Characterization of Early EDEM1 Protein Maturation Events and Their Functional Implications

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The endoplasmic reticulum (ER) quality control factor EDEM1 associates with a number of ER proteins and ER-associated degradation (ERAD) substrates; however, an understanding of its role in ERAD is unclear. The early maturation events for EDEM1 including signal sequence cleavage and glycosylation were analyzed, and their relationship to the function of EDEM1 was determined. EDEM1 has five N-linked glycosylation sites with the most C-terminal site recognized poorly cotranslationally, resulting in the accumulation of EDEM1 containing four or five glycans. The fifth site was modified post-translationally when bypassed cotranslationally. Signal sequence cleavage of EDEM1 was found to be a slow and inefficient process. Signal sequence cleavage produced a soluble form of EDEM1 that efficiently associated with the oxidoreductase ERdj5 and most effectively accelerated the turnover of a soluble ERAD substrate. In contrast, a type-II membrane form of EDEM1 was generated when the signal sequence was uncleaved, creating an N-terminal transmembrane segment. The membrane form of EDEM1 efficiently associated with the ER membrane protein SEL1L and accelerated the turnover of a membrane-associated ERAD substrate. Together, these results demonstrated that signal sequence cleavage functionally regulated the association of EDEM1-soluble and membrane-integrated isoforms with distinct ERAD machinery and substrates.

The signal hypothesis states that proteins are targeted to the eukaryotic secretory pathway by hydrophobic signal sequences that are frequently positioned at the N terminus of the protein (1). These proteins are cotranslationally targeted and translocated across the endoplasmic reticulum (ER)2 membrane where the vast majority of the maturation process occurs. Maturation events include signal sequence cleavage, protein folding, covalent modification, and assembly (2). The ER contains a number of factors that aid in these steps for secretory and membrane proteins. As protein maturation is an error-prone process, the ER also possesses a quality control process that monitors the successfulness of these steps (3, 4). Quality control factors dedicated to the evaluation and sorting of maturing proteins target defective proteins for destruction by the cytoplasmic proteasome after their dislocation through the ER membrane by a process termed ER-associated degradation (ERAD). Although many of the players involved in ER quality control have been uncovered over the past decade, there is still much to be learned about the mechanism of the quality control process.

Signal sequences that target proteins to the ER generally consist of the first 20–30 amino acids of a protein organized into three domains (5). Signal sequences start with a basic N-terminal domain (N-domain) followed by an extended hydrophobic domain (H-domain; 7–13 amino acids in length) and a polar domain (C-domain) that contains low molecular weight amino acids near the cleavage site. Recent evidence indicates that signal sequences do not solely provide transient targeting information. These sequences can affect translocation efficiencies to create the dual localization of cytoplasmic and organelle proteins. This is the case for the ER molecular chaperone calreticulin (CRT), the prion protein, and precore protein from the hepatitis B virus (6–8). In addition, the timing of cleavage may also affect later steps in biogenesis such as glycosylation or maturation efficiency (9). A key issue is whether slowed or inefficient cleavage of signal sequences can produce biologically relevant proteins with dual topologies. Signal sequence cleavage could create a soluble protein, whereas if the hydrophobic signal sequence remains intact or uncleaved, it could support membrane anchoring to generate a type II membrane protein or a polytopic protein with an additional transmembrane region.

The majority of proteins that traverse the secretory pathway receive multiple N-linked carbohydrates composed of terminal glucose and mannose residues organized into three branches. N-Linked carbohydrates are generally transferred cotranslationally by the oligosaccharyltransferase; however, recently a second isoform of the catalytic subunit STT3, termed STT3B, was discovered that supports post-translational modification (10). N-Linked glycans act as maturation and quality control signals that recruit factors to aid in various biogenesis events (11, 12). The ER carbohydrate binding molecular chaperones calnexin (CNX, type I membrane protein) and CRT (soluble protein) bind monoglycosylated glycans on the maturing pro-
tein to aid the folding process (13, 14). These homologous proteins possess similar functions but have different substrate specificities dictated by their contrasting topologies (15–17). The mannose content of the side chains provides quality control information as mannose trimming targets aberrant structures for ERAD (3, 18, 19). This process requires mannosidases to trim the glycan to a glycoform that is recognized by mannose binding lectins that then target the protein to an ER membrane ERAD complex for dislocation to the cytoplasm. In Saccharomyces cerevisiae, Htm1p and Yos9p appear to act as the mannosidase and the mannose binding lectin, respectively (20, 21). Mammalian cells have homologues for these proteins; however, these roles appear to be diversified as they are performed by a larger number of proteins, likely due to the expanded cargo population and larger secretory load associated with multicellular organisms.

