A stringent quality control process selects misfolded polypeptides generated in the endoplasmic reticulum (ER) for ER-associated degradation (ERAD). Here we assessed the maintenance of efficient glycoprotein folding in cells with defective ERAD caused by lack of adaptation of the intralumenal level of ER degradation-enhancing α-mannosidase-like protein (EDEM) to an increase in the ER cargo load. When these cells were converted into factories for production of high levels of human β-secretase, maturation of this N-glycosylated aspartic protease progressed as in wild-type cells initially to gradually become less efficient. Up-regulation of EDEM to strengthen the ERAD machinery (but not up-regulation of calnexin to reinforce the folding machinery) was instrumental in maintaining folding efficiency and secretory capacity. Our data underscore the important role that the degradation machinery plays in maintaining a functional folding environment in the ER.

Only a fraction of the polypeptide chains expressed in the ER is transported to its final destination. A stringent quality control process prevents export of orphan subunits of oligomeric complexes, folding-incompetent products of mutated genes, and by-products of protein biosynthesis that do not acquire the native conformation (1–3). Polypeptides that do not pass quality control are either retained by the ER chaperone machinery because it prevents clogging of the ER chaperone network to be used for further folding attempts or are sorted out for cytosolic degradation. The mechanisms regulating ER quality control are better understood for N-glycosylated polypeptides. N-glycans are added to asparagines in nascent chains as pre-assembled tri-antennary oligosaccharides composed of three terminal glucose, nine mannoses, and two N-acetylgalcosamines. The two outermost sugars are rapidly trimmed by ER glucosidases I and II. Polypeptides exposing mono-glycosylated intermediates of N-glycan trimming associate with the ER lectins calnexin (Cnx) and calreticulin (Crt).

The opposing actions of glucosidase II that trim the innermost glucose residue (thereby releasing the newly synthesized polypeptide from Cnx/Crt) and of UDP-glucose-glycoprotein glycosyltransferase that adds back one glucose to non-native conformers sending them back to Cnx/Crt, drive the so-called Cnx cycle (1, 2). For folding-incompetent and terminally misfolded glycopolypeptides, termination of unproductive folding attempts coincides with their definitive release from the Cnx cycle and deviation into the ERAD machinery. Interruption of unproductive folding attempts is a crucial step of ER quality control because it prevents clogging of the ER chaperone system.

Emerging evidence underscores the importance of a tightly controlled extraction of folding-incompetent glycopolypeptides from the Cnx cycle to promote their degradation. Extraction is regulated by the ER α-mannosidase I (4) and by EDEM (ER degradation-enhancing α-mannosidase-like protein (5)), an enzymatically inactive mannosidase-like protein. The ER α-mannosidase I cleaves one (6) or more (7) mannose residues from protein-bound N-glycans. Mannose trimming makes glucosidase-mediated protein deglucosylation (8) and UDP-glucose-glycoprotein glycosyltransferase-mediated protein reglucosylation (9) less efficient. This slows down the on/off cycles of substrate binding to/release from Cnx. Moreover, N-glycans with a reduced number of mannoses if coupled to misfolded protein determinants may serve for association with EDEM. Mannose trimming may therefore eventually lead to active extraction of folding incompetent glycoproteins from the folding attempts phase in the Cnx cycle (10–17). Consistently, selective inhibition of ER α-mannosidase I with the alkaloid kifunensine (4, 18) prevents hydrolysis of the Man9 structure (7), retards release of terminally misfolded glycoproteins from the Cnx cycle (19) and delays their degradation (reviewed in (11)). These findings are all in keeping with a mannose-timer model first proposed by Helenius (20) and refined in the last decade to include the kifunensine-sensitive ER α-mannosidase I as an essential factor determining the fate of glycoproteins with defective folding (1, 2, 4, 21). ERAD candidates may eventually associate with other ER chaperones that drive the unfolding and dislocation steps necessary for the proteasome-mediated destruction occurring in the cytosol (19, 22).

