Unassembled immunoglobulin light chains expressed by the mouse plasmacytoma cell line NS1 (κNS1) are degraded in vivo with a half-life of 50–60 min in a way that closely resembles endoplasmic reticulum (ER)-associated degradation (Knittler et al., 1995). Here we show that the peptide aldehydes MG132 and PS1 and the specific proteasome inhibitor lactacystin effectively increased the half-life of κNS1, arguing for a proteasome-mediated degradation pathway. Subcellular fractionation and protease protection assays have indicated an ER localization of κNS1 upon proteasome inhibition. This was independently confirmed by the analysis of the folding state of κNS1 and size fractionation experiments showing that the immunoglobulin light chain remained bound to the ER chaperone BiP when the activity of the proteasome was blocked. Moreover, kinetic studies performed in lactacystin-treated cells revealed a time-dependent increase in the physical stability of the BiP–κNS1 complex, suggesting that additional proteins are present in the older complex. Together, our data support a model for ER-associated degradation in which both the release of a soluble nonglycosylated protein from BiP and its retrotranslocation out of the ER are tightly coupled with proteasome activity.

INTRODUCTION

Folding and assembly of many cellular and extracellular proteins occur in the endoplasmic reticulum (ER). Molecular ER chaperones are believed to monitor both processes, ensuring that only when they are completed are proteins allowed to exit the ER and to follow the secretory pathway to their final destination. As an integral part of such a quality control system (Hammond and Helenius, 1995; Leitzgen and Haas, 1998), polypeptides that fail either to fold or to assemble correctly are retained in the ER by the chaperones and are eventually degraded. This process is called ER-associated degradation (ERAD) because it usually is rapid and nonlysosomal and occurs in a pre-Golgi compartment (Brodsky and McCracken, 1997; Sommer and Wolf, 1997). A growing number of ERAD substrates have been identified, among them a mutant form of carboxypeptidase Y (CPY*), unglycosylated prepro-α-factor, α1-antitrypsin, unassembled T-cell receptor (TCR) subunits, unassembled MHC class I heavy chain, mutant ribophorin, and the cystic fibrosis transmembrane conductance regulator (Jensen et al., 1995; Ward et al., 1995; Knop et al., 1996a; McCracken and Brodsky, 1996; Qu et al., 1996; Hughes et al., 1997; Huuppa and Ploegh, 1997; Yu et al., 1997; deVirgilio et al., 1998).

In a remarkable convergency of mammalian cell biology and yeast genetics, many components of the ERAD machinery have been uncovered (Hampton et al., 1996; Wiertz et al., 1996b; Plemper et al., 1997). The current model proposes that ERAD substrates are retrotranslocated from the ER to the cytosol via the Sec61 protein channel for degradation by the proteasome (Wiertz et al., 1996a; Kopito, 1997; Pilon et al., 1997). In many cases, the degradation is ubiquitin dependent (Hiller et al., 1997; Biederer et al., 1997; deVirgilio et al., 1998). The ER chaperones Kar2p (the yeast homologue of BiP) and calnexin and the DnaJ homologue Sec63p seem also to be involved, at least for some substrates (Qu et al., 1996; Plemper et al., 1997). Other genes, to be characterized, have been discovered in two independent genetic screens for mutants with impaired ERAD (DER and HERD genes; Hampton et al., 1996; Bordallo et al., 1998). However, despite the rapid increase in the knowledge of the ERAD machinery, much less is clear about the precise mechanistic roles of its components. Noteworthy, the delivery steps to the retrotranslocation channel and the driving force for this backward movement are still unresolved issues. Obvious candidates for the delivery are ER chaperones, in particular calnexin (see Qu et al., 1996; Liu et al., 1997), and possibly some of the DER and HERD genes, but no conclusive data are available. From genetic studies in yeast, cytosolic hsp70s have been eliminated as ERAD players (Brodsky et al., 1999), and some suggestive results have been reported in the case of integral
membrane proteins, pointing to the proteasome itself as the driving force for retrotranslocation (Mayer et al., 1998; Plemper et al., 1998).

We are specially interested in the role of BiP in ERAD. For this purpose, we are using the immunoglobulin (Ig) light (L) chain κ_{NS1}, a soluble and unglycosylated polypeptide synthesized by NS1 plasmacytoma cells, as a model protein. It represents a particularly suitable model for analyzing the role of BiP in degradation, because unassembled κ_{NS1} is quantitatively bound to BiP in a 1:1 complex, and its half-life nicely correlates with the rate of BiP–κ_{NS1} complex dissociation when κ_{NS1} is expressed in the absence of Ig heavy (H) chains (Knittler and Haas, 1992; Knittler et al., 1995; Cremer et al., 1994). This Ig L chain is a natural substrate, because it completely folds and is secreted when allowed to assemble into antibody molecules. Furthermore, the BiP binding site has been mapped to the unfolded N-terminal variable domain of this as well as of another unassembled Ig L chain, whereas the C-terminal constant domain is folded and not bound to BiP (Skowronnek et al., 1998; Hellman et al., 1999). The half-lives of different unassembled Ig L chains have been correlated with their variable domains (Skowronnek et al., 1998), suggesting an important role for BiP in the degradation of these molecules.

