Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 2505–2508, March 1993
Biochemistry

Dissociation of glucose-regulated protein Grp78 and Grp78–IgE Fc complexes by ATP

(chaperon/heat shock/endoplasmic reticulum/binding protein BiP)

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Contributed by Herbert Weissbach, December 17, 1992

ABSTRACT **Recent studies have shown that ATP can dissociate dimers of the glucose-regulated protein Grp78 to monomers. In the present study, we have used purified recombinant Grp78 from *Escherichia coli* to investigate this reaction in more detail. During the course of the Grp78 dimer–monomer conversion, a stable Grp78 monomer–ATP complex is formed. Upon removal of the ATP, the Grp78 dimer is reformed. ADP, nonhydrolyzable ATP analogues, and GTP do not effect the dissociation of Grp78 dimers. A cell line that overproduces IgE Fc has been used to examine the nature of the Grp78–IgE Fc complexes present and the effect of ATP on them. Grp78–IgE Fc complexes ranging from 100 kDa to 300 kDa were observed by sucrose gradient analysis, suggesting that aggregate forms of Grp78 may be present in some of these complexes. Treatment of the extracts with ATP resulted in release of a Grp78 monomer from the complex. These results suggest that the dissociation of Grp78 oligomers by ATP may be involved in the function of Grp78 in protein translocation through the endoplasmic reticulum.**

Immunoglobulin heavy chain binding protein (BiP) or glucose-regulated protein (Grp78) is localized in the endoplasmic reticulum (ER) and is a member of the highly conserved family of heat shock-related proteins known as Hsp70 (1). In mammalian cells, Grp78 is not induced by heat (1); however, treatments increasing the amount of malfolded proteins in the ER stimulate a dramatic increase in its synthesis (2, 3). For instance, inhibitors of N-linked glycosylation induce Grp78 synthesis and this induction is correlated with an increased binding of Grp78 to underglycosylated proteins (4, 5). Furthermore, Grp78 has been shown to bind tightly to mutant proteins, to proteins with incorrect disulfide bonds, and to incompletely assembled multimeric proteins (6–10). From these data, the view has emerged that Grp78 binds tightly to malfolded proteins, retaining them in the ER and, thus, preventing their secretion. In contrast, under normal conditions, it appears that Grp78 binds transiently to nascent proteins and promotes their proper folding and assembly (for review, see ref. 11). Grp78, like other Hsp70 proteins, binds tightly to ATP (12–14) and possesses a weak ATPase activity (13, 15, 16). ATP is required for disruption of Grp78 complexes (1, 2, 13, 15) and ATP hydrolysis is thought to be required for this dissociation (13). In addition, Grp78 has an autophosphorylation activity *in vitro* (14, 16).

Purified Grp78, from liver or an *Escherichia coli* recombinant (r) protein (rGrp78), was found to be primarily a mixture of monomers and dimers with a trace of higher molecular weight aggregates (15). ATP was shown to dissociate the dimers and heavier species to monomers. This observation raised the possibility that the different oligomeric forms of Grp78 might be involved in regulating its activity in the ER and that the role of ATP in dissociating Grp78–protein complexes may be to disaggregate Grp78 dimers and higher molecular mass oligomers. Recently, Freiden *et al.* (17) reported that Grp78 not complexed to proteins could be detected principally as a dimer and a small amount of monomer, the dimer being posttranslationally modified. Their data also indicated that Grp78–IgG heavy chain complexes have an apparent molecular mass of ≈350 kDa. The structure of the complex, with respect to the organization of Grp78, however, is unknown. Others have reported a Grp78–IgG light chain complex with a molecular mass of ≈110 kDa, consistent with the presence of a Grp78 monomer in the complex (18).

In the present report, we have extended our previous studies on the interaction of Grp78 with ATP to identify the species of Grp78 that functions in protein binding and to determine the role of ATP in this process.

MATERIALS AND METHODS

ATP Binding. rGrp78 was isolated and purified as described (15). Separation of Grp78 monomers and dimers was carried out by HPLC gel filtration using a Bio-Sil SEC-250 silica column (0.75 × 60 cm; Bio-Rad). Chromatography was at 4°C at a flow rate of 0.3 ml/min. The mobile phase was 20 mM Tris·HCl (pH 7.0) containing 200 mM KCl. Typical elution volumes for the Grp78 dimer and monomer were 13.5 and 16.5 ml, respectively. Detection was by absorbance at 215 nm. Liquid scintillation counting was performed using a Beckman LS-6800 spectrometer.

