A Shared Endoplasmic Reticulum-associated Degradation Pathway Involving the EDEM1 Protein for Glycosylated and Nonglycosylated Proteins*

Marina Shenkman1,1, Bella Groisman1, Efrat Ron1, Edward Avezov1, Linda M. Hendershot1, and Gerardo Z. Lederkremer1,2

From the 1Department of Cell Research and Immunology, George Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel and the 2Department of Tumor Cell Biology, St. Jude Children’s Research Hospital, Memphis, Tennessee 38105

Background: N-Glycan processing and interactions with lectins regulate the quality control and endoplasmic reticulum-associated degradation (ERAD) of glycoproteins. Results: Most of the same machinery targets nonglycosylated misfolded proteins. Conclusion: They share some membrane and luminal ERAD machinery but not all cytosolic components. Significance: ER glycan-interacting proteins must possess a dual specificity for glycan structure and for exposed misfolded polypeptide domains.

Studies of misfolded protein targeting to endoplasmic reticulum-associated degradation (ERAD) have largely focused on glycoproteins, which include the bulk of the secretory proteins. Mechanisms of targeting of nonglycosylated proteins are less clear. Here, we studied three nonglycosylated proteins and analyzed their use of known glycoprotein quality control and ERAD components. Similar to an established glycosylated ERAD substrate, the uncleaved precursor of asialoglycoprotein receptor H2a, its nonglycosylated mutant, makes use of calnexin, EDEM1, and HRD1, but only glycosylated H2a is a substrate for H2a, its nonglycosylated mutant, makes use of calnexin, component. Our results suggest a shared ERAD pathway for glycosylated and nonglycosylated proteins composed of luminal lectin machinery components also capable of protein-protein interactions.

Research on ER3 quality control and disposal of misfolded glycoproteins has attracted much attention in recent years. A pathway has been delineated, which involves lectin chaperones, glycosidases, and components of a putative retrotranslocation machinery, some of which also possess lectin activity, for delivery to the cytosolic proteasomes (1–5). The chain of events that leads to nascent glycoprotein through ER quality control and toward proper folding or ERAD involves processing of its N-glycans and their interactions. There is initially removal of the terminal glucose residue from the Glc3Man9GlcNAc2 precursor and also of the following glucose that allows binding to the chaperone/lectins calnexin and calreticulin. Removal of the third and last glucose causes dissociation from these lectins, although re-addition of a single glucose by the folding sensor UDP-Glc-glycoprotein glucosyltransferase triggers reassociation until folding is complete (6, 7). If proper folding cannot be achieved in a certain time frame, the glycoprotein molecules are targeted to ERAD by removal of more mannose residues (three or four) than occurs for folded molecules that export to the Golgi (one or two) (8, 9). This trimming of mannose residues involves ER mannosidase I and the ER degradation-enhancing α-mannosidase-like proteins (EDEMs) and is essential for delivery to ERAD (2, 10, 11). The mannose trimming regulates misfolded glycoprotein association with the lectins OS-9 and XTP3-B at the ER quality control compartment (ERQC), a staging ground for ERAD (8, 12, 13). OS-9 and XTP3-B are in turn associated with a protein complex involved in substrate ubiquitination, retrotranslocation, and targeting to the proteasomes. This complex includes E3 ubiquitin ligases like HRD1 and gp78 and interestingly a family of cytosolic ER-associated SCF ligases that include F-box proteins (Fbs1 and Fbs2) with a lectin moiety that recognizes N-glycans of misfolded glycoproteins for ubiquitination (14). Recognition by these lectin components of the ligases requires prior retrotranslocation of the substrate glycoprotein to expose its glycan to the cytosol.