In this study, we have analyzed by using different methods the fate of κ_{NS1} in NS1 cells after proteasome inhibition. Our findings strongly support a model in which dissociation of κ_{NS1} from BiP immediately precedes backward movement of the unglycosylated protein out of the ER lumen. Proteasome activity is required for retrotranslocation, either for the movement through the retrotranslocation channel or for the dissociation of the BiP–Ig L chain complex or for both steps.

MATERIALS AND METHODS

Chemicals and Antibodies

Chemicals were obtained from Sigma (St. Louis, MO), except MG132 and lactacystin, which were purchased from Calbiochem (La Jolla, CA), and the gel filtration molecular weight standards, from Amersham Pharmacia Biotech (Uppsala, Sweden). PS1 was a kind gift from P. Kloetzel (Humboldt University, Berlin, Germany). Affinity-purified goat antibodies to mouse κ Ig L chains were from Southern Biotechnologies (Birmingham, AL); polyclonal anti-Grp78 (PA1–014) and anti-calreticulin antibodies (PA3–900) were from Affinity Bioreagents (Golden, CO); the rabbit anti-rodent BiP antisera was a kind gift from L. Hendershot (St. Jude Children’s Research Hospital, Memphis, TN). Anti-tubulin monoclonal antibody was a kind gift from J. Wehland (Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany) (Wehland and Weber, 1987), and anti-calnexin antisera was a kind gift from E. Ivesa (University and Biocenter, Vienna, Austria) (de Virgilio et al., 1998).

Cell Culture

NS1 is a murine plasmacytoma, which synthesizes but does not secrete Ig L chains of the κ isotype (Köhler et al., 1976). The cells were routinely maintained in RPMI 1640 medium supplemented with 10% FCS, 1000 U/ml penicillin, and 1 mg/ml streptomycin.

Biosynthetic Labeling, Immunoprecipitation, and Western Blot Analysis

Cells (2 × 10⁶ cells/ml) were starved for 2 h in methionine-free RPMI 1640 medium containing 10% dialyzed FCS before [³⁵S]Met was added for 30 min (120 μCi/ml). The chase was initiated by addition of excess unlabeled methionine (2 mM). Aliquots were removed at various times of chase, and cells were separated from supernatants and washed once with ice-cold PBS before lysis in NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.5% NP-40) (Kessler, 1975) in a final concentration of 2 × 10⁶ cells/ml. After 30 min of solubilization, a postnuclear supernatant was obtained by 10 min of 10,000 × g centrifugation at 4°C. Inhibitors (25 μM lactacystin, 50 μM MG132, and 60 μM PS1) and DMSO as a control were included from the beginning of the starving period to the end of the chase, except for MG132, which was added immediately after the chase was initiated. In some experiments N-ethylmaleimide (NEM; 20 mM) was included in the PBS wash and in the lysis buffer to prevent postlysis oxidation of free sulfhydryl groups and allowing monitoring of the folding state of the Ig L chains.

Immunoprecipitations were performed from equivalent amounts of cell lysates by adding an equal volume of immunosorbent buffer (200 mM H₄BO₄, 50 mM Na₂B₄O₇, 150 mM NaCl, 1% NP-40, and 0.1% ovalbumin, pH 8.3) and polyclonal antibodies to BiP or goat antibodies to mouse Ig L chains, in combination with protein A-Sepharose. Precipitates were washed four times with borate-NaCl buffer (0.5% NP-40, 1 M NaCl, 25 mM Na₂B₄O₇, and 0.1 M H₂BO₃, pH 8.3) and twice with 40 mM HEPES, pH 8. Samples were run on SDS-PAGE under reducing or nonreducing conditions. Gels were stained with Coomassie brilliant blue to control for precipitating antibodies, the labeled proteins were visualized by autoradiography, and Ig L chain and BiP bands were quantified with a phosphorimagery (Fuji, Tokyo, Japan) BAS1000 using the program MACBAS version 1.0).

For Western blotting, proteins from equivalent amounts of either lysate or the different subcellular fractions (see below) were separated by SDS-PAGE and transferred onto nitrocellulose membranes that were blocked in PBS, 0.5% dry milk powder, and 0.05% Tween 20. Subsequently, the membranes were incubated with the following dilutions of antibodies: 1:500 for anti-α, 1:1000 for anti-BiP (PA1–014), 1:5000 for anti-calnexin, 1:2000 for anti-tubulin, and 1:1000 for anti-calreticulin. Proteins were visualized, after incubating with the corresponding HRP-conjugated secondary antibodies, with the ECL chemiluminescence blotting substrate (Boehringer Mannhein, Mannheim, Germany). Signals from Western blot were quantified by standard scanning densitometry using the NIH Image program.