Approximately 500 μg of rGrp78 was incubated either at 4°C for 15 min or 1 min at 37°C in a final volume of 600 μl of 20 mM Tris·HCl, pH 7.4/1 mM $MgCl_2$/50 μM [γ-^{32}P]ATP (specific activity, 20,000 cpm/pmol). At the end of the incubation, an aliquot containing 30 μg of Grp78 was applied to the HPLC gel-filtration column. Three-hundred-microliter fractions were collected, of which 25 μl was removed for determination of radioactivity. The remainder of the reaction mixture was brought to 80% $(NH_4)_2SO_4$ saturation, mixed for 45 min at 4°C, and centrifuged to collect the protein. The precipitated protein was washed twice with 80% $(NH_4)_2SO_4$ and then dialyzed against 20 mM Tris·HCl (pH 7.4). After dialysis, 30 μg of Grp78 was applied to the gel-filtration column, and 200 μl of each fraction was removed for determination of radioactivity.

DNA Constructions and Transfections. The cDNA for the human IgE Fc (Cε2-Cε4) was isolated by PCR from total peripheral blood lymphocyte cDNA. The DNA fragment encoding amino acids 235–547 was isolated by PCR and cloned into the expression vector pBC12-pSP36 (19). This vector allows the cloning of cDNA fragments adjacent to the

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Abbreviations: r, recombinant; ER, endoplasmic reticulum.

interleukin 2 receptor signal peptide, thereby allowing translocation of the protein into the ER. pMJ1386, the resulting cDNA expression construct, was verified by sequence analysis. The conditions for CHO duk$^-$ cell growth and permanent transfection have been described (20). Permanent cell lines were amplified by treatment with stepwise increases of amethopterin (0.1–1.0 μM). Stable clones expressing high levels of rIgE Fc fragments were isolated by limited-dilution cloning and designated CHO/1C11. This cell line produces but does not secrete IgE Fc (amino acids 235–547).

Separation of Grp78–IgE Fc Complexes. For *in vivo* labeling of proteins, cells were grown to 90% confluency at 37°C in Iscove's modified Dulbecco's medium (JRH Biosciences, Lenexa, KS) supplemented with 10% (vol/vol) fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml); starved in methionine-free Dulbecco's modified Eagle's medium (GIBCO) for 1 hr; and incubated for 2–3 hr in the same medium containing L-[^{35}S]methionine (600 Ci/mmol, 400 μCi/ml of medium; 1 Ci = 37 GBq). Cells were lysed in buffer containing 20 mM Tris·HCl (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$, and 0.2% Triton X-100 and centrifuged at 16,000 × *g* for 10 min at 4°C to remove insoluble material. Where indicated the extracts were incubated with 100 μM ATP for 10 min at 25°C. The extract was loaded onto a 13-ml 5–20% (wt/vol) sucrose gradient containing 20 mM Hepes·KOH (pH 7.4), 150 mM NaCl, and 0.2% Triton X-100 with or without 1 mM ATP. Gradients were prepared with a BioComp Gradient Master (Fredericton, NB, Canada). Centrifugation was at 244,000 × *g* for 19 hr at 4°C in a SW40.1 rotor. Four-hundred-microliter fractions were collected, from which IgE Fc was immunoprecipitated by incubation with rabbit anti-human IgE (ε-chain) polyclonal antibody (DAKO, Carpinteria, CA) and adsorbed onto protein A-Sepharose CL-4B (Sigma). The adsorbed immunoprecipitates were washed three times with gradient buffer without detergent, solubilized by boiling with SDS/gel loading buffer, and resolved on 10% SDS/polyacrylamide gels (21), and the labeled proteins were visualized by fluorography with Enlightning (NEN). Immunoprecipitations from unfractionated extracts were performed similarly.