EDEM1 (ER degradation-enhancing α-mannosidase-like protein 1) is the mammalian homologue of Htm1p (22). Over-expression of EDEM1 accelerates the extraction of proteins from the CNX binding cycle and their subsequent degradation by the proteasome (23, 24). In contrast, knockdown of EDEM1 stabilizes glycosylated ERAD substrates (24, 25). In addition to ERAD substrates, EDEM1 has been reported to associate with a number of quality control factors including CNX, SEL1L, ERdj5, ER mannosidase I, and Derlin-2/3 (27, 30). Point mutations at the hypoglycosylation and generation of EDEM1 possessing class I heavy chain H2-Kb signal peptide (kbss; MNSMVPCT-LLLLAAALAPTQ) (31). EDEM1 signal sequence was also replaced with that of the signal anchor sequence from influenza virus neuraminidase (NA) to produce a type II membrane form of EDEM1 (MNPNKILCTSATALVIGTIAVLIGITNLGLNLH11032; R14L, 5’-GTACAGTTGCTGCCAGGCGGAGGTTGATGACCTTTAACT-3’; R14L, 5’-GTACAGTTGCTGCCAGGCGGAGGTTGATGACCTTTAACT-3’). To create signal sequence-cleavable EDEM1-FLAG, the N-terminal 31 amino acids of EDEM1 were replaced with the signal sequence from the mouse class I heavy chain H2-Kb signal peptide (kbss; MNSMVPCT-LLLLAAALAPTQ) (31). EDEM1 signal sequence was also introduced before the KDEL motif at its C terminus. T cell receptor α (TCRα) expression plasmid fused with a HA tag at its C terminus was a kind gift from Dr. S. Fang (University of Maryland). Dr. M. Ziak and J. Roth (Zurich) generously provided the human α1-antitrypsin expression vectors.

Transfections, Metabolic Radiolabeling, Immunoprecipitation, and Immunoblotting—For transfections, plasmid DNA and LipofectAMINE2000 reagent (Invitrogen) were mixed in 1 μg per 2-μl ratio. For protein expression, EDEM1 and empty vector (pcDNA 3.1; Invitrogen) were used in a 1:3 ratio (0.25:0.75 μg with a total of 1 μg). When co-transfections were performed, the null Hong Kong mutant variant of α1-antitrypsin (NHK) or TCRα was used in equal amounts to EDEM1, whereas ERdj5-myc was used in 1:3 ratio (EDEM1-FLAG: ERdj5-myc with 0.25:0.75 μg). Near confluent cells grown on a 35-mm dish (3.0 × 10⁵ cells) were pulse-labeled for 15 min or 1 h as previously described without prior amino acids starvation (27, 32). After washing twice with PBS, the cells were chased for the indicated times. Radiolabeled cells were washed with PBS containing 10 mM NEM on ice for 10 min followed by lysis with 500 μl of MNT buffer (20 mM MES, 150 mM NaCl, 0.5% (w/v) Triton X-100, 50 mM Tris-HCl, 0.5 mM PMSF, pH 7.5). Lysates were precleared with 10% Zysorbin for 1 h at 4 °C then incubated with antibody and protein-A-Sepharose overnight at 4 °C. The immunopellets were washed (0.1% SDS, 0.01% Triton X-100, 10 mM Tris-HCl, 150 mM NaCl, pH 8.5, 900 μl), then SDS-PAGE was performed. Radiolabeled samples were visualized by phosphorimaging (FLA-500; Fujifilm) and quantified using MultiGauge software (Fujifilm).

For co-immunoprecipitations, the cell lysis procedure was performed with 1% CHAPS replacing Triton X-100/SDS in cell lysis and wash buffers to preserve protein-protein interactions. Cells were collected in 1% CHAPS lysis buffer (400 μl) and centrifuged at 14,000 rpm for 5 min. Interactions were then probed by immunoblotting. A fraction of the post-nuclear supernatant (5% for FLAG or 10% for SEL1L and myc immunoblots) was used to determine protein levels in the total cell lysates. The remainder of the cell lysates was divided into two equal fractions that represented one-third of the total cell lysates. One fraction was immunoprecipitated with α-FLAG.
Antibodies. The other fraction was immunoprecipitated with either α-SEL1L or α-myc antibodies. Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with the indicated antibodies using standard procedures. Densitometric quantification of immunoblots was performed using ImageJ software (National Institutes of Health). Percentage of EDEM1-FLAG bound to SEL1L was calculated as ((EDEM1-FLAG proteins after α-SEL1L immunoprecipitation)/(EDEM1-FLAG cell lysates) × 20) × 100. Percentage of SEL1L bound to EDEM1-FLAG was calculated as ((SEL1L protein after α-FLAG immunoprecipitation)/(SEL1L cell lysates) × 10) × 100. Percentage of EDEM1-FLAG bound to ERdj5-myc was calculated as ((EDEM1-FLAG proteins after α-myc immunoprecipitation)/(EDEM1-FLAG cell lysates) × 20) × 100. Percentage of ERdj5-myc bound to EDEM1-FLAG was calculated as ((ERdj5-myc protein after α-FLAG immunoprecipitation)/(ERdj5-myc cell lysates) × 10) × 100.