Much less is known about the processes that govern the fate of nascent nonglycosylated proteins. There is at least some distinction from the most upstream components of the glycoprotein pathway, as many of these proteins interact with the chaperone BiP instead of calnexin or calreticulin (15, 16), and at least...
some of these nonglycosylated ERAD substrates require the ERAD machinery-associated protein Herp (17). We recently found that, surprisingly, EDEM1, a central player in glycoprotein ERAD (10, 18, 19), can bind a glycoprotein ERAD substrate in a glycan-independent manner and that its overexpression or up-regulation during the unfolded protein response overrides the mannos trimming requirement for ERAD. This hinted to a potential role in nonglycosylated protein ERAD. Here, we set out to investigate the involvement of EDEM1 and of other lectin ERAD components for the disposal of three nonglycosylated unfolded proteins. As a glycosylated ERAD model control, we included the well characterized, uncleaved precursor of asialoglycoprotein receptor (ASGPR) H2a. This glycoprotein is expressed naturally in hepatocytes as a membrane precursor, which undergoes efficient cleavage producing a 35-kDa secreted form (20). When H2a is expressed in other cell lines, the membranous precursor is inefficiently cleaved, and the uncleaved precursor, as well as most of the cleaved fragment, is retained in the ER and degraded by the ubiquitin-proteasome system (12, 21). As a model of a naturally nonglycosylated ERAD substrate, we examined the unassembled nonglycosylated NS-1k light chain (NS-1 kLC). This protein is degraded by the 26 S proteasome (22), through an ERAD pathway that involves Herp, HRD1, and Derlin1 (17). We also analyzed a truncated Ig heavy chain, γ V-C_{14}, (23), which, like NS-1 kLC, is a nonglycosylated BiP substrate and is also targeted to the Herp/HRD1 pathway (17). We also included a nonglycosylated mutant of H2a. We found involvement of several luminal components of the glycoprotein ERAD machinery but not of the cytosolically localized SCP {\textsuperscript{ABC}}, a glycan-dependent E3 ligase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rainbow {\textsuperscript{14}}C-labeled methylated protein standards were obtained from GE Healthcare. Promix cell labeling mix ([{\textsuperscript{35}}S]Met plus [{\textsuperscript{35}}S]Cys), >1000 Ci/mmol, was from PerkinElmer Life Sciences. Protein A-Sepharose was from Repligen (Needham, MA). Lactacystin (Lac) and kifunensin were from Cayman Chemicals (Ann Arbor, MI). MG-132 and other common reagents were from Sigma. S-protein-agarose was from Novagen. Streptavidin linked to Texas Red was from Jackson ImmunoResearch (West Grove, PA). Protein G-agarose was from Santa Cruz Biotechnology.

**Plasmids and Constructs**—H2a and calnexin were subcloned in pCDNA1 (Invitrogen) (12). H2a{\textsuperscript{Δgly}} in pCDNA1 was described previously (24). Myc-H2a{\textsuperscript{Δgly}} was subcloned in pCDNA4. BIP-HA in pXM was a kind gift of Peter Murray (Memphis, TN). H2a170 and H2a102 were created by site-directed mutagenesis, substituting asparagine to glutamine (N102Q and N170Q). In H2a305 threonine 305 was substituted by isoleucine (T305I).

The pSUPER vector carrying short hairpin RNAs (shRNA) for human ER mannosidase I or human EDEM1 and EDEM1-HA in pCMVsport2 were used previously (13, 25). EDEM1ACRD was described previously (26).

Mouse Fbs2 F box deletion mutant (Fbs2ΔF) and human hsHRD1 RING finger mutant were those used previously (24). S-tagged XTP3-B and OS-9.1 and OS-9.2 (27) were those used in Ref. 26. H2a G78R uncleavable mutant fused through its C terminus to monomeric red fluorescent protein (H2a-RFP) was described previously (24, 28). NS-1 kLC and HA-γ V-C_{14} constructs were described previously (Refs. 16, 23, respectively).

**Primers and RT-PCR**—Total cell RNA was extracted with TRIzol reagent (Invitrogen). Reverse transcription was performed with a VersoTM cDNA kit (Thermo Fisher Scientific, Barrington, IL) as described previously (13).

