize the specificity of the peptide-induced depolymerization process of the isolated recombinant C-terminal domain (C30) of the molecular chaperone BiP, in the presence of specific synthetic peptides and with the neuropeptide Substance P. In the absence of peptidic ligand, C30 self-associates readily into multiple oligomeric species. Upon peptide addition, C30 oligomers convert into dimers, then into monomers. Our data indicate that the algorithm we previously developed to predict putative BiP binding sites in any protein sequence is also a good indicator as to whether a peptide can efficiently induce depolymerization of the C-terminal peptide binding domain and stimulate the ATPase activity of the full-length protein.

BiP is a molecular chaperone resident of the endoplasmic reticulum that belongs to the highly conserved HSP70 family. BiP performs function in protein folding and assembly [1], calcium homeostasis [2], translocation of nascent polypeptides [3], and in retargeting irreversibly misfolded proteins to the cytosol through the retro-translocation process [4]. BiP carries a weak ATPase activity that is stimulated by the binding of unfolded polypeptides onto the C-terminal substrate-binding pocket. It has been shown that BiP binds to specific hydrophobic sequences rich in tryptophan, leucine and phenylalanine exposed in unfolded polypeptides, but buried in the hydrophobic core of native proteins [5, 6]. Recent *in vivo* studies have shown that stable interactions with BiP cannot be detected in polypeptides that rapidly adopt a stable native conformation *in vitro* [7]. One model stipulates that BiP's chaperone activity would mainly be the result of its ability to prevent irreversible aggregation by binding specifically to these hydrophobic sequences prior to the protein folding and assembly processes [8]. A second model has been proposed in which the functions of BiP and of other HSP70s would be specified by interaction with accessory proteins [9–12].

BiP and other HSP70 functions are also regulated through an oligomerization/depolymerization equilibrium [13–18]. *In vivo*, substrate-free BiP is mostly oligomeric, as peptide-bound BiP is monomeric [13]. *In vitro*, ATP-bound HSP70 bind and release substrate with high association-dissociation rates, and upon ATP hydrolysis, HSP70 proteins are locked in stable complex with their substrates [19]. Monomeric BiP species are catalytically more active than oligomeric BiP [14]. We have recently reported that full-length murine BiP is in chemical equilibrium between multiple oligomeric and monomeric species [15]. We showed that the C-terminal domain of BiP was essential for oligomerization and peptide-induced depolymerization [15, 18–19]. To further understand the molecular mechanisms involved in the regulation of BiP activity, we have expressed in *Escherichia coli* the isolated C-terminal domain of BiP (C30). The carboxy-terminal fragment used contains the 18 kDa substrate-binding domain followed by the 10 kDa C-terminal tail, whose function is still unknown. The complete histidine-tagged carboxy-terminal domain oligomerizes into multiple oligomeric species and depolymerizes upon binding to specific short peptides or the neuropeptide Substance P. Our data indicate that peptides predicted to have a high affinity for full-length BiP stimulate efficiently its ATPase activity and induce conversion of the carboxy terminal oligomers into monomeric species. Our data support a model where accumulation of unfolded polypeptides in stressed cells induces depolymerization and stabilization of BiP monomeric species that can then exert its function as molecular chaperone.

Abbreviations used: ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; HSP70, heat shock protein of 70 kDa; PBS, phosphate buffered saline; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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MATERIALS AND METHODS

Plasmids

pC30 and p(His)10-C30. The cDNA encoding the complete C-terminal domain, i.e. C30 (residues 391-636), of murine BiP [20] was amplified by PCR from the plasmid pSecB115 [14] using the primers C19I (5′-GGAATTCCATATGGATACAGGTGATCTG GTA-3′) and pET12-2 (3′-TCGACTCAACCGACGACGGT-5′). The 820 bp PCR product was then cleaved by the restriction enzymes *NdeI* and *BamH1*, purified and subcloned in a dephosphorylated pET12a/*NdeI-BamHI* expression vector (Novagen, Inc.) to yield the pC30 vector. Subsequently, the *NdeI-BamHI* cassettes from pC30 were subcloned in the pET19b vector (Novagen, Inc.) digested with the same enzymes, in frame with an N-terminal ten histidine tag to yield p(His)10-C30 plasmids. The *NdeI-BamHI* cassettes were totally sequenced using two external primers (T7 primer, Promega and pET12-2 described above) and two internal primers (P13 5′-CCGATAATCAGCCAAC-3′; P14, 5′-CCGCCTGACACCTGAAG-3′). No mutations were identified. The plasmid $p(His)_{10}$-BiP, expressing $(His)_{10}$-BiP, was described previously [11].