For glycosidase digestion, immunoprecipitated samples were digested with Endo H as per the manufacturer’s instructions. For peptide N-glycosidase F treatment, post-nuclear cell lysates prepared by MNT lysis buffer were denatured with SDS-PAGE sample buffer. After adjusting pH to neutrality and adding Nonidet P-40 to 1%, samples were incubated in the presence or absence of peptide N-glycosidase F at 37 °C for 1 h.

Alkaline Extraction of Membranes—Alkaline extraction experiments were conducted at 4 °C. Near confluent 35-mm dishes of cells (~3.0 × 10^7 cells) were suspended in 100 μl of homogenization buffer (20 mM HEPES, pH 7.5, 5 mM KCl, 120 mM NaCl, 1 mM EDTA, and 0.2 mM sucrose) and passed through a 22-gauge needle 20 times. After centrifugation at 1000 × g for 10 min, the post-nuclear supernatant was centrifuged at 45,000 rpm in Beckman rotor (TLA 120.2) for 10 min to isolate cellular membranes. The membrane pellet was suspended in PBS (30 μl) and then incubated with freshly prepared 0.1 M Na_2CO_3 (pH 11.5; 700 μl) for 30 min on ice. After the alkaline treatment, samples were divided into two equal fractions; that is, total membranes and a fraction to be further separated by ultracentrifugation. The separated fractions were centrifuged at 65,000 rpm for 15 min through a sucrose cushion (50 mM triethanolamine, 0.3 M sucrose, pH 7.5, 100 μl). For immunoblotting, proteins in the supernatant and total membrane fractions were precipitated with trichloroacetic acid, whereas the membrane pellets were denatured directly with SDS-PAGE sample buffer. For immunoprecipitations, the pH of the total membrane fractions and the supernatants was adjusted by adding 1 M Tris-HCl, pH 7.5, and Triton X-100. Membrane pellets were directly lysed in MNT buffer.

Sucrose Density Gradient Ultracentrifugation—Transfected HEK 293T cells were washed and lysed in 500 μl of 1% CHAPS buffer. The post-nuclear supernatant was applied to the top of a 10–40% linear sucrose gradient (10 ml) with a 60% cushion (100 μl) in 1% CHAPS buffer (27). After ultracentrifugation at 38,000 rpm (145,000 g) for 17 h at 4 °C, samples were fractionated from top to bottom. Proteins were precipitated with trichloroacetic acid (final 10%) followed by separation by reducing SDS-PAGE and immunoblotting.

RESULTS

Post-translational N-Linked Glycosylation of the C-terminal EDEM1 Consensus Site—EDEM1 has five N-linked glycosylation consensus sites (Fig. 1A). 35S-Labeled EDEM1 migrated as a protein doublet after resolution by SDS-PAGE (Fig. 1B, lane 1). As previously demonstrated (30), this heterogeneity was due to the inefficient recognition of its five glycosylation sites, as the protein doublet coalesced as a single band upon Endo H treatment (Fig. 1B, lane 2). Endo H sensitivity also indicated that the protein did not reach the Golgi, as it did not obtain Endo H-resistant complex carbohydrates. One of the five glycosylation sites is located near the C terminus of EDEM1 (Asn-624). The modification of the C-terminal glycosylation sites would require post-translational transfer, as the stop codon is positioned ~30 amino acids after Asn-624. The oligosaccharyltransferase cotranslationally modifies sites when they are ~70 amino acids past the ribosomal P-site, accounting for ~40 amino acids to span the ribosome, ~20 to span the ER membrane, and ~10 to reach the oligosaccharyltransferase active site within the ER lumen (33).

To determine if the C-terminal glycosylation site of EDEM1 was responsible for the observed heterogeneity, this site was deleted by replacing Ser-626 with an Ala (EDEM1-S626A). EDEM1-S626A was resolved as a single band (S626A; Fig. 1B, lane 3). As previously demonstrated (Fig. 1B, lane 3), proteins were resolved by SDS-PAGE. EDEM1 glycosylation status: five glycans (EDEM1^WT), four glycans (EDEM1^S626A), and de-glycosylated forms (EDEM1^WT) are indicated on the left. Molecular mass markers are shown at right in kDa. C, HEK 293T cells were transfected with WT and EDEM1-S626A-FLAG and pulse-labeled 15 min followed by chase for indicated times. After immunoprecipitation with α-FLAG antibodies, samples were resolved by reducing SDS-PAGE.
When the glucosidase inhibitor DNJ was added to remove any heterogeneity created by glucose trimming, EDEM1-S626A migrated with the same mobility as the lower band observed with the WT doublet that corresponded to EDEM1 containing four glycans (Fig. 1B, lanes 7 and 8) (30).