Subcellular Fractionation

Cells were labeled as stated above and chased for 3 h in the presence or absence of lactacystin. Cells (1.4 × 10⁷) were washed with PBS and gently resuspended in an isotonic buffer (250 mM sucrose, 10 mM triethanolamine, and 1 mM EDTA, pH 7.4). Homogenization was performed by passing the cells 10 times through a 25-gauge needle and six times through a 27-gauge needle. Unbroken cells and debris were removed by two consecutive 1000 × g centrifugations (10 min, 4°C). The supernatant was pelleted again by centrifugation at 100,000 × g (90 min, 4°C), and the membrane vesicles (P3) and the cytosolic fraction (SN) were obtained. Proteins from equivalent amounts of the different fractions were used for immunoprecipitations or for Western blot.

Trypsin Protection Assays

The vesicle fraction (P3) obtained from the subcellular fractionation was resuspended in the isotonic buffer and incubated for 30 min at 30°C in the absence or presence of 15 μg/ml trypsin. Another aliquot of the fraction was incubated similarly in parallel in the presence of trypsin and 0.5% NP-40. Trypsin digestions were stopped by the addition of 500 μg/ml trypsin inhibitor. The different samples were used for either immunoprecipitation or Western blot.
Cells were biosynthetically labeled as above and chased for 3 h in the absence or presence of lactacystin. Lysates were obtained at the beginning of the experiment. At each of the time points indicated, postnuclear supernatant was prepared from equal numbers of cells (with the same incorporation of radioactivity) and used to immunoprecipitate the κNS1 chains as stated in MATERIALS AND METHODS. The calculated half-lives in minutes are indicated.

Size Fractionation

Cells were biosynthetically labeled as above and chased for 2 h in the presence of lactacystin or PS1, labeled for 30 min, and chased up to 4 h in the continuous presence of the inhibitors. MG132 was added after the pulse, because we observed a decrease in protein synthesis when it was present from the beginning of the experiment. At each of the time points indicated, postnuclear supernatant was prepared from equal numbers of cells (with the same incorporation of radioactivity) and used to immunoprecipitate the κNS1 chains as stated in MATERIALS AND METHODS. The calculated half-lives in minutes are indicated.

RESULTS

Ig L Chains κNS1 Are Degraded by the Proteasome

Previous studies from our laboratory (Knittler and Haas, 1992; Knittler et al., 1995) and the laboratory of Y. Argon (Gardner et al., 1993) showed that in the absence of Ig H chain expression, some Ig L chains are not secreted but degraded in vivo. This degradation occurs in a pre-Golgi compartment and is not inhibited by lysosomotropic agents. These features are shared by a growing number of proteins recently shown to be degraded in a proteasome-dependent manner (Ivesa et al., 1999). We set out to determine whether the unassembled Ig L chains were degraded by the proteasome by performing pulse–chase experiments in NS1 cells followed by anti-κ immunoprecipitations (Figure 1). As previously described, the half-life of κNS1 was ~1 h in untreated cells. In the presence of the proteasome inhibitors PS1, MG132, and lactacystin, an increase in the half-life of κNS1 was observed varying from two- to fivefold depending on the experiment and/or the inhibitor used. However, there was no increase in the amount of secreted Ig L chains; we found <5% in 4 h of chase in control as well as inhibitor-treated cells (our unpublished data), suggesting that the κNS1 chains were still degradation targets. This was confirmed by experiments in which the inhibitor MG132 was washed away after 4 h of chase; under these conditions degradation of κNS1 parlayed (our unpublished results). Thus, the data indicated that, as expected, κNS1 degradation was mediated by the proteasome, implying that the Ig L chain must be retrotranslocated to the cytosol where the proteasome resides (Enenkel et al., 1998; Rivett, 1998). Further experiments were performed with lactacystin because of its higher specificity for proteasome inhibition (Fenteany and Schreiber, 1998; Lee and Goldberg, 1998).