EDEM is a stress-regulated protein (5). Depletion of the proximal ER stress sensorIRE1α abolished ER stress-induced EDEM up-regulation and prevented ERAD of a model glycopeptide (23). Down-regulation of EDEM and inhibition of its up-regulation in cells exposed to small interfering RNA targeting EDEM transcripts also affected ERAD (16). Although a role of EDEM for efficient degradation of mutated gene products has been shown (10–17, 23), no link has yet been established.
between ERAD capacity and maintenance of functional protein folding environment in the mammalian ER.

Based on the severe glycoprotein ERAD (GERAD) phenotype characterizing cells depleted of IRE1α (23), we also expected a block of degradation of terminally misfolded glycopolypeptides in cells depleted of the IRE1α-activated transcription factor Xbp1 that are also unable to adjust the level of EDEM in response to ER stress (24). Therefore we made use of mouse embryonic fibroblasts (MEF) derived from Xbp1 knock-out mice generated in the laboratory of Laurie Gimcher and co-workers (25) to assess the importance of GERAD activity for maintenance of functional folding environment in the ER lumen.

Comparison of GERAD in wt cells and in cells depleted of Xbp1 showed that the Ire1/Xbp1-regulated adaptation of the intralumenal level of EDEM is actually not essential for GERAD but is required for progression to its highest efficiency. Assessment of capacity of wt cells and of cells depleted of Xbp1 to productively assist maturation of model viral glycoproteins expressed upon infection with influenza virus or Semliki forest virus did not reveal differences. Consistently, when wt cells and cells lacking Xbp1 were converted into factories for production of human β-secretase, folding and secretion occurred initially with the same efficiency. Failure to efficiently accomplish GERAD led to a slow accumulation of by-products of β-secretase biosynthesis in the ER lumen of Xbp1-depleted cells as disulfide-bonded, detergent-insoluble aggregates and progressively compromised function of the ER folding machinery. Up-regulation of EDEM but not up-regulation of Cnx prevented deposition of β-secretase in disulfide-bonded aggregates, thus restoring folding efficiency and sustaining secretion capacity at levels comparable with those of wt cells. These findings underscore the crucial role of GERAD and in particular of the regulation of the intralumenal level and activity of EDEM in coupling disposal of misfolded by-products of protein biosynthesis to maintenance of a functional protein folding environment in the ER.

MATERIALS AND METHODS

Infections and Transfections—Cells were infected as described in (26) or transiently transfected with LipofectAMINE 2000 (Invitrogen) according to the instructions of the manufacturer.

Pulse-Chase and Immunoprecipitation—Cells were starved for 30 min in Met/Cys free medium, pulsed for 10 min with 150 μCi of 35S-labeled Met/Cys in a 1-ml starvation medium/dish, and chased for the times indicated in the figures with Dulbecco’s modified Eagle’s medium supplemented with 5 μM cold Met/Cys. Postnuclear supernatants were prepared by solubilization of cells in 800 μl/dish ice-cold 2% CHAPS in Hepes buffer saline, pH 6.8, containing 20 mM ice-cold N-ethylmaleimide, protease inhibitors (HBS). Cell extracts were prepared by 10 min of centrifugation at 10,000 × g and analyzed by reducing SDS-PAGE. Immunoprecipitations were performed by adding protein A beads (Sigma), 1:10 (v/v) swollen in HBS) and the selected antibody to the cell extracts. Incubations were 1–4 h in a cold room. The immunoprecipitates were extensively washed three times with HBS, 0.5% CHAPS and resuspended in sample buffer for SDS-PAGE. Relevant bands were quantitated by ImageQuant software (Amersham Biosciences). Gels were also exposed to BioMax (Eastman Kodak Co.) films and scanned with an Agfa scanner.

Analysis of the Detergent-insoluble Protein Aggregates—To determine the presence of BACE in CHAPS-insoluble pellet, the pellet was washed once with 1 ml of phosphate-buffered saline, boiled in 100 μl of 1% SDS in HBS to denature the precipitate, and supplemented with 1 ml of Triton X-100 (1% in HBS) before the addition of protein A beads and antibody to a linear epitope of BACE. At least three independent experiments were analyzed. For Endo H (Roche Applied Science) treatment, BACE501 was immunoprecipitated from the cell extracts before incubation for 1 h at 37 °C with 1 milliunit of Endo H.