To determine if the Asn-624 site can be post-translationally glycosylated, 293T cells were transfected with WT and EDEM1-S626A. The cells were pulsed for 15 min with [35S]Met/Cys and chased for various times (Fig. 1C). Although a doublet was observed immediately after the pulse for WT EDEM1, a single EDEM1 band formed after 1 h of chase, and this band migrated with the slower mobility EDEM1 band or EDEM1 containing five glycans (Fig. 1C, lanes 1–3). This time-dependent decrease in mobility was not observed for EDEM1-S626A that persistently contained four glycans (Fig. 1C, lanes 4–6). The missing glycan found with EDEM1-S626A did not affect its ability to accelerate the turnover of NHK (data not shown). We concluded that the C-terminal glycosylation site of EDEM1 was modified post-translationally.

Delayed Signal Sequence Cleavage Creates Soluble and Membrane-anchored Forms of EDEM1—EDEM1 possesses an N-terminal hydrophobic sequence that is responsible for its targeting to the ER. EDEM1 was initially identified as a membrane protein as it localized to the membrane fraction using an alkaline floatation assay (22). The N-terminal hydrophobic domain was proposed to act as an integral membrane-spanning segment to create a type II membrane protein. More recent results have indicated that EDEM1 was, at least partially, present as a soluble protein (30, 34). Analysis of signal sequence cleavage sites for EDEM1 using the SignalP 3.0 algorithm suggested three possible low probability cleavage sites (supplemental Fig. 1) (35). To reconcile this apparent discrepancy, cells expressing radiolabeled EDEM1-FLAG were chased for various times to support maturation. Cells were homogenized, and membrane fractions were isolated by centrifugation. The membranes were then alkaline extracted to separate membrane and soluble forms of the protein after centrifugation (Fig. 2A).

Immediately after the radioactive pulse, the majority of EDEM1 appeared in the membrane fraction (Fig. 2A, lanes 2 and 3). However, the fraction of EDEM1 in the soluble fraction increased with increasing chase times until two-thirds of the protein was soluble after 4 h of chase (Fig. 2A, lane 11). CNX (membrane chaperone) and CRT (soluble chaperone) were localized to membrane and soluble fractions, respectively, regardless of chase time, indicating the effectiveness of the separation procedure (Fig. 2A). These results indicated that the signal sequence of EDEM1 was slowly cleaved post-translationally.

To determine if the N-terminal signal sequence of endogenous EDEM1 was also slowly cleaved, a pulse-chase experiment was performed in HEK 293T cells with endogenous EDEM1 immunoprecipitated after alkaline extraction. Similar to the ectopically expressed tagged protein, the majority of the protein was membrane-associated immediately after the chase, with the soluble fraction reaching greater than 50% after 2 h of chase (Fig. 2B). Altogether, these results indicate that the slow cleavage of the signal sequence of EDEM1 creates two isoforms of EDEM1; one that is associated with the membrane as a type II membrane protein and a second that is a soluble protein.

EDEM1 Signal Sequence Cleavage Regulates Its Topology—Because the signal sequence cleavage of EDEM1 was slow or insufficient, resulting in the generation of a heterogeneous population, constructs were designed to individually create membrane and soluble forms of EDEM1. This permitted the separate characterization of the two forms. To produce a soluble form, the EDEM1 signal sequence was replaced with the signal sequence from the mouse class I heavy chain H2-Kb signal peptide (termed kbss-EDEM1; Fig. 3A). This signal sequence supports the efficient translation, ER targeting, and cleavage of a variety of secretory cargo (supplemental Fig. 1)(31, 36).

Inefficient EDEM1 Glycosylation and Signal Sequence Cleavage

![Image](Image 362x611 to 551x708)

FIGURE 2. Post-translational signal sequence cleavage creates soluble and membrane-anchored forms of EDEM1. A, HEK 293T cells were transfected with WT EDEM1-FLAG, pulse-labeled for 15 min, and chased for the indicated times. Membrane preparations were alkaline-treated and centrifuged to separate membrane and soluble fractions. Total membranes (T), the membrane pellet (P), and soluble proteins found in the supernatant (S) fractions after alkaline extraction were separated as described under “Experimental Procedures.” After immunoprecipitation using α-CNX, -CRT or -FLAG antibodies, samples were resolved by reducing SDS-PAGE. The percentage of EDEM1 observed in the supernatant and pellet fractions is shown below the EDEM1-FLAG panel. B, 293T cells were pulse-labeled for 1 h and chased for indicated times. Total cell lysates (T), the membrane pellet (P), and soluble proteins found in the supernatant (S) after alkaline extraction were separated by centrifugation, and immunoprecipitations were performed. Samples were resolved by reducing SDS-PAGE. Molecular mass markers are displayed to the right. The percentage of EDEM1 observed in the supernatant and pellet fractions is shown below.

