The Unfolded Protein Response Transducer ATF6 Represents a Novel Transmembrane-type Endoplasmic Reticulum-associated Degradation Substrate Requiring Both Mannose Trimming and SEL1L Protein*

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Background: SEL1L, a partner protein of the E3 HRD1, is not required for degradation of known transmembrane-type endoplasmic reticulum-associated degradation (ERAD-Lm) substrates.

Results: Degradation of ATF6, a type II transmembrane glycoprotein, is blocked in SEL1L knock-out cells.

Conclusion: ATF6 represents a novel ERAD-Lm substrate requiring SEL1L for degradation.

Significance: ERAD-Lm substrates are degraded through much more diversified mechanisms in higher eukaryotes than previously thought.

Proteins misfolded in the endoplasmic reticulum (ER) are cleared by the ubiquitin-dependent proteasome system in the cytosol, a series of events collectively termed ER-associated degradation (ERAD). It was previously shown that SEL1L, a partner protein of the E3 ubiquitin ligase HRD1, is not required for degradation of misfolded luminal proteins (ERAD-Ls substrates) but not misfolded transmembrane proteins (ERAD-Lm substrates) in both mammalian and chicken DT40 cells. Here, we analyzed ATF6, a type II transmembrane glycoprotein that serves as a sensor/transducer of the unfolded protein response, as a potential ERAD-Lm substrate in DT40 cells. Unexpectedly, degradation of endogenous ATF6 and exogenously expressed chicken and human ATF6 by the proteasome required SEL1L. Deletion analysis revealed that the luminal region of ATF6 is a determinant for SEL1L-dependent degradation. Chimeric analysis showed that the luminal region of ATF6 confers SEL1L dependence on type I transmembrane protein as well. In contrast, degradation of other known type I ERAD-Lm substrates (BACE1,57, T-cell receptor-α, CD3-δ, and CD147) did not require SEL1L. Thus, ATF6 represents a novel type of ERAD-Lm substrate requiring SEL1L for degradation despite its transmembrane nature. In addition, endogenous ATF6 was markedly stabilized in wild-type cells treated with kifunensine, an inhibitor of α1,2-mannosidase in the ER, indicating that degradation of ATF6 requires proper mannose trimming. Our further analyses revealed that the five ERAD-Lm substrates examined are classified into three subgroups based on their dependence on mannose trimming and SEL1L. Thus, ERAD-Lm substrates are degraded through much more diversified mechanisms in higher eukaryotes than previously thought.

Secretory and transmembrane proteins are able to leave the endoplasmic reticulum (ER),4 the first organelle they encounter after synthesis on membrane-bound ribosomes, for their destination only when they gain correct tertiary and quaternary structures. If they remain unfolded or misfolded even after assistance from molecular chaperones and folding enzymes abundantly expressed in the ER, they are recognized and delivered to the ER membrane, retrotranslocated to the cytosol, and then ubiquitinated and degraded by the proteasome, a series of events collectively termed ER-associated degradation (ERAD) (1, 2).

Three degradation pathways are differentially utilized, depending on the location of the particular substrate lesion, namely ERAD-L (lesion in the ER lumen), ERAD-M (lesion inside the ER membrane), and ERAD-C (lesion on the cytosolic side of a transmembrane protein). The ERAD-L pathway is further classified into the ERAD-Ls (for degradation of soluble luminal proteins) and ERAD-Lm (degradation of transmembrane proteins) pathways. In yeast, the E3 ubiquitin ligase

