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Sequential assistance of molecular chaperones and transient formation of covalent complexes during protein degradation from the ER

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BACE457 is a recently identified pancreatic isoform of human β-secretase. We report that this membrane glycoprotein and its soluble variant are characterized by inefficient folding in the ER, leading to proteasome-mediated ER-associated degradation (ERAD). Dissection of the degradation process revealed that upon release from calnexin, extensively oxidized BACE457 transiently entered in disulfide-bonded complexes associated with the lumenal chaperones BiP and protein disulfide isomerase (PDI) before unfolding and dislocation into the cytosol for degradation. BACE457 and its lumenal variant accumulated in disulfide-bonded complexes, in the ER lumen, also when protein degradation was inhibited. The complexes were disassembled and the misfolded polypeptides were cleared from the ER upon reactivation of the degradation machinery. Our data offer new insights into the mechanism of ERAD by showing a sequential involvement of the calnexin and BiP/PDI chaperone systems. We report the unexpected transient formation of covalent complexes in the ER lumen during the ERAD process, and we show that PDI participates as an oxidoreductase and a redox-driven chaperone in the preparation of proteins for degradation from the mammalian ER.

Introduction

The quality control operating in the ER allows export of only correctly folded and completely assembled polypeptides. Terminally misfolded proteins are transported into the cytosol and degraded in processes collectively defined as ER-associated degradation (ERAD)* (McCracken and Brodsky, 1996; Kopito, 1997; Ellgaard et al., 1999). The importance of unraveling the mechanism(s) of ERAD in mammalian cells is emphasized by the recent description of the decisive role played by ERAD in hereditary human conformational diseases, such as cystic fibrosis and α1-antitrypsin deficiency (Plemper and Wolf, 1999; Kopito and Ron, 2000). Moreover, pathogens exploit the host ERAD machinery to escape immunosurveillance, as in the case of cytomegalovirus or Epstein-Barr virus (Wiertz et al., 1996a; Levitskaya et al., 1997), or to invade the host cytosol, as shown for bacterial and plant toxins (Hazes and Read, 1997; Tsai et al., 2001).

Models depicting the ERAD mechanism(s) rely on investigation in yeast and mammalian cells of not more than a dozen model glycoproteins and of few nonglycosylated proteins (for reviews see Cabral et al., 2001; Fewell et al., 2001). The picture emerging from these studies is that degradation of aberrant ER products is mainly performed by the cytosolic proteasome (Kopito and Sitia, 2000 and references therein). The topological distinction between site of production and site of degradation of ERAD substrates implies the export of misfolded proteins through the translocon complex (dislocation) (Wiertz et al., 1996b; Pilon et al., 1997; Plemper et al., 1997). There is accumulating evidence that proteasomes are strategically placed as a sentinel on the cytosolic face of dislocons and that normally dislocation and degradation are coupled events (Mayer et al., 1998; Mancini et al., 2000).

In mammalian cells, besides the overwhelming number of studies on calnexin, whose role remains nevertheless controversial (for review see Parodi, 2000), and the role of BiP in the retention of misfolded or orphan polypeptides in the ER lumen (Knittler et al., 1995), little is known about the involvement of lumenal molecular chaperones and enzymes in the ERAD process. In particular, although it has been

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*Abbreviations used in this paper: 2-D, two dimensional; DJ, *N*-butyldeoxynojirimycin; ERAD, ER-associated protein degradation; HEK, human embryonic kidney; Kif, kifunensine; L, clasto-lactacystin β-lactone; MA, 3-methyladenine; MG, MG132; N, ammonium chloride; PDI, protein disulfide isomerase.

Key words: ER-associated protein degradation; molecular chaperones; oxidoreductases; disulfide-bonded complexes; β-secretase

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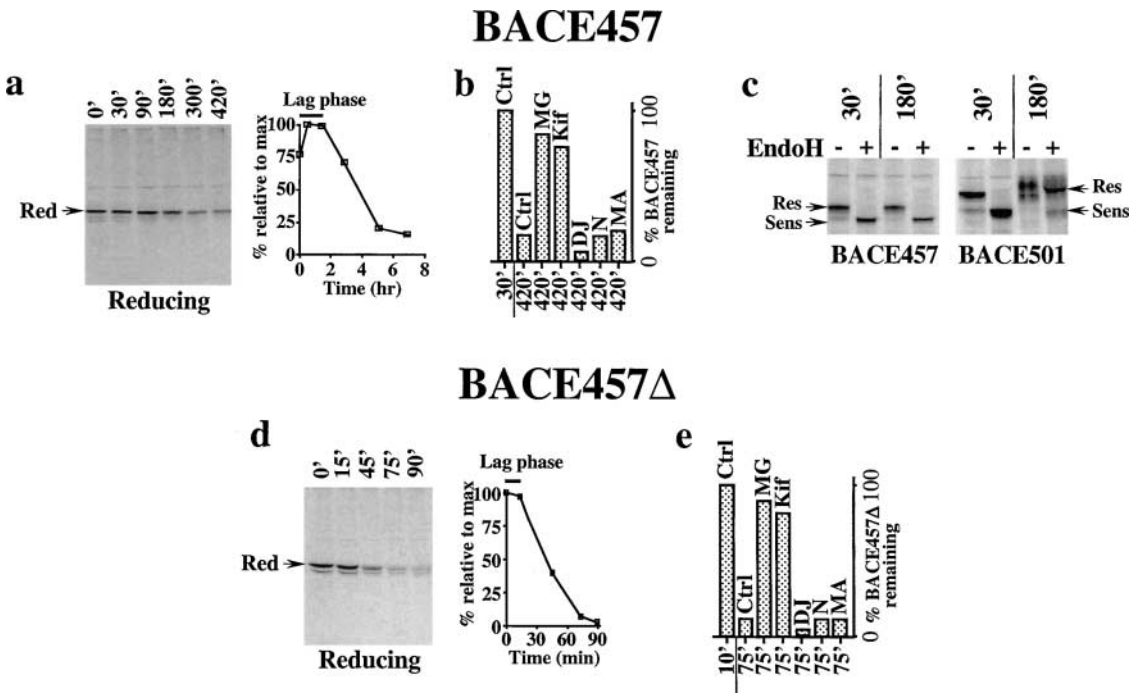

Figure 1. **BACE457 and BACE457Δ are ERAD substrates.** (a) Degradation of BACE457 during the chase is shown in the reducing gel. Kinetics of degradation were determined by densitometric analysis of the labeled protein. The lag phase of 90 min preceding the onset of BACE457 degradation is shown at the top of the graph. (b) To determine the effect of various ERAD inhibitors on degradation of BACE457, cells were mock treated (30 or 420 min) or incubated with MG (20 μM), Kif (1–10 μg/ml), DJ (1 mM), N (5 mM), or MA (10 mM) during starvation, pulse, and a 420-min chase. Refer to Fig. 3 for analysis of the inhibitory effects by SDS-PAGE. (c) BACE457 or BACE501 were transiently expressed in HEK293 cells. EndoH sensitivity of the proteins was assessed 30 and 180 min after their synthesis. Most of BACE501 was transported to the Golgi compartment, where it became resistant to EndoH treatment (Res). BACE457 was efficiently retained in the ER and remained sensitive to the endoglycosidase (Sens) (d and e), as in a and b for BACE457Δ.

shown that protein disulfide isomerase (PDI) is involved in the ERAD process in yeast (Gillece et al., 1999) and in protein dislocation in isolated microsomes (Tsai et al., 2001), and that ERAD is regulated by redox potential (Stafford and Bonifacino, 1991; Young et al., 1993; Courageot et al., 1999; Wilson et al., 2000), a direct implication of lumenal oxidoreductases in mammalian ERAD has not been shown yet.

