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Consequences of Individual N-glycan Deletions and of Proteasomal Inhibition on Secretion of Active BACE

Omar Vanoni,* Paolo Paganetti,† and Maurizio Molinari*

*Institute for Research in Biomedicine, CH-6500 Bellinzona, Switzerland; and †Novartis Institutes for Biomedical Research Basel, Neuroscience Research, Novartis Pharma AG, CH-4002 Basel, Switzerland

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BACE is an aspartic protease involved in the production of a toxic peptide accumulating in the brain of Alzheimer's disease patients. After attainment of the native structure in the endoplasmic reticulum (ER), BACE is released into the secretory pathway. To better understand the mechanisms regulating protein biogenesis in the mammalian ER, we determined the fate of five variants of soluble BACE with 4, 3, 2, 1, or 0 N-linked glycans. The number of N-glycans displayed on BACE correlated directly with folding and secretion rates and with the yield of active BACE harvested from the cell culture media. Addition of a single N-glycan was sufficient to recruit the calnexin chaperone system and/or for oligosaccharide de-glucosylation by the ER-resident α-glucosidase II. Addition of 1–4 N-glycans progressively enhanced the dissociation rate from BiP and reduced the propensity of newly synthesized BACE to enter aberrant soluble and insoluble aggregates. Finally, inhibition of the proteasome increased the yield of active BACE. This shows that active protein normally targeted for destruction can be diverted for secretion, as if for BACE the quality control system would be acting too stringently in the ER lumen, thus causing loss of functional polypeptides.

INTRODUCTION

Roughly one-third of the eukaryotic gene products are cotranslationally translocated into the ER lumen. These include all secretory proteins, the proteins displayed at the cell surface and all proteins operating in endocytic and exocytic compartments. Most of them are covalently modified at asparagines in Asn-Xxx-Ser/Thr motifs by the addition of preassembled glucose$_3$-mannose$_9$-*N*-acetylglucosamine$_2$- core oligosaccharides. N-linked oligosaccharides increase the solubility of as yet unfolded nascent polypeptide chains and recruit a dedicated machinery that assists the protein folding process, the calnexin chaperone system. Rapid removal of the two outermost glucose residues from the polypeptide-bound oligosaccharide elicits association with the lectin chaperones calnexin and calreticulin and the associated oxidoreductase ERp57. This facilitates the formation of intra- and intermolecular disulfide bonds, a rate-limiting step of the polypeptide folding process (Ellgaard *et al.*, 1999; Caramelo and Parodi, 2007, 2008). Cycles of substrate release/reassociation with calnexin facilitate folding and, for a restricted number of proteins, they may even be required to attain the native structure (Caramelo and Parodi, 2007, 2008; Soldà *et al.*, 2007). They are regulated by sequential removal and readdition of the innermost glucose residue on branch A of the oligosaccharide operated by the glucosidase II and the UDP-glucose:glycoprotein glucosyltransferase, respectively (Hammond *et al.*, 1994). A structure-based protein quality control operates in the ER lumen to prevent release into the secretory pathway of the majority of nonnative polypeptides. After a limited number of unsuccessful folding cycles, terminally misfolded proteins are translocated through the ER membrane and are degraded by cytosolic proteasomes (Hebert *et al.*, 2005; Lederkremer and Glickman, 2005; Meusser *et al.*, 2005; Nakatsukasa and Brodsky, 2008).

Systematic studies aiming at understanding mechanisms regulating protein biogenesis in living cells are rare and rely on a very short list of select model substrates (Pearse *et al.*, 2008 and references therein). Characterization of the mechanisms that regulate protein folding, quality control, and disposal from the ER lumen is crucial because defects in polypeptide maturation are often linked to highly debilitating *conformational* diseases (Aridor, 2007). The capacity to intervene in protein biogenesis will lead to development of therapeutic approaches aiming at delaying the progressive worsening of disease conditions or even at reverting disease phenotypes by using chemical and pharmacological chaperones that enhance protein folding or delay degradation of intermediates of protein folding programs (Molinari, 2007 and references therein). It will also facilitate more efficient and rentable production of recombinant proteins to be used in the clinics or in the industry (Baldi *et al.*, 2007).

Here we performed a thorough analysis of the fate of a disease-regulating glycoprotein, the soluble form of human BACE (BACEs, see Figure 1A). BACEs was selected as model substrate because it can be produced in *Escherichia coli* and refolded in active form from inclusion bodies (Hong *et al.*, 2000; Sardana *et al.*, 2004; Shimizu *et al.*, 2008). In principle therefore, this protein can attain an active form independent of

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Address correspondence to: Maurizio Molinari (maurizio.molinari@irb.unisi.ch).

Abbreviations used: BACE, Beta-site APP-cleaving enzyme 1; BACEs, soluble form of BACE; bDNJ, *N*-butyl-deoxynojirimycin; CHO, Chinese hamster ovary cells; DBA, disulfide-bonded aggregates; EndoH, endoglycosidase H; ER, endoplasmic reticulum; ERAD, ER-associated degradation; HEK, human embryonic kidney cells.

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N-glycosylation. Several variants of BACEs with 4, 3, 2, 1, or 0 N-linked glycans were ectopically expressed in human cells. We studied the dependency of the glycosylation state for parameters such as secretion rate and efficiency, chaperone use, formation of byproducts of the folding program, enzymatic activity, and enhancement of production yield upon manipulation of the ER folding and degradation capacity.

MATERIALS AND METHODS

Expression Plasmids, Antibodies, and Transfections

pRK7-based plasmid expressing the soluble form of wild-type BACE was generated by PCR with appropriate primers with consequent deletion of the 48 amino acids of the transmembrane and cytosolic region at the C-terminal of BACE1. Removal of the different N-glycosylation sites was performed by site-directed mutagenesis: codon AAC (asparagine coding) was substituted with codon CAA (glutamine coding). In this article we describe the case of the nonglycosylated BACEs0, of the mono-glycosylated BACEs1 (glycan at position 172, see Figure 1A), of the di-glycosylated BACEs2 (172 and 223), of the tri-glycosylated BACEs3 (153, 172, and 354), and of the tetra-glycosylated BACEs4 (153, 172, 223, and 354). Most of the experiments shown in this article have also been performed with the mono-glycosylated variants displaying the oligosaccharide in each one of the possible positions (153, 172, 223, or 354) and with the di-glycosylated variants with oligosaccharides at position 153–172 or 153–223 or 153–354 or 172–354 or 223–354). Anti-ubiquitin antibody was from DAKO (Carpinteria, CA), anti-human BiP from Stressgen (San Diego, CA), HRP-conjugated anti-mouse and anti-rabbit from Amersham Biosciences (Piscataway, NJ). HEK293 and CHO cells were grown in DMEM and αMEM, respectively, supplemented with 10% FBS. Cells were transiently transfected with Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer.

Radiolabeling and Immunoprecipitations

Eighteen hours after transfection, cells were starved for 15 min in Met/Cys free medium, pulsed for 10 min with 50 μCi [^{35}S]Met/Cys in 1 ml starvation medium/dish, and chased for the indicated times with DMEM supplemented with 5 mM cold Met/Cys. Extracellular medium was centrifuged (10 min at 10,000 $\times$ *g*) before immunoisolation of BACEs. Postnuclear supernatants (PNS) were prepared by solubilization of cells in 800 μl/dish ice-cold 2% CHAPS in HEPES-buffered saline (HBS), pH 6.8, containing 20 mM *N*-ethylmaleimide, protease inhibitors, and 10 U/dish of apyrase for BiP immunoprecipitations. CHAPS-insoluble material was separated by 10-min centrifugation at 10,000 $\times$ *g*. CHAPS-insoluble material was solubilized by boiling in 1% SDS and subsequent addition of 10 volumes of 1% Triton X-100. Immunoprecipitations were performed by adding protein A beads (Sigma; 1:10, w/v swollen in HBS) and the selected antibody to extracellular medium or cell extracts. Incubations were 1–4 h at 4°C. The immunoprecipitates were extensively washed, three times, with 0.5% CHAPS in HBS and resuspended in sample buffer for SDS-PAGE. Relevant bands were quantified by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Gels were also exposed to BioMax (Eastman-Kodak, Rochester, NY) films and scanned with an AGFA scanner (Mortsel, Belgium).

Endoglycosidase H Treatment

Secreted BACEs1 was immunoisolated and denatured in 0.5% SDS, 40 mM DTT and incubated for 2 h with recombinant endoglycosidase H (EndoH, New England BioLabs, Beverly, MA) at 37°C.

Immunoblotting

Forty hours after transfection cells were grown in the absence/presence of 9 μM PS-341 (Velcade, kind gift of R. Sitia, Milan) for 150 min. The extracellular media were collected, separated in reducing SDS-PAGE, and transferred onto PVDF membranes. Membranes were decorated with a monoclonal anti-BACE or a polyclonal anti-ubiquitin (1:1000). Secondary antibodies were HRP-conjugated anti-mouse IgG (1:5000) and HRP-conjugated anti-rabbit IgG (1:5000). Detection and protein amount analysis were performed with the ECL-Plus detection system (Amersham GE Healthcare, Waukesha, WI) and with the LAS4000 software.