**Antibodies**—Rabbit polyclonal C-terminal anti-H2 antibody was used in earlier studies (20, 29), as well as rabbit polyclonal anti-Derlin-1 (13). Mouse monoclonal anti-H2a C-terminal
antibody B9 was raised in our laboratory (30). Rabbit polyclonal anti-rodent BiP antiserum was described previously (31). Goat anti-mouse 
/H9260 was from SouthernBiotech (Birmingham, AL). Mouse monoclonal antibodies used were as follows: anti-FLAG (M2), anti-HA, anti-
/H9252-tubulin, anti-calnexin, rabbit anti-GM-130, anti-EDEM1, and anti-HA were from Sigma, anti-Myc was from Cell Signaling (Beverly, MA), and anti-S tag was from 
Novagen (Gibbstown, NJ). Goat anti-rabbit IgG antibody conjugated to Cy2, goat anti-mouse IgG DyLight549, goat anti-mouse IgG FITC, goat anti-rabbit and anti-mouse IgG conjugated to HRP, donkey anti-goat IgG linked to Cy2, and donkey anti-mouse IgG conjugated to Cy3 were from Jackson ImmunoResearch (West Grove, PA). Goat anti-rabbit IgG DyLight650 was from Abcam. Goat anti-rabbit IgG 
DyLight650 was from Abcam. Goat anti-mouse IgG conjugated to agarose was from Sigma. Biotinylated donkey anti-rabbit IgG was from IBA (Olivette, MO).

Cell Culture and Transfections—Human embryonic kidney (HEK) 293 cells were grown in DMEM plus 10% fetal calf serum (FCS) and NIH 3T3 cells in DMEM plus 10% newborn calf serum. All cells were grown at 37 °C under an atmosphere of 5% CO₂.

Transient transfection of NIH 3T3 cells was performed using an MP-100 Microporator (Digital Bio) according to the manufacturer’s instructions. Transient transfection of HEK 293 cells was done according to the calcium phosphate method. The experiments were performed 24–48 h after the transfection.

Metabolic Labeling, Immunoprecipitation, SDS-PAGE, and Quantitation—Subconfluent (90%) cell monolayers in 60-mm dishes were labeled with [³⁵S]Cys, lysed, and immunoprecipitated with anti-H2 antibodies, as described previously (20, 21), or using mouse anti-H2a B9 antibody and goat anti-mouse IgG immobilized on agarose beads where indicated. The same procedure was used for kLC but labeling with 100 μCi of [³⁵S]Met plus [³⁵S]Cys mix, lysis with a buffer containing 0.15 M NaCl, 10 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 0.1% SDS, 0.2% deoxycholate, and immunoprecipitation with goat anti-kLC and protein G-agarose. Reducing SDS-PAGE was performed on 10% Laemmli gels if not stated otherwise. The gels were analyzed by

FIGURE 2. H2aAgly is a substrate of EDEM1. A, similar to Fig. 1B, except that cells expressing H2aAgly were incubated with 40 μM MG-132 and 10% of the lysates were run on SDS-PAGE and immunoblotted with anti-CNX antibody (bottom panel). Phosphorimager quantitation values of H2aAgly bound to CNX (upper panel) divided by total H2aAgly (middle panel) are shown at the bottom relative to the pulse labeling. B, similar to A but with cells expressing H2aAgly and EDEM1-HA and cell lysis in buffer containing 1% Triton X-100 and 0.5% sodium deoxycholate. EDEM1-HA was immunoprecipitated (IP) from 70% of the cell lysates using anti-HA and H2aAgly from 25% of the lysates with anti-H2a. The remaining 5% of the lysates were run on SDS-PAGE and immunoblotted with anti-HA (bottom panel). C, similar to B but with WT H2a instead of H2aAgly. Note that the fully glycosylated band is shifted to a faster migration due to trimming of mannose residues, whereas the underglycosylated lower species is degraded (8). D, similar to B (middle panel) but with cells expressing H2aAgly together with control GFP or with EDEM1-HA and without MG-132. Quantitations of the percent of H2aAgly remaining after the chase relative to the pulse are shown at the bottom. E, similar to D but with cells expressing H2aAgly together with control anti-LacZ shRNA or anti-EDEM1 shRNA. F, in parallel with E, RNA was extracted from the cells and used for RT-PCR with primers for EDEM1 mRNA (upper panel) compared with GAPDH (lower panel). No RNA template was added in lane 3.
fluorography using 20% 2,5-diphenyloxazole and were exposed to Biomax MS film using a TransScreen-LE from Eastman Kodak (Vancouver, BC). Quantitation was performed in a Fuji-film FLA 5100 phosphorimager (Japan).