To verify that the two EDEM1 constructs behaved as designed, membranes from cells transfected with these constructs
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acted as a type II membrane protein containing an N-terminal EDEM1 generated a soluble protein, whereas NA-EDEM1 kbss-EDEM1 (Fig. 3) retention of the signal sequence for NA-EDEM1 but not for F treatment was slower than the kbss-EDEM1, indicative of the soluble and membrane fractions, respectively. Furthermore, the EDEM1 and NA-EDEM1 were predominantly found in the soluble protein controls, respectively. Molecular mass are shown at the in kDa.

were alkaline-extracted, and EDEM1 was visualized by immunoblotting. EDEM1 possessing the WT signal sequence was found equally in the membrane pellet and the supernatant at steady-state levels (Fig. 3B, lanes 5 and 6). As expected, kbss-EDEM1 and NA-EDEM1 were predominantly found in the soluble and membrane fractions, respectively. Furthermore, the mobility of NA-EDEM1 before and after peptide N-glycosidase F treatment was slower than the kbss-EDEM1, indicative of the retention of the signal sequence for NA-EDEM1 but not for kbss-EDEM1 (Fig. 3C). These results demonstrated that kbss-EDEM1 generated a soluble protein, whereas NA-EDEM1 acted as a type II membrane protein containing an N-terminal membrane anchor.

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The Topology of EDEM1 Influences Its Interactions with ERAD Machinery—EDEM1 has been found to associate with a number of proteins within the ER lumen including SEL1L, ERdj5, and CNX (23, 27, 28). SEL1L is an ER membrane adapter protein, which nucleates the formation of a large ERAD complex in the ER membrane that supports dislocation of aberrant cargo to the cytoplasm for degradation by the proteasome (37). ERdj5 is a soluble ER oxidoreductase that possesses four thioredoxin domains and a J-domain that supports the recruitment of the ER Hsp70 chaperone BiP (28). CNX is an ER carbohydrate binding chaperone that helps in the folding and ER retention of non-native proteins (13). To determine if the topology of EDEM1 influences interactions with ERAD machinery, the ability of the various forms of EDEM1 to associate with these ERAD factors was analyzed.

Transfected 293T cells were lysed with CHAPS, and immunoprecipitations were conducted with α-FLAG or α-SEL1L followed by immunoblotting. As previously reported (27), WT EDEM1-FLAG associated with endogenous SEL1L (Fig. 4A, lanes 6, bottom panel, and 10, upper panel). The association between EDEM1 and SEL1L was enhanced severalfold for NA-EDEM1 and diminished for kbss-EDEM1 (Fig. 4A, lanes 7 and 8, bottom panel, and 11 and 12, upper panel). Regardless of whether the interactions were monitored by SEL1L or FLAG immunoprecipitations followed by immunoblotting. In agreement with previous results (27), interactions between EDEM1 and SEL1L were abolished in the presence of the mannosidase inhibitor kifunensine (KIF; supplemental Fig. 2A, lane 12, lower panel). Altogether, these results suggested that membrane anchoring of EDEM1 augmented its glycan-dependent association with the type-I membrane protein SEL1L.

Next, the ability of the various EDEM1 constructs to interact with ERdj5 was analyzed. WT EDEM1 associated with ERdj5 as previously reported (Fig. 4, C, lanes 6 and 10, and D) (28). The interaction level increased significantly with kbss-EDEM1 and decreased for NA-EDEM1 (Fig. 4C, lanes 7 and 8, lower panel, and D) (28). These results indicated that soluble EDEM1 interacted more efficiently with the soluble ER oxidoreductase ERdj5 in a mannos trimming-independent manner.

EDEM1 has also been reported to bind CNX, which positions it to extract aberrant proteins from the CNX binding cycle for ERAD (23). These results were verified through co-precipitation studies whereby EDEM1-FLAG immunoprecipitations were found to pull down CNX (Fig. 5A, lane 10). As EDEM1 contains five N-linked glycosylation sites, it might associate with CNX as a chaperone to aid in its maturation. This possibility was supported by the inhibition of EDEM1 binding to CNX after glucosidase I and II inhibition with DNJ, which accumulated triglucosylated glycans (Fig. 5A, lane 11). Furthermore, the turnover of EDEM1 was accelerated in the presence of the glucosidase inhibitor, consistent with EDEM1 misfolding in the absence of CNX association (Fig. 5, B and C). These results were supportive of the monoglucosylated EDEM1 associating with CNX as a maturation substrate.

