Misfolded BiP is degraded by a proteasome-independent endoplasmic-reticulum-associated degradation pathway

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Misfolded proteins are removed from the ER (endoplasmic reticulum) by retrotranslocation to the cytosol and degradation by the ubiquitin–proteasome system in a process designated ERAD (ER-associated degradation). Analysing the turnover of a misfolded form of the ER-resident chaperone BiP (heavy-chain binding protein) (BiP Δ A), we found that the degradation of BiP Δ A did not follow this general ERAD pathway. In transfected cells, BiP Δ A was degraded, although proteasome-dependent ERAD was inactivated either by proteasome inhibitors or by ATP depletion. In semi-permeabilized cells, which did not support the degradation of the proteasomal substrate α 1-antitrypsin, the degradation of BiP Δ A was still functional, excluding the Golgi apparatus or lysosomes as the degradative compartment. The degradation of

BiP Δ A was recapitulated in biosynthetically loaded brain microsomes and in an extract of luminal ER proteins. In contrast with proteasome-dependent ERAD, degradation fragments were detectable inside the microsomes and in the extract, and the degradation was prevented by a serine protease inhibitor. These results show that the degradation of BiP Δ A was initiated in the ER lumen by a serine protease, and support the view that proteasome-independent ERAD pathways exist.

Key words: degradation, endoplasmic reticulum, endoplasmic-reticulum-associated degradation (ERAD), heavy-chain binding protein (BiP), misfolded protein, proteasome.

INTRODUCTION

Misfolded secretory and membrane proteins are recognized by quality-control mechanisms in the ER (endoplasmic reticulum) and the early secretory pathway (for reviews, see [1–3]). Most of them are retained in the ER or retrieved to the ER and are finally removed by a series of events named ERAD (ER-associated degradation). According to the current model of ERAD, the misfolded proteins are not degraded in the ER itself, but are translocated back to the cytosol, where they are degraded by the ubiquitin–proteasome system (for reviews, see [4–6]). Since the seminal reports on the degradation of the cystic fibrosis transmembrane conductance regulator [7,8], this concept has been validated by a large number of reports on different proteins in yeast, as well as in mammalian cells. Thus proteasome-dependent ERAD apparently constitutes the degradation pathway for the majority of misfolded secretory proteins.

Nevertheless, a small number of misfolded secretory proteins are reported to be degraded by proteasome-independent mechanisms (for a review, see [9]). In most cases, the proteasome contributes to the degradation of the respective protein, but is supplemented by additional proteases that operate in a parallel proteasome-independent pathway. The existence of such a two-fold degradation pathway has been reported for human thyroid peroxidase [10] and a mutant form of yeast carboxypeptidase Y when expressed in mammalian cells [11]. The Z variant of α 1-antitrypsin (piz) is degraded in most cells by a proteasome-dependent pathway [12,13] that is, in liver cells, supplemented by a proteasome-independent pathway [14]. ER-localized amyloid β -peptide is degraded in the cytosol by two parallel pathways: one using the proteasome and the other using insulin-degrading enzyme [15].

For the other proteins mentioned above, the proteases, as well as the degradative compartment, have not yet been identified.

To our knowledge, there is as yet only one example of ERAD that seems completely independent of the proteasome. It is the degradation of the ER-resident membrane protein SCD (stearoyl-CoA desaturase), which is mediated by a plasminogen-related protease [16,17]. However, SCD is constitutively short-lived and thus its degradation does not appear to result from misfolding. Accordingly, a distinct degradation signal was identified in the cytosolic domain of this protein [18].

Whereas the degradation of misfolded secretory and membrane proteins that travel through the ER has been investigated intensely, studies on the degradation of ER-resident proteins have as yet been limited to membrane proteins such as SCD (see above), hydroxymethylglutaryl-CoA reductase [19] or a mutant form of Sec61p [20]. Degradation pathways of ER-luminal proteins are as yet largely unknown. This is, however, an important aspect, because mutations of ER-luminal molecular chaperones or other folding factors would severely compromise the function of the secretory pathway if the mutant proteins were not disposed of. To address this question, we analysed the degradation of BiP Δ A, a misfolded form of the ER-luminal chaperone BiP (heavy-chain binding protein). We found that the mutant was disposed of by a proteasome-independent degradation pathway and that the degradation occurred, at least partially, in the ER lumen.