Coimmunoprecipitation and Immunoblotting—Cell lysis and immunoprecipitation of H2a and related constructs were done as described previously (21). Cell lysis was performed in the presence of protease inhibitor mixture (Roche Applied Science). For immunoprecipitation from HEK 293 cells, cell lysis was done in 1% Nonidet P-40, 50 mM Tris-HCl (pH 8), 150 mM NaCl or as indicated for 30 min on ice, and debris and nuclei were pelleted in a microcentrifuge for 30 min at 4 °C. The samples were immunoprecipitated with appropriate antisera and protein A-agarose. For immunoprecipitation with anti-HA monoclonal antibody, goat anti-mouse IgG-agarose was used. After overnight precipitation, the beads were washed three times with lysis buffer diluted 1:5, followed by elution of the bound proteins by boiling with sample buffer containing β-mercaptoethanol at 100 °C for 5 min. For communoprecipitation with NS-1 κLC, cells were lysed in a buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 0.1% SDS, 0.2% deoxycholate followed by immunoprecipitation with anti-κLC and protein G-agarose (if not stated otherwise).

Coimmunoprecipitation with anti-calnexin antibody was performed as described previously (32). Briefly, after metabolic labeling, cells were lysed in HBS buffer (pH 7.5), containing 2% sodium cholate; cell lysates were immunoprecipitated with anti-CN antibody, boiled in 1% SDS, then diluted with 10 volumes of 1% Triton X-100, 0.5% sodium deoxycholate in HBS, and reimmunoprecipitated with anti-H2a antibody.

Immunoblotting and detection by ECL were done as described previously (12), except for exposure and quantitation in a ChemiDoc XRS Imaging System (Bio-Rad).

Immunofluorescence Microscopy—The procedures employed were as described previously (12, 25) Confocal microscopy was done on a Zeiss laser scanning confocal microscope (LSM 510; Carl Zeiss, Jena, Germany) as described previously (25).

Statistical Analysis—Data are expressed as means ± S.E. Student’s t test (unpaired, two-tailed) was used to compare the two groups, and the p value was calculated in GraphPad Prism 5 (GraphPad software). p < 0.05 was considered as statistically significant.

RESULTS

Components of the Glycoprotein ERAD Pathway Target a Nonglycosylated Mutant of the ERAD Substrate ASGPR H2a Precursor to the ERQC and Are Required for Its Degradation—We previously reported that ASGPR H2a precursor associates after synthesis with the ER chaperone calnexin, dissociating slowly compared with its fast dissociation from the calnexin-interacting oxidoreductase ERP57 (32). We created three constructs where two alternative N-glycosylation sites of the three that exist in H2a were abrogated, with a single N-glycan remaining in either position 102, 170, or 305 (Fig. 1A). In addition, we made a construct with all glycosylation sites removed (H2aΔgly). We had seen previously that wild-type H2a is...
degraded soon after dissociation from calnexin (32). Therefore, in a pulse-chase analysis of H2a in the absence of proteasomal inhibition, followed by either direct immunoprecipitation or coimmunoprecipitation with anti-calnexin, a similar fraction of H2a is found associated with calnexin before or after the chase period (Fig. 1B, lanes 1 and 2, and graph). Although there were slight variations, all H2a mutants retaining a single N-glycan had similar half-lives and interacted with calnexin similarly to wild-type H2a, except for H2a102, which associated to a greater extent than WT H2a both initially and after the 4-h chase (Fig. 1B). Unexpectedly, the nonglycosylated H2agly showed association with calnexin similar to WT H2a. Therefore, we focused on characterizing the involvement of other factors considered to be part of the glycoprotein ER quality control machinery on the targeting to degradation of H2agly. EDEM1 showed a significant association with H2agly under steady state conditions, both in the presence and absence of the proteasomal inhibitor MG-132 (Fig. 1C). We then examined the kinetics of calnexin and EDEM1 association with H2agly in the presence of the proteasomal inhibitor to minimize degradation. The association of EDEM1 with H2a gly was more prolonged than that with calnexin (Fig. 2A and B). However, unlike the association of EDEM1 with WT H2a (Fig. 2C) and other glycoprotein ERAD substrates (33, 34), it did not increase with time (Fig. 2B). Overexpression of EDEM1 significantly accelerated the degradation of H2a gly, whereas knockdown of EDEM1 inhibi-