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4 The abbreviations used are: ER, endoplasmic reticulum; ERAD, ER-associated degradation; NHK, null Hong Kong variant of α1-proteinase inhibitor; UPR, unfolded protein response; hATF6, human ATF6; TAP, tandem affinity purification; Endo H, endoglycosidase H; TCR-α, T-cell receptor-α.
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Hrd1p, coupled with its binding partner Hrd3p, mediates ERAD-L and ERAD-M, whereas the E3 ubiquitin ligase Doa10p mediates ERAD-C (3). Interestingly, mammals express SEL1L as a sole homolog of Hrd3p but Hrd1 and gp78 as homologs of Hrd1p (4). In mammals, degradation of ERAD-Ls substrates, namely the null Hong Kong (NHK) variant of α,γ-proteinase inhibitor, BACE476Δ, and CD3-ΔΔ, is blocked by knocking down Hrd1, whereas degradation of the ERAD-Lm substrate BACE476 is blocked only when both Hrd1 and gp78 are knocked down, and degradation of another ERAD-Lm substrate (CD3-Δ) is blocked by knocking down gp78. Accordingly, knockdown of SEL1L, which is a partner protein of Hrd1 but not gp78, is sufficient to block degradation of ERAD-Ls substrates but not ERAD-Lm substrates (5).

We have recently utilized chicken DT40 cells in the analysis of ERAD mechanisms in higher eukaryotes because the chicken genome encodes the same set of genes as homologs of yeast ERAD components as encoded by mammalian genomes and because the exceptionally high efficiency of these cells in homologous recombination and their stable phenotype and availability of six selection markers provide a unique opportunity to create single, double, or triple knock-out at the cellular level with relative ease. We knocked out the SEL1L gene in DT40 cells and showed that SEL1L is required for degradation of ERAD-Ls substrates (NHK and its non-glycosylated version NHK-QQO) but not for ERAD-Lm substrates (NHK-BACE and CD3-Δ) (6), as reported in mammals (5).

Here, during characterization of SEL1L knock-out DT40 cells, we discovered a novel type of endogenous ERAD-Lm substrate, ATF6, whose degradation requires SEL1L. ATF6 is a type II transmembrane protein in the ER that serves as a sensor/transducer of the unfolded protein response (UPR), together with two other UPR transducers, IRE1 and PERK (protein kinase RNA-like endoplasmic reticulum kinase), which are type I transmembrane proteins in the ER (7).

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—DT40 cells were cultured at a density of $1 \times 10^5$ to $1 \times 10^6$ cells/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% chicken serum, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 39.5°C in a humidified 5% CO₂ and 95% air atmosphere. SEL1L knock-out cells were established previously (6). DT40 cells were transfected by electroporation using a MicroPorator (Digital Bio) with two pulses at 1500 V for 15 ms according to the manufacturer’s instructions. HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (4.5 g/liter glucose) supplemented with 10% fetal bovine serum, 2 mM glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C in a humidified 5% CO₂ and 95% air atmosphere. Transfection was performed using X-tremeGENE 9 DNA transfection reagent (Roche Applied Science) according to the manufacturers’ instructions.

Construction of Plasmids—Recombinant DNA techniques were performed according to standard procedures (8), and the integrity of all constructed plasmids was confirmed by extensive sequencing analyses. Site-directed mutagenesis was carried out using DpnI. Myc-hATF6α and Myc-hATF6α(402) were derived from pCGN-HA-ATF6(670) and pCGN-HA-ATF6(402) (9), respectively. hATF6α(C)-TAP is identical to ATF6α(C)WT-TAP (10).

RT-PCR—Total RNA prepared from wild-type and SEL1L knock-out cells (~5–10⁶ cells) by the acid guanidinium/phenol/chloroform method using ISOGEN (Nippon Gene) was converted to cDNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Unspliced and spliced XBP1 cDNA was amplified using Ex Taq DNA polymerase (Takara) and primer pair 5’-GGCGGAGTCTACGGATGTTGA-3’ and 5’-AAGGCGAACAGGATCATCA-3’.