To determine the form of Grp78 released by dissociation of the complex by ATP, labeled extracts were passed over an anti-IgE column, prepared by attaching IgE antibody to Affi-Gel HZ (Bio-Rad) according to the manufacturer's recommendations. The column was washed extensively with gradient buffer without Triton X-100 and then treated with ATP to release Grp78. The released Grp78 was analyzed on the gel-filtration column described above. Proteins were precipitated with 10% (vol/vol) CCl_3COOH with ovalbumin (30 μg) as carrier. Half of the sample was analyzed by gel electrophoresis as described above and the other half was analyzed on a Western blot. Proteins were transferred to Hybond-ECL (Amersham) and probed for Grp78 by using affinity-purified rabbit anti-rGrp78. Immunoreactive bands were detected using the ECL system (Amersham).

RESULTS

We have reported (15) that dimers of Grp78 can be converted *in vitro* to monomers by ATP. These earlier experiments used Grp78 purified from bovine liver or rGrp78 purified from *E. coli*. Both preparations were primarily a mixture of dimers and monomers with small amounts of higher molecular mass species. In the present experiments, we initially attempted to determine whether the dimer-to-monomer conversion is reversible and to identify which forms of Grp78 form stable complexes with ATP.

Fig. 1 shows the results of an experiment in which rGrp78 was incubated with [γ-^{32}P]ATP and then applied to an HPLC gel-filtration column. The initial pattern of the protein consisted of 30–40% dimers and 60–70% monomers (Fig. 1*A*). After incubation with ATP (Fig. 1*B*), there was almost complete conversion to the monomer as reported (15), and the radioactivity was associated mostly with the monomer. The radioactivity was noncovalently bound since it was released by acid precipitation. To determine the reversibility of this reaction, the incubation mixture, after treatment with ATP, was precipitated with $(NH_4)_2SO_4$, extensively dialyzed to remove ATP, and then rechromatographed. Fig. 1*C* shows that after removal of the ATP, the monomer/dimer ratio was similar to the original preparation seen in Fig. 1*A*, and the amount of radioactivity bound to the monomer species was decreased by >90%.

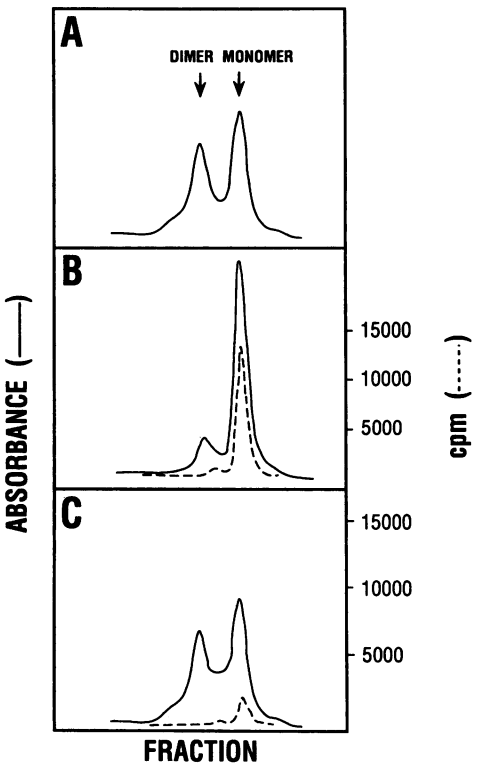

FIG. 1. Binding of ATP to rGrp78 and interconversion of rGrp78 monomers and dimers. rGrp78 (500 μg) was incubated in the presence of [γ-^{32}P]ATP, and the protein was precipitated with $(NH_4)_2SO_4$ and then dialyzed. Protein (30 μg) from each step was subjected to HPLC gel filtration and the A_{215} and radioactivity were assayed. (*A*) Before incubation with ATP. (*B*) After incubation with ATP. (*C*) After $(NH_4)_2SO_4$ precipitation and dialysis.

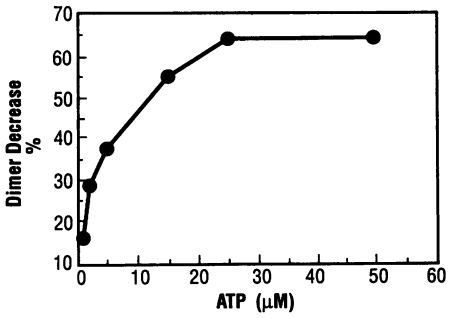

FIG. 2. Effect of ATP concentration on the conversion of Grp78 dimers to monomers. Grp78 (30 μg) was incubated for 1 min at 37°C in a buffer containing 20 mM Tris·HCl (pH 7.4), 1 mM $MgCl_2$, and various concentrations of ATP. The samples were applied to a HPLC gel-filtration column and the A_{215} was monitored. The area under the dimer peak and the percentage decrease in the amount of dimer were determined.