Unassembled Ig L chains occur as partially folded BiP-bound molecules in the cell, and BiP binds to the N-terminal variable domain in which the intrachain disulfide bond is not formed. The intrachain disulfide bond is formed in the C-terminal constant domain, which does not bind to BiP (Skowronek et al., 1998; Hellman et al., 1999). Under nonreducing conditions, immunoprecipitated κNS1 chains are found in two forms in control cells (Figure 2; Knittler et al., 1995): a partially oxidized form (I; reflecting a molecule with an oxidized constant domain and a reduced, BiP-bound variable domain) and a completely oxidized form (II) most probably a product of postlysis oxidation of the variable domain after dissociation from BiP during immunoprecipitation. We reasoned that an indirect measure of the localization to the ER lumen of the Ig L chain (as BiP-bound κNS1) would be the presence of form I. A pulse–chase experiment was performed in which the cells were lysed in the presence of the alkylating agent NEM. The folding state and the relative amounts of the two forms during the chase were similar in control and proteasome-inhibited cells (Figure 2). Some investigators have reported the detection of completely reduced molecules upon inhibition of their degradation (Tortorella et al., 1998). We did not detect any reduced Ig L chains (which should be visible as a slower migrating band above the partially oxidized form) even with longer exposures of the autoradiogram shown in Figure 2 or by Western blotting of overloaded gels. To rule out the possibility that we failed to detect reduced molecules because the antibodies used would not bind to an Ig L chain with a reduced constant domain, we tested the antibodies for their capacity to immunoprecipitate reduced κNS1 Chains. In comparison with partially folded Ig L chains, 40–50% of the molecules could still be immunoprecipitated when com-

![Figure 1](image-url)

**Figure 1.** Effect of proteasome inhibitors on the half-life of Ig L chains. A representative pulse–chase experiment is shown. NS1 cells were starved for 2 h in the presence of lactacystin or PS1, labeled for 30 min, and chased up to 4 h in the continuous presence of the inhibitors. MG132 was added after the pulse, because we observed a decrease in protein synthesis when it was present from the beginning of the experiment. At each of the time points indicated, postnuclear supernatant was prepared from equal numbers of cells (with the same incorporation of radioactivity) and used to immunoprecipitate the κNS1 chains as stated in MATERIALS AND METHODS. The calculated half-lives in minutes are indicated.

![Figure 2](image-url)

**Figure 2.** Folding state of IgκNS1 chains upon lactacystin treatment. A pulse–chase experiment similar to that shown in Figure 1 was performed, with the following modifications: before lysis, cells were washed 5 min with PBS in the presence of 20 mM NEM, and the same NEM concentration was included during lysis. Ig L chains were immunoprecipitated under the usual conditions (see MATERIALS AND METHODS). SDS-PAGE was performed under nonreducing conditions, which allows distinction of two different folding forms of the κNS1 chains (I, partially oxidized; and II, completely oxidized).
no significant difference in the relative amounts of was used for immunoprecipitations. Nevertheless, there was a diolabeled proteins from control or lactacystin-treated cells and 6, respectively), because the same amount of total ra-

chains recovered from lactacystin-treated cells reflects the extent of proteasome inhibition (Figure 3, compare lanes 1 and 6, respectively), because the same amount of total radiolabeled proteins from control or lactacystin-treated cells was used for immunoprecipitations. Nevertheless, there was no significant difference in the relative amounts of \( \kappa_{\text{NSI}} \) chains recovered from the various fractions when both conditions were compared (Figure 3, compare lanes 2–5 with lanes 7–10). We repeatedly detected \( \kappa_{\text{NSI}} \) in the cytosolic fraction (SN), most likely a result of ER membrane leakage, because a part of other soluble ER markers such as BiP or calreticulin was also found in that fraction (Figure 3, lanes 5 and 10). The vesicle fraction P3 contained little or no cyto-

Table 1. Ig L chain localization after proteasome inhibition

<table>
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<tr>
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<th>Actual values(^a)</th>
<th>Theoretical values(^b)</th>
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<tr>
<td></td>
<td>P3</td>
<td>SN</td>
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<td></td>
<td>DMSO</td>
<td>Lactacystin</td>
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<td></td>
<td>72.2 ± 3</td>
<td>27.8 ± 3</td>
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\(^a\) Signals of labeled \( \kappa \) chains immunoprecipitated from equivalent amounts of the vesicular fraction (P3) and the cytosol (SN) prepared from lactacystin-treated or control (DMSO) cells as described in the legend to Figure 3 were quantified by phosphorimaging. For each of the three independent experiments and conditions, the total amount of \( \kappa \) chains (P3 + SN) was set to 100%, and the percentage of \( \kappa \) chains present in P3 \((\%P3 = P3/(P3 + SN))\) and in SN \((\%SN = 1 - \%P3)\) was determined.

\(^b\) Theoretical values were deduced from the data of the three pulse–chase experiments (with or without lactacystin) used for the fractions. The amount of \( \kappa \) chains after 3-h lactacystin treatment minus the amount of \( \kappa \) chains in the respective control represented the amount of nondegraded chains accumulated after 3 h of chase. This value, given as a percentage of the total amount of \( \kappa \) chains at 3 h of chase (plus lactacystin), represented the fraction of chains expected to be in the cytosol (SN) if retrotranslocation was not coupled to proteasome activity. In the control, these theoretical values were set to P3 = 100 and SN = 0, because these are the values most approximated to the situation in vivo.