Immunological Techniques—Immunoblot analysis was carried out according to the standard procedure (8). Approximately 2 x 10⁶ cells were collected by centrifugation at 3000 rpm for 2 min, washed with PBS, suspended in Laemmli sample buffer, and boiled for 5 min. Samples were subjected to SDS-PAGE. Chemiluminescence obtained using Western blotting Luminol reagent (Santa Cruz Biotechnology) was detected using a LAS-3000 mini Luminograph analyzer (Fuji Film). Rabbit anti-chicken ATF6 antibody was raised against the N-terminal region (amino acids 22–293). Rabbit anti-chicken XBP1 antibody was raised against the N-terminal region (amino acids 1–61). Anti-c-Myc antibody (9E10) was obtained from Wako, anti-β-actin antibody from Chemicon International, anti-KDEL antibody from Medical and Biological Laboratories, anti-FLAG antibody from Sigma; anti-HA antibody from Recentec, and anti-ATF4 antibody from Santa Cruz Biotechnology.

Immunofluorescence analysis was carried out as described previously (10). Anti-GM130 monoclonal antibody was obtained from BD Bioscience. Metabolic labeling and immunoprecipitation was carried out as described previously (6, 11).

Analysis of N-Glycans of Total Cellular Glycoproteins—Pyridylation and structural identification of N-glycans of total cellular glycoproteins were performed as described previously (12). The pyridylated glycans were fractionated using a TSKgel Amide-80 column (Tosoh Bioscience). Each fraction was further applied to a Shim-pack HRC-ODS column (Shimadzu) to isolate each isomer. The elution times of the individual peaks on the amide-silica and ODS columns were normalized with respect to the degree of polymerization of pyridylated isomalto-oligosaccharide and are represented in units of glucose. N-Glycan structures were identified based on their elution positions on the two HPLC columns (see above) compared with the elution positions of pyridylaminated glycans described in the literature (13).

RESULTS

The UPR Is Constitutively Activated in SEL1L Knock-out Cells—We examined whether deletion of SEL1L affects the homeostasis of the ER by checking the splicing status of XBP1 mRNA, an event immediately downstream of activation of the UPR transducer IRE1, which is a sensitive marker for the occurrence of ER stress (14). As shown in Fig. 1A, the addition of tunicamycin, which potently evokes ER stress by inhibiting protein N-glycosylation (15), markedly induced splicing of XBP1 mRNA (compare lane 4 with lane 2); 26 nucleotides were removed from pre-mRNA in wild-type DT40 cells, as in mam-
We found that the level of \( \text{XBP1} \) mRNA splicing was enhanced in \( \text{SEL1L} \) knock-out cells compared with wild-type cells, albeit only slightly (compare lane 3 with lane 2). Immunoblotting revealed that induction of p\( \text{XBP1} \) (S), the product of spliced \( \text{XBP1} \) mRNA, in \( \text{SEL1L} \) knock-out cells was slight (~2-fold) compared with induction by tunicamycin treatment of wild-type cells (Fig. 1B). Immunoblotting also revealed slight induction of ATF4, an event immediately downstream of activation of the UPR transducer PERK, in \( \text{SEL1L} \) knock-out cells (~1.5-fold) compared with marked induction of ATF4 in wild-type cells.
in wild-type cells treated with tunicamycin (Fig. 1C). It should be noted that induction of ATF4 is rapid, as ATF4 is translationally induced after PERK activation. In contrast, because pXBPI(S) must be translated from spliced XBPI mRNA, induction of pXBPI(S) initiates only after recovery from PERK-mediated translational attenuation. Thus, ATF4 was much more
robustly induced than pXBPI(S) at 1.5 h after the addition of tunicamycin to wild-type cells.

These results indicated that SEL1L knock-out cells experienced elevated ER stress compared with wild-type cells, most likely due to blockage of ERAD-Ls. On the other hand, the levels of the two ER chaperones BiP (GRP78) and GRP94, major transcriptional targets of the UPR, were much higher in SEL1L knock-out cells (~4–5-fold) than in wild-type cells, and their levels were nearly equal to those in wild-type cells treated with tunicamycin for 8 h (Fig. 1D). This constitutively enhanced expression of BiP explains the suppressed activation of IRE1 and PERK in SEL1L knock-out cells compared with wild-type cells treated with tunicamycin for 1.5 h, as BiP is a negative regulator of IRE1 and PERK (16). Despite enhanced expression of BiP and GRP94, SEL1L knock-out cells were found to be much more sensitive to tunicamycin compared with wild-type cells (Fig. 1E), indicating the importance of ERAD-Ls in maintaining the homeostasis of the ER.