EXPERIMENTAL

Plasmids

The cDNA of hamster BiP was amplified by RT (reverse transcriptase)-PCR from CHO (Chinese-hamster ovary) cells and

Abbreviations used: AEBF, 4-(2-aminoethyl)benzenesulphonyl fluoride; BiP, heavy-chain binding protein; BiP Δ A, misfolded BiP; BiPwt, wild-type BiP; CHO, Chinese-hamster ovary; ER, endoplasmic reticulum; ERAD, ER-associated degradation; HA, haemagglutinin; NLVS, 4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-Leu-vinylsulphone; piz, α 1-antitrypsin variant Z; SCD, stearoyl-CoA desaturase.

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cloned into the vector pSVSport-1 (Invitrogen). To insert an HA (haemagglutinin) epitope between amino acids 640 and 641 of BiP, two complementary oligonucleotides (5'-GCCCTTACCCA-TACGACGTCCCAGACTACGCTG-3' and 5'-GGCCAGCGTA-GTCTGGGACGTCGTATGGGTAAG-3') coding for the HA epitope (YPYDVPDYAGP) were annealed in such a way that they could be inserted into the Eco109I site of the BiP coding sequence (pSPBiP). BiP containing an HA tag at this position has been shown to be fully functional [21]. For the construction of BiP Δ A, 72 amino acids were deleted from the ATPase domain of BiP by digesting pSPBiP with MscI and NgoMI, filling in the overhanging ends with Klenow enzyme and re-ligating the construct (pSPBiP Δ A). For bacterial expression, the coding sequences of BiP and BiP Δ A respectively were amplified by PCR without the signal sequence and inserted into the vector pASK-IBA4 (IBA) which provides a StrepTagII at the N-terminus of the proteins (pIB4BiP and pIB4BiP Δ A). pSPpiz coding for the Z allele of α 1-antitrypsin was generated by cloning the cDNA of human α 1-antitrypsin (A.T.C.C.) into pSVSport-1, followed by PCR mutagenesis of codon 366: GAG, coding for glutamate, was changed to AAG, coding for lysine. All constructs were verified by sequencing (GATC Biotech, Konstanz, Germany). For *in vitro* transcription, pSPBiP and pSPBiP Δ A were linearized and transcribed with SP6 polymerase (Epicentre).

Cell culture experiments

CHO-K1 cells were transfected with pSPBiP, pSPBiP Δ A or pSPpiz using FuGENE 6 (Roche) or Metafectene (Biontex). For pulse-chase experiments, the cells were labelled 2 days after transfection for 30 min with [^{35} S]methionine. The labelling medium was removed, and the chase was performed in medium containing 15 mg/l unlabelled methionine and, when indicated, one of the following inhibitors: 100 μ M NLVS (4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-Leu-vinylsulphone), 40 μ M lactacystin or 100 μ M MG-132 (Calbiochem). When cellular ATP was depleted, the chase was performed in the presence of 20 μ M antimycin A (Sigma) and 1 mM 2-deoxy-D-glucose. To determine the degree of the ATP depletion, the ATP content was measured in a parallel experiment using the CellTiter Glo Luminescent Cell Viability assay (Promega), according to the manufacturer's protocol. For calibration, a standard curve was prepared by diluting ATP in culture medium. For the detection of BiP, the cells were lysed in PBS, 1% Triton X-100, 10 μ M E-64, 1 μ M pepstatin A and 1 mM PMSF. BiP was immunoprecipitated with anti-HA antiserum (Santa Cruz Biotechnology), resolved by SDS/PAGE (10% gels) and visualized using a Cyclone Storage Phosphor System (Packard) with quantification using the OptiQuant 3.0 software (Packard). Statistical significance was determined by the two-sample Student's *t* test, using the Origin 7.0 software (OriginLab Corporation). For cycloheximide-chase experiments, 2 days after transfection, the cells were pre-incubated for 30 min with 100 μ g/ml cycloheximide, and then chased in the presence of cycloheximide and, where indicated, 0.3 mM AEBSF [4-(2-aminoethyl)benzenesulphonyl fluoride]. The cells were lysed, and BiP Δ A and actin were detected by immunoblot and enhanced chemiluminescence using anti-HA antiserum and anti-actin monoclonal antibody (Chemicon) respectively. For densitometric analysis, OptiQuant was used, and the BiP Δ A signals were corrected for equal actin signals.