Protein Purification

(His)10-C30 and (His)10-BiP were expressed in *E. coli* BL21(DE3) cells (Novagen, Inc., Madison, WI), purified by affinity chromatography on Talon resin (Clontech, Palo Alto, CA) or Nickel-agarose (Novagen, Inc., Madison, WI) according to the manufacturer's instructions. Large inactive aggregates were eliminated by ion exchange chromatography on MonoQ (Amersham Pharmacia Biotech, Piscataway, NJ). (His)10-C30 and (His)10-BiP were eluted with a 0.05-1 M KCl linear gradient in 20 mM Tris (pH = 8) buffer. Fractions containing C30 or BiP proteins were pooled and further dialyzed against Binding Buffer (20 mM HEPES pH 7, 75 mM KCl, 5 mM $MgCl_2$). The content of secondary structure in (His)10-C30 (30 μM) was 25.2% α-helix, 64.6% β-sheet, 0.3% turns and 10.9% random coil as estimated by circular dichroism.

Peptides

The tryptophan-containing peptides pp12 (LFWPFEWI), pp28 (HWDFAWPW), pp32 (WTWWEWLA), pp37 (FTYGSRWL), and pp52 (YVDRFIGW), as well as the neuropeptide Substance P (RPKPQQFFGLM), were identified to bind to full-length BiP in previous studies [6, 21]. The following peptides were identified to bind to full-length FLAG-BiP through the screening of a Trp-less and Met-less phage display library (will be described elsewhere) and were synthesized: pep-2 (LSVKFLT), pep-3 (LSIFSVT), pep-6 (FASQKFI) and pep-7 (FYRYGVI). The peptide pep-5 (AQAGSQS), identified in the random library and classified as non-reactive, was also synthesized as a negative control. Peptide synthesis and purification were carried out as described [6].

RESULTS

Peptide binding induces dissociation of recombinant (His)10-C30 oligomers into mostly dimeric and monomeric species, in a process that is peptide concentration dependent. To study the dynamics and mechanisms of the peptide-induced depolymerization process in the absence of interaction with the catalytic ATPase domain, we directly expressed the carboxy-terminal domain of BiP as an isolated Histidine tagged recombinant fragment (His)10-C30 (see Materials and Methods). When analyzed on size-exclusion chromatography, (His)10-C30 (40 μM) elutes as a broad peak, characteristic of a mixture of multiple oligomeric species (data not shown). On native electrophoresis gel, (His)10-C30 migrates as a mixture of oligomeric forms (Fig. 1). Concentration dependence experiments indicate that (His)10-C30 monomeric species co-exist with (His)10-C30 oligomers at low concentration (0.8-10 μM), while large oligomers become preponderant at 25 μM and above, regardless of the method being used to analyze the degree oligomerization, i.e. size-exclusion chromatography, native electrophoresis or cross-linking with glutaraldehyde followed by SDS-PAGE (data not shown).

Eleven peptides were tested for their ability to induce depolymerization of His10-C30 oligomers (Fig. 1). The peptides pp28, pp52, pp32, pp12 and pp37 were previously identified to bind to full-length BiP with variable affinities by screening of a 8-mer phage-display peptide library [6]. In the presence of high concentrations of pp28 and pp52, His10-C30 oligomers tend to disappear and monomeric species accumulate. At the highest concentrations of pp28 and pp52, the bands corresponding to dimeric species decrease in intensity. The peptides pp52, pp32 and pp12 are very insoluble, therefore we were not able to use them at high concentrations. However, pp32 is able to induce significant accumulation of monomeric species even at low concentration. The peptide pp37 is soluble but very inefficient in promoting dissociation of (His)10-C30 oligomers.