BACE Activity Assay

Extracellular medium (10 μl) was directly incubated for 30 min in a reaction buffer (20 mM acetic acid, pH 4.5, 0.01% CHAPS, and 0.01% TOP BLOCK to improve BACEs solubility during the activity essay) containing 1 μM_{fin} ALEXA-substrate. Activity was measured in Corning 96-well microplates (Corning Glass Works, Corning, NY) using the SPECTRAmax GEMINIXPS spectrofluorometer (Molecular Devices). The increase in fluorescence upon substrate hydrolysis was measured (every 50 s) with the SoftMax Prosoftware. Relative activities represent the slope of the straight line obtained by plotting the increase in fluorescence over time.

RESULTS

A Direct Correlation between Extent of BACEs Glycosylation and Secretion

To determine whether the extent of protein glycosylation affects protein folding and secretion efficiency, we analyzed the fate of BACEs displaying 0 (BACEs0) to 4 N-glycans (BACEs4, Figure 1A). The polypeptides were ectopically expressed, individually, in HEK293 cells. Eighteen hours after transfection, cells were metabolically labeled for 10 min with [^{35}S]methionine and cysteine. Incubations were prolonged after radioactivity washout for 5–240 min when the secretion plateau was reached for all glycosylated mutants (Figure 1, the chase was prolonged up to 480 min only for BACEs0, panel A). At the end of each chase time, the cell culture media were harvested and the secreted, labeled BACEs variants were immunoisolated with a specific antibody (Figure 1B, secreted). Cells were solubilized with 2% CHAPS, a zwitterionic detergent, and the lysates were centrifuged to separate detergent-soluble material (PNS) from detergent-insoluble material. The labeled BACEs variants present intracellularly at the end of each chase time were immunoisolated from the detergent-soluble fraction (Figure 1B, intracellular) and were separated in 10% polyacrylamide gels under reducing conditions. Labeled bands were quantified by densitometric analysis.

The amount of labeled BACEs immunoisolated from cell lysates decreased (Figure 1, B–F, intracellular) as a function of protein secretion with progression of the chase (Figure 1, B–F, secreted). This showed that for all glycosylation mutants, a variable fraction of the newly synthesized, labeled protein was secreted in the extracellular media.

The amount of detergent-soluble labeled BACEs0 retained intracellularly decreased to ~25% of the initial amount after 240 min and to ~10% after 480 min of chase (Figure 1B, intracellular). The amount of labeled BACEs0 immunoisolated from the cell culture media did not exceed the 5% of the initial synthesized pool of protein (Figure 1B, secreted). Thus, secretion of nonglycosylated BACEs0 was very inefficient; the vast majority of newly synthesized BACEs0 underwent degradation or entered in detergent-insoluble aggregates (see below).

The mono- and di-glycosylated BACEs variants disappeared with faster kinetics from the intracellular fractions (Figures 1, C–D, intracellular). Secretion of BACEs1 (38%, Figure 1C, secreted) and of BACEs2 (44%, Figure 1D, secreted) was much more efficient compared with secretion of the nonglycosylated version of the model protein (5%, Figure 1B, secreted).

The more extensively glycosylated forms of BACEs (BACEs3, Figure 1E, and BACEs4, Figure 1F) disappeared even faster from the detergent lysates and were secreted with the highest efficiency (~60%, Figure 1, E and F).

These data demonstrated the existence of a direct correlation between the extent of BACEs glycosylation and the efficiency of secretion.

A Direct Correlation between Extent of BACEs Glycosylation and Secretion Kinetics

The data for the secretion rates and secretion efficiencies are summarized for the five glycosylation mutants in Figure 1G. These data confirm the direct correlation existing between the extent of BACEs glycosylation, the secretion efficiency and the secretion rate (Figure 1G). Secretion of the nongly-

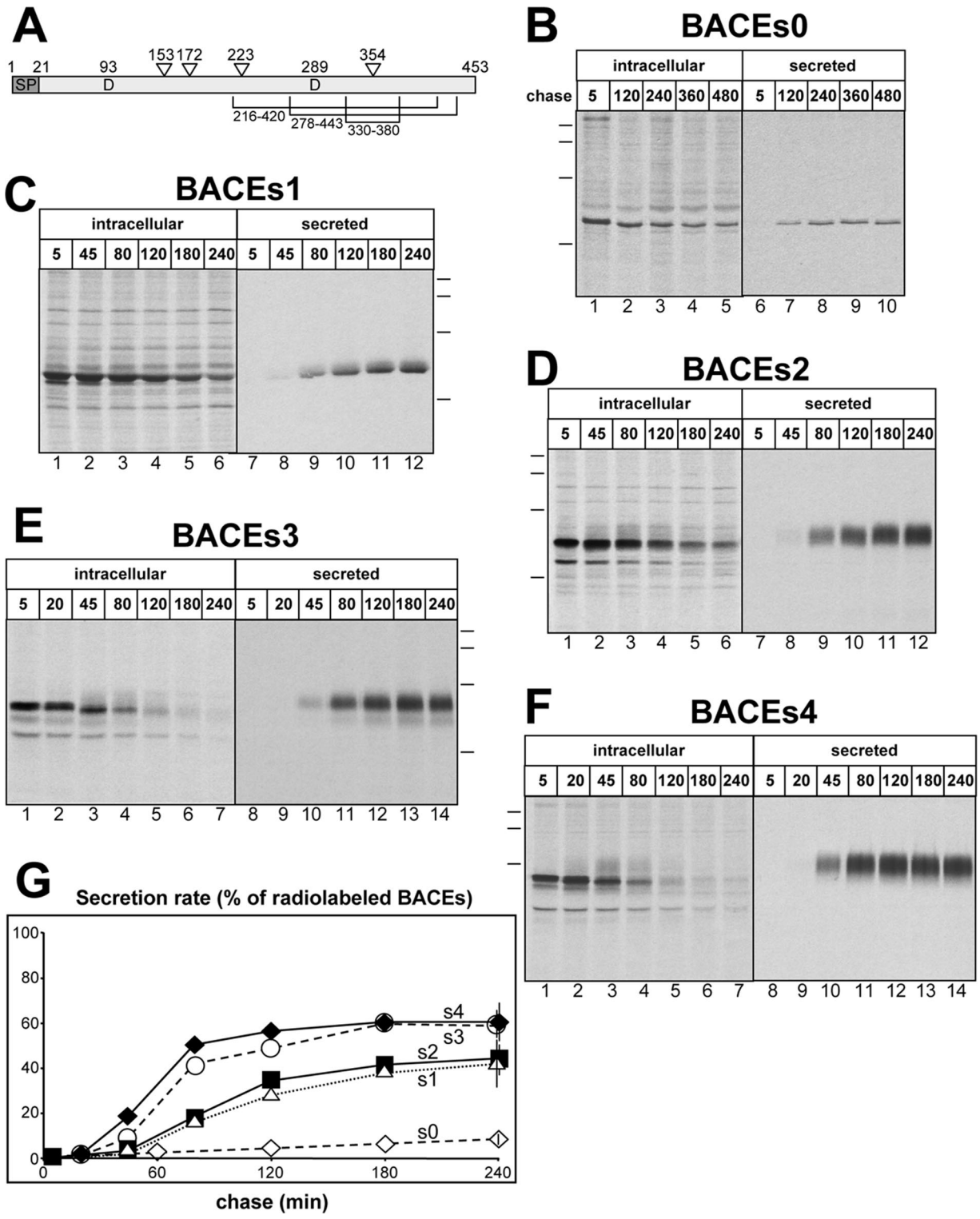

Figure 1. Secretion rate and efficiency correlate with the extent of BACEs glycosylation. (A) Schematic representation of BACEs. SP is the signal peptide, and D are the two catalytic aspartic acid residues (their position in the sequence is shown). Open triangles show the position of the 4 N-glycosylation sites. The position of cysteines engaged in intramolecular disulfide bonds is also shown. (B) Radioactively labeled BACEs0 has been immunoisolated from cell lysates (intracellular, lanes 1–5) or from the cell culture media (secreted, lanes 6–10) after each chase time. Proteins have been separated in reducing SDS-PAGE and labeled BACEs0 has been quantified. BACEs0 immunoisolated after a 5-min chase is considered the *initial amount* (100%). The residual intracellular BACEs0 (25%) and the fraction of labeled BACEs0 harvested from the culture media after 240 min of chase (5%) are shown in lanes 3 and 8, respectively. Only for this mutant, chase was prolonged up to 480 min. Lines show the position of the 116-, 97-, 66-, and 41-kDa mobility markers. (C) Same as B for BACEs1. (D) Same as B for BACEs2. (E) Same as B for BACEs3. (F) Same as B for BACEs4. (G) Quantifications of secretion of the five glycosylation mutants as shown in B–F (secreted).