Semi-permeabilized cells

At 2 days after transfecting CHO cells as above, the plasma membrane was permeabilized by incubating the cells for 5 min

on ice with 30 μ g/ml digitonin, followed by 10 min incubation in PBS without digitonin [22]. For the chase, the cells were incubated in PBS containing either an ATP-regenerating system (4 mM MgCl₂, 2 mM ATP, 10 mM creatine phosphate and 100 μ g/ml creatine kinase) or an ATP-depleting system (4 mM MgCl₂, 5 mM glucose and 60 units/ml hexokinase). The cells were lysed as above. The proteins were visualized by immunoblot and enhanced chemiluminescence using HA antiserum for BiP and BiP Δ A, and α 1-antitrypsin antiserum (Sigma) for piz.

Microsomal degradation system

Microsomes were prepared from the grey matter of porcine brain obtained from the local slaughterhouse [23]. To determine the degree of contamination of the microsomes by other membranes, the activities of the plasma membrane marker alkaline phosphatase and the lysosomal marker β -hexosaminidase were measured using *p*-nitrophenyl-thymidine-5'-phosphate and *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide as substrates [24]. Grp94 was visualized using a rat monoclonal anti-Grp94 antibody (StressGen). For quantification, the blot was scanned and evaluated using the OptiQuant software. *In vitro* translation reactions contained 50% reticulocyte lysate (Promega), 0.5 μ Ci/ μ l [^{35} S]methionine, 50 ng/ μ l mRNA and 0.1 eq/ μ l microsomes (1 eq is the amount of microsomes corresponding to a protein concentration yielding a D_{280} of 50; for a full definition of eq, see [23]). After 30 min of incubation at 30°C, the translation was stopped with puromycin (2 mM final concentration). The microsomes were isolated by centrifugation at 9000 *g* for 10 min at 4°C, and washed in 0.5 M potassium acetate, pH 7.4, for 20 min on ice. After centrifugation, the microsomes were resuspended in PBS containing an ATP-depleting system (see above), divided into aliquots (corresponding to 10 μ l of translation reaction mixture) and chased at 37°C. At the end of the chase, the proteins were separated by SDS/PAGE (10% gels) and visualized by autoradiography.

Protease-protection analysis

BiP Δ A-loaded microsomes were resuspended in PBS containing 60 μ g/ml proteinase K and, where indicated, 1% Triton X-100. After incubation for 30 min on ice, the products were analysed as above.

Degradation in ER extract

An extract of luminal ER proteins was prepared from porcine brain microsomes as described in [25], with the only modification that the ammonium sulphate precipitation was performed at 60% saturation. The precipitated proteins were solubilized in 50 mM Tris/HCl, pH 7.5, 50 mM KCl, 5 mM MgCl₂ and 250 mM sucrose. An extract of 1 eq refers to the amount resulting from 1 eq of microsomes. For the degradation experiments, 0.3 μ g of BiP or BiP Δ A was incubated at 37°C in PBS containing 8 eq of extract. Where indicated, PMSF was used at 1 mM. The reaction mixture was separated by SDS/PAGE (10% gels), and the proteins were detected by immunoblot using Streptactin-peroxidase (IBA).

Purification of recombinant BiP and BiP Δ A

BiP and BiP Δ A were expressed in *Escherichia coli* transformed with pIB4BiP or pIB4BiP Δ A respectively, purified on a Strep-tactin-agarose column (IBA), according to the manufacturer's protocol, and concentrated using Centricon 50 columns (Millipore).