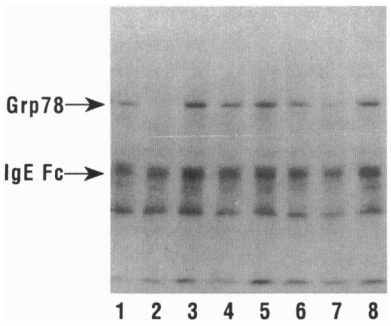

FIG. 3. Effect of various nucleotides and Mg^{2+} on Grp78–IgE Fc complex stability. Grp78–IgE Fc complexes were immunoprecipitated from labeled cell extracts and then treated for 10 min at 25°C with a buffer containing 20 mM Hepes·KOH (pH 7.4) and 150 mM NaCl, and various nucleotides (100 μM) and/or $MgCl_2$ (1 mM) was added to some samples. The immunoprecipitates were then washed with buffer and the bound proteins were analyzed. Lanes: 1, buffer only; 2, ATP plus Mg^{2+}; 3, ADP plus Mg^{2+}; 4, GTP plus Mg^{2+}; 5, adenosine 5'-[β,γ-methylene]triphosphate plus Mg^{2+}; 6, adenosine 5'-[β,γ-imido]triphosphate plus Mg^{2+}; 7, ATP but no Mg^{2+}; 8, Mg^{2+} but no ATP.

The effect of ATP concentration on the dimer-to-monomer conversion is shown in Fig. 2. In these experiments, the protein was detected by measuring the A_{215}. Since ATP also absorbs at this wavelength, any increase in the A_{215} in the monomer peak could be due to ATP binding to the monomer and not to an increase in the amount of monomer resulting from the conversion of the dimer to the monomer. Therefore, the ATP-dependent dimer-to-monomer conversion was measured by determining the decrease of the dimer peak. At concentrations of ATP >25 μM, there was a maximal decrease of the dimer species of ≈65%. ADP, GTP, and nonhydrolyzable ATP analogues could not substitute for ATP (data not shown).

The ability of ATP to dissociate Grp78 dimers raised the possibility that this reaction might be involved in the dissociation of Grp78–protein complexes in the ER. To examine this, we attempted to identify the type of Grp78 complexes in transfected CHO cells that produce a nonsecreted IgE Fc fragment. After labeling the cells with [^{35}S]methionine, IgE Fc was immunoprecipitated from total cell extracts and from extracts that were fractionated on sucrose gradients. The presence of Grp78 in the immunoprecipitates was indicative of complex formation. As shown in Fig. 3, lane 1, the immunoprecipitate from the total cell extract contained a cluster of radiolabeled bands in the 40-kDa region that corresponds to IgE Fc and a band at 78 kDa. The 78-kDa protein in the immunoprecipitate was identified as Grp78 by Western blot analysis (data not shown). Presumably, the immunoprecipitate contained both free IgE Fc and Grp78–IgE Fc complexes. When the immunoprecipitate was treated with 100 μM ATP in the presence of 1 mM $MgCl_2$, virtually all of the 78-kDa protein disappeared (Fig. 3, lane 2). As shown in Fig. 3, lanes 3–6, ADP, GTP, and nonhydrolyzable ATP analogues could not disrupt the complex. In the absence of $MgCl_2$, ATP was less effective in dissociating Grp78 from IgE Fc (lane 7). $MgCl_2$ alone had no effect on the stability of the complex (lane 8).