RESULTS

Xbp1 Depletion Delays Degradation from the ER of Folding-incompetent Glycopolypeptides—Generation of active Xbp1 occurs by unconventional mRNA splicing operated by the ER stress sensor IRE1α in response to intraluminal accumulation of misfolded proteins (27, 28). Awareness of the block in glycoprotein ERAD in IRE1α-depleted cells (23), we determined the severity of the GERAD phenotype in cells lacking Xbp1. To this end, we compared the degradation of BACE457Δa, a folding-incompetent glycoprotein previously used as a GERAD model substrate (16, 19), in wt and Xbp1−/− MEF.

Seventeen h after transfection, cells were metabolically labeled for 10 min with [35S]methionine and cysteine. After various chase times, cells were detergent-solubilized and post-nuclear supernatants were supplemented with a specific antibody to isolate labeled BACE and compare kinetics of degradation in the two cell lines (Fig. 1A). In wt cells, the half-life of BACE457Δa was 45 min (Fig. 1A, wt and average of three independent experiments). In cells lacking Xbp1 degradation was significantly delayed (Fig. 1A, Xbp1−/−, the half-life of the protein was 105 min as determined with experiments with longer chase times), but it was not blocked as one could have expected based on the phenotype described for the cells lacking IRE1α (23). The GERAD defect was partially corrected and degradation of BACE457Δa in the Xbp1−/− MEF was substantially accelerated with a reduction of the protein half-life to 60 min upon back-transfection with an Xbp1-expression vector (Fig. 1A) or with an EDEM-expression vector (Fig. 1B). Overexpression of Cnx did not accelerate nor delay BACE457Δa degradation in cells lacking Xbp1, showing that variations in the intraluminal level of Cnx were not affecting the capacity of these cells to operate GERAD (Fig. 1C).

Exposure of Xbp1-depleted cells to castanospermine, a glucose analogue that prevents the entry of glycoproteins in the Cnx cycle (29), restored efficient removal of terminally misfolded glycoproteins from the ER lumen (Fig. 1D). Bypass of the Cnx cycle has been shown to emancipate glycoprotein degradation from the intraluminal level of EDEM (16) because EDEM operates as an extraction factor for terminally misfolded glycoproteins from the Cnx cycle (15, 16). Taken together, these data therefore confirm that the GERAD phenotype of Xbp1−/− MEF is indeed mainly related to the suboptimal level of EDEM.

Thus, lack of EDEM up-regulation in cells depleted of Xbp1 delayed degradation of terminally misfolded glycoproteins. In contrast to what was shown previously for cells in which aber-
EDEM, ERAD, and Maintenance of Folding Efficiency

Fig. 2. Consequences of Xbp1 depletion on maturation of viral and cellular glycoproteins. A, SFV glycoproteins have been immunoprecipitated from detergent extracts of SFV-infected cells. E2 is generated by convertase-mediated cleavage of p62 in the Golgi compartment. Generation of E2 can therefore be used to determine kinetics of SFV glycoproteins release from the ER. B, detergent extracts have been immunoprecipitated with anti-Cnx. Densitometric analysis of the labeled E1/p62 shows that the glycoproteins are released from the lectin chaperone with the same kinetics in wt and in Xbp1−/−MEF. C, same as in B for Crt. D, BACE501 maturation 17 h after transfection of wt and Xbp1−/−MEF. Arrow G shows the complex glycosylated form generated when native BACE501 is released from the ER; arrow ER shows the immature form of BACE501. For this panel the entire gel is presented. (In Fig. 3, A, C, E, and G, only the relevant bands will be shown.) E, sensitivity of BACE501 to Endo H has been assessed to determine kinetics of release from the ER. Initially, BACE501 is fully sensitive to Endo H treatment (10-min and 1-h chase). Later on, the faster migrating ER form of the glycoprotein remains Endo H sensitive, and the Golgi form acquires Endo H-resistant N-glycans. Kinetics of BACE501 export from the ER is the same in wt and in Xbp1−/−MEF.