We then examined the activation status of the UPR transducer ATF6, which is responsible for transcriptional induction...
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of various ER chaperones in mammals (17, 18) and DT40 cells. ATF6 is constitutively synthesized as a type II transmembrane glycoprotein in the ER (the precursor form designated pATF6(P)) and is activated by regulated intramembrane proteolysis in response to ER stress to produce the active and nuclear form designated pATF6(N) (Fig. 1F). Although most vertebrate genomes encode the ATF6α and ATF6β isoforms, the chicken genome appears to encode a single ATF6 gene. Immunoblotting analysis of endogenous ATF6 revealed that pATF6(P) was cleaved to produce pATF6(N) when wild-type DT40 cells were treated for 30 min with 1 mM dithiothreitol, which misfolds proteins in the ER by reducing disulfide bonds (Fig. 2A). Immunoblotting also revealed a low level of pATF6(N) in SEL1L knock-out cells (Fig. 2B, lane 2), and this level was lower than that in wild-type cells treated with tunicamycin for 1.5 h (lane 3). Thus, activation of ATF6 in SEL1L knock-out cells was suppressed by an increased level of BiP, similar to IRE1 and PERK, as BiP is also a negative regulator of ATF6 (19). Nonetheless, the level of pATF6(N) in SEL1L knock-out cells was comparable with that in wild-type cells treated with tunicamycin for 8 h (compare lane 2 with lane 4). Constitutive mild activation of ATF6 was considered to lead to markedly increased levels of BiP and GRP94 in SEL1L knock-out cells (Fig. 1D). It should be noted that the unglycosylated precursor form of ATF6 (designated pATF6(P*)) was produced in wild-type cells treated with tunicamycin (Fig. 2B, lanes 3 and 4) but not with dithiothreitol (Fig. 2A, lane 2).

ATF6 Represents a Novel Type of Endogenous ERAD-Lm Substrate—We also noticed that the level of pATF6(P) in SEL1L knock-out cells was much higher than that in wild-type cells (Fig. 2B, compare lane 2 with lane 1). We therefore compared the stability of pATF6(P) in wild-type and SEL1L knock-out cells. Cycloheximide chase experiments revealed that pATF6(P) was degraded with a half-life of ~2 h in wild-type cells, whereas pATF6(P) was markedly stabilized in SEL1L knock-out cells (Fig. 2C). Degradation of pATF6(P) in wild-type cells was blocked by treatment with the proteasome inhibitor MG132 (Fig. 2D). Both cycloheximide chase (Fig. 2E) and pulse-chase (Fig. 2F) experiments revealed that exogenously expressed chicken ATF6 tagged with c-Myc was also degraded in an SEL1L-dependent manner. These results indicate that SEL1L is required for ERAD of pATF6(P).