In summary, the topology of EDEM1 played a role in determining its interactions with ERAD machinery including SEL1L and ERdj5, whereas EDEM1 appeared to interact with CNX as a maturation substrate. The membrane form of EDEM1 (NA-EDEM1) associated more efficiently with the membrane
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FIGURE 4. The association of EDEM1 with ERAD machinery is regulated by the cleavage of its signal sequence. A, 293T cells were transfected with the indicated cDNAs for 24 h and then lysed with buffer containing 1% CHAPS. 5 and 10% of the total cell lysate samples were used for immunoblotting directly with α-FLAG or α-SEL1L antibodies, and then lysed with buffer containing 1% CHAPS. After immunoprecipitation with α-FLAG (lanes 5–8) or α-SEL1L (lanes 9–12) antibodies, all samples were separated by reducing SDS-PAGE following immunoblotting with antibodies indicated to the right of the immunoblots. Molecular mass markers are displayed at the right in kDa. B, EDEM1-FLAG and SEL1L interactions in A were quantified. White bars indicate immunoprecipitations with SEL1L antisera followed by immunoblotting with antisera directed against the FLAG epitope of EDEM1. Black bars designate immunoprecipitations with the FLAG antisera followed by immunoblotting with α-SEL1L antibodies. Error bars represent S.D. from at least three independent experiments. C, cells were transfected with ERdj5-myc and EDEM1-FLAG constructs for 24 h and then lysed in CHAPS buffer. 5 and 10% of the total cell lysate samples were used for immunoblotting directly with α-FLAG or α-myc antibodies, respectively (lanes 1–4). Two fractions, each containing one-third of the cell lysates, were immunoprecipitated with α-FLAG or α-myc antibodies, respectively (lanes 5–8). Immunoprecipitations with myc antisera followed by immunoblotting with antisera directed against the FLAG epitope of EDEM1. White bars indicate immunoprecipitations with α-FLAG (lanes 5–8) or α-myc (lanes 9–12) antibodies. All samples were separated by reducing SDS-PAGE followed by immunoblotting with the antibodies indicated to the right of the immunoblots. The asterisk indicates background bands observed in the cell lysates. D, quantification of EDEM1-FLAG and ERdj5-myc interactions from C are presented. White bars indicate immunoprecipitations with myc antisera (ERdj5-myc) followed by immunoblotting with antisera directed against the FLAG epitope of EDEM1. Black bars designate immunoprecipitations with the FLAG antisera followed by immunoblotting with α-SEL1L antibodies. Black bars designate immunoprecipitations with the FLAG antisera followed by immunoblotting with α-myc antibodies. Error bars represent S.D. from at least three independent experiments.

FIGURE 5. Glucosidase trimming supports EDEM1 binding to calnexin. A, HEK 293T cells were transfected with empty vector (lanes 1, 5, 9, and 13) or WT EDEM1-FLAG (lanes 2–4, 6–8, 10–12, and 14–16). Cells were treated with 500 μM DNJ or 100 μM Kif for 18 h where designated and then lysed in a buffer containing 1% CHAPS. After immunoprecipitation with α-FLAG or α-CNXX antibodies, samples were separated by reducing SDS-PAGE following immunoblotting (WB) with α-FLAG or α-CNXX antibodies. B, HEK 293T cells were transfected with WT EDEM1-FLAG followed by a 15-min pulse-labeling and chase for indicated periods. Before pulse-labeling and during the chase, cells were treated with or without 500 μM DNJ. Immunoprecipitations with α-FLAG were processed. Molecular mass markers are displayed to the right in kDa. C, shown is quantification of EDEM1 levels in B. The initial time point (0 min) of each experiment was set to 100%. Pulse-chase experiments with no drug (square) or DNJ (triangle) are shown. Error bars represent the S.D. for three independent experiments.

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adapter SEL1L, whereas soluble EDEM1 (kbss-EDEM1) interacted more efficiently with soluble ERdj5. Therefore, signal sequence cleavage altered the topology of EDEM1 and influenced its binding profile to ER partner proteins.

Membrane Topology of EDEM1 Affects ERAD Substrate Selectivity—EDEM1 constructs were analyzed to determine if EDEM1 possessing contrasting topologies could differentially accelerate the turnover of soluble and membrane-anchored ERAD substrates. α1-Antitrypsin is a soluble glycosylated protein, and the null Hong Kong (NHK) variant is a truncation mutation associated with rapid turnover by ERAD (38, 39). Co-expression of NHK with all three forms of EDEM1 accelerated the turnover of NHK when compared with empty vector (Fig. 6, A and B). The turnover of NHK was accelerated most extensively with kbss-EDEM1 co-expression where the half-life for NHK decreased to 43 min, compared with ~80 min with WT or NA-EDEM1. In addition, the mobility of NHK increased slightly in a time-dependent manner in the presence of kbss-EDEM1, whereas the mobility of NHK was unchanged in the presence of NA-EDEM1 (Fig. 6A, upper panel lanes 7 and 9 and lanes 10 and 12). The increase in mobility was likely caused by the mannosidase activity of EDEM1 or through its association with ER mannosidase I (29, 40, 41). The soluble form of EDEM1 was most effective at accelerating the turnover of the soluble ERAD substrate NHK.