The peptides previously identified were eight residue-long, most of them containing tryptophan residues, an amino acid incorporated at low frequency in most protein sequences [6]. BiP and other HSP70s exhibit maximal peptide-stimulated ATPase activity for seven-residue long peptides [5]. The three-dimensional structure of the *E. coli* homologue DnaK complexed with the NR peptide (NRLLLTG) indicates that tryptophan is too bulky to fit into the DnaK major anchor site at the so-called "site 0" position, which corresponds to residue L4 of the NR peptide [22]. To further investigate the preference for tryptophan residues and the optimum requirement of peptide length in BiP recognition of unfolded polypeptides, the peptides pep2, pep3, pep6, pep7 were isolated from screening a tryptophan- and methionine-less 7-mer peptides phage-display library (see Materials and Methods). Most of the 7-mer peptides were much more soluble that those previously identified allowing their use in peptide-binding assays at millimolar concentrations. The peptide pep2 induces depolymerization of the full-length BiP [15] and also induces efficient depolymerization of His10-C30 (Fig. 1, panel F). The peptide pep7 is also a very good substrate for His10-C30 (Fig. 1, panel I). Some activity, as revealed by an increase of monomeric species, is also evident for high concentrations of pep3 (Fig. 1, panel G). Substance P, an 11 residue-long neuropeptide described to bind to full-

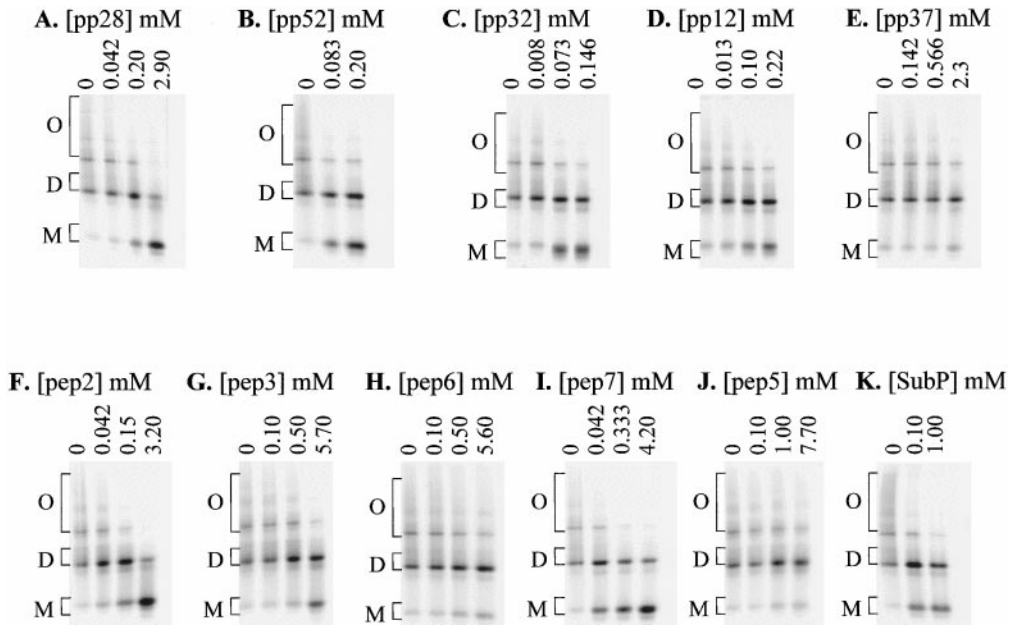

FIG. 1. Specificity of peptide-induced depolymerization of (His)10-C30. (His)10-C30 (10 μM) was incubated with or without varying concentrations of the following peptides: pp28 (HWDFAWPW), pp52 (YVDRFIGW), pp32 (WTWWEWLA), pp12 (LFWPFEWI), pp37 (FTYGSRWL), pep 2 (LSVKFLT), pep 3 (LSIFSVT), pep 6 (FASQKFI), pep 7 (FYRYGVI), pep 5 (AQAGSQS), and Substance P (RPKPQQFFGLM). After 12–16 h incubation at 37°C, 12 μl aliquots of the samples were loaded on a 10% non-denaturing polyacrylamide gel [26]. After electrophoresis in non-denaturing conditions, the proteins were detected using staining with Coomassie-Blue R-250. O, oligomer, D, dimer, M, monomer.