To determine which region of pATF6(P) is responsible for SEL1L-dependent degradation, we transfected wild-type and SEL1L knock-out cells with Myc-tagged wild-type hATF6α and its truncated forms. It should be noted that the defect in ERAD-Ls in SEL1L knock-out DT40 cells was rescued by transfection of not only the chicken but also the human SEL1L gene (6). Cycloheximide chase experiments showed that Myc-tagged full-length hATF6α was degraded in wild-type cells and that its degradation was blocked in SEL1L knock-out cells (Fig. 3B), as in the case of chicken endogenous pATF6(P). hATF6α(C)-TAP, consisting of the luminal region of hATF6α (amino acids 405–670), which is preceded by the signal sequence of the immunoglobulin κ chain for translocation into the ER and which is followed by three tandem copies of the c-Myc epitope, the tobacco etch virus protease recognition site, and two copies of the IgG-binding domain, was constructed previously (10). Similarly, hATF6α(C)-TAP was degraded in wild-type DT40 cells more slowly than Myc-hATF6α (Fig. 3, compare D with B), and this degradation required SEL1L (Fig. 3D). Endoglycosidase H (Endo H) digestion showed that both Myc-hATF6α and hATF6α(C)-TAP were glycosylated in wild-type and SEL1L knock-out cells as expected (Fig. 3, C and E, respectively). Myc-hATF6α(402) is a modified version of the previous construct HA-hATF6α(402), which consists of the N-terminal cytosolic region and the transmembrane domain and which is localized in the ER (9). Myc-hATF6α(402) was degraded in wild-type DT40 cells more slowly than Myc-hATF6α (Fig. 3, compare D with B); however, this degradation did not depend on SEL1L (Fig. 3F). Thus, the luminal region of hATF6α is a determinant for SEL1L-dependent degradation. In other words, the luminal region of hATF6α confers SEL1L dependence on the SEL1L-independent substrate Myc-hATF6α(402) when fused to make Myc-hATF6α, whereas the N-terminal cytosolic and transmembrane regions cannot confer SEL1L independence to the SEL1L-dependent substrate hATF6α(C).

We previously showed that degradation of NHK, a soluble luminal misfolded glycoprotein, depends on SEL1L in DT40 cells, whereas NHK-BACE, in which NHK is fused to the N terminus of the transmembrane domain of the type I trans-

5 T. Okada and K. Mori, unpublished data.
membrane protein BACE501, does not (6), as reported in mammalian cells (5). We thus fused the luminal region of hATF6 to the N terminus of the transmembrane domain of BACE501 (BACE(TM)) (Fig. 4A). This chimera was functional with regard to ER stress sensing and subsequent transport to the Golgi apparatus. Thus, when expressed in HeLa cells by transfection, hATF6(C)-BACE(TM)-TAP was localized in the ER under normal conditions and relocated to the Golgi apparatus when HeLa cells were treated with 1 mM dithiothreitol for 1 h (Fig. 4B). On degradation, hATF6(C)-BACE(TM)-TAP was found to become SEL1L-dependent (Fig. 5B), indicating that the luminal region of hATF6 confers SEL1L dependence on degradation to both type I and type II transmembrane proteins. We further investigated SEL1L dependence on degradation of other known type I ERAD-Lm substrates, namely human BACE457, a splicing variant of BACE501 similar to BACE476 (5), mouse T-cell receptor-α (TCR-α) (20), and the recently identified human CD147 (21). Cycloheximide chase experiments showed that HA-tagged human BACE457, FLAG-tagged mouse TCR-α, and Myc-tagged human CD147 were degraded similarly in both wild-type and SEL1L knock-out cells (Fig. 5, C–E). Thus, ATF6 represents a novel type of endogenous ERAD-Lm substrate whose degradation requires SEL1L despite its transmembrane nature.

**ERAD-Lm Substrates Are Categorized into Three Subgroups**—As high mannose-type oligosaccharide structures are important for ERAD of glycoproteins (1), we determined the N-glycosylation profiles of total cellular glycoproteins prepared from

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**FIGURE 5.** Effect of deleting SEL1L on degradation of various type I ERAD-Lm substrates. A, schematic structures of human (h) BACE457-HA, mouse (m) TCR-α-FLAG, and human CD147-Myc are shown. s.s, signal sequence; Ψ, potential N-glycosylation site; TMD, transmembrane domain. B–E, wild-type and SEL1L knock-out cells were transfected with plasmid to express one of the four proteins as indicated and analyzed as described for Fig. 2E.
wild-type and SEL1L knock-out cells. We focused on high mannosetype oligosaccharides with the elution positions indicated in Fig. 6A. To facilitate comparison, the amount of each high mannose-type oligosaccharide relative to the total amount of high mannose-type oligosaccharides was calculated and is shown in Fig. 6B. In wild-type cells, the major population was the M8B form, followed by the M9 form, which is consistent with the results obtained with HepG2 cells (12, 22). We found that the level of the M9 form was decreased and that the levels of the M7A, M6H1, M6, and M5 forms were increased in SEL1L knock-out cells compared with wild-type cells (Fig. 6B). These forms expose 1,6-linked mannose residues, which are bound by the terminal recognition lectins OS-9 and/or XTP3-B for delivery of ERAD substrates to the SEL1L-HRD1 complex in mammals (1). The accumulation of glycoproteins with exposed 1,6-linked mannose residues in SEL1L knock-out cells (25% of total) compared with wild-type cells (17% of total) strongly suggests that a similar ERAD mechanism, namely mannose trimming-dependent degradation of glycoproteins, operates in DT40 cells also.