The type I membrane glycoprotein BACE457 is a recently identified pancreatic isoform of human β-secretase. Compared with the brain isoform (BACE501), BACE457 is short of a 44–amino acid region located between the two catalytic aspartyl residues. In contrast to BACE501, when BACE457 is expressed in cells, it is retained in the ER in an immature state (Bodendorf et al., 2001; Tanahashi and Tabira, 2001). BACE501 cleaves the amyloid precursor protein to initiate the processing pathway leading to the generation of the β-amyloid peptides deposited in the senile plaques associated with Alzheimer's disease (Selkoe, 2001). BACE457 does not contribute significantly to amyloid precursor protein processing and its physiologic relevance remains to be established.

In this report, we show that folding of BACE457 and of its soluble variant, BACE457Δ, is inefficient. Most of the protein transiently expressed in human cells is retained in and degraded from the ER. Detailed analysis revealed that BACE457 and BACE457Δ entered first the ER folding machinery, namely the calnexin cycle, to explore conformations characterized by the variable extent of intramolecular disulfide bonding. After release from calnexin, the terminally misfolded polypeptides did associate with the lumenal chaperones BiP and PDI before dislocation into the cytosol and proteasome-mediated degradation. Importantly, after release from calnexin, most BACE457 entered into intermolecular disulfide-bonded complexes that had to be disassembled in the ER lumen to allow dislocation. Temporary inhibition of the ERAD machinery revealed disulfide-bonded complexes for the soluble variant BACE457Δ as well. We show that in the mammalian ER, the calnexin and the BiP/PDI chaperone systems work sequentially during the preparation of ERAD substrates for dislocation and degradation. We report that disulfide-bonded complexes may be formed in the ER lumen as transient intermediates during the ERAD process and that PDI acts as an oxidoreductase and as a redox-driven molecular chaperone during protein degradation from the mammalian ER.

Results

BACE457 and BACE457Δ are ERAD substrates

BACE457 or the soluble variant BACE457Δ were expressed in transiently transfected human embryonic kidney (HEK) 293 cells. To determine the fate of these proteins, cells were metabolically labeled, chased for various times, and cell extracts were analyzed after immunoprecipitation with BACE-specific antibody by reducing SDS-PAGE. BACE was the major labeled protein visible in the gels (Fig. 1, a and d, Red).

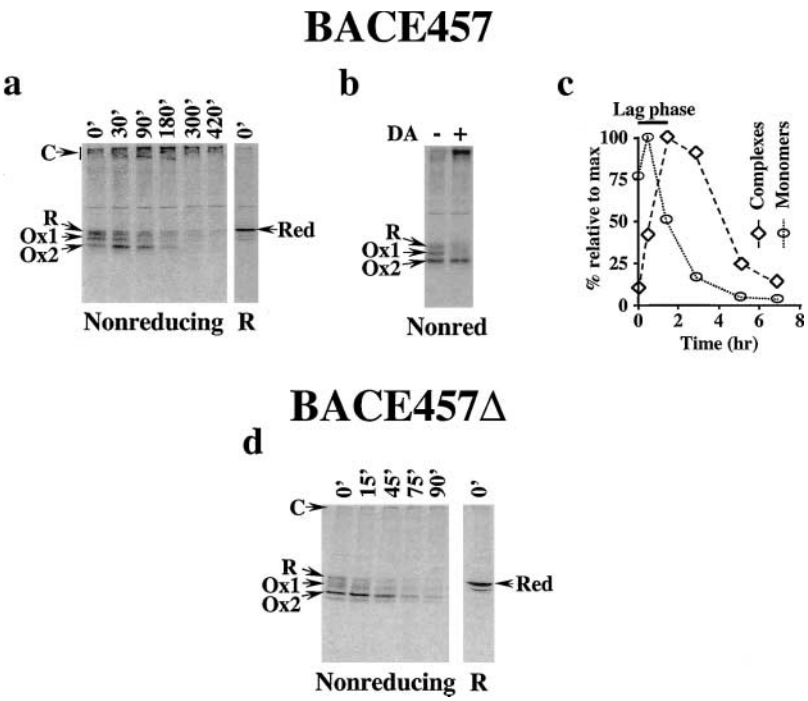

Figure 2. **BACE457 and BACE457Δ are extensively oxidized before onset of degradation.** (a) Samples were also analyzed by nonreducing SDS-PAGE to assess the oxidation state of BACE457 during the experiment. The oxidation intermediates of BACE457 (R, Ox1, and Ox2) and the disulfide-bonded complexes (C) are indicated; upon reduction, BACE457 migrated as a single band (reducing lane, Red). (b) Cells expressing BACE457 were metabolically labeled and chased for 30 min. The chase was concluded with a 5-min incubation with (+) or without (−) 5 mM ice-cold diamide (diazenedicarboxylic acid bis[*N*,*N*-dimethylamide]), a cell permeable oxidant that induces aggregation of folding intermediates or misfolded proteins containing free SH groups (Tortorella et al., 1998). Aggregation of BACE475 upon treatment with diamide was determined by nonreducing SDS-PAGE. (c) Quantification of BACE457 monomers (R+Ox1+Ox2, dotted line, circles) and BACE457 complexes (broken line, diamonds) was performed from nonreducing gels as described in the Materials and methods. (d) As in panel a for the soluble variant BACE457Δ.

After a lag phase of ~90 min, the amount of labeled BACE457 decreased with a half-life of 4 h (Fig. 1 a). As a consequence, after 420 min of chase, ~90% of the labeled protein had been degraded (Fig. 1, a and b). Treatment with ERAD inhibitors, such as the proteasome inhibitors MG132 (MG) and clasto-lactacystin β-lactone (L) or the mannosidase I inhibitor kifunensine (Kif; Liu et al., 1999), prevented BACE457 degradation (Fig. 1 b and Fig. 3). Treatment with inhibitors of lysosomal degradation or of autophagocytosis (ammonium chloride [N] and 3-methyladenine [MA], respectively; Fig. 1 b) did not protect BACE457 from degradation.

BACE457Δ was degraded faster ($t_{1/2}$ = 40 min). The lag phase for the soluble variant of BACE457 was only 15 min (Fig. 1 d). Again, the 90% degradation observed after 75 min of chase in control cells (Fig. 1, d and e) was significantly delayed by MG or Kif (Fig. 1 e and Fig. 3) or L (not depicted) but not by the lysosomal or autophagocytosis inhibitors (Fig. 1 e). BACE457 and BACE457Δ were retained in the ER before being degraded. This was shown by the preservation of EndoH sensitivity (Fig. 1 c), colocalization with ER markers by immunofluorescence, lack of processing of the propeptide, and absence of protein at the cell surface or in the conditioned medium (unpublished data; Bodendorf et al., 2001; Tanahashi and Tabira, 2001). The brain splicing variant, BACE501, expressed at similar levels in transiently transfected HEK293 cells (Fig. 1 c), showed a very efficient folding, was transported along the secretory pathway, as shown by acquisition of EndoH resistance (Fig. 1 c), and was properly targeted at the plasma membrane (Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999).