length BiP [21], also induces depolymerization of His10-C30 oligomers at relatively low concentrations (Fig. 1, panel K). Pep5, a peptide selected as a negative control (Materials and Methods), does not induce (His)10-C30 depolymerization, even at concentrations reaching about 8 mM (Fig. 1, panel J). Pep6 is very inefficient at inducing dissociation of (His)10-C30 oligomers (Fig. 1, panel H). The peptides pp28, pp52, pp32, pep2, pep7 and Substance P appear to be the most efficient peptides, promoting noticeable (His)10-C30 depolymerization at concentrations below 200 μM, and almost complete disappearance of large oligomers at concentrations close to their solubility limit.

Concentration-dependence experiments were carried out using the peptide-induced depolymerization assay on native electrophoresis with three of the most efficient peptides pp28, pep2 and pep7 (Fig. 2, upper panels). Monomeric species accumulate with increasing concentrations of synthetic peptides. Oligomers almost completely disappear at high concentrations. Dimeric species tend to decrease and almost totally convert into monomers at high concentrations of pep7. The concentration of monomers was estimated by densitometry of the Coomassie Blue stained native gel and plotted as a function of peptide concentration (Fig. 2, lower panels). The proportion of monomer remains fairly constant at high concentration of peptide, indicating that peptides are in large excess over the carboxy terminal fragment and that an equilibrium has been reached. Apparent affinities were determined as the concentration of peptide leading to 50% of the total amount of monomeric species. Apparent affinity of pep 2 for (His)10-C30 monomer estimated from Fig. 2 was found to be equal to 125 μM, which is in the same range as the $K_m \cdot$ pep2 determined in ATPase stimulation assays of the full-length protein [15]. Apparent affinities of pp28 and pep7 for (His)10-C30 monomer were found to be in the 200–400 μM range. Similar peptide concentration-dependent experiments were carried out with the other peptides and reported in Table 1, together with the apparent affinity determined in peptide-stimulated ATPase assays for the full-length (His)10-BiP. Values obtained for the peptides pp52, pep2 and Substance P are in the same range by using the two assays. For all the other peptides, apparent affinity constant determined in His10-C30 peptide-induced depolymerization assay are significantly higher (from three to ten times) than $K_m \cdot$ peptide determined in ATPase assays. This may be due to a fast exchange rate of these peptides for the His10-C30 and a partial dissociation and re-oligomerization of the fragment that occurs during electrophoretic migration.

DISCUSSION

BiP/GRP78 oligomerizes into multiple oligomeric species that interconvert with each other in a concentration- and temperature-dependent process