Ig L Chains Remain in the ER Lumen When Proteasome Activity Is Inhibited

We addressed ER localization of \( \kappa_{\text{NSI}} \) chains after lactacystin treatment more directly by cell fractionation and protease protection assays. To this end, NSI cells were labeled for 30 min and chased for 3 h in the presence or absence of lactacystin. Cells were mechanically homogenized in isotonic buffer and fractionated by differential centrifugation. Ig L chains were immunoprecipitated from equivalent amounts of solubilized fractionated or unfraccionated material and separated by SDS-PAGE, and the signals were quantified (Figure 3 and Table 1). The higher amount of labeled Ig L chains recovered from lactacystin-treated cells reflects the extent of proteasome inhibition (Figure 3, compare lanes 1 and 6, respectively), because the same amount of total radiolabeled proteins from control or lactacystin-treated cells was used for immunoprecipitations. Nevertheless, there was no significant difference in the relative amounts of \( \kappa_{\text{NSI}} \) chains recovered from the various fractions when both conditions were compared (Figure 3, compare lanes 2–5 with lanes 7–10). We repeatedly detected \( \kappa_{\text{NSI}} \) in the cytosolic fraction (SN), most likely a result of ER membrane leakage, because a part of other soluble ER markers such as BiP or calreticulin was also found in that fraction (Figure 3, lanes 5 and 10). The vesicle fraction P3 contained little or no cytosolic contaminants, as indicated by the absence of detectable cytosolic marker tubulin (our unpublished data). Quantification of the signals obtained showed the relative distribution of \( \kappa_{\text{NSI}} \) in the vesicle (P3) and cytosolic (SN) fractions obtained from cells treated with or without lactacystin (Table 1). Based on the extent of inhibition, we calculated the values expected for a situation in which all \( \kappa_{\text{NSI}} \) molecules accumulating in lactacystin-treated cells had been translocated to the cytosol (Table 1). From these results, we conclude that \( \kappa_{\text{NSI}} \) chains are not translocated to the cytosol but remain associated with ER membranes after proteasome inhibition.

The fractionation data did not exclude that at least some of the Ig L chains were located at the cytosolic face of the ER membrane, as has been reported for other proteins in similar conditions (Hiller et al., 1996; Meerovitch et al., 1998). To control for this possibility, trypsin protease protection experiments were performed with the vesicle fraction P3 (Figure 4A). Ig L chains were then immunoprecipitated and quantified, and general leakage of soluble proteins from the lumen of the vesicles was monitored by Western blot of calreticulin, a soluble ER chaperone. Under optimal conditions, one would expect to find calreticulin completely protected from trypsin digestion on the one hand, whereas both calreticulin and the Ig L chains should be completely degraded upon detergent treatment, on the other hand. We never achieved such a condition, even when we used BiP as a luminal marker or proteinase K instead of trypsin (our unpublished data). Regardless of these technical difficulties, however, the protection of Ig L chains from trypsin digestion was the same in control and lactacystin-treated samples and did not significantly differ from that found for calreticulin.
Ig L Chain Is Quantitatively Bound to BiP in Lactacystin-treated Cells

An independent way to study localization of Ig L chains is to determine whether the molecules are bound to the luminal ER chaperone BiP, as was already demonstrated for κNS1 in untreated cells (Knittler et al., 1995). For this purpose, size fractionation experiments were performed. Samples were applied onto a Superdex 200 column, and eluted fractions were used to immunoprecipitate κNS1. Under control conditions, the BiP–κNS1 complex is well preserved as the Ig L chains were recovered in fractions corresponding to the expected molecular mass of a stoichiometric BiP–κNS1 complex, and no other major peak appeared (Figure 5A). From the gels used to quantify the relative amount of Ig L chains, it is seen that BiP coprecipitated with κNS1 chains in these fractions (Figure 5B). In the presence of MgATP that leads to dissociation of BiP–ligand complexes, the Ig L chains were recovered in fractions corresponding to monomeric molecules (Knittler et al., 1995; our unpublished data). We repeated the experiment in parallel with samples chased for 3 h in the presence of lactacystin. The eluting profile of Ig L chain was undistinguishable from that of the control sample (Figure 5A), and MgATP caused the same effect as in the control.
The combination of the results presented in Figures 3–5 indicated that, even after proteasome inhibition, κNS1 chains were quantitatively bound to BiP in a partially oxidized form. As a consequence, these results independently confirmed that κNS1 was localized in the ER when degradation was blocked, despite the cytosolic location of the proteasome. An important corollary of this is that the retrotranslocation of the Ig L chains is tightly coupled with the catalytic activity of the proteasome itself.