We concluded that the deletion of 44 residues in the ectodomain of the pancreatic variant of BACE457 impaired folding and rendered most of the protein expressed in human cells an ERAD substrate, as defined by the requirement of mannose trimming and the involvement of the cytosolic proteasome for degradation.

BACE457 and BACE457Δ are extensively oxidized before the onset of degradation

To analyze the behavior of BACE457 and BACE457Δ before and during degradation, samples were also analyzed by nonreducing SDS-PAGE. Initially, the labeled proteins were distributed in multiple bands that differed in the extent of intrachain disulfide bonding and had therefore distinct electrophoretic mobility (R, Ox1, and Ox2; BACE457, Fig. 2 a; BACE457Δ, Fig. 2 d). The labeled species indicated with R had the same mobility as BACE run under reducing conditions (Fig. 2, a and d, Red), indicating that most of its cysteines were still not disulfide bonded. Two additional oxidation intermediates of BACE (Ox1 and Ox2) were resolved in nonreducing gels. Ox2 appeared later, had the fastest mobility, and was the only form of BACE that was diamide resistant (BACE457, Fig. 2 b). This showed that all cysteines of Ox2 were in intramolecular disulfides and/or protected from further oxidation.

BACE457 monomers (R+Ox1+Ox2) persisted for ~30 min and then disappeared with a half-life of ~90 min (Fig. 2 a and Fig. 2 c, Monomers). Monomers disappeared therefore during the lag phase before the onset of degradation (Fig. 1 a and Fig. 2 c). The disappearance of BACE457 monomers correlated with the formation of disulfide-bonded complexes of heterogeneous size, visible as a broad band of labeled material at the top of the nonreducing gels (Fig. 2 a, C). Full-length BACE457 was the major labeled component of the complexes, as shown upon their disassembly in reducing gels (Fig. 1 a). The formation of disulfide-bonded complexes was a clear symptom of protein misfolding. Complexes accumulated during the lag phase and disappeared with the same kinetics observed for BACE457 degradation (Fig. 2 a and Fig. 2 c, Complexes). We concluded that BACE457 and BACE457Δ were extensively oxidized during a lag phase preceding proteasome-mediated degradation. Intriguingly, degradation of

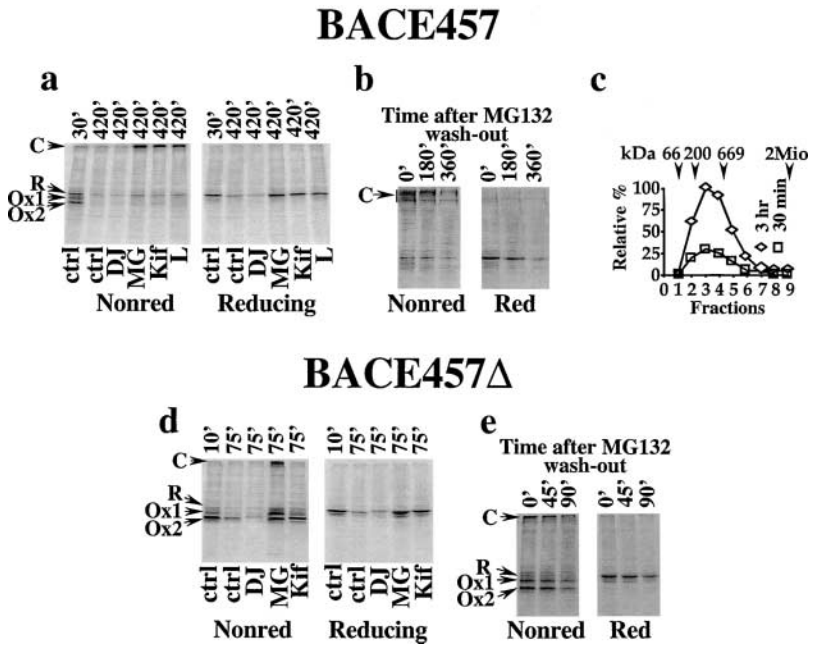

Figure 3. **ERAD substrates accumulate in disulfide-bonded complexes and remain in a degradation-competent state upon inhibition of the ERAD machinery.** (a) About 90% of labeled BACE457 was degraded in control cells after a 420-min chase (compare labeled BACE after 30 min and 420 min, Reducing, ctrl). Incubation with DJ reproducibly accelerated BACE457 degradation, whereas MG, Kif, and L significantly prevented degradation (Reducing). Most of the protein remained in disulfide-bonded complexes upon inhibition of the ERAD machinery (Nonreducing). The slightly slower mobility of BACE457 observed when the Kif sample was run under reducing conditions confirmed that mannose trimming had been efficiently inhibited. (b) Cells were incubated for 1.5 h in the presence of the reversible proteasome inhibitor MG to induce accumulation of misfolded, disulfide-bonded BACE457 (0 min). Upon MG washout, the chase was continued for the times indicated. (c) To determine the size of the disulfide-bonded complexes, cells expressing BACE457 were pulsed with radioactivity and chased for 30 min (squares) or 3 h (diamonds). Cell extracts were sedimented through 10–25% sucrose gradients with calibration markers. Fractions were collected, lighter first, precipitated with BACE457-specific antibody, and subjected to nonreducing SDS-PAGE to determine the sedimentation coefficient of monomers and disulfide-bonded complexes. In the figure, only the position in the gradient of the complexes is shown. (d) As in panel a for BACE457Δ. (e) As in panel b for BACE457Δ.

BACE457 seemed to occur from a pool of disulfide-bonded protein.

BACE remains degradation competent upon formation of disulfide-bonded complexes

We next investigated the fate of BACE457 and BACE457Δ when the ERAD machinery had been inhibited. Detergent extracts of cells mock treated or treated with ERAD inhibitors were immunoprecipitated and subjected to nonreducing or reducing SDS-PAGE (Fig. 3, a and d).

ERAD inhibitors did not prevent the conversion of BACE457 monomers in disulfide-bonded complexes, but efficiently blocked the disappearance of the complexes (Fig. 3 a, Nonreducing, MG, L, and Kif) and the degradation of the protein (Fig. 3 a, Reducing, MG, L, and Kif). Importantly, when the ERAD machinery was reactivated, e.g., upon washout of MG after the formation of disulfide-bonded complexes, the protein contained in the complexes was efficiently cleared from the cells (Fig. 3 b).

These data showed that aggregation of BACE457 was a reversible process and that, for BACE457, disulfide-bonded complexes represented a pool from which the protein was degraded.

The soluble form, BACE457Δ, was retained in the monomeric, fully oxidized Ox2 status when degradation was inhibited with Kif (Fig. 3 d). When the proteasome was inhibited, BACE457Δ accumulated in the oxidized monomers Ox1 and Ox2 as well as in disulfide-bonded complexes (Fig. 3 d, MG). As shown for BACE457, also the misfolded BACE457Δ contained in the complexes was efficiently cleared from the cells when degradation was resumed upon washout of the inhibitors (Fig. 3 e).