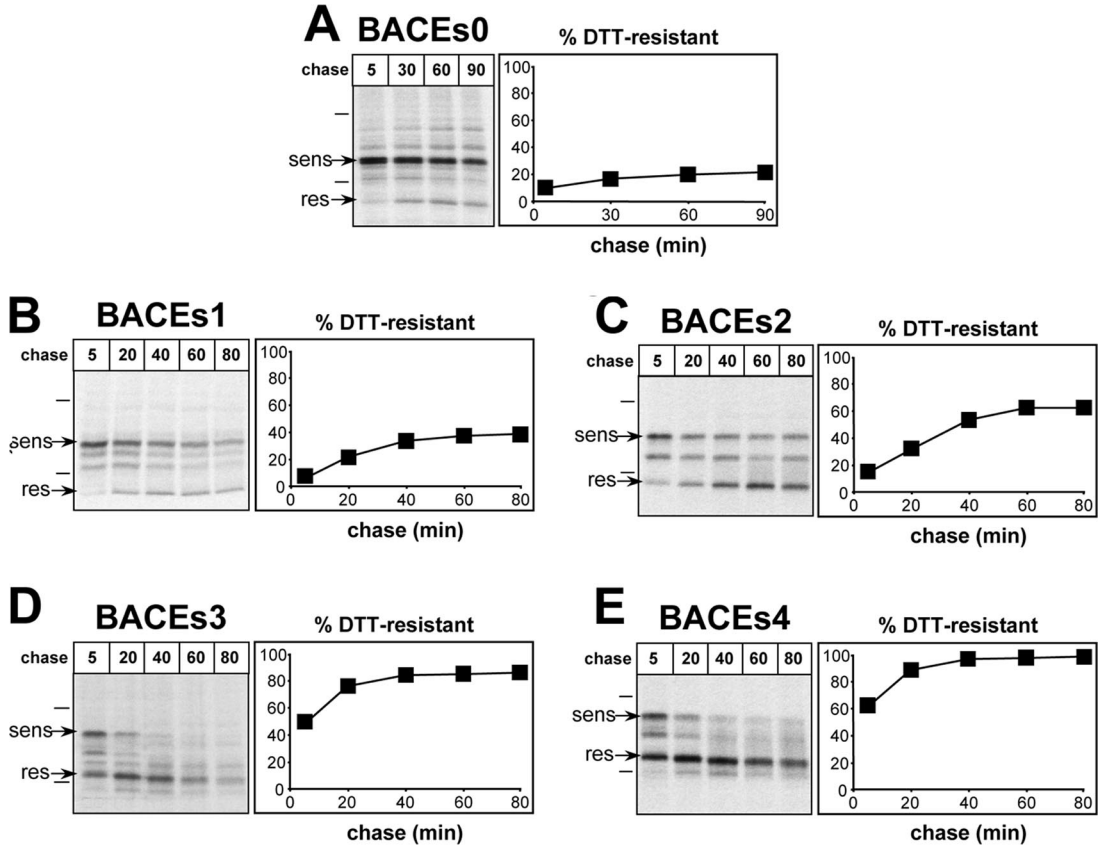

Figure 2. Efficiency of attainment of DTT-resistant structure correlates with the extent of BACEs glycosylation. (A) At the end of the chase times indicated, cells were incubated for 5 min on ice with 5 mM DTT in PBS to reduce nonnative disulfide bonds. After cell lysis, intracellular BACEs0 was immunoisolated and separated in nonreducing SDS-PAGE. The ratio DTT-sensitive BACEs0 (→sens): DTT-resistant BACEs0 (res) is plotted. (B) Same as A for BACEs1. (C) Same as A for BACEs2. (D) Same as A for BACEs3. (E) Same as A for BACEs4.

cosylated mutant (BACEs0) was very slow and inefficient. BACEs1 and BACEs2 had similar secretion kinetics, with a t_{50} (time of half-maximal secretion) of ~90 min. The more extensively glycosylated BACEs3 and BACEs4 showed a t_{50} of ~60 min.

A Direct Correlation between Extent of BACEs Glycosylation and Rate of Acquisition of a DTT-resistant Conformation

As a rule, newly synthesized polypeptides are released from the ER only upon attainment of the native structure (Ellgaard *et al.*, 1999). Unfortunately, conformational antibodies discriminating native from nonnative BACEs are not available. Thus, to confirm that kinetics and extent of secretion were dependent on the acquisition of the native structure, we monitored the rate of generation of DTT-resistant disulfides in the five glycosylation mutants. In most folded proteins, intramolecular disulfide bonds are solvent inaccessible and are not reduced by moderate DTT concentrations (Thornton, 1981).

At the end of each chase time shown in Figure 2 and before lysis, cells were incubated for 5 min on ice in a medium containing 5 mM DTT. Cell incubation with this concentration of DTT readily reduces nonnative, solvent exposed, intramolecular disulfides without affecting native disulfides (Tatu *et al.*, 1993). The labeled BACEs mutants were immunoisolated from PNS, and the proteins were separated under nonreducing conditions. Disulfide bonds in nonnative conformers were fully reduced by the DTT treatment on ice. Consequently, nonnative conformers showed slower electrophoretic mobility (→ sens in Figure 2) when compared with native ones. In the latter, preservation of covalent bonds linking cysteines resulted in faster electrophoretic mobility (→ res in Figure 2). The DTT-sensitive and the DTT-resistant labeled polypeptide bands were quantified and the ratio DTT-resistant:DTT-sensitive was plotted (Figure 2).

For intracellular BACEs0, the conversion from the DTT-sensitive into the more compact DTT-resistant conformation was very slow and inefficient (Figure 2A). This was consistent with the very slow and inefficient secretion shown for this protein in Figure 1, B and G. Even after a 90-min chase, ~80% of the intracellular BACEs0 was fully reduced upon exposure of cells to DTT. Addition of 1, 2, 3, and 4 N-glycans progressively accelerated the formation of DTT-resistant conformers that reached the 37, 62, 86, and 98% after the 80 min of chase shown in Figure 2, B–E, respectively. As discussed above, the fraction of labeled, DTT-resistant form of the proteins eventually decreased during the chase because the native (and DTT-resistant) conformers are rapidly secreted in the extracellular media (Figure 1).

An Inverse Correlation between Number of Glycans and Persistence of BiP Binding

Next, we assessed whether BACEs variants did associate with BiP, an abundant ER-resident chaperone that has been

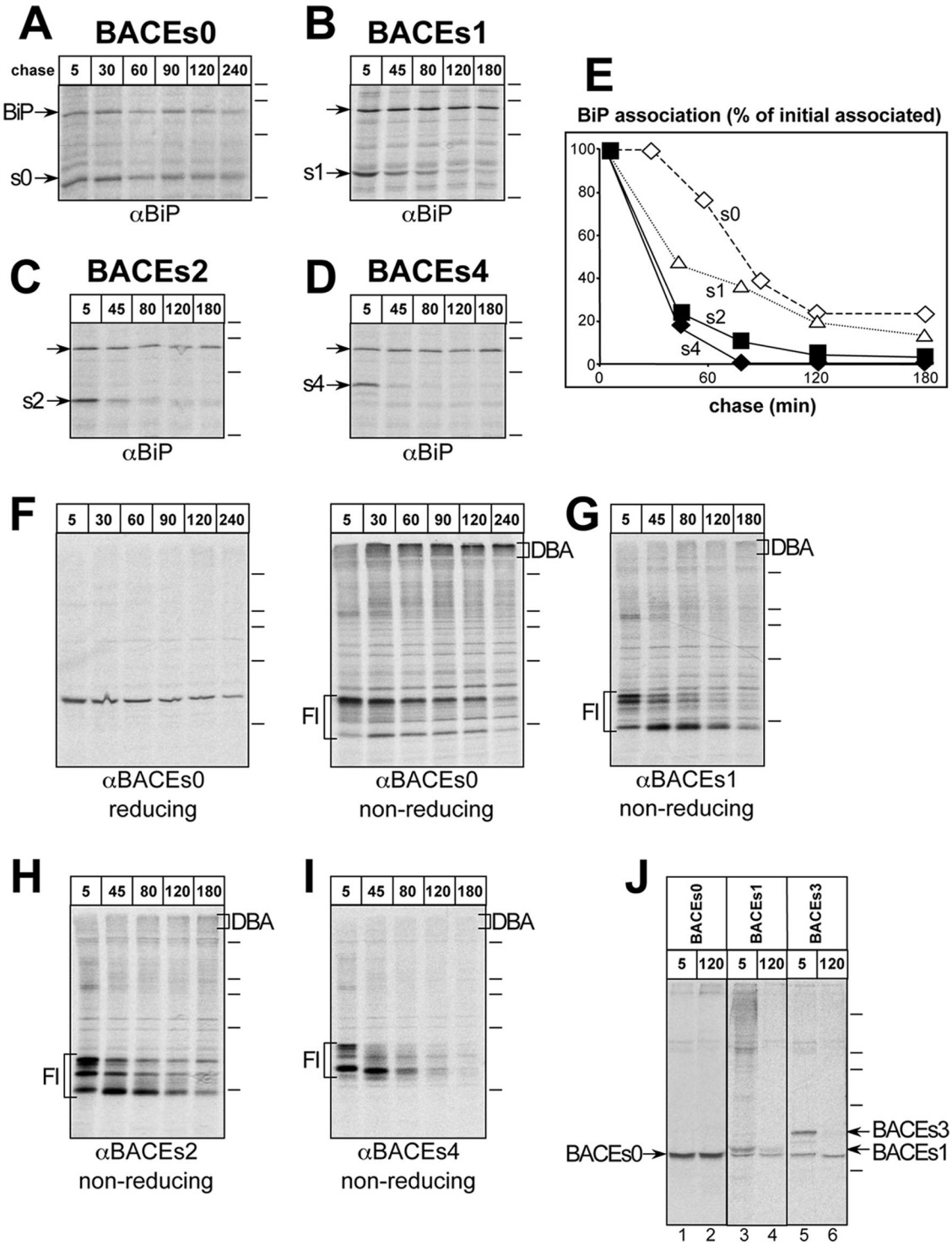

Figure 3. BiP association and propensity to form detergent-soluble and -insoluble aggregates. (A) Cells were solubilized in the presence of apyrase to rapidly consume cellular ATP, thus preventing substrate release from BiP during processing of the samples. BiP was immunoisolated from cell lysates with the associated BACEs0 at the end of the indicated chase times. The proteins were separated in reducing SDS-PAGE. BACEs0 coimmunoisolated with BiP after a 5-min chase is considered the *initial amount* (100%). (B) Same as A for BACEs1. (C) Same as A for BACEs2. (D) Same as A for BACEs4. (E) Kinetics of BACEs release from BiP. (F) The labeled BACEs0 was separated in nonreducing gel to monitor formation of disulfide-bonded (CHAPS-soluble) aggregates (DBA). The same samples were also run under reducing conditions. (G) Same as F for BACEs1. (H) Same as F for BACEs2. (I) Same as F for BACEs4. (J) The CHAPS-insoluble material was solubilized in 1% SDS, was immunoisolated with BACEs-specific antibodies, and was separated in reducing SDS-PAGE.

shown to assist polypeptide maturation in the ER (Hendershot, 2004). To compare kinetics of BiP association, lysis of cells expressing ectopic BACEs0, BACEs1, BACEs2, and BACEs4 was performed at the end of the chase times shown

in Figures 3, A–D, in the presence of apyrase. Apyrase rapidly depletes cellular ATP and allows preservation of the noncovalent complexes engaging BiP and cellular substrates (Munro and Pelham, 1986).