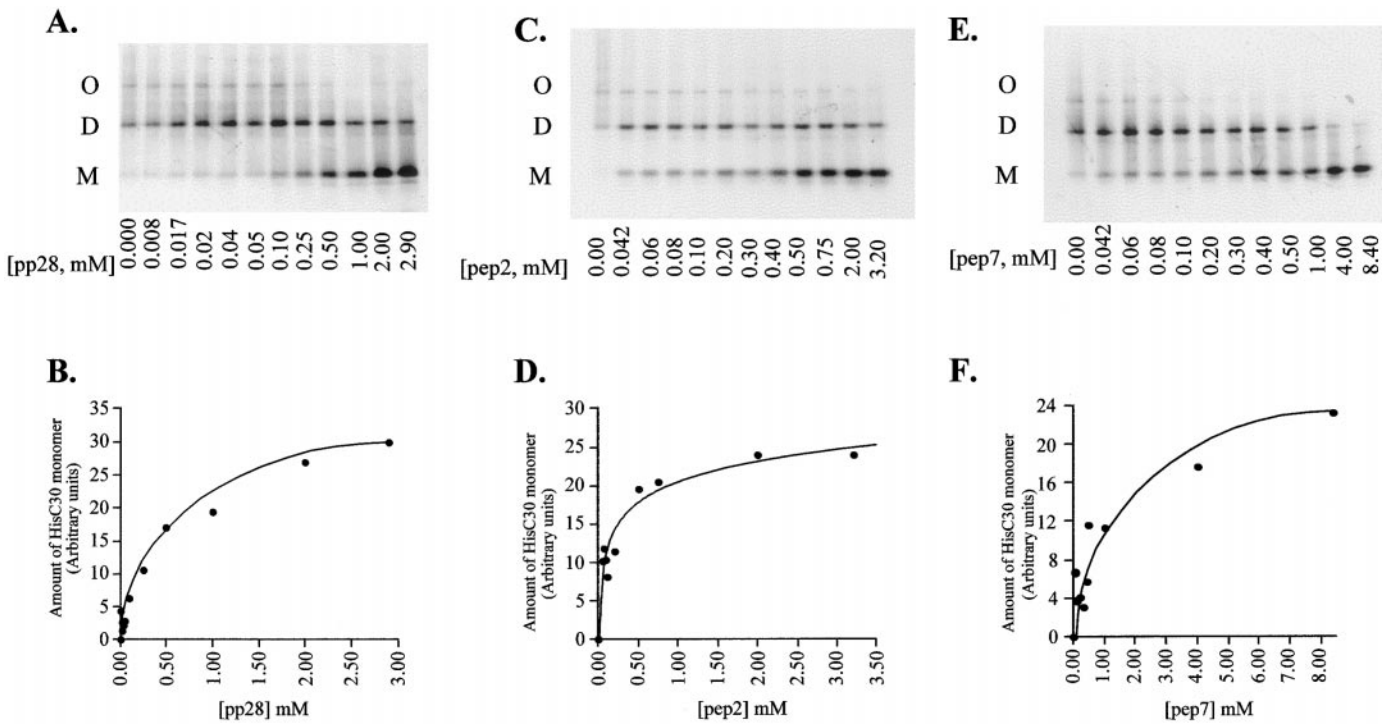

FIG. 2. Apparent affinity of pp28, pep 2, and pep7 for (His)10-C30 monomers determined by native electrophoresis. Top panels: (His)10-C30 (10 μM) was incubated with increasing concentrations of pp28 (**A**), pep2 (**C**) or pep7 (**E**). After 12–16 h incubation at 37°C, the samples were analyzed by native electrophoresis as described in Fig. 1. The amount of (His)10-C30 monomers (bottom panels) was estimated by densitometry scanning of the Coomassie stained gels (AMBIS, Inc., San Diego, CA), and plotted as a function of peptide concentration. Apparent affinities were estimated as the concentration of peptide that produces 50% of (His)10-C30 monomers. O, oligomer, D, dimer, M, monomer.

[15]. Binding of ATP or of a synthetic peptide induces depolymerization of BiP oligomers and stabilization of monomeric species [15]. We and others [5–6] have previously shown that peptides containing hydrophobic amino acids such as tyrosine, phenylalanine, leucine and tryptophan are the most efficient at stimulating the ATPase activity of BiP. Here we show that the polyhistidine tagged C-terminal domain of BiP self-associates into multiple oligomeric species and depolymerizes into monomeric forms upon peptide binding. Similar results have been obtained with the C30 fragment expressed without the N-terminal extension of ten Histidine residues. In this study, we choose two groups of peptides, one group contains tryptophan residues in 8-mer sequences and has already been used in peptide binding and ATPase stimulation studies with the full-length BiP protein [6, 14–15], and the other group contain seven residue long peptides isolated from screening of a tryptophan-less, methionone-less peptide phage-display library. Except for pp37, peptides that contain tryptophan residues and/or are of eight residues or more (Substance P), are the most efficient in stimulating BiP's ATPase activity and depolymerizing (His)-C30 oligomers (Table 1).