**Proteasome Inhibition Increases the Physical Stability of the BiP–κNS1 Complex**

Unassembled Ig L chains are quantitatively bound to BiP in a 1:1 complex in vivo (Cremer *et al.*, 1994; Knittler *et al.*, 1995; Figure 5). Moreover, the dissociation kinetics of κNS1 from BiP matches the half-life of the Ig L chain itself (Knittler and Haas, 1992) pointing to an important function of BiP in the degradation of unassembled Ig L chains. We followed the fate of the BiP–κNS1 complex in the absence or presence of lactacystin by coimmunoprecipitation experiments. BiP and coimmunoprecipitated κNS1 chains were visualized in the same gel (no second immunoprecipitation is necessary, because the Ig L chain is the major BiP substrate in NS1 cells; Figure 6A). In the control situation, only a small portion (2–5%) of the κNS1 chains present in the sample was coimmunoprecipitated with BiP. This was partly due to incomplete immunoprecipitation of BiP. In fact, only 30–40% of the total amount of BiP was isolated in these experiments. In addition, the V domain of the κNS1 does not exhibit a very stable interaction with BiP during immunoprecipitation, in contrast to BiP–IgL chain complexes seen with size chromatography (Figure 5; Knittler and Haas, 1992; Skowronek *et al.*, 1998). Nevertheless, and as expected, the kinetics of κNS1 dissociation from BiP was identical to that of κNS1 degradation (Figure 6, A and B). In contrast, the dissociation of κNS1 from BiP did not match the half-life of κNS1 when the proteasome inhibitor was present (Figure 6, A and B). Instead, the Ig L chain signal remained constant or even slightly increased during the chase, depending on the experiment. The coimmunoprecipitation of Ig L chains with BiP was specific, as it was abolished by treatment with MgATP before immunoprecipitation (our unpublished data). We quantified the Ig L chain signals to determine the percentage of coprecipitated κNS1 and consistently found a two- to threefold increase after 2 h of chase when lactacystin was present (Figure 6C).

These results were surprising. Certainly, the presence of the proteasome inhibitor should lead to a net increase in the amount of total Ig L chains. But even if this caused the formation of additional BiP–κNS1 complex during the chase, one would not expect to find increasing levels of labeled Ig L chains coprecipitating with BiP, because elevated levels of κNS1 would decrease the specific activity in the total Ig L chain pool to the same extent. Therefore, a shift in the equilibrium toward the BiP–κNS1 complex recovered by immunoprecipitation of BiP could at most be due to an increase in the total amount of BiP, which could escape our analysis because additional BiP would not be labeled. Indeed, it has been reported that proteasome inhibition leads to up-regulation of chaperones, BiP among them (Bush *et al.*, 1997; Mathew *et al.*, 1998). However, we found that the total amount of BiP remained constant in NS1 cells even after 8 h
of lactacystin treatment (our unpublished data). Therefore, the increase in the amount of BiP–κNS1 complex cannot be explained by a simple shift in the equilibrium toward the complex caused by an increase in the concentration of Ig L chains or BiP. From these results, we conclude that proteasome inhibition leads to a higher physical stability of the BiP–κNS1 complex.

DISCUSSION

Preconditions for ERAD to occur are 1) recognition of the misfolded protein in the ER, 2) translocation of the substrate to the cytosol, and 3) proteasomal degradation. The first task is most likely assumed by the various chaperones present in the ER. An important question is whether these proteins also assume a function in the process of substrate delivery to the cytosol. A number of publications address the role of the lectin-like transmembrane chaperone calnexin in the degradation of misfolded glycoproteins (Knop et al., 1996b; deVirgilio et al., 1998; Keller et al., 1998). Genetic analysis provided evidence for a role of the soluble chaperone BiP in the degradation of a mutant form of the glycoprotein carboxypeptidase Y (Plemper et al., 1997). We have presented here biochemical studies aimed at elucidating the role of BiP in the degradation of a soluble unglycosylated protein using the unassembled Ig L chain κNS1 as a model substrate. We found that inhibition of proteasome activity led to a prolonged half-life of the κNS1 chains, establishing that this protein is an ERAD substrate. Furthermore, our data indicated that dissociation from BiP and retrotranslocation of the BiP-bound substrate are tightly coupled to proteasome activity.