A single polypeptide chain is expressed in the ER lumen of cells infected with SFV. After a co-translational cleavage generating two glycoproteins (E1 and p62), E1 initiates the folding process in association with BiP and PDI and completes maturation after joining p62 in association with Cnx, Crt, and ERp57 (26). Release from the ER occurs after oligomerization in trimers of E1/p62 heterodimers. Kinetics of SFV glycoproteins maturation in wt and Xbp1−/−MEF was determined by measuring the association of E1 and p62 with the lectin chaperones Cnx and Crt and by monitoring the generation of E2, a cleavage product of p62 generated in the Golgi after completion of the folding phase.

Analysis of E1 and p62 confirmed that Xbp1 depletion did not affect significantly the maturation process because the oxidative phase of folding (Fig. 2A) and kinetics of release from Cnx and Crt (Fig. 2, B and C, respectively), and release from the ER as shown by the generation of E2 (Fig. 2A) occurred at similar rates in wt and Xbp1−/−MEF. The objection could be raised that viral glycoproteins have evolved to optimize folding efficiency under condition of ER overload with viral products, thus minimizing the use of cellular chaperones. Therefore we decided to convert wt and Xbp1−/−MEF in factories producing a cellular glycoprotein, the active variant of human β-secretase (BACE501). BACE501 processes the amyloid precursor protein originating the β-amyloid, an aggregation-prone peptide deposited in the brain of Alzheimer’s disease patients (30). The aim of our experiments was to verify the capacity of wt MEF and of MEF with defective ERAD as a consequence of Xbp1 depletion to sustain production of a secretory protein of interest. The recombinant glycoprotein was expressed in transfected cells and metabolically labeled as described above.

Native BACE501 is released from the ER and is subjected to complex glycosylation in the Golgi that decreases electrophoretic mobility (31). Both the molecular weight increase (Fig. 2D, arrows. ER for the immature, and G for the mature, complex glycosylated forms of BACE501) and the acquisition of Endo H resistance as a further estimate of the rate of BACE501 export from the ER (Fig. 2E) occurred with similar kinetics in wt and Xbp1−/−MEF when analyzed 17 h after transient transfection. Thus, normal maturation of the viral glycoproteins E1 and p62 (and influenza virus hemagglutinin, not shown) and of the cellular glycoprotein BACE501 showed that cells depleted of Xbp1 were fully competent to assist glycoprotein folding and secretion.

Loss of Protein Folding Efficiency and Secretory Capacity in Cells Depleted of Xbp1—To summarize, under normal conditions wt MEF and cells lacking Xbp1 have similar growth rates and viability. Upon viral infection or transient transfection, both cell lines proved at first sight fully competent in assisting efficient maturation and secretion from the ER of the increased amount of client protein entering the ER lumen. Aware that protein folding is error-prone and that increased production of secretory protein must correlate with higher generation of aberrant by-products of protein biosynthesis that need to be rapidly cleared from the ER lumen to avoid constipation, we wondered if cells depleted of Xbp1 and characterized by moderate defect in ERAD were able to maintain high output of secretory proteins.

To assess this, we tested and compared maintenance of folding efficiency and transport along the secretory line of...
BACE501 expressed in transiently transfected wt and Xbp1−/− MEF. Folding efficiency was assessed by determining the presence of terminally misfolded labeled BACE501 in the detergent-insoluble fraction upon CHAPS lysis of cultured cells. Transport along the secretory line was assessed by quantification of the Golgi versus ER form of BACE501 as determined 5 h after synthesis of the recombinant protein. These tests were performed in cells expressing the recombinant protein for 17 h after transfection (Fig. 3, A and B) or 72 h after transfection (Fig. 3, C–H). Briefly, cells were metabolically labeled 17 or 72 h after transfection with a BACE501 expression plasmid as described above, chased for 5 h, and detergent-solubilized. The postnuclear supernatant was supplemented with BACE-specific antibody and analyzed in reducing SDS-PAGE. The detergent-insoluble material was analyzed as described under "Materials and Methods" to determine whether it contained terminally misfolded BACE501.