We previously developed an algorithm that allows the identification of putative BiP binding sites in any protein sequences [6]. The program is based on experimental findings and calculates a BiP score for seven residue-long peptidic sequences. The higher the score, the more likely the peptide will contain a high affinity binding site for BiP. BiP scores were calculated for all peptides tested in this study and reported in Table 1. The BiP algorithm appears to be also a good indicator for the ability of a peptidic substrate to induce depolymerization of the carboxy-terminal domain of BiP (Table 1). Peptides with high scores ($\geq +5$) promote depolymerization of the carboxy terminal domain of BiP at relatively low concentrations. Peptides with low scores (≤ -5) are inefficient at millimolar concentrations. Intermediates scores have less predictive values: only three peptides, e.g. pep7, pp52 and pep2, out of six tested, efficiently depolymerize (His)10-C30 oligomers and stimulate BiP ATPase activity with K_M in the 100 μM range or below. These findings are in agreement to what has been reported for the ability of peptides to bind to full-length protein and stimulate its ATPase activity [6, 23–25]. This report indicate that the efficiency of peptide-induced depolymerization of the carboxy-terminal domain of BiP correlates with a peptides ability to stimulate BiP ATPase activity, and these two properties can be predicted to some extent by using the BiP algorithm described previously [6].

TABLE 1

Peptide-Dependent ATPase Stimulation of (His)10-BiP and Peptide-Induced Depolymerization of (His)10-C30

Peptides	BiP scores	Average BiP score	K_M · peptide (mM)	K_{app} · peptide (mM)
pp28 (HWDFAWPW)	+3/+32	+17.5	0.010 ± 0.002[a]	0.200 ± 0.050
pp32# (WTWWEWLA)	+24/−2	+13	0.005	0.045 ± 0.015
pp12# (LFWPFEWI)	+15/+13	+14	0.008	0.070 ± 0.020
Substance P (RPKPQQFFGLM)	−3/+2/−2/+7/+7	+7	0.050	0.045 ± 0.020
pep 7 (FYRYGVI)	+5	+5	0.105 ± 0.010	0.400 ± 0.050
pp52 (YVDRFIGW)	+2/+5	+3.5	0.033 ± 0.010*[a]	0.020 ± 0.010
pp37 (FTYGSRWL)	−1/+7	+3	0.250	0.600 ± 0.250
pep 2 (LSVKFLT)	−3	−3	0.110 ± 0.010[b]	0.125 ± 0.025
pep 6 (FASQKFI)	−3	−3	0.600 ± 0.200	1.0 ± 0.100
pep 3 (LSIFSVT)	−9	−9	0.800 ± 0.200	0.450
pep 5 (AQAGSQS)	−17	−17	>5.0	>5.0

Note. BiP scores, calculated as described [6], represent the ability of a peptide to bind to full-length BiP (see text). The scoring system has been compiled for seven-residues-long peptides. For longer peptides, each score has been calculated, as well as the average score. ATPase assays were performed as described [15]. Values reported for apparent affinities (K_{app}) were estimated as described in Fig. 2 and are the average of two or more experiments.

* Real dissociation constant.

[a] From Blond-Elguindi *et al.* 1993 [6].

[b] From Chevalier *et al.* 1998 [15].

Indicate very insoluble peptides.

Full-length BiP depolymerizes rapidly and totally into all monomeric species in the presence of ATP or a non-hydrolyzable analogue, but not in the presence of ADP, indicating that ATP binding but not its hydrolysis promotes a change in conformation leading to the stabilization of the catalytically active monomeric form [15]. In this study, we show that in absence of the catalytic N-terminal domain, all monomeric species cannot be obtained for any of the peptides tested, even for the most soluble ones. Instead, at saturation, an equilibrium was reached, where dimeric and monomeric species coexist, indicating that the ATPase domain is not essential but may play a role in the stabilization of monomeric BiP. Interdomain interactions may also accelerate the rate of peptide-induced depolymerization *in vivo*. Other data supporting these views are: (i) BiP proteolytic carboxy-terminal fragment prepared from all monomeric BiP readily oligomerizes upon dissociation from the N-terminal domain [15] and (ii) monomerization of the full-length BiP from trimeric/dimeric BiP was achieved within 5 min with ATP at 37°C [15]. However, the rate of peptide-induced depolymerization of full-length BiP in absence of ATP [$\tau_{1/2} > 120$ min, ref. 15] or in the absence of physical contact with the catalytic domains [$\tau_{1/2} > 120$ min, L.K. data not shown] is very slow. Mutants affected in the self-association/depolymerization process have been isolated (L.K. unpublished). The biochemical characterization of such mutants should unravel the role of such an equilibrium in the functions of BiP as a molecular chaperone.