The oxidation states of BACE457 and BACE457Δ showed that they were not dislocated into the reducing cytosol when degradation was blocked. Consistently, only ~10% of the labeled proteins was isolated from the cytosolic fraction obtained by ultracentrifugation of cell homogenates (unpublished data). This is in keeping with emerging evidence showing that dislocation and degradation are coupled events both in yeast and mammalian cells (Mayer et al., 1998; Mancini et al., 2000).

Accumulation of misfolded BACE457 in the ER causes an increase in the number, and not in the size, of disulfide-bonded complexes

To better characterize the disulfide-bonded complexes transiently formed during BACE457 degradation, their size was determined after a 30-min chase, when very little BACE457 was disulfide bonded, and after a 3-h chase, when approximately three times more protein was disulfide bonded (Fig. 2 c, Complexes). Cell extracts were prepared as described above and sedimented through 10–25% sucrose gradients. Fractions were collected, lighter first, precipitated with BACE-specific antibody, and analyzed by SDS-PAGE. The disulfide-bonded complexes were heterogeneous in size, ranging from 120 to 800 kD, as determined by cosedimentation of calibration markers (Fig. 3 c). Surprisingly, complexes formed when the aggregation process had just begun (i.e., after a 30-min chase) sedimented in the same fractions as complexes isolated from cells in which most of the labeled BACE was disulfide bonded (Fig. 3 c, 3-h chase). We concluded that the accumulation of misfolded BACE457 in the ER resulted in the formation of more complexes of the same size rather than in the enlargement of preexisting complexes. Taken together, the data presented in Figs. 1–3 showed that complexes were units with a limited capacity for accepting misfolded BACE457 and in which BACE457 remained degradation competent.

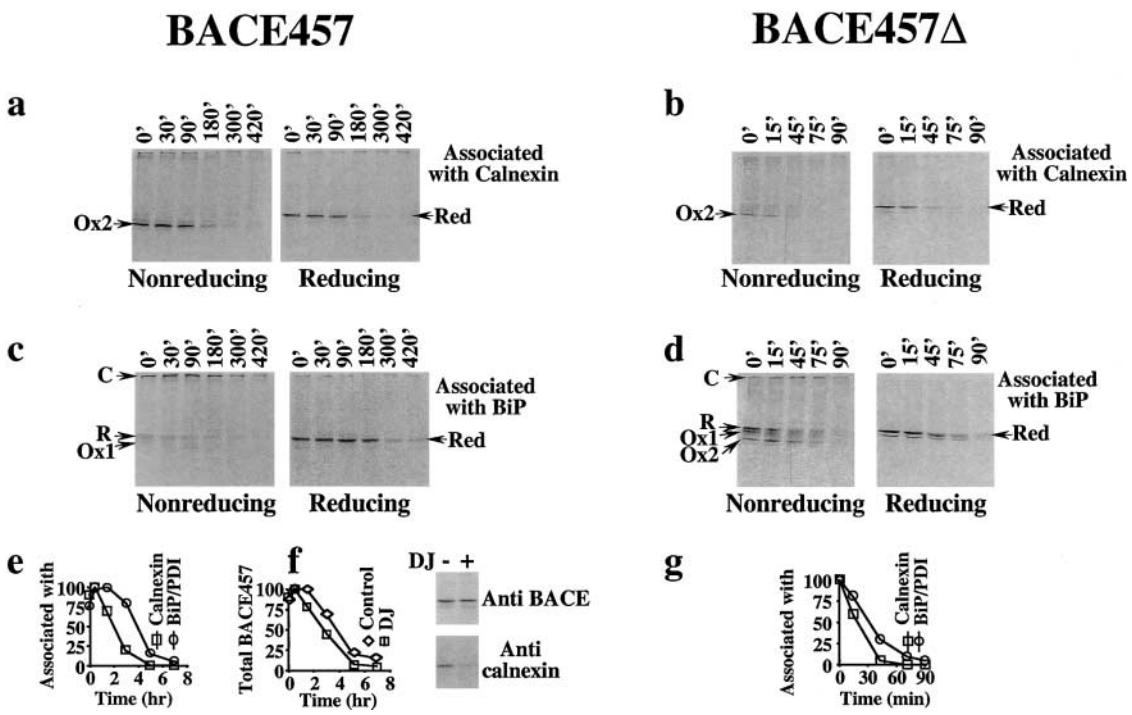

Figure 4. **Association of BACE457 and BACE457Δ with calnexin and BiP.** (a) Cell extracts were immunoprecipitated with antibody to calnexin. After extensive washings, proteins were denatured and the BACE457 associated with the chaperone was extracted with an antibody to a linear BACE epitope. Samples were then subjected to SDS-PAGE. (b) The same for BACE457Δ. (c and d) As in panels a and b, but with antibody to BiP. (e) Kinetics of BACE457 association with calnexin, BiP, and PDI was determined from reducing gels. Kinetics was indistinguishable for BiP and PDI. Association of PDI with BACE is shown in Fig. 5. (f) Kinetics of BACE457 degradation was determined in cells treated during starvation, pulse, and chase with 1 mM DJ to prevent BACE association with calnexin (DJ) or in mock-treated cells (Control). DJ efficiently prevented BACE457 association with calnexin. (g) Kinetics of BACE457Δ association with calnexin, BiP, and PDI, as in panel e.

Involvement of ER resident chaperones in the ERAD of BACE

To establish which ER resident protein associated with BACE457 and BACE457Δ, cell extracts were immunoprecipitated with antibody to calnexin and calreticulin, lectin-like molecular chaperones assisting the folding of glycoproteins (Trombetta and Helenius, 1998), to BiP, which assists protein folding but also retains misfolded proteins in the ER (Gething, 1999), and to several ER resident oxidoreductases (PDI, ERp57, ERp72, and ERp29; Ferrari and Soling, 1999), which may regulate the reduction of inter- and intrachain disulfide bonds to allow dislocation of BACE457 or BACE457Δ for degradation. After the first immunoprecipitation with chaperone-specific antibody, the samples were reimmunoprecipitated with an antibody to a linear epitope of BACE. As discussed in detail below, of the chaperones tested, only calnexin (Fig. 4, a and b), BiP (Fig. 4, c and d), and PDI (Fig. 5) associated with a detectable amount of BACE.

Calnexin associates with monomeric BACE during the lag phase preceding the onset of degradation

Calnexin associated transiently only with the fully oxidized Ox2 form of BACE457 and BACE457Δ (Fig. 4, a and b). The half-life of calnexin–BACE457 complexes was ~90 min (Fig. 4 e). It was significantly shorter for calnexin–BACE457Δ complexes (~20 min; Fig. 4 g). Both proteins were associated with calnexin during the lag phase before degradation. They were not degraded directly after release from calnexin because kinetics of association with the chaperone (Fig. 4, e and g) was clearly distinct from kinetics of BACE degradation (Fig. 1 a and d). Degradation was inhibited when the glucosidase inhibitor *N*-butyl-deoxynojirimycin (DJ) was used to delay BACE457 and BACE457Δ release from calnexin (see Materials and methods), showing that both proteins were shielded from the degradation machinery when associated with the chaperone (unpublished data). On the other hand, when DJ was used to prevent access of BACE to the calnexin cycle (Fig. 4 f, gel), the half-life of both proteins was reduced (Fig. 1, b and e, DJ; Fig. 3, a and d, DJ; Fig. 4 f, DJ; Fig. 6 a, DJ). This was a consequence of a significantly shorter lag phase. For example, upon incubation with DJ, the lag phase before the onset of BACE457 degradation was only 30 min (90 min in mock-treated cells) and the protein half-life was <3 h (4 h in mock-treated cells) (Fig. 4 f).