After sucrose-gradient centrifugation Grp78–IgE Fc complexes were observed throughout a large region of the gradient as shown in Fig. 4*A*. To obtain an approximate molecular mass of the complexes, several protein standards were used as markers. The heaviest complexes (fractions 10–15) appeared to be 200–300 kDa, whereas the intermediate ones (fractions 16–20) were 150–200 kDa, and the lightest complexes (fractions 21–25) were ≈100 kDa or less. When the extract was treated with ATP (1 mM) before centrifugation, there was no longer any Grp78 present in the immunoprecipitates of the gradient fractions (Fig. 4*B*). It should be noted that a small amount of a 55-kDa protein of unknown origin was seen in the light region of both gradients. Also, the IgE Fc in the ATP-treated extract, presumably not complexed, shifted to the light region of the gradient, supporting the view that ATP can dissociate high molecular mass Grp78–IgE Fc complexes, causing release of the Grp78 and IgE Fc. Based on our previous finding that ATP could dissociate dimers of purified Grp78 to monomers (15), it was important to also determine whether the Grp78 released from the Grp78–IgE Fc complexes was a monomer or dimer. For these experiments, the cell extract containing the Grp78–IgE Fc complex

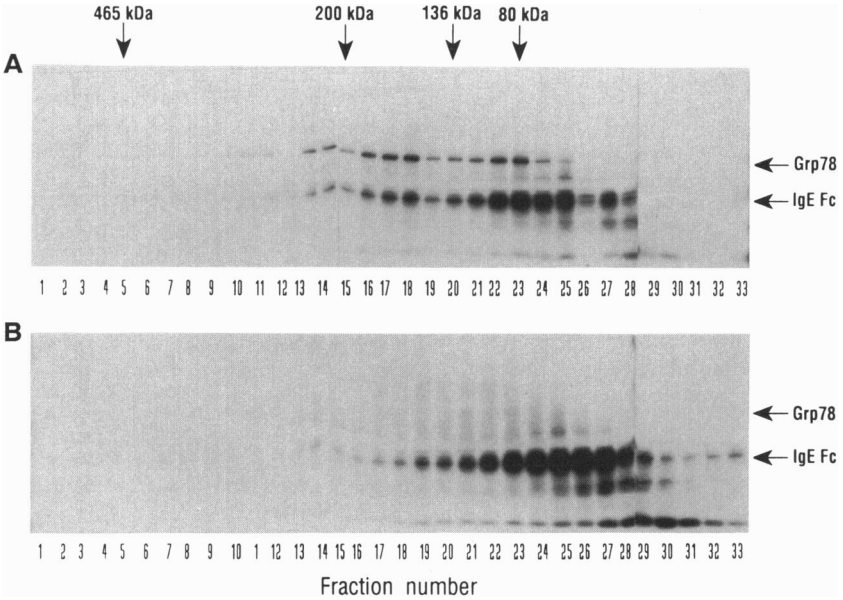

FIG. 4. Effect of ATP on the molecular size of Grp78–IgE Fc complexes. Labeled cell extract (200 μg) either treated or untreated with ATP (1 mM) for 10 min at 25°C was loaded onto a 5–20% sucrose gradient with or without ATP (1 mM). Fractions were collected, Grp78–IgE Fc complexes were immunoprecipitated, and the bound proteins were analyzed by SDS/PAGE and fluorography. Sedimentation positions of several protein markers are indicated above the autoradiograms (80 kDa, *E. coli* elongation factor G; 136 kDa, *E. coli* MetH B_{12} enzyme; 200 kDa, β-amylase; 465 kDa, *E. coli* β-galactosidase). (*A*) No ATP treatment. (*B*) ATP treatment.

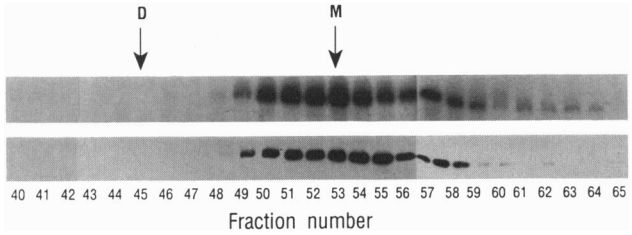

FIG. 5. Form of Grp78 after ATP disruption of Grp78–IgE Fc complexes. Labeled cell extract (≈200 μg of protein) was passed over an anti-IgE affinity column. The column was treated with ATP (1 mM), and the eluate was applied to a gel-filtration column. Half of each column fraction was analyzed by SDS/PAGE and fluorography (*Upper*). The other half was probed for Grp78 by Western blot analysis (*Lower*).

was passed over an affinity column containing the IgE antibody, the column was washed with buffer, and Grp78 was released from the complex by treating the column with ATP (1 mM). The form of the released Grp78 was shown to be a monomer by gel filtration (Fig. 5) and native polyacrylamide gel analysis (data not shown).