Immunoisolation of BiP from cell lysates and protein separation in reducing gels revealed that association of BACEs0 with BiP was long lasting. For at least 30 min after synthesis there was no release of BACEs0 from BiP (compare lane 1 and lane 2 in Figure 3A). BACEs0 release from BiP progressed slowly during the chase and even after a 240-min of chase roughly 25% of the initial amount was still associated with the chaperone (Figure 3E). In contrast, BACEs1 (54% of labeled protein released from BiP in 45 min, compare lanes 1 and 2 in Figure 3B), BACEs2 (75% release in 45 min, Figure 3C) and BACEs4 (85% release in 45 min, Figure 3D) were rapidly and efficiently released from this luminal chaperone. Dissociation rates from BiP are shown in Figure 3E. Because BiP associates with hydrophobic regions in the polypeptide backbone (Blond-Elguindi *et al.*, 1993; Hendershot, 2004), it is conceivable that the presence of bulky hydrophilic N-glycans reduces the room available for BiP to bind and concomitantly facilitates the recruitment of lectin chaperones (Molinari and Helenius, 2000). Also, because the presence of a single N-glycan was sufficient to substantially accelerate substrate release from BiP, it is possible that the presence of an oligosaccharide per se and/or the intervention of sugar processing enzymes (α-glucosidases) and lectins (e.g., calnexin) may sterically interfere with BiP binding and/or may contribute to BiP displacement from newly synthesized polypeptides (see *Discussion*).

In the case of BACEs, it was not possible to prove or to exclude a sequential intervention of BiP and of the calnexin chaperone system. Sequential intervention of these two classes of chaperones was previously shown in the case of canonical ER-associated degradation (ERAD) substrates (Hebert and Molinari, 2007 and references therein). These are subjected to a first phase of "folding attempts" in the calnexin cycle from where they are subsequently extracted to be deviated into the BiP chaperone system and then degraded (reviewed in Olivari and Molinari, 2007). It has also been possible to clearly distinguish two phases in which the BiP and the calnexin chaperone systems operate sequentially in the case of efficient folders such as viral gene products (Hebert and Molinari, 2007 and references therein). In the case of the proteins analyzed in this study, a fraction of the newly synthesized chains is subjected to productive folding, and a fraction fails to attain the native structure and is retained in the ER and eventually degraded. It is conceivable therefore, that the calnexin and the BiP chaperone systems act in parallel (rather than sequentially) on different populations of newly synthesized BACEs molecules.

An Inverse Correlation between Extent of BACEs Glycosylation and Formation of Detergent-soluble and -insoluble Aggregates

N-glycosylation determines the fate of the associated polypeptide chain in many different ways (Molinari, 2007). The highly hydrophilic N-glycans enhance solubility of intermediates of the folding process and allow entry of nascent chains into the calnexin chaperone system. Significantly, separation of the glycosylation mutants in nonreducing gels (Figures 3, F–I) revealed that the extent of glycosylation inversely correlated with the propensity of a given model polypeptide to form aberrant, high-molecular-weight disulfide-bonded aggregates (DBA).

For BACEs0, only a fraction of the labeled protein entered the running gel and was separated in few intermediates of the oxidative BACEs0 folding program (folding intermediates [FI], Figure 3F, nonreducing). A substantial amount of the labeled protein progressively entered in complexes that remained at the interface between stacking and running gel when the samples were separated under nonreducing conditions (Figure 3F, DBA). After a 240 min of chase, the vast majority of labeled BACEs0 was in DBA (Figure 3F, nonreducing). Under reducing conditions, the BACEs0-containing, high-molecular-weight complexes were disassembled, confirming that they were disulfide-bonded aggregates (Figure 3F, reducing). Formation of disulfide-bonded aggregates is symptom of extensive protein misfolding and explains both the long intracellular persistence of BACEs0 (Figure 1B, intracellular) as well as the low secretion efficiency (Figure 1B, secreted, and Figure 1G). Only a minor fraction of labeled BACEs1 and s2 was trapped in disulfide-bonded aggregates (DBA in Figure 3, G and H, respectively), whereas aggregates were virtually absent in the case of BACEs4 (Figure 3I, DBA), consistent with the higher folding efficiency and secretion yield of the glycosylated variants of BACEs.

Solubilization of cells with CHAPS allows the separation of a soluble fraction, from which proteins are immunoisolated with specific antibodies as done for the figures shown so far and of a detergent-insoluble fraction. CHAPS-insoluble proteinaceous aggregates were dissolved by boiling the pellet of the CHAPS solubilization in 1% SDS. Subsequent immunoisolations with the BACEs-specific antibody were performed upon sample dilution with 10 volumes of 1% Triton X-100 to visualize the fraction of labeled, extensively misfolded, insoluble BACEs. Consistent with the data shown in Figure 3, F–I, a substantial amount of BACEs0 (20% of the synthesized protein) remained as insoluble material throughout the chase (Figure 3J, lanes 1 and 2). A fraction of the labeled glycosylated variants BACEs1 and BACEs3 was detergent-insoluble immediately after synthesis (Figure 3J, lanes 3 and 5, respectively). At longer chase times, however, the entire population of glycosylated BACEs was soluble in CHAPS (Figure 3J, lanes 4 and 6).

To summarize, the extent of BACEs glycosylation correlates directly with rate and efficiency of secretion and inversely with the propensity of the model protein to form detergent-soluble and -insoluble aggregates in association with BiP.

Blocking Substrate Release from Calnexin Differently Affects Secretion of BACEs Glycosylation Mutants

Most, if not all, newly synthesized glycoproteins expressed in the mammalian ER attain their native structure within the calnexin chaperone system. Substrate association with calnexin is mediated by mono-glucosylated N-glycans transiently displayed by nascent and newly synthesized polypeptides (Hammond *et al.*, 1994). The association signal is generated by the coordinated action of two ER-resident glycanases, the α-glucosidase I and the α-glucosidase II. They sequentially remove two of the three glucose residues from the polypeptide-bound oligosaccharide branch A. On substrate release from calnexin, removal of the third glucose residue by α-glucosidase II is required to prevent immediate substrate reassociation with calnexin. Calnexin interactions with glycopolypeptides are normally investigated by coimmunoprecipitation (Hammond *et al.*, 1994). This is a reliable assay for multiglycosylated polypeptides, but interactions of mono- or di-glycosylated polypeptides with calnexin are often lost during processing of the samples possibly generating false negatives. To circumvent this technical issue, we