The data showed a temporal correlation between release of BACE457 monomers from calnexin (Fig. 4 e) and conversion of the monomers into disulfide-bonded complexes (Fig. 2 c) before degradation. Altogether, association with calnexin was not required for targeting membrane-bound and soluble BACE457 to the ERAD machinery. When association with calnexin was prevented, degradation started earlier. It was the time of association with calnexin that determined the length of the lag phase during which BACE457 and BACE457Δ were protected from premature aggregation and degradation.

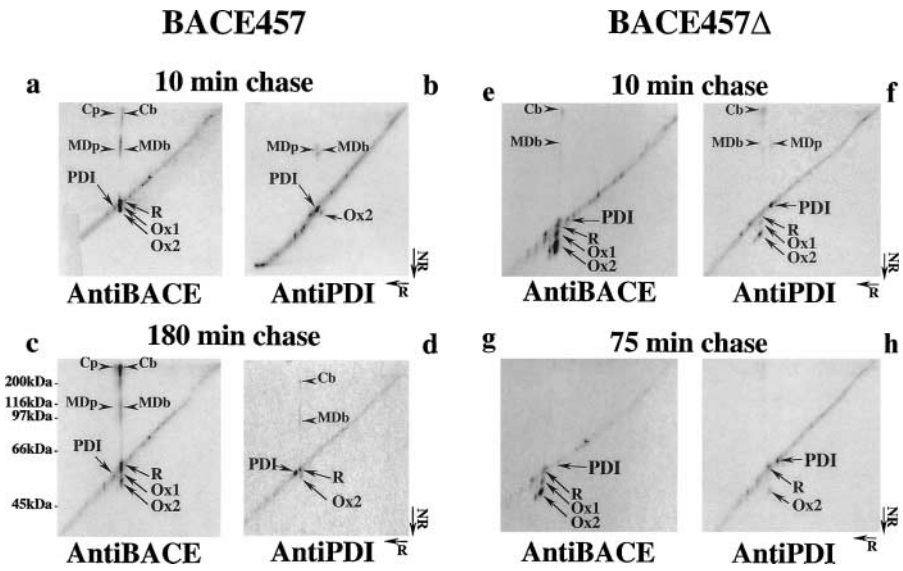

Figure 5. **Diagonal gel analysis of the association of PDI with BACE457 and BACE457Δ.** Extract of cells expressing BACE457 or BACE457Δ were immunoprecipitated with antiBACE (a, c, e, and g) or antiPDI (b, d, f, and h). After stringent washing to eliminate nonspecific interactions, samples were subjected to diagonal SDS-PAGE as described by Molinari and Helenius (1999). BACE in different oxidation states (shown with arrows) was the major labeled protein in the gels loaded with antiBACE precipitates. PDI was the major labeled protein in gels loaded with the antiPDI precipitates. Immunoprecipitations were highly specific because abundant cytosolic (actin) or ER proteins (calreticulin and ERp72; unpublished data) did not coprecipitate with BACE or PDI. High molecular weight disulfide-bonded complexes are located above the diagonal of the gels and are shown with Cp (for their PDI component) and with Cb (for their BACE component). Mixed disulfides are shown with MDp and MDb, respectively.

BiP and PDI associate with misfolded and disulfide-bonded BACE

BiP (Fig. 4, c and d) and PDI (Fig. 5) associated transiently with both variants of BACE457. The half-life of BACE457 complexes with BiP and with PDI was 4 h (Fig. 4 e); the half-life of complexes containing BACE457Δ was 40 min (Fig. 4 g). Kinetics of BACE457 and BACE457Δ association with BiP and PDI were indistinguishable from kinetics of BACE degradation, showing that release from the BiP/PDI chaperone system and degradation were tightly coupled events.

BiP associated at first with the partially oxidized forms of the newly synthesized ERAD candidates (Fig. 4, c and d, R and Ox1, 0-min chase). Later on, and throughout their presence in the ER lumen, BiP was associated with disulfide-bonded complexes generated upon release of misfolded BACE457 from calnexin (Fig. 4 c) or with misfolded BACE457Δ in different oxidation states (Fig. 4 d). The association of BiP with the early folding intermediates R and Ox1 and that of calnexin with the more oxidized species Ox2 showed a sequential involvement of these two chaperones during the folding attempts before degradation, as shown previously for a number of newly synthesized proteins (Molinari and Helenius, 2000). Normally, proteins complete their folding in the calnexin cycle, and then are transported along the secretory pathway. This was observed, for example, for BACE501 under the same experimental conditions (Fig. 1 c). For BACE457 and BACE457Δ, however, folding attempts failed and upon release from calnexin, the misfolded products did associate with BiP and PDI to be eventually degraded. These data show for the first time that the glycoprotein-specific calnexin cycle and the BiP/PDI chaperone system may act sequentially not only to assist protein folding but also to warrant ERAD of misfolded products.

Analysis of PDI association with BACE by diagonal gel electrophoresis

PDI was the only lumenal oxidoreductase (among those tested, see above) found to associate transiently with BACE457. The involvement of PDI in the ERAD of both BACE457 variants was analyzed in detail with a powerful two-dimensional (2-D) SDS-PAGE technique (Fig. 5) previously used to establish the involvement of oxidoreductases in protein oxidation during folding in vivo (diagonal gel electrophoresis; Molinari and Helenius, 1999, 2002). Cell extracts were prepared before the onset of BACE degradation (after a 10-min chase), or when degradation was in progress (after a 180-min chase for BACE457 and after a 75-min chase for BACE457Δ, respectively; Fig. 5). After immunoprecipitation with antibodies to BACE or PDI and stringent washing, labeled proteins were separated in diagonal gels.