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**FIGURE 4.** Upon proteasomal inhibition H2a gly colocalizes with EDEM1 and ERAD factors at the ERQC. A, plasmids encoding for H2a-RFP and myc-tagged H2a gly were cotransfected in NIH 3T3 cells. One day after transfection, cells were incubated for 3 h without (upper panels) or with Lac (25 μM) (lower panels), fixed, permeabilized, and incubated with mouse anti-Myc and FITC-conjugated goat anti-mouse IgG. Representative confocal optical slices are shown. B, similar to A but with endogenous GM130 detected with rabbit anti-GM130 and Cy3-conjugated goat anti-rabbit IgG. C, cells cotransfected with HA-tagged EDEM1 and nonglycosylated H2a gly and incubated with rabbit anti-H2a and Cy3-conjugated goat anti-rabbit IgG. D, similar to B; endogenous Derlin-1 was visualized using rabbit anti-Derlin-1 and Cy3-conjugated goat anti-rabbit IgG. E, similar to C but with Myc-tagged HRD1 instead of EDEM1-HA. Mouse anti-Myc antibodies and FITC-conjugated goat anti-mouse IgG were used to visualize HRD1. Bar, 10 μm. F, similar to C with cells expressing H2a gly and FLAG-tagged Fbs2, detected with mouse anti-FLAG and FITC-conjugated goat anti-mouse IgG. G, cells transfected with Myc-tagged H2a gly and GalT-YFP, incubated with mouse anti-Myc and goat anti-anti mouse DyLight549 IgG. Endogenous BiP was visualized using rabbit anti-Bip and goat anti-rabbit DyLight650 IgG, pseudocolored blue. A section was enlarged for better detail of the lack of colocalization.
A previous study found that H2a is a substrate for two E3 ubiquitin ligases, HRD1 and SCFFbs2 (13). Fbs2 contains a substrate-recognition subunit Fbs2, which possesses a high mannose glycan binding domain (35). H2a and WT H2a showed similar association with HRD1 (Fig. 3A). However, H2a did not bind to Fbs2 (Fig. 3B). Consistently, overexpression of a dominant-negative RING mutant of HRD1 blocked ERAD of H2a but not of Fbs2 (Fig. 3C). Both dominant-negative proteins were shown to block the degradation of WT H2a (13). Conversely, interactions of Fbs2 with the chaperone BiP showed negligible binding to H2a, whereas it associated significantly with H2a (Fig. 3D). We next determined whether H2a accumulates like WT H2a and other glycoprotein substrates in the juxtanuclear ERQC (8, 12, 28). Indeed, proteasomal inhibition caused accumulation of H2a from an initial dispersed ER pattern to the ERQC, where it colocalized with the glycoprotein ERAD substrate H2a linked to a monomeric red fluorescent protein (H2a-RFP) (Fig. 4A) and with a Golgi marker, GM130 (Fig. 4B). Accumulated H2a also colocalized to a fair extent with EDEM1, Derlin-1, HRD1, and Fbs2 (Fig. 4C–F). We had seen previously that these ERAD machinery components are recruited to the ERQC upon proteasomal inhibition or glycoprotein substrate overexpression (13, 28). Although H2a does not appear to bind to or require SCFFbs2 for its degradation (Fig. 3), both proteins are still recruited to the ERQC, SCFFbs2 apparently from the cytosolic side and H2a on the membrane. This suggests that the ERQC may be a staging ground for ERAD of glycoproteins (13, 28) as well as nonglycoproteins and that SCFFbs2 is recruited either independent of the glycan nature of the substrate or that sufficient amounts of endogenous glycoproteins exist to recruit it. H2a remained separate from a Golgi marker, GM130, as well as from most of the abundant ER chaperone BiP, which is not concentrated in the ERQC (Fig. 4G). This result is similar to the segregation of BiP from overexpressed ERQC-localized glycosylated H2a (12, 28).