As already mentioned, our previous work on Ig chains had pointed to an important function of BiP in the retention in the ER and consequent degradation of unassembled molecules (Knittler and Haas, 1992; Knittler et al., 1995; Skowronnek et al., 1998). Similar findings were reported for a Xenopus transmembrane protein, the P-type Na, K-ATPase. Both the multimembrane-spanning α-subunit and the type II transmembrane glycoprotein β subunit bind to BiP, and this interaction correlates with the stability of the subunits when expressed in the absence of the partner chain (Begghah et al., 1996). All the data presented here consistently showed that κNS1 is trapped in the ER lumen in a BiP-bound form when proteasome activity is impaired. First, the folding state of κNS1 chains did not change upon proteasome inhibition (Figure 2), indicating that the Ig L chains were still bound to BiP. In this context, it may not be too surprising that we failed to detect Sec61p-bound Ig L chains by coimmunoprecipitation experiment (our unpublished data). Together, these data argue for the Ig L chain being completely translocated into the ER. Moreover, one must take into account that the second cysteine participating in the constant domain disulfide bond is placed only 20 amino acids away from the C terminus. In addition, it has recently been shown that CPY* is also entirely in the ER before retrotranslocation starts (Plemper et al., 1999). Second, subcellular fractionation and protease digestion experiments indicated that, regardless of proteasome activity, Ig L chains reside within a membrane-bounded compartment (Figures 3 and 4). Last, size fractionation of cellular proteins revealed that Ig L chains prevented from degradation remain in a BiP-bound state (Figure 5). In addition, we found that degradation of accumulated Ig L chains is resumed with the removal of a reversible proteasome inhibitor, indicating that the conditions used led to the arrest of a defined step in the normal degradation pathway. These results also showed that the substrate is not translocated to the cytosol in the absence of proteasome function, strongly suggesting that extraction of unassembled Ig L chains from the ER is concomitant with proteolysis. The analysis of a model transmembrane protein revealed a membrane-embedded intermediate lacking the cytoplasmic domain when mutant proteasomes were acting (Mayer et al., 1998). Because the special design of this model protein may have precluded the direct extension of this finding to other proteins, it was important to show that proteasome activity is indeed needed for extraction of at least some transmembrane proteins (Plemper et al., 1998), a hypothesis also suggested by Yang et al. (1998), who showed that TCR δ is retained mostly in the ER after proteasome inhibition.

Our data are not in contrast with other reports on retrotranslocation that occurs in the presence of proteasome inhibitors. A review of the literature reveals that most of the available data support or at least do not dispute the direct involvement of the proteasome in the retrotranslocation event. However, a more careful quantification of fractionation and protease protection experiments presented is needed. For instance, although TCRs and mutant ribophorin are partly found in the cytosol in a deglycosylated state (Huppa and Ploegh, 1997; Yu et al., 1997; deVirgilio et al., 1998; Yang et al., 1998), the amount detected is clearly lower than expected given the extent of the inhibition of degradation, as already stated by Yu and coworkers (Yu et al., 1997). We have calculated the amount of Ig L chains expected to be in the cytosol if retrotranslocation had occurred in the absence of proteasome function (Table 1). From these data, it is clear that κNS1 does not leave the ER when the proteasome is inactive. This may simply reflect that the equilibrium between ER and soluble cytosolic forms is dependent on the nature of the protein (Dusseljee et al., 1998; Yang et al., 1998) and/or on its interaction with ER-resident chaperones. On the other hand, the translocation pore may be in a “closed” state (Hamman et al., 1998) and needs an opening signal to allow an ordered process such as translocation or retrotranslocation to occur. This signal could be provided by the active proteasome.

The first pioneering studies on cytomegalovirus protein-induced MHC class I H chain degradation show that most of the H chain appears in the cytosol upon expression of the US2 and US11 proteins (Wiertz et al., 1996a,b). However, as the same authors point out, this could just be due to an intrinsic difference in the mechanism of retrotranslocation induced by the viral proteins. The in vitro studies with yeast microsomes from the laboratories of F. Brodsky, A. McCracken, and K. Römisch (Werner et al., 1996; Pilon et al., 1997) also find significant retrograde transport of unglycosylated prepro-α-factor under certain conditions, for instance in the presence of mutant proteasomes. However, it is not clear whether this reflects a particular property of the substrate or whether the in vitro system is leaky in the control of retrotranslocation.