The reduced form of BACE (R) is devoid of disulfide bonds. It had therefore the same mobility under nonreducing and reducing conditions and was on the diagonal of the gels. Ox1 and Ox2 were below the diagonal, as expected for proteins with intramolecular disulfide bonds (Fig. 5, AntiBACE and AntiPDI). Radioactivity was also visible above the diagonal of the gels, showing that labeled proteins were involved in intermolecular disulfide-bonded complexes (Fig. 5, a–f). Complexes were more abundant for BACE457, as already shown in Fig. 2, and were distributed along a vertical line in two distinct regions above the diagonal of the gels (arrows C and MD), showing that they were heterogeneous in size (as shown in Fig. 3 c) and variable in disulfide bonding. Complexes labeled with C had an estimated molecular mass above 200 kD; those labeled as MD were of 100–120 kD. Their location on the vertical of BACE showed that both C and MD contained disulfide-bonded BACE molecules (Fig. 5, a–f). In some of the gels (Fig. 5, a–c and f), the complexes above the diagonal were clearly characterized by the presence of two traces of radioactivity running parallel and very close to each other. This showed that a second labeled protein was disulfide bonded to BACE in the complexes. This protein was PDI because complexes were decorated in immunoblots by a monoclonal antibody to PDI (unpublished data), because the coordinates in the 2-D gels corresponded to those of labeled PDI immunoprecipitated with specific antibody

from cell extracts (Fig. 5, AntiPDI), and because the complexes containing disulfide-bonded BACE were also isolated from cell extracts with PDI-specific antibody (Fig. 5, AntiPDI). We concluded that both C and MD disulfide-bonded complexes had a BACE component (Cb and MDb) and a PDI component (Cp and MDp) associated covalently via interchain disulfide bonds. The complexes indicated with MD were of particular interest because, according to their electrophoretic mobility, they consisted of a mixed disulfide between one PDI and one BACE molecule. Because mixed disulfides are formed between oxidoreductases and substrates during catalysis of redox reactions (Huppa and Ploegh, 1998; Molinari and Helenius, 1999), the data supported the involvement of PDI as an oxidoreductase during attempts of BACE folding and during reduction of intra- and intermolecular disulfide bonds to prepare misfolded BACE for dislocation and degradation.

PDI is preferentially associated with oxidized BACE before the onset of degradation and with the reduced form of BACE during degradation

PDI was also noncovalently associated with BACE457 and BACE457Δ, because a significant amount of monomeric BACE in various oxidation states was visible on and below the diagonal of gels loaded with the antiPDI precipitates.

After a 10-min chase, monomeric BACE457 was equally distributed in the R, Ox1, and Ox2 states (Fig. 5 a). Of the three oxidation states, Ox2 coprecipitated preferentially with PDI (Fig. 5 b). Oxidation of BACE457Δ was faster and after a 10-min chase, ~70% of the labeled protein was fully oxidized (Fig. 5 e, Ox2). All of the BACE457Δ oxidation forms present in the cells coprecipitated with PDI in amounts that reflected their relative abundance in the cells. During the lag phase, PDI (with BiP and calnexin; Fig. 4) was most likely involved in the fruitless attempts to fold BACE and/or convert BACE monomers into disulfide-bonded complexes.

During the degradation phase (after a 180-min chase for BACE457 in Fig. 5 d and a 75-min chase for BACE457Δ in Fig. 5 h), PDI was preferentially associated with the reduced form of BACE457 and BACE457Δ (Fig. 5, d and i, R) and only poorly with Ox2, though this was the more abundant oxidation form present in the cells at this time of chase (Fig. 5, c and g, AntiBACE).

Overall, involvement of PDI in mixed disulfides with BACE, and preferential association of the lumenal oxidoreductase with distinct oxidation forms of the ERAD candidates in different phases of the ERAD process, indicated a dynamic and active role for PDI during the preparation of BACE for dislocation into the cytosol. The fully reduced form of BACE457 and BACE457Δ associated with PDI in the last moment of BACE permanence in the ER may represent dislocation-competent entities trapped in the act of being dislocated into the cytosol.

DTT inhibits dislocation and degradation of BACE

Reducing agents may accelerate protein degradation from the ER (Stafford and Bonifacino, 1991; Young et al., 1993; Courageot et al., 1999; Mancini et al., 2000; Wilson et al., 2000), whereas oxidants may inhibit this process (Stafford and Bonifacino, 1991; Mancini et al., 2000). These results

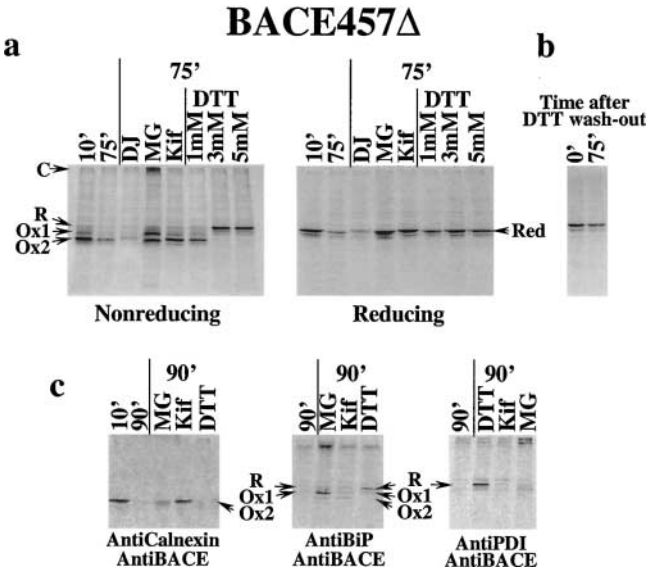

Figure 6. **Molecular chaperones involved in the retention of BACE upon inhibition of ERAD machinery.** (a) Cells were mock treated or incubated for the times indicated with DJ, MG, Kif, or DTT at different concentrations. BACE457Δ was precipitated with specific antibody and analyzed by SDS-PAGE. (b) Cells were incubated for 90 min with 5 mM DTT. Upon DTT washout, the incubation was continued for the times indicated. (c) Cell extracts were precipitated with anticalnexin, antiBiP, or antiPDI, and then with antiBACE upon denaturation.

could be ascribed to a facilitated dislocation of reduced, unfolded ERAD substrates.

To determine whether changes in the ER redox environment also affected BACE degradation, cells were exposed to low millimolar concentrations of diamide or DTT. As expected, the oxidant diamide inhibited degradation of BACE457 (unpublished data). Surprisingly, the reducing agent DTT inhibited BACE degradation too (shown for BACE457Δ in Fig. 6 a where the effect of DTT is also compared with other ERAD inhibitors). After a 75-min chase, 30% of BACE457Δ was protected from degradation already in the presence of 1 mM DTT (vs. 10% in control cells; Fig. 6 a). At higher DTT concentrations (3 or 5 mM), protection from degradation was comparable to that obtained with MG and Kif (Fig. 6 a). At these concentrations of DTT, the intramolecular disulfide bonds of BACE had been reduced because the electrophoretic mobility of BACE457Δ was the same under nonreducing and reducing conditions (Fig. 6 a). BACE, theoretically in a fully unfolded state, was nevertheless not dislocated into the cytosol, as determined by ultracentrifugation of cell homogenates (unpublished data), and remained protected from degradation (Fig. 6 a). Importantly, the DTT block was reversible and BACE degradation from the ER was resumed, with somewhat slower kinetics, upon DTT washout (Fig. 6 b).

Association of BACE with lumenal chaperones upon inhibition of the degradation process

To assess the mechanism by which BACE457 was retained in the ER, inaccessible to the cytosolic ERAD machinery, upon DTT treatment, we determined whether modification of the ER redox environment resulted in the stable association of the fully reduced protein with ER resident lumenal

chaperones. Detergent extracts of cells mock treated or treated with DTT, or with other ERAD inhibitors, were subjected to immunoprecipitation with antibodies to calnexin, BiP, or PDI as described above.