Figure 1 BiPΔA is misfolded

(A) Schematic representation of BiPwt and BiPΔA having 72 amino acids deleted in the ATPase domain. The N-terminal StrepTag epitope and the C-terminal HA epitope are indicated. pept.-bind., peptide-binding region. (B) BiPwt and BiPΔA were incubated with the indicated amounts of proteinase K (prot K) in the absence (– ATP) or presence (+ ATP) of ATP. Proteins were visualized by Coomassie Blue staining.

Protease-resistance analysis

Recombinant BiP or BiPΔA (3 μg) was incubated for 20 min at 37°C in 20 mM Hepes/KOH, pH 7.0, 25 mM KCl and 2 mM MgCl₂ containing the indicated amounts of proteinase K, and, where indicated, 1 mM ATP. The proteins were visualized by Coomassie Blue staining.

RESULTS

BiPΔA is degraded independently of the proteasome

To study the degradation of loss-of-function mutations of BiP, a mutant form of the protein, BiPΔA, was generated by deleting 72 amino acids from the ATPase domain of wild-type BiP (BiPwt), including residues required for ATP binding and ATP hydrolysis (Figure 1A). Protease-resistance analysis was used to show that BiPΔA was indeed misfolded and therefore a degradation-prone protein. For this purpose, both proteins were expressed in *E. coli*, purified and incubated with proteinase K (Figure 1B). BiPwt was rather stable in the presence of low concentrations of proteinase K, and this stability was increased further by the addition of ATP. In contrast, BiPΔA was readily degraded, even at the low proteinase K concentration, independently of the presence or absence of ATP.

To analyse the turnover of wild-type and mutant BiP, CHO cells were transfected with each protein, labelled with [³⁵S]methionine, and the disappearance of the proteins was followed by immunoprecipitation (Figure 2A). Whereas BiPwt was stable, BiPΔA was degraded with a half-life of between 2.5 and 3 h (Figure 2B). Thus BiPΔA is recognized by the ER quality-control system as a misfolded protein and is targeted for degradation.

To determine whether BiPΔA was degraded by the proteasome, cells were treated with the proteasome inhibitor NLVS [26]. Unexpectedly, the degradation of BiPΔA was not affected by NLVS (Figure 3A). Neither the extent nor the kinetics of degradation were changed (Figure 3B). Two additional proteasome inhibitors, lactacystin [27] and MG-132 [28], were also ineffective in inhibiting the degradation of BiPΔA (Figure 3C).

Proteasomal degradation of ER-localized proteins is an ATP-dependent process. The ATP requirement results from the translocation of the proteins across the ER membrane, from the ubiquitination reaction and from the unfolding of the proteins when

Figure 2 BiPΔA is selectively degraded in CHO cells

(A) CHO cells transfected with BiPwt or BiPΔA were labelled with [³⁵S]methionine and chased for the indicated times (in h). BiP was immunoprecipitated using an anti-HA antibody and visualized by autoradiography. u, untransfected cells. (B) Graphic representation of eight independent experiments performed as in (A). Results are means ± S.D.

they are fed into the proteasomal cavity. To test the ATP-dependence of the degradation of BiPΔA, CHO cells were transfected with BiPΔA or a mutant form of α1-antitrypsin (piz), which is degraded in CHO cells by the proteasome [12]. During the chase, the cells were treated with 2-deoxy-D-glucose and antimycin A to inhibit ATP synthesis, which resulted in approx. 90% depletion of the cellular ATP after 1 h, and almost complete ATP depletion thereafter (Figure 4A). The loss of ATP in the treated cells at 0 h chase is a result of the handling time required for the ATP measurement (20 min). In the presence of the inhibitors, the inhibition of the degradation of piz was statistically significant ($P < 0.05$). In contrast, BiPΔA was degraded almost as efficiently as in the absence of the inhibitors. The slight inhibition of the degradation was statistically not significant (Figures 4B and 4C).