We thus examined the effect of treatment of wild-type cells with kifunensine, which inhibits ER 1,2-mannosidase (23), on degradation of various ERAD-Lm substrates. We found that although kifunensine treatment did not significantly affect the apparent molecular weight of chicken ATF6 (Fig. 6C), chicken ATF6 was markedly stabilized in kifunensine-treated cells (Fig. 6D), indicating that degradation of chicken ATF6 requires proper mannose trimming. In contrast, cycloheximide chase experiments revealed that kifunensine treatment did not affect the degradation of human BACE457 and mouse TCR-β but delayed that of mouse CD3-δ and human CD147 (Fig. 7, A–E), consistent with the results reported for human CD147 (21). Endo H treatment showed that all were glycosylated as expected (Fig. 7, F–I). These results indicate that ERAD-Lm substrates are classified into three subgroups based on their dependence on mannose trimming and SEL1L (Table 1).

**DISCUSSION**

The molecular mechanisms of ERAD have been elucidated mostly by analyzing transfected cells overexpressing various
transmembrane proteins that alone are unable to assemble into a mature complex, such as CD3-δ and TCR-α, or that are considered to be misfolded by alternative splicing, such as BACE457 and BACE476, as well as various luminal proteins that are truncated and thus misfolded, such as truncated ribophorin (RI332), NHK, CD3-δΔ, and BACE476Δ. Only recently have two endogenous ERAD-Lm substrates been successfully identified. One is the C-terminal fragment derived from the Hedgehog precursor molecule (24). The Hedgehog precursor molecule is processed in the ER by self-cleavage into the N-terminal Hedgehog ligand and the C-terminal fragment. The C-terminal fragment is a luminal glycoprotein constitutively degraded via the SEL1L-HRD1 pathway, indicating that it is the first endogenous ERAD-Lm substrate identified to date. The other is CD147, a type I transmembrane glycoprotein in the ER that functions as an obligatory assembly factor for monocarboxylate transporters (21). CD147 is constitutively degraded in an HRD1-dependent manner and accumulates in a complex with OS-9 in SEL1L-depleted cells, and its degradation is inhibited by kifunensine and by knocking down OS-9 or XTP3-B, indicating that its lesion is located in the ER lumen. Thus, CD147 is the first endogenous ERAD-Lm substrate identified to date. However, it was also reported that the effect of knock-

TABLE 1

| Classification of ERAD-Lm substrates | Requirement | Mannose trimming | SEL1L | Substrate |
|-------------------------------------|-------------|-----------------|-------|-----------|
| Class I                             | No          | No or little    | BACE457, TCR-α |
| Class II                            | Yes         | No or little    | CD3-δ, CD147 |
| Class III                           | Yes         | Yes             | ATP6α  |

FIGURE 7. Effect of inhibition of mannose trimming on degradation of various type I ERAD-Lm substrates. Wild-type cells transfected with plasmid to express the indicated proteins were untreated (−) or treated (+) with 5 μg/ml kifunensine (kif). One hour later, cycloheximide (CHX) chase experiments were carried out without removing kifunensine. Cell lysates were analyzed as described for Fig. 3B (A–D) and Fig. 3C (F–I). E, the amount of each protein after a 3-h chase in cells untreated (−) or treated (+) with kifunensine is presented as percentage remaining (n = 3). h, human; m, mouse.
ing down SEL1L on degradation of CD147 was much weaker than that of knocking down HRD1, which is consistent with our results obtained in DT40 cells (Fig. 5E). These results indicate that CD147 is categorized into classical ERAD-Lm substrates, which are degraded independently of SEL1L. In addition, exogenously expressed, epitope-tagged versions of CD147 reportedly do not behave like endogenous CD147 (21), which unfortunately may hamper further detailed analysis of the ERAD-Lm mechanisms using CD147 as a model substrate.