After a 90-min chase, BACE457Δ had been almost completely degraded and was therefore not found in association with chaperones (Fig. 6 c, 90'). Analysis of the DTT-treated cells revealed that after a 90-min chase, BACE was still associated with PDI, to a lesser extent with BiP, but not with calnexin (Fig. 6 c, DTT, AntiPDI, AntiBiP, and AntiCalnexin). These data showed that BACE dislocation and degradation were inhibited in response to DTT treatment mainly because DTT prevented BACE release from PDI. Upon DTT washout, BACE was released from PDI and degradation was resumed as shown in Fig. 6 b.

Interestingly, PDI was only involved significantly in the retention of BACE in the ER when the ERAD process was inhibited by DTT. The Ox1 form of BACE457Δ and disulfide-bonded complexes were in fact mainly retained by BiP, when degradation was prevented by MG (Fig. 6 c, AntiBiP, MG). It remains to be established how Ox2 was retained in the ER when degradation was inhibited by MG, because we only found a small fraction of this oxidation form associated with a chaperone, namely calnexin (Fig. 6 c, AntiCalnexin, MG).

The effect of Kif was to retard BACE457Δ release from calnexin (Fig. 6 c, AntiCalnexin, Kif). A possible explanation for this latter finding is that Man9 glycans are more efficiently reglucosylated by the ER folding sensor glucosyl transferase than Man8 sugars (Parodi, 2000). The persistence of Man9 glycans in the presence of Kif would therefore favor a prolonged retention of ERAD candidates in the calnexin cycle. Association with calnexin was not sufficient, alone, to explain the inhibitory effect of Kif on ERAD because upon longer incubation, BACE457Δ was eventually released from the chaperone, but protection from degradation persisted. These data implied that Man9 glycans, per se, protect proteins from degradation and/or that generation of Man8 is required to tag misfolded polypeptides for degradation.

We concluded that MG, Kif, and DTT treatments prevented the dislocation of misfolded BACE into the cytosol, confirming that degradation and dislocation are coupled events. The retention mechanism was different for each one of the inhibitors tested, involving the formation of disulfide-bonded complexes and/or association with calnexin, BiP, or PDI as the major retention mechanism.

Discussion

BACE457 is the splice variant of β-secretase found in the pancreas. Compared with the brain isoform, BACE501, BACE457 lacks 44 residues in the lumenal domain. Although maturation of BACE501 transiently expressed in HEK293 cells was efficient (this study; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999), we found that most of the BACE457 expressed under similar conditions was retained in the ER and became a substrate for ERAD.

The fate of BACE457 and of the soluble mutant BACE457Δ was characterized by a lag phase during which the proteins were extensively oxidized and remained protected from premature degradation during nonproductive attempts at folding in association with the molecular chaperone calnexin. Upon release from calnexin, misfolded BACE457 (and to a lesser extent BACE457Δ) transiently entered into disulfide-bonded complexes before degradation from the ER. Because the lumenal portion of the two proteins is identical, it is likely that attachment at the membrane, or the transmembrane region itself, which contains two cysteines, determined the longer association with calnexin, the slower degradation, and the increased susceptibility to aggregation observed for BACE457. Both BACE457 and BACE457Δ remained trapped in disulfide-bonded complexes when their degradation was inhibited, but their degradation was resumed upon washout of the ERAD inhibitors. This, and the transient formation of complexes during normal degradation of BACE457 implied disassembly of the complexes and unfolding of BACE by reduction of intra- and intermolecular disulfides to allow dislocation and proteasome-mediated degradation. Dislocation of ERAD substrates through the dislocon (Kopito, 1997) as well as access to the proteasomal cavity (Rubin and Finley, 1995) require, in fact, extensive unfolding of the polypeptide chain. Accumulation of extensively oxidized species upon inhibition of the ERAD machinery also implied that besides dislocation (Mayer et al., 1998; Mancini et al., 2000), unfolding of ERAD candidates before dislocation relies on active degradation machinery.

BACE457 or BACE457Δ were the major components of the disulfide-bonded complexes. However, by coprecipitation and diagonal gel analysis, we established that lumenal chaperones, such as BiP and PDI (but not calnexin), were also associated with the complexes. Based on the relatively low molecular weight, we estimated that the disulfide-bonded complexes contained up to 10 BACE457 or BACE457Δ molecules in addition to fewer BiP and PDI molecules. The number (and not the size) of these chaperone-containing complexes increased in the ER lumen when degradation of misfolded BACE was inhibited, indicating that they were units with limited capacity for accepting misfolded polypeptides.

Recent data showed that chaperone systems, such as BiP–Jem1p–Scj1p, may prevent the formation of protein aggregates in yeast (Fewell et al., 2001; Nishikawa et al., 2001). In mammalian cells, at least for the substrates presented in this study, association with BiP and PDI was not sufficient to prevent the formation of disulfide-bonded complexes. BiP and PDI might however play an important regulatory role in keeping the size of the complexes under control, thereby preventing their irreversible deposition. Protein aggregation and deposition in intracellular compartments or within tissues is in fact often an end point of protein misfolding associated with invalidating human pathologies (conformational diseases; Kopito and Ron, 2000). The data presented in this paper show that the formation of disulfide-bonded complexes might be a reversible process in the mammalian ER, and that complexes may represent an intermediate state in which ERAD candidates are maintained as degradation competent. Reversible aggregation of ERAD candidates in the ER lumen may actually have a number of advantages. First, it could represent a general mechanism to effectively prevent transport of misfolded proteins along the secretory pathway or their dislocation into the cytosol when

prompt degradation is not warranted. This is important for cell viability, because it has recently been shown that aggregation-prone polypeptides accumulating in the cytosolic compartment damage the proteasome system and trigger cell death (Bence et al., 2001). Second, it could facilitate the dislocation process by concentrating misfolded products in specific regions of the ER lumen, possibly close to dislocons. Concentration of misfolded products would also minimize deleterious interference with the activity of the folding machinery or with nascent chains emerging in the ER lumen. Third, the packaging of ERAD candidates would reduce the number of lumenal chaperone molecules involved in the regulation of protein degradation from the ER. This could have important implications, because excessive sequestration of lumenal chaperones by misfolded products triggers unfolded protein responses and leads to impaired cell viability (Urano et al., 2000). It was an important observation in this contest that the reversible aggregation of misfolded BACE457 during its ERAD did not trigger unfolded protein responses (as determined by lack of BiP up-regulation; unpublished data).

Despite the significant difference in the rate of degradation for both BACE457 and BACE457Δ, the different kinetics of association with calnexin and with BiP/PDI clearly supported a two-phase model in which the two-chaperone systems acted sequentially during ERAD. Calnexin protected BACE457 and BACE457Δ from aggregation and premature degradation, and assisted the newly synthesized glycoproteins in fruitless attempts at folding. BiP and PDI were also involved in this phase and then played an essential role after the release of terminally misfolded BACE from calnexin to prepare the covalently bonded and/or extensively oxidized polypeptides for dislocation during ERAD, and to maintain covalent complexes as degradation competent when the ERAD machinery was inactive. Our data showed therefore that the glycoprotein-specific calnexin cycle and the BiP/PDI chaperone system may act in a coordinate fashion not only to assist the maturation of newly synthesized polypeptides as shown by Molinari and Helenius (2000), but also to warrant the degradation of aberrant products that are unable to reach a transport-competent conformation.