The α-subunit of the TCRα is a type I membrane glycoprotein that forms a larger heterocomplex in T lymphocytes (42).
Unassembled subunits are rapidly degraded through the ERAD pathway (43). As was the case for NHK, coexpression of TCRα with any of the three forms of EDEM1 supported the accelerated turnover of TCRα (Fig. 6, C and D). However, the stability of TCRα was decreased most extensively by the presence of NA-EDEM1. Therefore, EDEM1 was more effective at aiding in the turnover of substrates that possessed similar topologies to the ERAD factor; soluble EDEM1 efficiently aided the turnover of a soluble protein, and the membrane form of EDEM1 was most efficient in assisting the degradation of a membrane-associated ERAD substrate.

Arg-14 Is Important for EDEM1 Signal Sequence Cleavage—The signal sequence of EDEM1 was examined to identify residues that may contribute to its inefficient cleavage. An Arg residue is located in the center of the hydrophobic cluster (H-domain) of the signal sequence of EDEM1 (Fig. 7A). The H-domain is required for membrane insertion, and it helps to position the cleavage site for efficient recognition and cleavage by the signal peptidase complex. To test the necessity for this charged residue, Arg-14 was changed to a Leu (R14L-EDEM1). The efficiency of signal sequence cleavage was analyzed by separation of membrane and soluble fractions after the membranes from HEK 293T-transfected cells were alkaline-extracted. R14L-EDEM1 was completely observed in the membrane pellet, indicating that signal sequence cleavage was abolished by this alteration (Fig. 7B, lane 9). Interestingly, this alteration was predicted to be more efficiently cleaved using SignalP 3.0 (supplemental Fig. 1). These results indicated that the Arg-14 was required for signal sequence cleavage, and this residue influenced the topology of EDEM1.

The topology of EDEM1 affected the association of EDEM1 with ER factors and substrates. To determine if the topology of EDEM1 had an impact on the size of EDEM1-containing complexes formed, the EDEM1 constructs with modified signal sequences were analyzed by density gradient ultracentrifugation after cell lysis with CHAPS. WT EDEM1-FLAG, which existed as soluble and membrane proteins at steady state, was widely distributed from a monomeric form (Fig. 7C, lane 3, 4.6S) to high molecular weight complexes (lane 10) (44). R14L-EDEM1, a membrane form of EDEM1, exhibited similar profiles as WT EDEM1. In contrast, kbss-EDEM1 or soluble EDEM1 was isolated in a restricted number of fractions corresponding to the formation of smaller size complexes (lane 5, 8.9S). SEL1L, a component of the ERAD retrotranslocation complex, formed large complexes as previously reported (lanes 9–11) (44). The SEL1L ERAD complex colocalized in the fractions with the larger EDEM1 complexes found most prominently for WT and R14L-EDEM1 that contained membrane-integrated EDEM1. These results demonstrated that the topology of EDEM1 had a large impact on the size of the EDEM1-containing complexes formed.

DISCUSSION

Elucidating the role for EDEM1 in ER quality control has been hindered, as conflicting or incomplete results have been obtained for its activities and general properties. First, EDEM1 was initially reported to be a membrane protein, but more recent results have indicated that it is also a soluble protein (22,
EDEM1–FLAG constructs were visualized by immunoblotting with antibodies, and endogenous SEL1L was identified with antibodies, and endogenous SEL1L was identified with antibodies. Molecular weight markers are shown to the right in kDa. C, cell lysates from transfected HEK 293T cells lysed in CHAPS were layered onto 10–40% sucrose gradients with a 60% sucrose cushion. After centrifugation and fractionation, proteins were denoted to the bottom of the gel: serum albumin, fraction 3; trypsinogen, fraction 5; and lysozyme, fraction 9. Molecular mass standards for the SDS-PAGE are denoted to the right in kDa.

FIGURE 7. Arg-14 is required for signal sequence cleavage. A, the N-terminal 30 amino acids of human EDEM1 are displayed with the Arg-14 mutation indicated. B, 293T cells were transfected with indicated vectors. Total membrane preparations (T) from the transfected cells were separated into membrane pellet (P) and soluble supernatant (S) after alkaline extraction, and protein samples were resolved by reducing SDS-PAGE followed by immunoblotting with indicated antibodies. Molecular weight markers are shown to the right in kDa. C, cell lysates from transfected HEK 293T cells lysed in CHAPS were layered onto 10–40% sucrose gradients with a 60% sucrose cushion containing 1% CHAPS. After centrifugation and fractionation, proteins were precipitated by trichloroacetic acid and resolved by reducing SDS-PAGE.

Inefficient EDEM1 Glycosylation and Signal Sequence Cleavage

In the absence of signal sequence cleavage, the N-terminal signal sequence of EDEM1 acted as a transmembrane domain to create a type II membrane protein as shown by alkaline extraction (Fig. 2). Signal sequence cleavage is generally a rapid cotranslational event (17, 49, 50). For EDEM1, the half-time for signal sequence cleavage was greater than 1 h for both ectopically and endogenously expressed EDEM1 (Fig. 2). As the half-life of EDEM1 is relatively short in a variety of cell lines (∼1 h) (45, 46), this brought up the questions of whether both forms of EDEM1 are active? Do they work in different complexes and have different substrate specificities? And what is the biological significance of EDEM1 possessing contrasting topologies?