Here, we identified the second endogenous ERAD-Lm substrate, namely ATF6, but the first ERAD-Lm substrate whose degradation requires SEL1L. Although it was shown previously that ATF6α was degraded by the proteasome via HRD1 in mammalian cells, its SEL1L dependence was not examined (25). Importantly, the exogenously expressed, epitope-tagged versions of chicken and human ATF6α behaved like endogenous chicken ATF6 in DT40 cells. Using this transfection system, we were able to show that the luminal region of ATF6α conferred SEL1L dependence on both type I and type II transmembrane proteins. Degradation of hATF6α(C)-TAP (Fig. 3D) and hATF6α(C)-BACE(TM)-TAP (Fig. 5B) appeared to be biphasic, which was not seen for degradation of endogenous chicken ATF6 (Fig. 2C), suggesting that an SEL1L-independent mechanism might occur at later time points in the degradation of artificial chimeric proteins. It should be noted that the luminal region of ATF6α is sufficient for sensing ER stress and subsequent transport to the Golgi apparatus (10), and we found that hATF6α(C)-BACE(TM)-TAP also relocates from the ER to the Golgi apparatus in response to ER stress (Fig. 4B). Thus, the luminal region of ATF6α is functional regardless of its topology, i.e. type I or type II transmembrane orientation, as far as ER stress-induced translocation from the ER to the Golgi apparatus is concerned.

In contrast, NHK does not confer SEL1L dependence on type I transmembrane protein, such as the transmembrane domain of the type I transmembrane protein BACE501 (6). Thus, the recognition mechanism of endogenous ERAD substrates might differ from that obtained by analysis of exogenously overexpressed misfolded proteins. Extensive comparison of the luminal regions of ATF6α and NHK will provide us new insights into the molecular mechanisms of ERAD-Ls and ERAD-Lm, particularly regarding how ERAD substrates are recognized in the lumen of the ER and delivered to the SEL1L-HRD1 complex for subsequent retrotranslocation to the cytosol.

We have also shown here that degradation of ATF6 requires proper mannose trimming. Our further analysis revealed that ERAD-Lm substrates are classified into three subgroups (Table 1): class I (BACE457 and TCRα), requiring neither mannose trimming nor SEL1L; class II (CD3-δ and CD147), requiring mannose trimming but not SEL1L; and class III (ATF6), requiring both mannose trimming and SEL1L. This opens up completely new questions as to how they are recognized and degraded differentially. It is now clear that ERAD-Lm substrates are degraded through much more diversified mechanisms in higher eukaryotes than previously thought.

To our knowledge, as subclassification of ERAD-Lm substrates has not been reported in yeast ERAD to date, complexity in the ERAD-Lm mechanism may exist only in higher eukaryotes and may be correlated with the redundant existence of various ERAD components, such as HRD1 and gp78, in higher eukaryotes compared with those in yeast. Investigation of the underlying molecular mechanisms using ATF6 as a key tool and exploration of advantages of having such complexity will enhance our understanding of the biological roles of ERAD in higher eukaryotes. Certainly, it remains to be determined whether an increased level of BiP in SEL1L knock-out cells (Fig. 1D) plays any role in the stabilization of ATF6. We will also identify a region in ATF6 that is required for SEL1L-dependent degradation. ATF6 might become a stable protein if such a region is removed. The biological significance of SEL1L-dependent constitutive degradation of ATF6 will be unraveled by introducing such “stable” ATF6 into ATF6α/β double knockout medaka fish, which we established recently (26), and by characterizing its phenotype.

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