The mammalian ER contains at least half a dozen oxidoreductases that could be implicated in the reduction of inter- and intramolecular disulfide bonds during the preparation of ERAD substrates for dislocation. Some of them are implicated in the oxidative folding of newly synthesized proteins in vivo (Molinari and Helenius, 1999), but none has been found to be implicated in yeast or mammals in the enzymatic unfolding of polypeptides. An involvement of PDI in the transport of proteins from the ER lumen into the cytosol has been, on the other hand, recently proposed. Yeast strains expressing PDI mutants were in fact defective in the dislocation of misfolded proteins without cysteines (Gillece et al., 1999). Moreover, experiments performed in vitro have implicated PDI in the dislocation of the catalytic subunit of cholera toxin during cellular intoxication (Tsai et al., 2001). Here, we extend our knowledge of PDI functions in the ER. Using a sensitive 2-D SDS-PAGE technique previously used to determine the involvement of PDI and ERp57 in protein folding (Molinari and Helenius, 1999), we established that only PDI, of the oxidoreductases examined in this work, associated with BACE457 and BACE457Δ before their dislocation into the cytosol. We identified mixed disulfides generated in the ER between PDI and BACE. This implied a redox activity of PDI in the generation of intra- and intermolecular disulfide bonds during and after the attempts-at-folding phase and/or in the reduction of disulfides to disentangle BACE-containing complexes and unfold the misfolded polypeptides to make them competent for dislocation.

The powerful 2-D technique used for our investigation also allowed for detection of the noncovalent association of PDI with fully reduced BACE, ready to be dislocated into the cytosol. This putative intermediate of BACE dislocation was stabilized, reversibly, upon modification of the ER redox environment. Reducing conditions generated in the ER by a low millimolar concentration of DTT, in fact, blocked the release of BACE from PDI, preventing both dislocation and degradation of the ERAD substrate. These data were somewhat surprising because they contradict observations made for other ERAD substrates; T cell receptor, major histocompatibility complex class I, and immunoglobulin chains are degraded faster under reducing conditions (Stafford and Bonifacino, 1991; Young et al., 1993; Courageot et al., 1999; Mancini et al., 2000; Wilson et al., 2000). This could reflect differences in the experimental models (semipermeabilized cells for T cell receptor and major histocompatibility complex class I degradation vs. living cells for our studies) and/or different mechanisms of preparation for degradation used by different proteins. Importantly, degradation of BACE was resumed when physiological redox conditions were reestablished upon DTT removal. These data hint at a redox-driven chaperone activity of PDI during ERAD of BACE, similar to that reported for PDI during dislocation of the A chain of cholera toxin into the cytosol (Tsai et al., 2001). Binding and release of ERAD substrates would be regulated by a redox cycle in which the reduced form of PDI would associate with substrates and substrate release would require reoxidation of PDI.

Materials and methods

Mammalian expression constructs and antibodies

Plasmids for expression of BACE457 and BACE501, and antibody to BACE are described by Bodendorf et al. (2001). Antibodies to PDI, calnexin, and ERp29 were gifts from Ineke Braakman (Utrecht University, Utrecht, Netherlands), Ari Helenius (ETH, Zurch, Switzerland), and Souren Mkrtchian (Karolinski Institute, Stockholm, Sweden). Antibody to ERp57 is described by Molinari and Helenius (1999). Antibodies to BiP, ERp72, calreticulin, and actin are commercially available (StressGen Biotechnologies).

Cell culture and transfection

HEK293 cells were grown in DME, 10% FBS, penicillin, streptomycin (GIBCO BRL). Cells were plated in 6-cm Petri dishes to 90% confluency and transfected with 3–6 μg plasmid DNA and 20 μl Lipofectamine2000 (GIBCO BRL) per dish. Transient expression was allowed for 15–24 h.

Metabolic labeling, preparation of cell extracts, immunoprecipitation, endoglycosidase treatment, diagonal electrophoresis, and sucrose gradients

Cells were starved for 30 min in Met/Cys-free medium, pulsed for 10 min with 150 μCi [^{35}S]Met/Cys in 1 ml starvation medium/dish and chased for the times indicated in the figures with DME supplemented with 5 mM cold Met/Cys. At the end of a chase, cells were flooded with 20 mM ice-cold *N*-ethylmaleimide to prevent postlysis oxidation of free cysteines. Postnuclear supernatants were prepared by solubilization of cells in 800 μl/dish of ice-cold 2% CHAPS-Hepes buffer saline, pH 6.8, containing *N*-ethylma-

leimide, protease inhibitors (HBS), and 20 U apyrase (Sigma-Aldrich) per dish when associations with BiP were investigated. Release of substrates from BiP requires ADP–ATP exchange. By rapidly depleting ATP from cell extracts, apyrase stabilizes BiP–substrate complexes before immunoprecipitation. Cell extracts were prepared by a 10-min centrifugation at 10,000 *g* and analyzed after immunoprecipitation with specific antibodies by nonreducing, reducing, or diagonal SDS-PAGE. Relevant bands were quantitated by ImageQuant software (Molecular Dynamics). At least three independent experiments were analyzed. All kinetics shown in the paper represent an average of the experiments considered. For EndoH (Roche Molecular Biochemicals) treatment, immunoprecipitated BACE457 or BACE501 were incubated for 1 h at 37°C with 5 mU of EndoH. Samples were then analyzed by reducing SDS-PAGE. For diagonal electrophoresis, the precipitates were first subjected to nonreducing SDS-PAGE in capillary tubes. The gels were extruded from the tubes, boiled for 10 min in reducing sample buffer, placed onto conventional slab gels, and subjected to a second electrophoresis under reducing conditions (this technique is described in detail in Molinari and Helenius, 2002). Immunoprecipitations were performed by adding protein A beads (PAB, 1:10 wt/vol swollen in HBS; Sigma-Aldrich) and the selected antibody to the cell extracts. Incubations were for 1–4 h in a cold room. The immunoprecipitates were extensively washed, three times, with HBS and 0.5% CHAPS and resuspended in sample buffer for SDS-PAGE. When association with lumenal chaperones was examined, after the first immunoprecipitation with chaperone-specific antibody, the beads were washed three times, boiled in 100 μl 1% SDS in HBS to denature the precipitate, and supplemented with 1 ml Triton X-100 (1% in HBS), fresh protein A beads, and antibody to a linear epitope of BACE to perform the second immunoprecipitation. After three extensive washes, beads were resuspended in sample buffer and subjected to SDS-PAGE. To determine the size of disulfide-bonded BACE457, 100 μl of detergent extracts were overlaid on a 10–25%, linear sucrose gradient prepared with a gradient master (Biocomp) and centrifuged for 3 h at 260,000 *g* (rotor MLS50; Beckman Coulter).

Inhibitors

L (20 μM; Calbiochem), MG (20 μM; Calbiochem), or Kif (1–10 μg/ml; Toronto Research Chemicals) was added 30 min before the pulse and was maintained during the pulse and throughout the chase unless specified. N was used at 5 mM and MA at 10 mM. The glucosidase inhibitor DJ (1 mM) was added to the cells after a 10-min chase and was maintained until the end of the chase to inhibit substrate release from calnexin. It was added during starvation, pulse, and chase to prevent substrate association with calnexin as described by Hammond et al. (1994).

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