The ATP-independence of the turnover of BiPΔA argued not only against an involvement of the proteasome, but also against lysosomal degradation or simple secretion of BiPΔA, because, in the absence of ATP, vesicular transport is inhibited. To exclude further a role of vesicular transport in the degradation of BiPΔA, semi-permeabilized cells were used. In these cells, vesicular transport is, in addition to ATP, strictly dependent on added GTP and cytosol [22]. Nevertheless, BiPΔA was degraded in the semi-permeabilized cells without the addition of GTP or cytosol (Figure 5A). As BiPwt remained stable, the degradation was selective for the misfolded BiPΔA. The degradation of the proteasomal substrate piz was inhibited completely, demonstrating that, under these experimental conditions, proteasome-dependent ERAD was no longer functional. In accordance with proteasome-independent degradation of BiPΔA, its degradation was not inhibited by MG-132 (Figure 5B). Thus the degradation

Figure 3 Degradation of BiPΔA is independent of proteasomes

(A) BiPΔA-transfected CHO cells were labelled with [^{35} S]methionine and chased for the indicated times (in h) in the absence (–) or presence (+) of the proteasome inhibitor NLVS. BiPΔA was immunoprecipitated and visualized by autoradiography. u, untransfected cells; *, endogenous BiP sticking to the agarose beads. (B) Graphic representation of three independent experiments performed as in (A). Results are means $\pm$ S.E.M. (C) BiPΔA-transfected CHO cells were labelled with [^{35} S]methionine and chased for 8 h in the absence (–) or presence of lactacystin (LC), NLVS or MG-132 (MG). Analysis was performed as in (A).

experiments in the semi-permeabilized cells not only excluded vesicular transport as a prerequisite for the degradation of BiPΔA, but also argued strongly against proteasomes being required for its degradation.

Degradation of BiPΔA is initiated inside the ER by a serine protease

With the exception of the small amyloid β -peptide [15], the translocation of proteins from the ER to the cytosol depends on ATP. Therefore the finding that the degradation of BiPΔA did not require ATP pointed to the possibility that BiPΔA was degraded inside the ER. To address this question, the degradation of BiPΔA was followed in ER-derived microsomes. The microsomes were not isolated from pancreas, as usual, but from brain to avoid the possible activation of trypsin and other proteases present in pancreas microsomes. To determine the degree of contamination of the microsomes by other membranes, the activities of the plasma membrane marker alkaline phosphatase and the lysosomal marker β -hexosaminidase were measured. The activities of both enzymes found in the microsomal preparation accounted for less than 0.1 % of the activities present in the homogenate (alkaline phosphatase, 0.06 %; β -hexosaminidase, 0.02 %). Grp94, which was used as a marker for the ER, was enriched in the microsomes, as compared with the homogenate, by a factor of 20 to 40,

Figure 4 Degradation of BiPΔA does not require ATP

BiPΔA- or piz-transfected CHO cells were labelled with [^{35} S]methionine and chased for the indicated times (in h) in the absence (–) or presence (+) of antimycin A and 2-deoxy-D-glucose. (A) The cellular ATP content of control cells (light grey) and ATP-depleted cells (dark grey) was measured at the indicated times (in h) using a luciferase-based assay. The ATP content found in control cells at 0 h incubation was set as 100 %. Results are means $\pm$ S.D. ($n = 4$). (B) BiPΔA and piz were immunoprecipitated and visualized by autoradiography. u, untransfected cells. (C) Graphic representation of three independent experiments performed as in (B). Results are means $\pm$ S.E.M.

depending on the individual preparation (Figure 6A). The microsomes were loaded by *in vitro* translation with BiP or BiPΔA and washed with high-salt buffer to remove cytosolic proteins adsorbed to their outside. The complete import of BiPΔA was verified by protease-protection analysis, which showed that the protein was inaccessible to trypsin added to the outside of the microsomes (Figure 6B). Upon incubation of the microsomes in cytosol-free buffer, BiPΔA was degraded, whereas BiPwt remained stable (Figure 6C). As in living cells, the degradation was independent of ATP. Interestingly, an additional band with an apparent molecular mass of approx. 30 kDa became visible during the degradation of BiPΔA (Figure 6D). As this band was not present directly after the translation, it possibly represented a degradation intermediate of BiPΔA.