![Figure 5](http://www.jbc.org/) - **DEME1 mutant devoid of its carbohydrate recognition domain (CRD) can interact with substrates and target them to degradation.** A, similar to Fig. 2D but with DEME1 mutant with most of its CRD deleted (DEME1ΔCRD) instead of WT DEME1. B, same procedure as in Fig. 1C was performed, but with cells expressing either HA-tagged DEME1 WT or DEME1ΔCRD, together with either H2a or H2aΔgly. Asterisks indicate C-terminal (HA-tagged) cleavage products of DEME1 and DEME1ΔCRD, observed in total lysates but almost absent from the coimmunoprecipitation. Molecular mass markers are indicated on the right in kDa. IP, immunoprecipitation. C, similar to B but with cotransfection with or without an anti-ER mannosidase I (ERManI) shRNA encoding p-SUPER plasmid as indicated. Two days post-transfection, cells were incubated in the presence or absence of 10 μM kifunensine (Kif) for 6 h. Immunoblots were with anti-H2a, anti-DEME1, and anti-GAPDH. D, RNA was extracted from cells transfected with the plasmids encoding anti-ER mannosidase I or control anti-LacZ shRNA, and used for RT-PCR with primers for ER mannosidase I mRNA (upper panel) compared with GAPDH (lower panel). Lane 3 shows a sample with no RNA template.

Overall, the results show a similar routing and requirement of ERAD pathway components for H2a as compared with WT H2a, including calnexin, DEME1, and HRD1. Notable exceptions are BiP, which binds strongly to nonglycosylated H2a but not to the glycoprotein, H2a, and SCFFbs2, which targets H2a but is not required for degradation of H2aΔgly.

**Glycan-independent Targeting of the Nonglycosylated Substrate by a Mutant DEME1 Lacking Its Carbohydrate Recognition Domain**—We had shown that when DEME1 is overexpressed or up-regulated by the UPR it bypasses the glycan...
dependence for glycoprotein ERAD. In these conditions, the carbohydrate recognition domain of EDEM1 was not required for it to target WT H2a (26). We tested whether a mutant EDEM1 (EDEM1ΔCRD), lacking most of its carbohydrate-recognition domain, which corresponds to the catalytic portion of homologous mannosidases (26), would target H2aΔgly for degradation. In a pulse-chase analysis, overexpression of EDEM1ΔCRD significantly increased the degradation of H2aΔgly (Fig. 5A). EDEM1ΔCRD associated to a similar degree with both WT H2a and H2aΔgly, and even more strongly than WT EDEM1 (Fig. 5B). Trimming of mannose residues of the substrate, in the case of WT H2a, or of any putative intermediate glycoprotein in the case of an indirect interaction was not required for the association of EDEM1ΔCRD with WT H2a or with H2aΔgly (Fig. 5C). Consistently, neither the expression nor activity of ER mannosidase I was necessary for this association (Fig. 5, C and D).

Targeting of a Naturally Nonglycosylated Substrate by EDEM1 and Routing to the ERQC—As the above experiments were done on a nonglycosylated mutant of a glycoprotein, we wondered whether a naturally nonglycosylated ERAD substrate would behave similarly. Therefore, we analyzed a nonglycosylated Ig κ light chain (NS-1 κLC), which utilizes several components of the ERAD machinery, Derlin-1, Herp, HRD1, and p97 (17), but is recognized by the ER chaperone BiP instead of calnexin (15, 16). The degradation of NS-1 κLC was accelerated by overexpression of EDEM1 and strongly inhibited by knockdown of EDEM1 (Fig. 6, A and B). In keeping with this finding, EDEM1 coimmunoprecipitated substantially with NS-1 κLC (Fig. 6C). This association did not change significantly upon inhibition of its degradation. Similar to H2aΔgly, NS-1 κLC also coimmunoprecipitated with EDEM1ΔCRD, the overexpression of which decreased NS-1 κLC levels (Fig. 6D).

NS-1 κLC coimmunoprecipitated with BiP, as seen previously (16), and in this case the association decreased upon proteasomal inhibition (Fig. 6E), suggesting release from BiP at late ERAD stages. However, it was not a significant decrease.