Seventeen h after transfection, the labeled BACE501 analyzed 5 h after the pulse was mostly in the ER form of BACE501 as determined 5 h after synthesis of the recombinant protein. These tests were performed in cells expressing the recombinant protein for 17 h after transfection (Fig. 3, A and B) or 72 h after transfection (Fig. 3, C–H). Briefly, cells were metabolically labeled 17 or 72 h after transfection with a BACE501 expression plasmid as described above, chased for 5 h, and detergent-solubilized. The postnuclear supernatant was supplemented with BACE-specific antibody and analyzed in reducing SDS-PAGE. The detergent-insoluble material was analyzed as described under "Materials and Methods" to determine whether it contained terminally misfolded BACE501.

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cells in maintaining folding efficiency and secretory capacity by facilitating clearance of the ER lumen from by-products of BACE501 biosynthesis.

Xbp1\(^{-/-}\) MEF were co-transfected with a BACE501 and an EDEM expression vector. Their capacity to productively assist BACE501 was assessed 72 h later as described above; cells were therefore metabolically labeled and chased for 5 h to determine the fraction of labeled BACE501 transported along the secretory pathway by quantifying the ratio of the Golgi versus the ER form of BACE501. Comparison with mock-transfected Xbp1\(^{-/-}\) MEF showed that whereas in these cells the ratio of the Golgi versus the ER form of BACE501 5 h after synthesis was less than 1:1, cells with an elevated level of EDEM had maintained a ratio of about 4:1 (Fig. 3E, mock versus EDEM), thus very close to the optimal ratio of BACE501 maturation found in wt cells (Fig. 3, A and C) and in Xbp1-depleted cells analyzed 17 h after transfection (Fig. 3A). That folding efficiency and therefore export from the ER was better preserved during the 72 h in cells overexpressing EDEM was confirmed by the lower amount of labeled BACE501 entering in disulfide-bonded aggregates during the 5 h of chase in these cells (Fig. 3F, compare BA and BM in mock and in EDEM-transfected cells). Thus artificial increase of the ERAD activity in Xbp1-depleted cells was sufficient to maintain their capacity to efficiently fold and secrete BACE501 for 72 h.

Next we determined whether the same results could have been obtained by strengthening the activity of the ER folding machinery upon Cnx up-regulation. Transfection of Xbp1\(^{-/-}\) MEF with a Cnx expression vector, a treatment that had no beneficial effect for ERAD capacity of these cells (Fig. 1C), did also not affect the ratio of the Golgi versus the ER form (Fig. 3G) and did not help in preventing loss of folding efficiency because the same amount of labeled BACE501 entered in disulfide-bonded complexes in mock- and in Cnx-transfected Xbp1-depleted MEF (Fig. 3H). Thus, elevation of the intraluminal level of EDEM (but not of Cnx) granted preservation of folding efficiency and secretory capacity in cells lacking the transcription factor Xbp1.

Altogether, in MEF converted into factories producing high amount of a secretory glycoprotein, depletion of Xbp1 did not affect folding and transport of the recombinant protein (or of model viral glycoproteins) initially (Fig. 2 and Fig. 3, A and B). However, the fraction of protein fulfilling quality control standards needed for ER exit was strongly reduced in the long run (Fig. 3, C–H) because the activity of the ERAD machinery could not be adjusted to the increased amount of client protein, and the ER lumen thus failed to be cleared from the by-products of glycoprotein biosynthesis. This led to their intracellular accumulation and deposition in detergent-insoluble disulfide-bonded complexes. EDEM was found to play a crucial role in preventing these pathologic events, and its overexpression was sufficient to maintain folding efficiency and sustain production of high levels of recombinant secretory proteins in cells depleted of Xbp1 (Fig. 3, E and F).