These findings suggested that the degradation of BiPΔA was initiated inside the ER. To determine whether ER-luminal or

Figure 5 BiP Δ A is degraded in semi-permeabilized cells

(A) CHO cells were transfected with BiPwt, BiP Δ A or piz. After permeabilizing the plasma membrane with digitonin, the cells were chased for the indicated times (in h) in the presence of an ATP-depleting (–ATP) or ATP-regenerating (+ATP) system. The proteins were visualized by immunoblotting. (B) BiP Δ A-transfected semi-permeabilized cells were chased for the indicated times (in h) in the absence (–MG) or presence (+MG) of the proteasome inhibitor MG-132. BiP Δ A was visualized by immunoblotting.

membrane-bound proteases were involved, an ER extract containing the ER-luminal proteins was prepared by mild-detergent extraction of the microsomes. BiPwt and BiP Δ A were expressed in *E. coli*, purified and incubated with the ER extract (Figure 7). Whereas BiPwt was stable in the ER extract, BiP Δ A was degraded. The degradation was dependent on the presence of the ER extract, excluding the possibility that the protease was co-purified from the bacteria. As in the microsomal system, an additional band of approx. 30 kDa was generated during the chase. As this band was recognized by Streptactin–peroxidase, it comprised the N-terminal part of BiP Δ A containing the StrepTag.

For a first classification of the proteolytic activity contained in the ER extract, different class-specific protease inhibitors were applied. Inhibitors specific for cysteine proteases (E-64), metalloproteases (phenanthroline) or aspartic proteases (pepstatin A) were ineffective (results not shown). Of the inhibitors tested, only PMSF stabilized BiP Δ A and prevented the generation of the N-terminal degradation intermediate (Figure 8A). With the caveat that PMSF is not totally specific for serine proteases, the inhibition by PMSF pointed to a serine protease being responsible for the initial degradation of BiP Δ A. To test whether the degradation of BiP Δ A in living cells was also mediated by a serine protease, CHO cells were treated with cycloheximide and chased in the presence of the serine protease inhibitor AEBSE. BiP Δ A and actin were detected by immunoblotting. As in the ER extract, BiP Δ A was stabilized by the serine protease inhibitor (Figure 8B). Taken together, the data showed that BiP Δ A was degraded selectively in a proteasome-independent process which was initiated in the ER lumen by a serine protease.

DISCUSSION

In the ER, a large variety of structurally unrelated secretory and membrane proteins that fail to adopt their correct state of folding and assembly have to be recognized and finally degraded. In addition, a substantial number of structurally unrelated ER-resident

Figure 6 BiP Δ A is degraded in ER-derived microsomes

(A) Microsomes were prepared from porcine brain. The enrichment of the ER marker Grp94 in the microsomal preparation (m), as compared with brain homogenate (h), was determined by Western blot analysis. Note that different amounts of protein were loaded. (B) Microsomes were loaded with BiP Δ A by *in vitro* translation. After re-isolation by centrifugation, the microsomes were treated with trypsin in the absence or presence of detergent. (C) BiP Δ A- or BiPwt-loaded microsomes were washed with high salt to remove adsorbed cytosolic proteins, resuspended in buffer containing an ATP-depleting system and chased for the indicated times (in h). Proteins were separated by SDS/PAGE (10% gels) and visualized by autoradiography. (D) BiP Δ A-loaded microsomes were chased for the indicated times (in h). Proteins were separated by SDS/PAGE (10% gels) and visualized by autoradiography. *, possible degradation intermediate of BiP Δ A. Molecular masses (in kDa) are indicated.

Figure 7 Degradation of BiP Δ A is initiated by an ER-luminal protease

Purified BiPwt or BiP Δ A was incubated for the indicated times (in h) with an ER extract prepared by mild-detergent extraction of brain microsomes. BiP was visualized by Streptactin–peroxidase. *, degradation intermediate of BiP Δ A. Molecular masses (in kDa) are indicated.