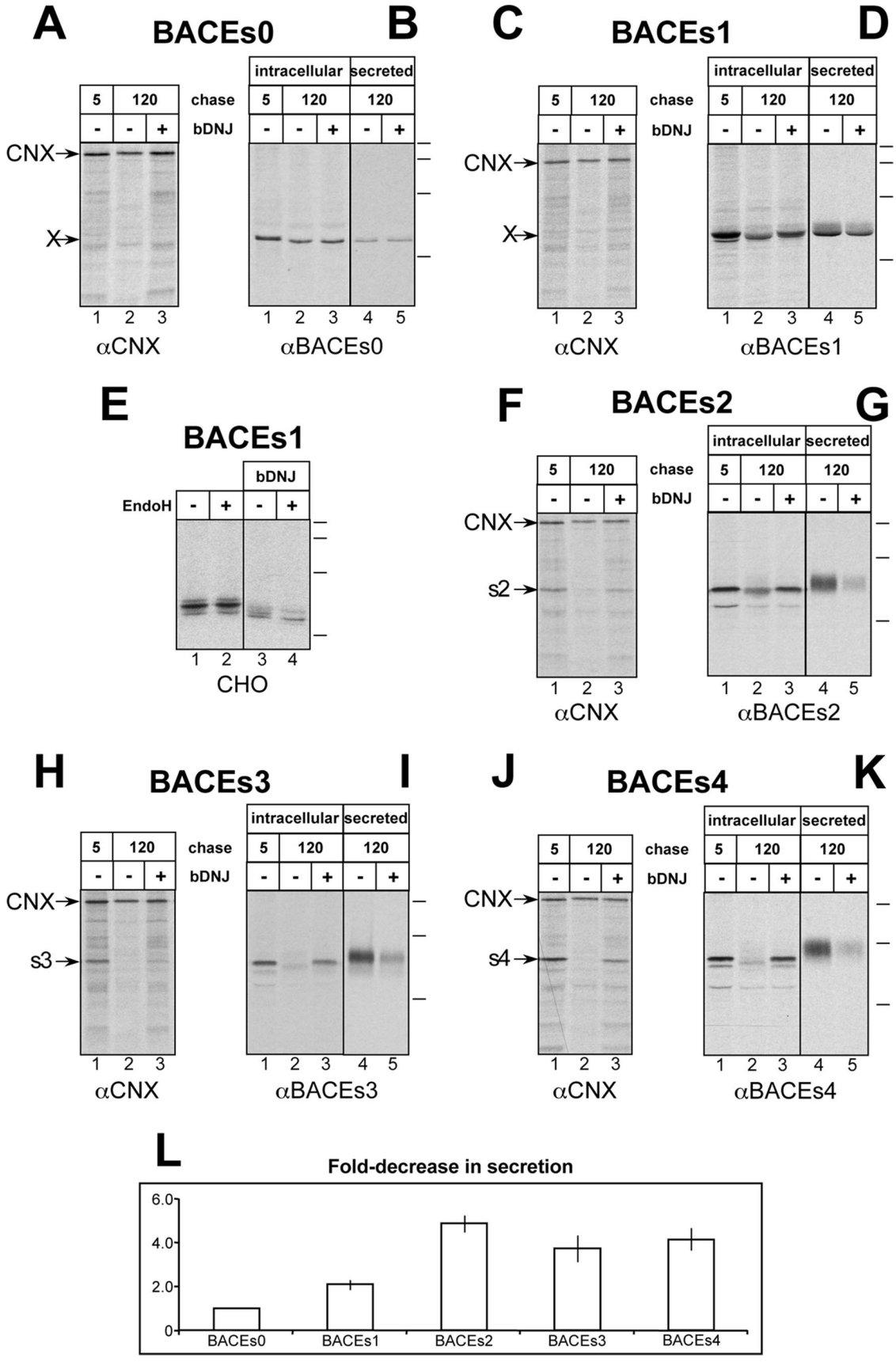

Figure 4. Consequences of inhibition of substrate release from calnexin on secretion of enzymatically active BACEs. (A) Calnexin (with associated substrates) was immunoisolated from cell lysates after a 5-min chase (lane 1), after 120 min of chase (lane 2), or after a 5-min chase

coupled conventional coimmunoprecipitation with a second assay in which we monitored variations in polypeptide secretion under conditions in which substrate association with calnexin progressed normally, but release was inhibited. To this end, cells expressing the five BACEs glycosylation mutants were exposed to the α-glucosidase inhibitor *N*-butyldeoxynojirimycin (bDNJ) a few minutes after the pulse with radioactivity. Posttranslational addition of bDNJ does not affect the removal of the two outermost glucose residues, which allows entry of nascent polypeptides into the calnexin chaperone system. Rather, it substantially inhibits the glucosidase II-operated de-glucosylation of labeled glycoproteins, which is required for their efficient release from calnexin (Hebert *et al.,* 1995). We anticipated that this treatment would substantially decrease secretion of those mutants that associate with calnexin during maturation (please note that soluble variants of BACE do not associate with calreticulin; Pieren *et al.,* 2005 and unpublished data). Cells were pulsed with radioactivity as described above and were chased for 5 min to allow BACEs association with calnexin. Incubation was prolonged to 120 min in the absence (− in Figure 4) or in the presence of bDNJ to inhibit substrate release from the chaperone (+ in Figure 4).

Immunoisolation of calnexin from detergent lysates of cells expressing BACEs0 revealed transient association of several labeled polypeptides. None of them had the electrophoretic mobility of ectopically expressed BACEs0 (→, X shows the calculated mobility of BACEs0, Figure 4A, lane 1). This was expected because N-glycans are required for substrate association with calnexin (Ou *et al.,* 1993). Most of the labeled polypeptides coprecipitating with calnexin disappeared after 120 min of chase, consistent with their maturation, resulting in dissociation from the lectin chaperone (lane 2). Addition of 1 mM bDNJ after a 5-min chase inhibited substrate release from calnexin as shown by the significant amount of labeled polypeptides still coprecipitated with the chaperone after additional 120 min of chasing (lane 3).

Analysis of intracellular (Figure 4B, lanes 1 and 2) and of secreted BACEs0 (Figure 4B, lane 4) confirmed the disappearance of the labeled polypeptide from cells and the secretion of ~4% of the labeled protein after 120 min of chase as shown in Figure 1. bDNJ treatment did not modify the intracellular content (Figure 4B, lane 3) and the amount of secreted BACEs0 (lane 5) as expected for a nonglycosylated polypeptide that does not associate with calnexin.

Similarly to the nonglycosylated variant, the mono-glycosylated BACEs1 did not coprecipitate with calnexin (Figure 4C). In this case, however, lack of coprecipitation must be ascribed to a disassembly of the BACEs1:calnexin complex during processing of the samples, as explained above. Analysis of the polypeptide fate, in fact, revealed that inhibition of substrates release from calnexin caused intracellular accumulation of labeled BACEs1 (compare lane 3 vs. lane 2 in Figure 4D) and a corresponding reduction of the polypeptide secretion by half from almost 30% of the total protein in untreated cells (Figures 4D, lane 4, and 1C, lane 10) to ~15% in bDNJ treated cells after 120 min (Figure 4D, lane 5). The case of BACEs1 is relevant because there is some confusion in the literature on whether the single oligosaccharide displayed on mono-glycosylated polypeptides is or is not de-glucosylated by the α-glucosidase II to generate the mono-glucosylated trimming intermediate required to associate with calnexin (Deprez *et al.,* 2005; Totani *et al.,* 2006; Wilkinson *et al.,* 2006). Our data show that despite the technical difficulty to coprecipitate BACEs1 with calnexin (Figure 4C), this protein actually recruits the calnexin chaperone system where it remains trapped when de-glucosylation is inhibited (Figure 4D). Thus, α-glucosidase II generates mono-glucosylated trimming intermediate in vivo even in proteins displaying a single N-glycan.

The action of the ER-resident α-glucosidase II on mono-glycosylated polypeptides was confirmed in another cell type, namely Chinese hamster ovary cells (CHO). CHO lack a Golgi-resident endo-α-D-mannosidase that de-mannosylates polypeptides released from the ER with terminal glucoses on the oligosaccharide branch A (Hiraizumi *et al.,* 1993). In these cells, only if de-glucosylation in the ER progresses normally, oligosaccharides are processed by Golgi enzymes to complex structures that cannot be cleaved by EndoH (Rothman *et al.,* 1984; Lubas and Spiro, 1987; Moore and Spiro, 1990; Hiraizumi *et al.,* 1993). Analysis of the BACEs1 secreted from CHO cells confirmed that this mono-glycosylated protein had attained an EndoH-resistant status. In fact, the electrophoretic mobility of the mock-treated (Figure 4E, lane 1) and of the EndoH-treated polypeptide (lane 2) was the same. As a control, glycans displayed on secreted BACEs1 remained EndoH sensitive, and the labeled polypeptide showed faster electrophoretic mobility upon EndoH treatment (compare lanes 3 and 4 in Figure 4E), when ER-glucosidase activity was inhibited by cell incubation with bDNJ. Thus, mono-glycosylated polypeptides are de-glucosylated by α-glucosidases and enter in the calnexin chaperone system in vivo, even though substrate association with calnexin cannot directly be monitored by coprecipitation.

Coprecipitation with calnexin was observed for di-glycosylated BACEs2 (Figure 4F), showing that two N-glycans are sufficient for preserving the substrate chaperone complex during isolation. Analysis of the cell extracts and of the secreted material confirmed that inhibition of substrate release from calnexin resulted in intracellular retention (Figure 4G, compare lane 3 vs. 2) and impaired secretion of BACEs2 (lane 5 vs. 4). Similarly, bDNJ treatment inhibited both release from calnexin and secretion of BACEs3 (Figure 4, H–I) and BACEs4 (Figure 4, J–K). Thus, analysis of the intracellular protein retention and of the secretion in the extracellular media revealed that all variants displaying at least one N-glycan entered the calnexin chaperone system. Inhibition of substrate release from calnexin variably affected secretion of the individual mutants (Figure 4L, quantification of three independent experiments).

Figure 4. (cont) followed by 115-min chase in the presence of 1 mM bDNJ to inhibit substrate release from calnexin. (B) Same as A but BACEs0 was immunoisolated from the cell lysates (lanes 1–3) or from the cell culture media (lanes 4 and 5). (C) Same as A for cells expressing BACEs1. (D) Same as B for cells expressing BACEs1. (E) BACEs1 was expressed in CHO cells. Secreted BACEs1 was immunoisolated from the cell culture media and was mock-treated (lane 1) or treated with EndoH (lane 2). Lanes 3 and 4 show the same treatment for the protein expressed in CHO cells incubated during starvation, pulse and chase with bDNJ. (F) Same as A for cells expressing BACEs2. (G) Same as B for cells expressing BACEs2. (H) Same as A for cells expressing BACEs3. (I) Same as B for cells expressing BACEs3. (J) Same as A for cells expressing BACEs4. (K) Same as B for cells expressing BACEs4. (L) Fold-decrease in secretion upon inhibition of substrate release from calnexin (average of at least three independent experiments).