ERAD seems to involve the same translocation channel as is used for protein translocation into the ER. The yeast
sec61-2 mutant was shown to be defective in the retrotranslocation of ERAD substrates (Plemper et al., 1997), and the group of H. Ploegh reported on the interaction of MHC class I H chains with the Sec61 protein during retrotranslocation (Wiertz et al., 1996a). Translocation of polypeptides from the cytosol to the ER is ensured by the presence of an N-terminal signal sequence that is cleaved off during or after translocation. One of the puzzling questions is, therefore, how lumenal substrates are reinserted into the translocation channel for retrograde movement. BiP-bound substrates could be positioned close to the translocation channel by binding of BiP to the DnaJ-like domain of the translocon component Sec63p (Corsi and Schekman, 1997). In fact, genetic data support a role for Sec63p in ERAD (Plemper et al., 1999). The existence of a human Sec63p homologue (Skowronek et al., 1999) strongly suggests a similar function in mammalian cells. Once the BiP–substrate complex is placed in close proximity to the channel, one could imagine a stochastic process for threading the polypeptide into the pore. We do not know whether cycles of BiP binding and release occur, but if so, the equilibrium is obviously completely shifted to the BiP-bound state, because no free Ig L chains were detected, even when proteasome activity was impaired. In fact, recent data indicate that few, if any, cycles of binding and release occur during the interaction of BiP with IgG H chains, suggesting that dissociation takes place only when the protein is either assembled with Ig L chains, if present, or marked for degradation, if not (Hendershot, personal communication). These findings could argue in favor of a signal required to introduce the BiP-bound substrate into the translocation machinery. Given our results, it is tempting to speculate that active proteasome signals the triggering of the retrotranslocation event. The group of D. Wolf has presented data implicating BiP/Kar2p in the degradation of CPY*. The Kar2 mutant used exhibited a defect in the ATPase domain of the chaperone, suggesting that ATP hydrolysis is important for release of the substrate to the cytosol. When Kar2p function is impaired, CPY* remains in the ER (Plemper et al., 1997). It would be interesting to know whether CPY* is bound to Kar2p under these conditions.

As yet undefined factors, possibly DER or HERD gene products (Hampton et al., 1996; Bordallo et al., 1998), could be involved in the final recognition of degradation substrates and their inevitable targeting to the degradative machinery by supporting threading of the substrate into the pore and retrotranslocation. By performing kinetic studies on BiP–\(\kappa_{\text{NS1}}\) interaction in lactacystin-treated cells, we found a time-dependent increase in the amount of labeled Ig L chains coimmunoprecipitated with BiP (Figure 6). This pointed to a stabilization of BiP substrate interaction, possibly because of additional components participating in the BiP–\(\kappa_{\text{NS1}}\) complex before the degradation process. Although there was no evidence for a shift in the elution profile for Ig L chains in the presence of lactacystin (Figure 5), a higher-molecular-weight complex composed of the BiP–\(\kappa_{\text{NS1}}\) complex and additional components could still exist and be detected under different conditions. We are currently trying to identify such putative components by different techniques: high resolution size chromatography, cross-linking, and coimmunoprecipitation with candidate proteins, but so far all of our efforts have failed. However, one cannot discard the possibility that the result of Figure 6 is due to a conformational change in the BiP–\(\kappa\) complex, which renders it more stable. Anyway, in both cases, proteasome inhibition allows the detection of a novel “state” of the BiP–\(\kappa\) complex. Because the arrest caused by proteasome inhibition was reversible, we think that this stabilization reflects a transient step in the normal degradation pathway, which became detectable because undegraded labeled Ig L chains prepared for retrotranslocation had accumulated.

Several models could well accommodate our data. First, a multiprotein complex (most probably including BiP and perhaps some DER gene products and Sec63p) could act as the retrotranslocation apparatus, which would be allosterically connected with the proteasome to ensure a high coupling between retrotranslocation and degradation. Another possibility is that the chemical energy of ubiquitination acts as a molecular ratchet to prevent the backward movement of the protein once the retrotranslocation has been initiated. Although this model seems to fit well for some proteins (Biederer et al., 1997; deVirgilio et al., 1998), we have not detected any trace of ubiquitination of our Ig L chains when proteasome activity is inhibited (our unpublished results). A more formal scenario (not yet excluded) is the existence of a rapidly degraded inhibitor of retrotranslocation that builds up when the proteasome is inhibited. Finally, we would like to present our particular model for the NS1\(\kappa\) L chain, which is closer to the first one proposed above: \(\kappa_{\text{NS1}}\) is bound to BiP, probably together with other ER-resident proteins but not with the Sec61 protein, in a partially folded state. BiP molecules carrying substrate might be positioned close to the translocation channel through binding to the Sec63p protein. Eventually, \(\kappa_{\text{NS1}}\) dissociates from the complex and is rapidly retrotranslocated to the proteasome for degradation. Proteasome activity is required for extracting the Ig L chains from the ER. Whereas the ATPase function of BiP could provide a driving force from the luminal side, the proteasome itself, by its AAA-ATPase subunits located at the 19S cap structure (Glickman et al., 1998; Leonhard et al., 1999), could energize the retrotranslocation process from the outer surface of the ER. This could be facilitated by a specific anchoring of the proteasome to the cytosolic face of the ER (in mammalian cells ~10% of the proteasomes are located at that place; Rivett, 1998). It will be interesting to investigate the role of the Sec63 protein in these processes, because with its large cytosolic domain and the BiP-binding luminal DnaJ portion, it represents an ideal molecule to allow cross-talk between the cytosolic and luminal sides of the ER.

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