In untreated cells, NS-1 κLC appeared in a diffuse ER pattern, and upon proteasomal inhibition it accumulated and colocalized significantly with EDEM1 at the ERQC (Fig. 7A), where a substantial fraction of NS-1 κLC also colocalized with H2a-RFP (Fig. 7B). The HRD1/SEL1L-associated lectin OS-9 (27) concentrated in the ERQC, as seen previously (26), and upon proteasomal inhibition, it colocalized with accumulated NS-1 κLC (Fig. 7C, lower panels).
We had seen that even in the absence of expression of an ERAD substrate, calnexin accumulates in the ERQC upon proteasomal inhibition, whereas BiP does not (12, 32). We wondered whether upon expression of NS-1/LC, a protein that associates strongly with BiP, BiP would now appear in the ERQC. As expected, in the absence of proteasomal inhibitors, NS-1/LC colocalized with BiP in a disperse ER pattern (Fig. 7D). However, upon proteasomal inhibition, although NS-1/LC is now concentrated in the ERQC, BiP remained mostly secluded from this region and neither protein colocalized with a Golgi marker (Fig. 7, E and F), similar to what we observed upon coexpression of H2aΔgly (Fig. 4G).

**Nonglycosylated Protein Targeting to ERAD by EDEM1**

We looked at another nonglycosylated BiP substrate, truncated Ig heavy chain (HA-γV-Cγ1), also an ERAD substrate (17). Once again, we found that EDEM1 knockdown caused accumulation of HA-γV-Cγ1, and EDEM1 overexpression caused a significant reduction of its level (Fig. 8A). HA-γV-Cγ1 coimmunoprecipitated specifically with EDEM1 (Fig. 8B). EDEM1ΔCRD also accelerated HA-γV-Cγ1 degradation (Fig. 8C).

**Nonglycosylated Substrates Associate with XTP3-B and to a Lesser Extent with OS-9**—We then explored whether the nonglycosylated substrates associate with the lectins OS-9 and its functional homolog XTP3-B. Coimmunoprecipitation experiments showed specific and significant interaction of both NS-1/LC and HA-γV-Cγ1 with XTP3-B (Fig. 9, A and B). However, OS-9 associated only weakly with NS-1/LC, and almost no interaction could be detected with HA-γV-Cγ1 (Fig. 9, A and B). Similarly, H2aΔgly associated strongly with XTP3-B, and in a much weaker fashion with OS-9 (Fig. 9C).
Nonglycosylated Protein Targeting to ERAD by EDEM1

**DISCUSSION**

Our results suggest that nonglycosylated misfolded secretory proteins are targeted by much of the so-called glycoprotein ER quality control and ERAD machinery. Indeed, calnexin has previously been implicated in the quality control of some nonglycosylated substrate proteins (36, 37). Here, we show that EDEM1 can interact in a glycan-independent manner with misfolded nonglycosylated H2aΔgly, which associates with both calnexin and BiP. In addition, we show that EDEM1 can bind to two nonglycosylated proteins, NS-1 kLC and γ V-C1, which associate with BiP instead of calnexin (15, 16). This is consistent with a suggested chaperone function of EDEM1 (38, 39), which could bind to the polypeptide moiety of misfolded glycoproteins (26). A possibility that should be evaluated in future studies is that the association of EDEM1 with the nonglycosylated substrates might occur through interaction of EDEM1 with the mannose residues (36, 37, 40), which also binds to BiP.

EDEM1 is involved in trimming of mannose residues from precursor N-glycans during glycoprotein ER quality control (26, 39, 41). However, this trimming is not necessary for EDEM1 binding to its substrates when EDEM1 is exogenously overexpressed (13, 42) or when EDEM1 is up-regulated during the unfolded protein response (26).