Figure 8 BiPΔA is degraded by a serine protease

(A) Recombinant BiPΔA was incubated for the indicated times (in h) with an ER extract in the presence (+) or absence (–) of PMSF and visualized by Streptactin–peroxidase. *, degradation intermediate of BiPΔA. Molecular masses (in kDa) are indicated. (B) BiPΔA-transfected CHO cells were treated with cycloheximide and chased for the indicated times (in h) in the absence (–) or presence (+) of the serine protease inhibitor AEBSF. BiPΔA and actin were visualized by immunoblotting using anti-HA and anti-actin antibodies respectively. The blots were densitometrically analysed, and the BiPΔA signal was normalized to equal actin signals.

proteins have to be degraded during regular protein turnover or because of protein defects. This enormous diversity of degradation substrates contrasts with the current understanding of ERAD, which is limited to one well-characterized pathway. This pathway utilizes the cytosolic ubiquitin–proteasome system after retrotranslocation of the degradation substrate to the cytosol. Our results support the view that the proteasome-based pathway, although being most probably the central ERAD pathway, may not be the only one.

To our knowledge, there is as yet only one report on the degradation of an ER-resident luminal protein. This study shows that in cells exposed to oxidative stress protein disulphide-isomerase is degraded by proteasomes [29], indicating that the proteasome-dependent ERAD pathway operates also in the turnover of soluble ER-resident proteins. The degradation of BiP or of BiP mutants has as yet not been examined.

Proteasome-dependent ERAD is inhibited by proteasome inhibitors and by ATP depletion. This has been shown in living cells [30,31], in semi-permeabilized cells [32], and in microsome-based degradation systems [33,34]. In contrast with these examples, the degradation of BiPΔA was inhibited neither by proteasome inhibitors nor by ATP depletion in any of these systems. The ATP requirement of proteasome-dependent ERAD is explained not only by the ATP-dependence of the ubiquitin–proteasome system itself [35], but also by the preceding retrotranslocation of the substrates from the ER to the cytosol also depending on ATP [32,36,37], although small proteins may be retrotranslocated independently of ATP [15]. In agreement with a degradation pathway independent of retrotranslocation, BiPΔA was not retrotranslocated in the microsome-based system (results

not shown), although this system supports the retrotranslocation of both proteins that are targeted to the proteasome and proteins that escape proteasomal degradation [15,25,38]. Thus the ATP-independent degradation of BiPΔA strongly argues against its retrotranslocation to the cytosol and its degradation by cytosolic proteasomes, but is in line with a degradative system inside the ER.

To date, proteasome-independent ERAD has been described for only a very limited number of proteins. As far as these examples have been characterized, the independence of proteasomes is the only feature these proteins and BiPΔA have in common. For misfolded carboxypeptidase Y and piz, the trimming of the N-glycans appears to determine whether these proteins are degraded by proteasomes or by other, as yet unidentified, proteases [11,14]. As BiPΔA is not glycosylated, similar regulatory mechanisms can be excluded.

Although the proteases which are responsible for the proteasome-independent ERAD are largely unknown, it seems unlikely that a single proteolytic system is responsible. A cysteine protease, ER-60, has been reported to degrade mutant human lysozyme [39]. Human thyroid peroxidase is degraded by a membrane-bound leupeptin-sensitive serine protease [10] as is SCD [40]. The SCD-degrading protease was identified as a plasminogen-like protease [17]. BiPΔA was degraded by a PMSF- and AEBSF-sensitive protease, also pointing to the involvement of a serine protease. This protease was, however, not inhibited by leupeptin (results not shown) and was a soluble protein, arguing against its identity with the plasminogen-like protease.

Our findings suggest the presence in the ER lumen of an active protease which has the ability to distinguish misfolded BiP from native BiP. The fulfilment of this selectivity does not necessarily require a specialized protease, because even proteinase K (see Figure 1B) is able to distinguish mutant forms of BiP from native BiP. The more demanding requirement is the discrimination between misfolded BiP and immature BiP, i.e. BiP which has not yet finished its folding process. It should be noted, however, that this requirement is not specific for the degradation of BiP or proteasome-independent ERAD in general, but pertains to the proteasome-dependent ERAD pathway as well. One possibility is that folding intermediates are protected from degradation, as long as they interact with molecular chaperones and other folding factors of the ER. In support of this model are reports on a negative correlation between chaperone binding and degradation [13,41–45]. Thus chaperone-mediated preferential protection of folding intermediates as compared with misfolded proteins could confer specificity to a protease which is by itself unable to discriminate misfolded proteins from folding intermediates.

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