DISCUSSION

The above results indicate that, *in vitro*, the monomer and dimer forms of Grp78 are in an equilibrium that shifts in the presence of ATP. Our purified rGrp78 preparations contain a mixture of dimers and monomers; however, when treated with ATP, the dimer species dissociates to a monomer–ATP complex. After ATP is removed, the monomer is converted back to a dimer–monomer mixture very similar to that present in the original preparation. Similar results were seen with Grp78 isolated from liver (data not shown).

There is conflicting information about the form(s) of Grp78 present in purified preparations of the protein as well as in crude extracts. Initially, it was reported that Grp78 purified from glucose-starved transformed fibroblasts exists as a 400-kDa oligomer (22). However, only monomers and dimers were found to be the predominant species present in Grp78 purified from bovine liver or rGrp78 from *E. coli* (15). Knittler and Haas (18) reported that immunoglobulin light chains were bound to Grp78 monomers in a cell line that synthesizes immunoglobulin light chains, but not heavy chains. In contrast, Freiden *et al.* (17) found that in cells synthesizing heavy chains, but not light chains, a Grp78 complex was found with a mass of ≈350 kDa. Although the form of Grp78 in this complex was not directly determined, the authors suggested, based on other evidence, that the monomer form of Grp78 is the form that binds to unfolded proteins.

Our data indicate that there are different complexes of Grp78–IgE Fc in cell extracts. The apparent molecular masses range from ≈100 kDa to ≈300 kDa (Fig. 4). These complexes could be normal intermediates found in the ER or might be artifacts produced *in vitro*. Our extracts, by necessity, are made with small amounts of detergent that may cause the release of weakly bound components. It also is not known whether the complexes are stable to high centrifugal forces. Therefore, it is possible that the complexes we observe are breakdown products of larger species. Thus, it should be stressed that the exact composition of the Grp78–IgE Fc complex present in the cell extract has not been determined. Recently, it has been shown by chemical crosslinking studies that Grp94 can be found in a complex with Grp78 and immunoglobulin chains (23). After ATP treatment of the extracts, the Grp78 is released from the complexes as a monomer (Fig. 5) and the IgE Fc originally present in the heavier fractions sediments as a lower molecular mass species. These results do not exclude the possibility that Grp78 was released from the complex as a dimer and then subsequently converted to the monomeric form.

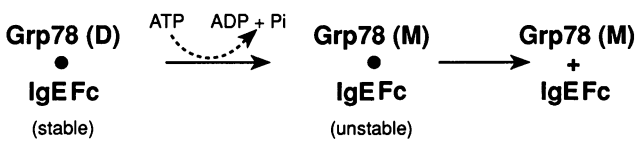

FIG. 6. Scheme proposing the dissociation of a Grp78–IgE Fc complex by ATP. D, dimer; M, monomer.

It is not yet clear whether the Grp78 in the heavier fractions binds to IgE Fc as an oligomer or as a monomer. A Grp78 dimer, if associated with an IgE Fc dimer, would give a complex close to 300 kDa. If so, our results are consistent with the scheme presented in Fig. 6. The Grp78–IgE Fc complex is thought to be composed of a Grp78 dimer (or higher molecular mass oligomer). This complex is pictured as being quite stable due to high-affinity interactions. In the presence of ATP, the complex dissociates primarily due to conversion of the Grp78 dimer to a monomer. ATP is required for the dissociation of Grp78–protein complexes, and ATP hydrolysis may occur during this reaction (1, 13). The resulting Grp78 (monomer)–IgE Fc complex is pictured as a less-stable intermediate that dissociates with the release of the Grp78 and free IgE Fc. The Grp78 found complexed to IgE Fc in the light region of the gradient (Fig. 4) could be part of a terminal complex containing the Grp78 monomer before dissociation (Fig. 6). In the presence of excess ATP, the released Grp78 should remain as a monomer. During this process, Grp78 is thought to facilitate proper folding of the bound protein so that it can undergo release from the ER, post-translational modification, or interaction with other proteins.

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