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REFERENCES

1. Craig, E. A. (1993) *Science* **260,** 1902–1903.
2. Liu, H., Bowes, R. C., van de Water, B., Sillence, C., Nagelkerde, J. F., and Stevens, J. L. (1997) *J. Biol. Chem.* **272,** 21751–21759.
3. Brodsky, J. L., and Schekman, R. (1993) *J. Cell Biol.* **123,** 1355–1363.
4. Plemper, R. K., Bohmler, S., Bordallo, J., Sommer, T., and Wolf, D. H. (1997) *Nature* **388,** 891–895.
5. Flynn, G. C., Chappel, T. G., and Rothman, J. E. (1989) *Science* **245,** 385–390.
6. Blond-Elguindi, S., Cwirla, S. E., Dower, W. J., Lipshutz, R. J., Sprang, S. R., Sambrook, J. F., and Gething, M.-J. H. (1993) *Cell* **75,** 717–728.
7. Hellman, R., Vanhove, M., Lejeune, A., Stevens, F. J., and Hendershot, L. (1999) *J. Cell Biol.* **144,** 21–30.
8. Hartl, F.-U, Hlodan, R., and Langer, T. (1994) *Trends Biochem.* **19,** 20–25.
9. James, P., Pfund, C., and Craig, E. A. (1997) *Science* **275,** 387–389.
10. Misselwitz, B., Staeck, O., and Rappoport, T. A. (1998) *Mol. Cell* **2,** 593–603.
11. McClellan, A. J., Endres, J. B., Vogel, J. P., Palazzi, D., Rose, M. D., and Brodsky, J. L. (1998) *Mol. Biol. Cell* **9,** 3533–3545.
12. Russell, R., Karzai, A. W., Mehl, A. F., and McMacken, R. (1999) *Biochemistry* **38,** 4165–4176.
13. Freiden, P. J., Gaut, J. R., and Hendershot, L. M. (1992) *EMBO J.* **11,** 63–70.

14. Blond-Elguindi, S., Fourie, A. M., Sambrook, J. F., and Gething, M.-J. H. (1993) *J. Biol. Chem.* **268,** 12730–12735.
15. Chevalier, M., King, L., Wang, C., Gething, M.-J., Elguindi, E., and Blond, S. Y. (1998) *J. Biol. Chem.* **273,** 26827–26835.
16. Burkholder, W. F., Zhao, X., Zhu, X., Hendrickson, W. A., Gragerov, A., and Gottesman, M. E. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 10632–10637.
17. Benaroudj, N., Triniolles, F., and Ladjimi, M. M. (1996) *J. Biol. Chem.* **271,** 18471–18476.
18. Benaroudj, N., Fouchaq, B., and Ladjimi, M. M. (1996) *J. Biol. Chem.* **272,** 8744–8751.
19. Gao, B., Eisenberg, E., and Greene, L. (1996) *J. Biol. Chem.* **271,** 16792–16797.
20. Kozutsumi, Y., Normington, K., Press, E., Slaughter, C., Sambrook, J., and Gething, M.-J. (1989) *J. Cell Sci. Suppl.* **11,** 115–137.
21. Oblas, B., Boyd, N. D., Luber-Narod, J., Reyes, V. E., and Leeman, S. E. (1990), *Biochem. Biophys. Res. Commun.* **166,** 978–983.
22. Zhu, X., Zhao, X., Burkholder, W. F., Gragerov, A., Ogata, C. M., Gottesman, M. E., and Hendrickson, W. A. (1996) *Science* **272,** 1606–1614.
23. Fourie, A. M., Sambrook, J. F., and Gething, M.-J. (1994) *J. Biol. Chem.* **269,** 30470–30478.
24. Knarr, G., Gething, M.-J, Modrow, S., and Buchner, J. (1995) *J. Biol. Chem.* **270,** 27589–27594.
25. Gething, M.-J., Blond-Elguindi, S., Buchner, J., Fourie, A., Knarr, G., Modrow, S., Nanu, L., Segal, M., and Sambrook, J. (1995) *Cold Spring Harbor Quant. Symp. Biol.* **60,** 417–428.
26. Laemmli, U. K. (1970) *Nature* **227,** 680–685.