The N-terminal signal sequence of EDEM1 was exchanged to individually generate soluble and membrane forms of EDEM1. These constructs were used to determine that the topology of EDEM1 played an important role in the selection of binding partners in the ER. The soluble form of EDEM1 associated most efficiently with the soluble ER oxido-reductase ERdj5 (Fig. 4, C and D). ERdj5 contains four thioredoxin domains that mediate the reduction of inter- or intramolecular disulfide bonds. ERAD substrate reduction is thought to render the substrate translocation competent for their dislocation through an ER membrane translocon (28). In addition, ERdj5 also possesses a J-domain that helps in the recruitment of the ER Hsp70 member BiP to the complex. BiP is envisioned to play a role in unfolding, dissolving of aggregates, and/or the dislocation process.

Co-immunoprecipitation studies found that the membrane form of EDEM1 (NA-EDEM1) associated most significantly with the membrane protein SEL1L when compared with the WT and soluble (kbss-EDEM1) forms of EDEM1 (Fig. 4, A and B). As previously observed, EDEM1 binding to SEL1L was inhibited by the mannosidase inhibitor kifunensine, suggesting that the association was, at least in part, mediated by the mannosidase domain of EDEM1 (27). SEL1L is a type I membrane protein that functions as an adapter protein to nucleate an ERAD translocation/ubiquitination complex in the ER membrane. Therefore, the binding of EDEM1 to SEL1L and ERAD
substrates has been proposed to play a role in substrate delivery to the ER membrane dislocation complex (27, 37).

The topology of ER factors has also been shown to influence substrate selection. The ER carbohydrate binding chaperones CNX and CRT both bind maturing proteins possessing monoglycosylated glycans, but their substrate profiles vary (51). Adding a membrane tail to CRT allowed it to bind to CNX-specific substrates (15). Together the two chaperones support the assistance of proteins that possess glycans localized proximal to the membranes as well as sites that reside deeper in the lumen (16). Furthermore, the topology of ERAD substrates dictates the ERAD route or the machinery utilized for their turnover (52, 53). Soluble ERAD substrates were found to be dependent on HRD1, SEL1L, OS-9, and XTP3-B for turnover, whereas these factors were not essential for the degradation of membrane proteins possessing a lesion in their luminal domain (53). The topology of EDEM1 influenced its ability to assist in the turnover of ERAD substrates. Soluble EDEM1 was more effective at accelerating the turnover of the soluble ERAD substrate NHK, whereas membrane EDEM1 was more effective with the membrane protein TCR-α. Therefore, the dual topology of EDEM1 appears to help augment the array of proteins that EDEM1 can efficiently assist in the ERAD pathway.

Inefficient signal sequence cleavage seems to be unique to mammalian EDEM1 among the EDEM-family members, as EDEM2, EDEM3, and Htm1p were shown to be efficiently cleaved to form soluble proteins in agreement with SignalP 3.0 predictions (supplemental Fig. 1) (34, 54–56). Arg-14 in the signal sequence of EDEM1 is, at least in part, responsible for the heterogeneity, as its alteration to Leu supported the generation of a completely membrane-anchored protein (Fig. 7). The placement of a charged residue in the center of the H-domain can support intramembrane interactions and complex formation (57). Positively charged Arg and Lys residues in the transmembrane regions of the α6 subunits of TCR interact with negatively charged residues in other TCR subunits to support complex assembly and cell surface transport (42). This suggests that charged residues that reside in transmembrane regions can contribute to complex formation. It would be of interest to characterize factors that interact with a membrane form of EDEM1 that possessed the transmembrane Arg residue. However, attempts to create a transmembrane form of EDEM1 that retained Arg-14 by altering the cleavage site area were unsuccessful (data not shown). This was likely due to the promiscuity of the protease, which cleaved at another site when an individual site was disrupted.

Inefficient or slow signal sequence cleavage appears to be a strategy utilized by the cell to generate soluble and membrane-anchored forms of EDEM1. Hegde and co-workers (6, 58) have shown that inefficient targeting to the ER of proteins containing suboptimal signal sequences supports the accumulation of both luminal and cytoplasmic biologically relevant proteins from a single transcript. Signal sequence cleavage also appears to be a process that has evolved as a mechanism to augment the limited size of the genome. Instead of creating genes that encode for proteins possessing similar activities with different topologies (as is the case with CNX and CRT), perhaps signal sequence cleavage levels can be modulated to create these two topologies from a single gene. Mutations that disrupt the cleavage of signal sequences have been found to be associated with a number of human diseases including diabetes, underscoring the importance of signal sequence cleavage in cell homeostasis (59). Further studies will be required to determine the role of signal sequence cleavage in protein maturation and the scope of substrates that possess dual topologies due to inefficient or slow cleavage of their targeting sequences.

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