**DISCUSSION**

Here, by taking advantage of a cell line with a defective capacity to adapt the ERAD machinery to the cellular need as a consequence of Xbp1-depletion, we investigated mechanistic and functional links between degradation machinery and protein production facility. We report that an Xbp1-regulated program responds to increase in ER cargo load leading to accumulation of by-products of protein synthesis. Activation of this program preserves efficiency of protein folding in the ER, sustains protein secretion, and prevents formation of intracellular deposits that could eventually compromise cell and/or organism viability. Elevation of the level of the α-mannosidase-like protein EDEM in cells depleted of Xbp1 was necessary to restore the highest efficiency of GERAD, to prevent intraluminal accumulation of misfolded BACE501, and to maintain efficiency of protein folding and secretion at levels comparable with those of wt cells.

Until recently, the unfolded protein response (UPR, Refs. 32–34) was mainly associated with (and studied under) pathological conditions. Cumulating evidences now show that ER stress transducers may also be selectively and/or sequentially activated during physiologic processes such as organogenesis or generation of professional secretors such as plasma cells (35). Particularly significant are the works showing a link between expression and activity of the IRE1-activated transcription factor Xbp1 and survival and/or differentiation of secretory cells and organs (25, 36–38). Together with the finding that depletion of IRE1 abolishes the capacity of cells to degrade misfolded glycoproteins accumulating in the ER lumen as a consequence of lack of Xbp1-induced adaptation of the intraluminal level of EDEM (23), these findings moved the spotlight back to the IRE1/Xbp1 UPR pathway, until then considered as “accessory” to the work of other players during mammalian UPR (39). The current models describing UPR and UPR-like events occurring in mammalian cells are still more focused on the involvement of these processes in preparation and adaptation of the folding/secrretion machinery to the increased demand of folding assistance upon rise of ER client proteins, as an example during B cell differentiation in antibody-producing plasma cells (40, 41). Seemingly, studies on the importance of adaptation of the ERAD machinery to maintain efficiency of protein folding and quality control in the mammalian ER and to sustain protein secretion have been neglected so far.

It is of great interest that when the activity of the ERAD machinery was artificially elevated by increasing the intraluminal level of ER α-mannosidase I, protein folding-intermediates were prematurely targeted for degradation before completion of chaperone-assisted maturation (17). This suggests that folding and ERAD machinery are to some extent competing for newly synthesized polypeptides. If hyperactivity of the ERAD machinery causes premature termination of chaperone-assisted folding attempts, an insufficient ERAD capacity leads to luminal accumulation of by-products of protein biosynthesis normally generated in the ER lumen because of the inefficiency of the protein folding process.

We assume that the mechanisms described in this work are of crucial importance for cells with potent secretory activity because the amount of by-products of protein biosynthesis that needs to be efficiently cleared from the ER lumen is likely to be proportional to the ER load in client proteins. It is of interest that Xbp1, and we believe that the same will be found true for EDEM, is expressed at high level in all secretory organs and cells and that Xbp1 depletion specifically affects generation and survival of secretory cells like hepatocytes and antibody-secreting plasma cells (25, 36–38).

For plasma cells, Xbp1 has been shown to intervene in terminal steps of B cell differentiation and to peak in activity well after ER expansion only when Ig synthesis has been boosted (35, 37, 38, 40). We found that the Xbp1 activation occurring late during the process of plasma cells maturation correlated with up-regulation of EDEM, thus with the reinforcement of the degradation machinery of the plasma cells. Two hints at a cellular response to the increased demand for efficient disposal of by-products of Ig production at a stage in which the secretory...

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K. K. Eriksson and M. Molinari, unpublished data.
factory of plasma cells is busy producing several thousands of Ig molecules per second (42).

The finding that increasing the ERAD capacity by elevating the intracellular level of EDEM not only enhanced protein degradation from the ER but also prevented accumulation of misfolded and insoluble protein aggregates is important because of the many human diseases caused by protein deposition (43). The adaptation mechanisms described in this paper are proposed to be relevant for survival and unperturbed activity of professional secretors, of cells devoted to expression of particularly problematic proteins (e.g. the cystic fibrosis channel that folds at very low efficiency normally (44)), and even more crucial for cells expressing mutated gene products with defective folding and for cell converted into factories producing recombinant secretory proteins of medical or industrial relevance.

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