Inhibiting Substrate Association with Calnexin Differently Affects Secretion of BACEs Glycosylation Mutants

Next, we determined for each glycosylation variant the importance of folding in the calnexin chaperone system to attain a transport-competent conformation. To this end, cells

were incubated in the presence of 1 mM bDNJ during starvation, pulse, and chase to preserve all N-glycans in the triglucosylated form that prevents substrate association with calnexin (Hammond *et al.*, 1994).

As expected, secretion of BACEs0, which is not glycosylated and does not associate with calnexin, was not affected by cell exposure to bDNJ (Figure 5A). All other glycosylation mutants showed a significant reduction of secretion efficiency in cells treated with the specific α-glucosidase inhibitor (Figure 5, B–E, and quantifications of three independent experiments in Figure 5F). Notably, secretion of BACEs1, which cannot be coimmunoisolated with calnexin (Figure 4C), was also substantially reduced when access into the calnexin chaperone system was inhibited (Figure 5B). Some confusion does exist on this issue in the available literature (Deprez *et al.*, 2005; Totani *et al.*, 2006; Wilkinson *et al.*, 2006). Our data clearly show that polypeptides displaying a single N-glycan are efficiently processed by ER α-glucosidases and attain a transport-competent conformation in association with calnexin. Surprisingly, inactivation of the calnexin cycle affects more dramatically the secretion of BACEs1 (fivefold reduction) and of BACEs2 (6.5-fold reduction) than the secretion of the more extensively glycosylated variants BACEs3 (threefold reduction) and BACEs4 (3.7-fold reduction; Figure 5F). These data show that the presence of 3 and 4 N-glycans has, per se, a positive effect on folding even when access to the lectin chaperone system is inhibited. By substantially increasing the hydrophilic surface, extensive glycosylation certainly reduces the propensity to enter in hydrophobic contacts with other unfolded chains that would irreversibly deviate the newly synthesized polypeptide into off pathways of the folding program.

Proteasome Inhibition Increases Secretion of Active BACEs Mutants

Secretion efficiency is a peculiar property of every polypeptide. The cystic fibrosis transmembrane conductance regulator (CFTR) is a paradigmatic example of a protein with very low secretion efficiency. More than 70% of the newly synthesized protein is deviated into the ER-associated degradation pathway, and it is extracted from the ER membrane and degraded by cytosolic proteasomes (Kopito, 1999). In the ER lumen, machineries that regulate protein folding and machineries that interrupt folding programs and deviate nonnative polypeptides into the ERAD pathway compete for newly synthesized polypeptides (Molinari, 2007). This is an important aspect because several human conformational diseases are caused by missense mutations that may not affect function but rather delay proper folding to such an extent to favor the disposal of the mutant protein before attainment of the native conformation. Also, competition between polypeptide folding and degradation may substantially reduce the yield of production of recombinant proteins of industrial or clinical interest.

Secretion efficiency for the BACEs proteins analyzed in this study ranged from 60% for the normally glycosylated polypeptide to ~5% for the nonglycosylated variant. The secretion-deficient glycosylation mutants were retained intracellularly and/or degraded.

To assess whether BACEs productivity was improved by inhibition of ERAD, secretion of the 5 BACEs variants was compared in cells mock-treated or treated during the chase with PS-341 (Velcade), a cell permeable tripeptide (Pyrazylcarbonyl-Phe-Leu-boronate) used as therapeutic agent for relapsed multiple myeloma (Adams and Kauffman, 2004). The boron atom of PS-341 binds the active site of the 26S proteasome, thereby specifically inhibiting proteasomal activity (Adams and Kauffman, 2004).

Cells were incubated for 2 h and 30 min without (− in Figure 6, A–E) or with PS-341 (+ in Figure 6, A–E). Cell lysates were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were probed with anti-ubiquitin antibodies to confirm proteasome inhibition and accumulation of poly-ubiquitylated polypeptides (poly-ub, Figure 6, A–E). The proteins in the extracellular media were also separated electrophoretically and transferred on PVDF membranes. These membranes were probed with the antibody to BACE to visualize the BACEs variants secreted from mock-treated cells (−) and from cells incubated with PS-341 (+).

For BACEs0, inhibition of the proteasome enhanced secretion by twofold (Figure 6A). For all other variants, enhancement of secretion was much more modest and ranged between 15 and 30% (Figure 6, B–E, Secreted protein, quantifications are an average of three independent experiments). Importantly, for all proteins, the enhancement in polypeptide secretion resulted in a corresponding increase in the enzymatic activity recovered from the cell culture media as determined by spectrofluorometric quantification of the cleavage of a fluorogenic ALEXA-conjugated substrate peptide (*Materials and Methods*). The enhancement of protein secretion upon inhibition of the proteasomal activity was confirmed with another experimental approach and by using a different inhibitor of the proteasome. To this end, cells were pulsed with radioactivity for 10 min. After radioactivity washout, incubation was prolonged for 4 h in the presence of MG132. At the end of the chase, the labeled BACEs variants secreted in the cell culture media were immunoisolated with the specific antibody and were separated in SDS-PAGE (Figure 6F). Quantifications confirmed the data shown in Figure 6, A–E, showing a doubling of the BACEs0 secretion and an increase of 15–40% of secretion of the glycosylated form of the polypeptide. In this case as well, the increase in secretion corresponded to an equivalent increase in the enzymatic activity recovered from the cell culture media. Hence, at least in the case of BACEs, inhibition of protein disposal resulted in an increase of the yield of enzymatically active protein. Under normal conditions, therefore, a variable fraction of the newly synthesized BACEs variants is degraded before attainment of the native structure. At least part of these prematurely degraded polypeptides can be rescued and secreted in active form upon delay of their disposal.

DISCUSSION

We have performed a thorough analysis of the maturation in mammalian cells of a model protein as a function of the number of glycan modifications present on the polypeptide backbone. In this study, we report data for the nonglycosylated BACEs0, for BACEs1 displaying the N-linked glycan at position 172, for BACEs2 (172 and 223), for BACEs3 (153, 172, and 354) and for BACEs4 (153, 172, 223, and 354). Experiments have also been performed with the mono-glycosylated variants displaying the single oligosaccharide in the three other positions (153, 223, or 354) and with the di-glycosylated variants with oligosaccharides in all possible combinations (positions 153–172 or 153–223 or 153–354 or 172–354 or 223–354). Although variation of the number of N-glycans clearly affected the fate of the newly synthesized polypeptide chains, their position had very little consequence on the outcome of the experiments (unpublished data).

The data show that the number of glycans displayed on BACEs correlated directly with rate and efficiency of

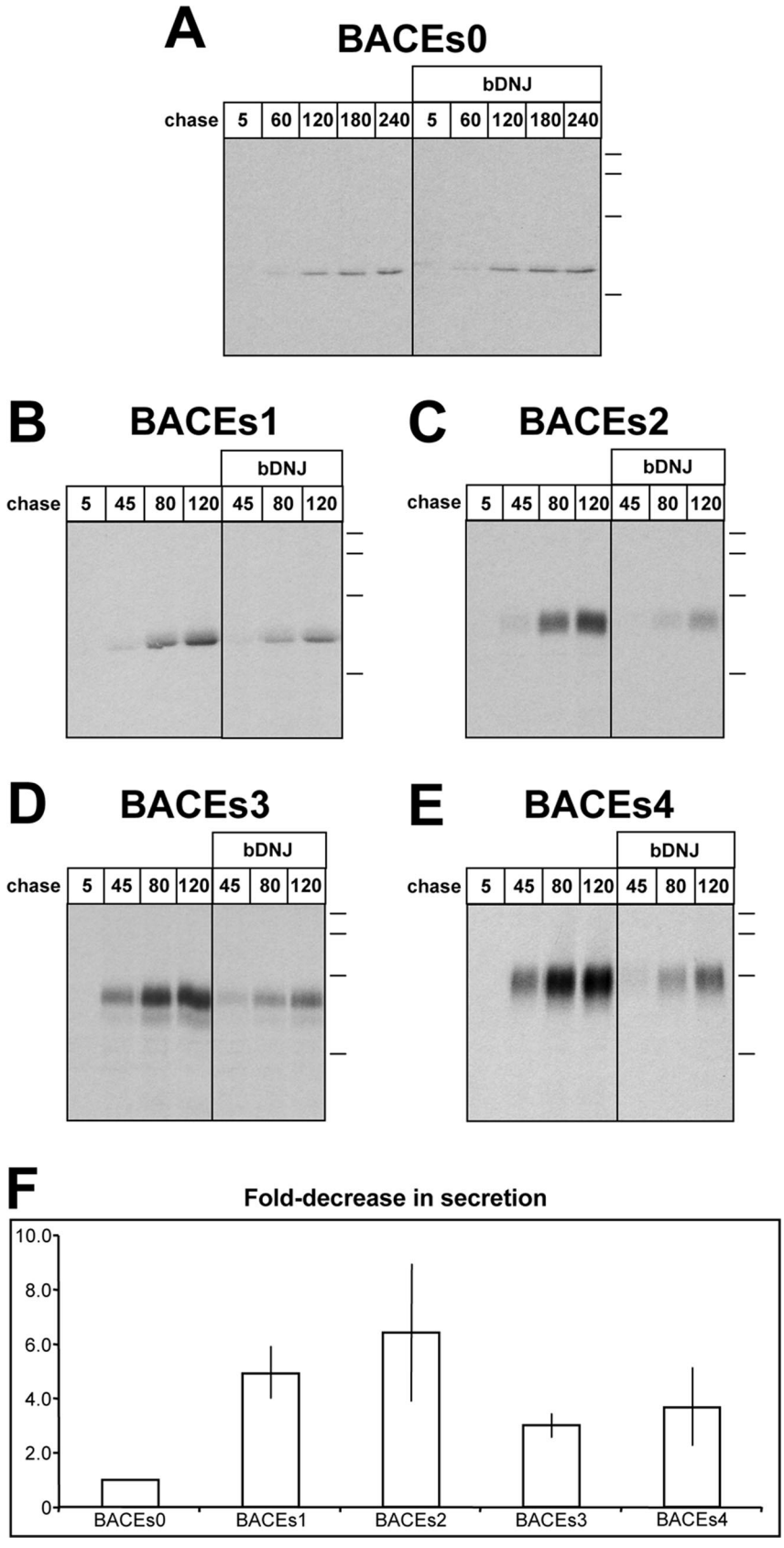

Figure 5. Consequences of inhibition of substrate association with calnexin on BACEs secretion. (A) Secretion of BACEs0 was compared in mock-treated versus bDNJ-treated cells. (B) Same as A for BACEs1. (C) Same as A for BACEs2. (D) Same as A for BACEs3. (E) Same as A for BACEs4. (F) Fold-decrease in secretion upon inhibition of substrate association with calnexin (average of at least three independent experiments).