EDEM1 had been implicated in the targeting of the nonglycosylated ricin toxin for retrotranslocation (43), although this toxin is not an ERAD substrate. Here, we show for a nonglycosylated mutant of a glycoprotein ERAD substrate and also for bona fide nonglycoprotein ERAD substrates that their degradation is dependent on EDEM1. In keeping with this, the interaction and targeting of H2aΔgly for degradation by EDEM1 does not require EDEM1’s carbohydrate recognition (mannosidase-like) domain (Fig. 5). A recent study of EDEM1 association with tyrosinase showed that the N terminus of EDEM1 interacts with this substrate (44). Consistently, C-terminal EDEM1 fragments showed almost no interaction with H2a or H2aΔgly (Fig. 5B). Interestingly, the N-terminal portion (after the signal peptide) is absent in the EDEM1 homolog in *Saccharomyces cerevisiae* Mnl1 (Htm1). Consistent with this difference, nonglycosylated yeast ERAD substrates do not seem to be targeted by Mnl1 (45–47).

Other components of “glycoprotein ERAD” that target nonglycosylated proteins for degradation are the lectins OS-9 and XTP3-B. We detected significant binding of XTP3-B but weak association of OS-9 with H2aΔgly, NS-1 kLC, and γ V-C1.1. It is unclear if XTP3-B binds directly to these substrates independently of their lectin activity or if it coprecipitates as part of a complex.

Similar to the nonglycosylated substrates that we have studied, nonglycosylated mutant amyloidogenic transthyretin was previously found to associate with XTP3-B but not OS-9 (27). However, a nonglycosylated mutant of another glycoprotein substrate, α1-antitrypsin NHK associated with both lectins (48, 49), suggesting that these two lectins may have different sets of preferred substrates. In *S. cerevisiae*, Yos9 was shown to be involved in the nonglycosylated protein ERAD (50, 51), underscoring the nonglycoprotein binding potential for a number of
these components that are often considered to be specific for the turnover of glycosylated proteins.

The concentration of NS-1 kLC and H2aAgly in the ERQC and relative depletion of BiP from this compartment upon proteasomal inhibition (Figs. 4 and 7) suggest that at advanced stages of targeting to ERAD, these substrates might dissociate from BiP and bind to EDEM1 and/or XTP3-B. Indeed, there was a relative decrease in the association of NS-1 kLC with BiP upon proteasomal inhibition (Fig. 6E). However, the effect on the association was not significant. This suggests that BiP is in large excess of the substrate, and upon proteasomal inhibition, BiP molecules in the peripheral ER not bound to NS-1 kLC would far exceed the amount of BiP in association with NS-1 kLC in the ERQC. This is consistent with a model that we had proposed, where binding of misfolded proteins to the relatively low amounts of BiP present in the ERQC would facilitate its displacement from the UPR sensors IRE1 and PERK, which accumulate in this compartment, leading to their activation (1, 28).

The F-box proteins Fbs2 and the similar but neural-specific Fbs1 are lectin subunits of the Skp, Cullin, F-box containing (SCF) E3 ligase, which target high mannose glycans of misfolded glycoproteins once they reach the cytosol (14). Their role is to possibly prevent premature deglycosylation of the substrates by peptide:N-glycosidase before ubiquitination (52). They do not seem to target a nonglycosylated protein \textit{in vitro} (14) nor in cells \textit{in vivo} as we observed here (Fig. 3). A deubiquitination step was reported as a requirement for dislocation of a substrate to the cytosol, which would imply a second ubiquitination step for the substrate to be recognized by the proteasome (53). We can speculate that this second ubiquitination step might be provided by SCFFbs2 for glycoproteins, and in the case of nonglycosylated substrates by E3 ligases involved in ubiquitination of cytosolic nonglycosylated proteins. Except for this cytosolic step in the targeting of glycoprotein ERAD substrates to the proteasomes, glycosylated and nonglycosylated substrates seem to use a similar luminal and membrane ERAD machinery. As most secretory proteins are glycosylated, with nonglycosylated proteins being a minority, it stands to reason that they would utilize at least some components of a shared machinery. It is important to emphasize that this machinery is used not only in the case of glycoprotein substrates with their glycosylation sites artificially abrogated but also by a naturally nonglycosylated ERAD substrate, NS-1 kLC.

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Marina Shenkman, Bella Groisman, Efrat Ron, Edward Avezov, Linda M. Hendershot and Gerardo Z. Lederkremer

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