polypeptide folding and secretion (Figures 1–3) and that one N-linked glycan was sufficient to recruit the calnexin chaperone system and/or to allow substrate de-glucosylation by the ER-resident α-glucosidase II (Figures 4 and 5). This latter

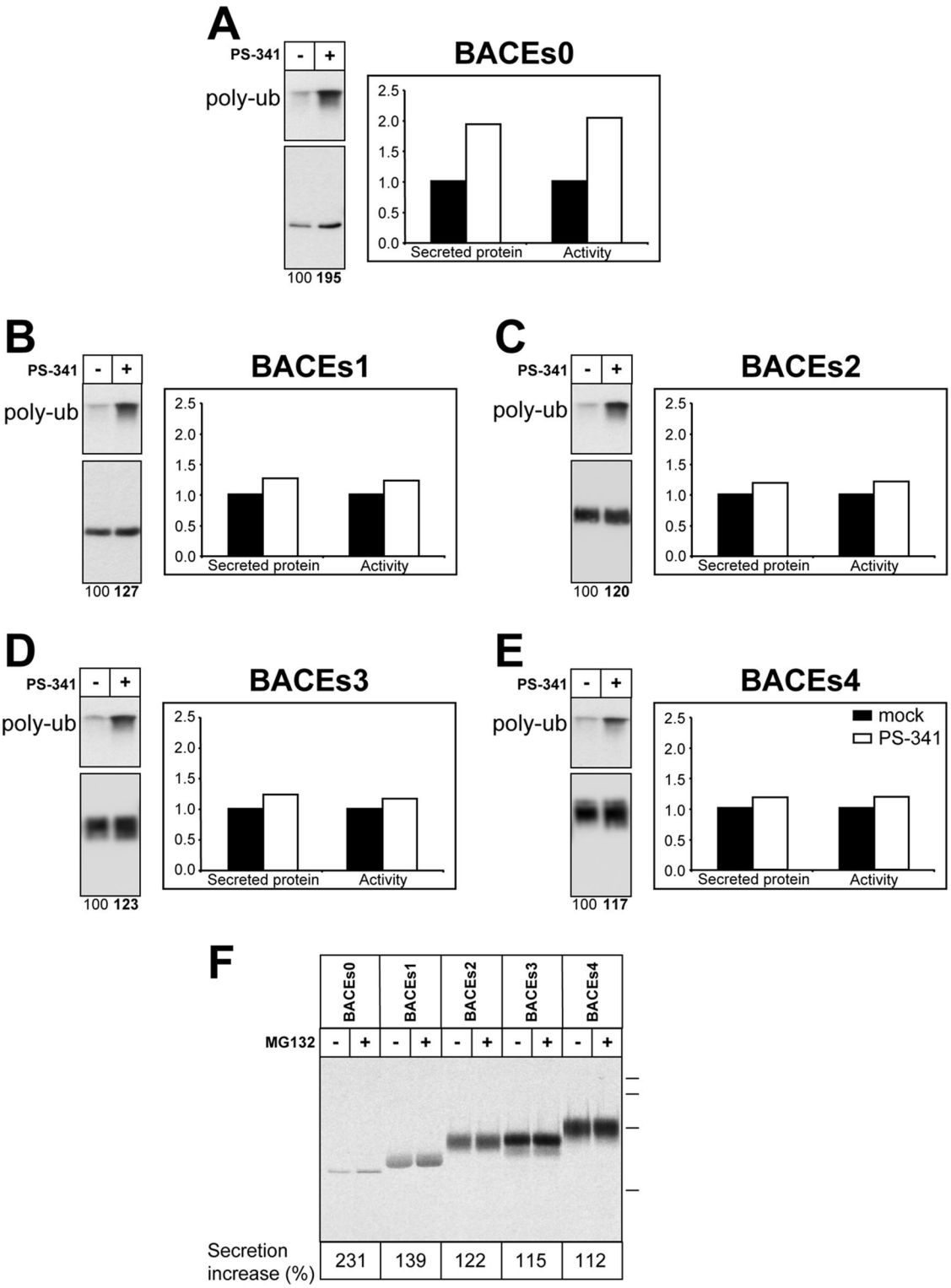

Figure 6. Consequences of proteasome inhibition on BACEs secretion. (A) Cells were mock-treated (−) or were incubated for 150 min with 9 μM PS-341 (+). At the end of the incubation, cell lysates were separated in reducing SDS-PAGE, and proteins were blotted on PVDF. The membranes were decorated with antibodies to ubiquitin to check accumulation of poly-ubiquitylated chains as an indication of proteasomal inactivation (top gel, poly-ub). The cell culture media were separated in reducing SDS-PAGE, and proteins were blotted on PVDF. The membranes were decorated with antibodies to BACE (100% is the amount of BACEs secreted from untreated cells in 150 min). The increase in secreted protein and in enzymatic activity recovered from the cell culture media is plotted. (B) Same as A for BACEs1. (C) Same as A for BACEs2. (D) Same as A for BACEs3. (E) Same as A for BACEs4. (F) Cells were immunolabeled and chased for 4 h without (−) or with (+) 50 μM MG132. The labeled BACEs variants were immunoisolated from cell culture media and separated in reducing SDS-PAGE. Percentages of increase in secretion for individual glycosylation mutants are shown below the gels.

finding merits a specific comment. It is well known that substrate association with calnexin is very weak (Kapoor *et al.*, 2003). A direct and transient interaction can reliably be monitored by coimmunoprecipitation of multi-glycosylated substrates with the lectin chaperone, whereas for mono- and even for di-glycosylated polypeptides association might be lost during sample processing. Moreover, in isolated microsomes, the ER α-glucosidase II can only very inefficiently generate the mono-glucosylated trimming intermediate of the N-glycan processing reaction that allows association of a mono-glycosylated arrested chain with calnexin (Deprez *et al.*, 2005). How and if mono-glycosylated polypeptides make use of the calnexin chaperone system is therefore unclear (Deprez *et al.*, 2005; Totani *et al.*, 2006; Wilkinson *et al.*, 2006). By coupling conventional coimmunoprecipitation with two assays in which we monitored variations in polypeptide secretion under conditions in which substrate association or release from calnexin were inhibited, our data convincingly show that mono-glycosylated polypeptides gain access to the calnexin system in living cells. The variants with 1 and 2 N-glycans actually suffered much more than the variants with 3 and 4 N-glycans upon inactivation of the calnexin chaperone system (Figure 5). Thus, hyper-glycosylation per se enhances the protein folding process, even in the absence of lectin assistance, possibly by preventing entry in off pathways of the polypeptide's folding program. Consistently, the number of glycans correlated inversely with the propensity of the polypeptide chain to enter in detergent-soluble or -insoluble aggregates (Figure 3).

Stepwise addition of N-glycans also correlated with a progressive increase in the rate of substrate release from BiP (Figure 3). This finding implies that not only chaperone selection (Molinari and Helenius, 2000), but also substrate release from specific chaperone machineries could be dictated by competition with other chaperones willing to act on the newly synthesized chain. In the specific case, we propose that the intervention of sugar processing enzymes (α-glucosidases) and lectins (e.g., calnexin) may contribute to BiP displacement from newly synthesized, folding polypeptides. An alternative explanation is that the faster release from BiP derives from an acceleration of the folding process that rapidly conceals binding determinants for this conventional chaperone, independent of the steric hindrance caused by the intervention of other chaperones to assist the maturation of the folding polypeptides.

Finally, one should notice that there is a good correlation between the kinetics of attainment of DTT-resistant structures and the rate of secretion of the individual BACEs variants (Figures 1 and 2). However, for none of the BACEs variants, the entire population of DTT-resistant conformers was secreted from cells as active enzyme. For example, the fraction of intracellular DTT-resistant BACEs0 reaches the 20% of the labeled protein, but not more than the 5% of it is actually secreted. This may indicate that only a fraction of the DTT-resistant conformers actually have native structure. Alternatively, or in addition to that, it is conceivable that the retention-based quality control operating in the ER is too stringent and inhibits export of a fraction of native (DTT-resistant) BACEs conformers. This implies that the manipulation of the ER quality control tightness or the intervention on the relative activities of the folding versus ERAD machineries operating in cells expressing ectopic proteins may offer an interesting opportunity to enhance productivity of functional polypeptides (Figure 6). Consistently, and similarly to what reported for folding-defective proteins such as mutated CFTR and dystrophin (Bonuccelli *et al.*, 2003; Farinha and Amaral, 2005; Vij *et al.*, 2006), proteasome inhibition increased the yield of active BACEs harvested from the cell culture media. In most of the cases, inactivation of the proteasome inhibits translocation of proteins from the ER lumen into the cytosol (reviewed in Hebert and Molinari, 2007). It is therefore likely that in the presence of proteasome inhibitors polypeptides that are *inappropriately* deviated into the degradation machinery at steady state are allowed to spend longer time in the ER lumen, thus eventually escaping aberrant retention by the quality control machinery.

It should be noted that, even a modest increase of the fraction of a mutated protein terminating the folding program as an active entity may considerably reduce the course of loss-of-function disorders caused by premature polypeptide disposal (Aridor, 2007; Hebert and Molinari, 2007). A 20–30% increase in the yield of active recombinant proteins may also offer a significant economic interest for industrial production of biomolecules (Baldi *et al.*, 2007).

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REFERENCES

Adams, J., and Kauffman, M. (2004). Development of the proteasome inhibitor Velcade (Bortezomib). Cancer Invest. *22*, 304–311.

Aridor, M. (2007). Visiting the ER: the endoplasmic reticulum as a target for therapeutics in traffic related diseases. Adv. Drug Deliv. Rev. *59*, 759–781.

Baldi, L., Hacker, D. L., Adam, M., and Wurm, F. M. (2007). Recombinant protein production by large-scale transient gene expression in mammalian cells: state of the art and future perspectives. Biotechnol. Lett. *29*, 677–684.

Blond-Elguindi, S., Cwirla, S. E., Dower, W. J., Lipshutz, R. J., Sprang, S. R., Sambrook, J. F., and Gething, M. J. (1993). Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. Cell *75*, 717–728.

Bonuccelli, G., Sotgia, F., Schubert, W., Park, D. S., Frank, P. G., Woodman, S. E., Insabato, L., Cammer, M., Minetti, C., and Lisanti, M. P. (2003). Proteasome inhibitor (MG-132) treatment of mdx mice rescues the expression and membrane localization of dystrophin and dystrophin-associated proteins. Am. J. Pathol. *163*, 1663–1675.

Caramelo, J. J., and Parodi, A. J. (2007). How sugars convey information on protein conformation in the endoplasmic reticulum. Semin. Cell Dev. Biol. *18*, 732–742.

Caramelo, J. J., and Parodi, A. J. (2008). Getting in and out from calnexin/calreticulin cycles. J. Biol. Chem. *283*, 10221–10225.

Deprez, P., Gautschi, M., and Helenius, A. (2005). More than one glycan is needed for ER glucosidase II to allow entry of glycoproteins into the calnexin/calreticulin cycle. Mol. Cell *19*, 183–195.

Ellgaard, L., Molinari, M., and Helenius, A. (1999). Setting the standards: quality control in the secretory pathway. Science *286*, 1882–1888.

Farinha, C. M., and Amaral, M. D. (2005). Most F508del-CFTR is targeted to degradation at an early folding checkpoint and independently of calnexin. Mol. Cell. Biol. *25*, 5242–5252.

Hammond, C., Braakman, I., and Helenius, A. (1994). Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. Proc. Natl. Acad. Sci. USA *91*, 913–917.

Hebert, D. N., Foellmer, B., and Helenius, A. (1995). Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. Cell *81*, 425–433.

Hebert, D. N., Garman, S. C., and Molinari, M. (2005). The glycan code of the endoplasmic reticulum: asparagine-linked carbohydrates as protein maturation and quality-control tags. Trends Cell Biol. *15*, 364–370.

Hebert, D. N., and Molinari, M. (2007). In and out of the ER: protein folding, quality control, degradation, and related human diseases. Physiol. Rev. *87*, 1377–1408.

Hendershot, L. M. (2004). The ER function BiP is a master regulator of ER function. Mt. Sinai. J. Med. *71*, 289–297.

Hiraizumi, S., Spohr, U., and Spiro, R. G. (1993). Characterization of endomannosidase inhibitors and evaluation of their effect on N-linked oligosaccharide processing during glycoprotein biosynthesis. J. Biol. Chem. *268*, 9927–9935.

Hong, L., Koelsch, G., Lin, X., Wu, S., Terzyan, S., Ghosh, A. K., Zhang, X. C., and Tang, J. (2000). Structure of the protease domain of memapsin 2 (beta-secretase) complexed with inhibitor. Science *290*, 150–153.

Kapoor, M., Srinivas, H., Kandiah, E., Gemma, E., Ellgaard, L., Oscarson, S., Helenius, A., and Surolia, A. (2003). Interactions of substrate with calreticulin, an endoplasmic reticulum chaperone. J. Biol. Chem. *278*, 6194–6200.

Kopito, R. R. (1999). Biosynthesis and degradation of CFTR. Physiol. Rev. *79*, S167–S173.

Lederkremer, G. Z., and Glickman, M. H. (2005). A window of opportunity: timing protein degradation by trimming of sugars and ubiquitins. Trends Biochem. Sci. *30*, 297–303.

Lubas, W. A., and Spiro, R. G. (1987). Golgi endo-alpha-D-mannosidase from rat liver, a novel N-linked carbohydrate unit processing enzyme. J. Biol. Chem. *262*, 3775–3781.

Meusser, B., Hirsch, C., Jarosch, E., and Sommer, T. (2005). ERAD: the long road to destruction. Nat. Cell Biol. *7*, 766–772.

Molinari, M. (2007). N-glycan structure dictates extension of protein folding or onset of disposal. Nat. Chem. Biol. *3*, 313–320.

Molinari, M., and Helenius, A. (2000). Chaperone selection during glycoprotein translocation into the endoplasmic reticulum. Science *288*, 331–333.

Moore, S. E., and Spiro, R. G. (1990). Demonstration that Golgi endo-alpha-D-mannosidase provides a glucosidase-independent pathway for the formation of complex N-linked oligosaccharides of glycoproteins. J. Biol. Chem. *265*, 13104–13112.

Munro, S., and Pelham, H. R. (1986). An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. Cell *46*, 291–300.

Nakatsukasa, K., and Brodsky, J. L. (2008). The recognition and retrotranslocation of misfolded proteins from the endoplasmic reticulum. Traffic *9*, 861–870.

Olivari, S., and Molinari, M. (2007). Glycoprotein folding and the role of EDEM1, EDEM2 and EDEM3 in degradation of folding-defective glycoproteins. FEBS Lett. *581*, 3658–3664.

Ou, W. J., Cameron, P. H., Thomas, D. Y., and Bergeron, J. J. (1993). Association of folding intermediates of glycoproteins with calnexin during protein maturation. Nature *364*, 771–776.

Pearse, B. R., Gabriel, L., Wang, N., and Hebert, D. N. (2008). A cell-based reglucosylation essay demonstrates the role of GT1 in the quality control of a maturing protein. J. Cell Biol. *181*, 309–320.

Pieren, M., Galli, C., Denzel, A., and Molinari, M. (2005). The use of calnexin and calreticulin by cellular and viral glycoproteins. J. Biol. Chem. *280*, 28265–28271.

Rothman, J. E., Urbani, L. J., and Brands, R. (1984). Transport of protein between cytoplasmic membranes of fused cells: correspondence to processes reconstituted in a cell-free system. J. Cell Biol. *99*, 248–259.

Sardana, V., Xu, B., Zugay-Murphy, J., Chen, Z., Sardana, M., Darke, P. L., Munshi, S., and Kuo, L. C. (2004). A general procedure for the purification of human beta-secretase expressed in *Escherichia coli*. Protein Expr. Purif. *34*, 190–196.

Shimizu, H., Tosaki, A., Kaneko, K., Hisano, T., Sakurai, T., and Nukina, N. (2008). Crystal structure of an active form of BACE1: an enzyme responsible for amyloid {beta} protein production. Mol. Cell. Biol. *28*, 3663–3671.

Soldà, T., Galli, C., Kaufman, R. J., and Molinari, M. (2007). Substrate-specific requirements for UGT1-dependent release from calnexin. Mol. Cell *27*, 238–249.

Tatu, U., Braakman, I., and Helenius, A. (1993). Membrane glycoprotein folding, oligomerization and intracellular transport: effects of dithiothreitol in living cells. EMBO J. *12*, 2151–2157.

Thornton, J. M. (1981). Disulphide bridges in globular proteins. J. Mol. Biol. *151*, 261–287.

Totani, K., Ihara, Y., Matsuo, I., and Ito, Y. (2006). Substrate specificity analysis of endoplasmic reticulum glucosidase II using synthetic high-mannose-type glycans. J. Biol. Chem. *281*, 31502–31508.

Vij, N., Fang, S., and Zeitlin, P. L. (2006). Selective inhibition of endoplasmic reticulum-associated degradation rescues DeltaF508-cystic fibrosis transmembrane regulator and suppresses interleukin-8 levels: therapeutic implications. J. Biol. Chem. *281*, 17369–17378.

Wilkinson, B. M., Purswani, J., and Stirling, C. J. (2006). Yeast GTB1 encodes a subunit of glucosidase II required for glycoprotein processing in the endoplasmic reticulum. J. Biol. Chem. *281*, 6325–6333.