AAA ATPase p97/Valosin-containing Protein Interacts with gp78, a Ubiquitin Ligase for Endoplasmic Reticulum-associated Degradation*

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Endoplasmic reticulum-associated degradation (ERAD) is a protein quality control mechanism that eliminates unwanted proteins from the endoplasmic reticulum (ER) through a ubiquitin-dependent proteasomal degradation pathway. gp78 is a previously described ER membrane-anchored ubiquitin ligase (E3) involved in ubiquitination of ER proteins. AAA ATPase (ATPase associated with various cellular activities) p97/valosin-containing protein (VCP) subsequently dislocates the ubiquitinated proteins from the ER and chaperones them to the cytosol, where they undergo proteasomal degradation. We now report that gp78 physically interacts with p97/VCP and enhances p97/VCP-polyubiquitin association. The enhanced association correlates with decreases in ER stress-induced accumulation of polyubiquitinated proteins. This effect is abolished when the p97/VCP-interacting domain of gp78 is removed. Further, using ERAD substrate CD3ε, gp78 consistently enhances p97/VCP-CD3ε binding and facilitates CD3ε degradation. Moreover, inhibition of endogenous gp78 expression by RNA interference markedly increases the levels of total polyubiquitinated proteins, including CD3ε, and abrogates VCP-CD3ε interactions. The gp78 mutant with deletion of its p97/VCP-interacting domain fails to increase CD3ε degradation and leads to accumulation of polyubiquitinated CD3ε, suggesting a failure in delivering ubiquitinated CD3ε for degradation. These data suggest that gp78-p97/VCP interaction may represent one way of coupling ubiquitination with retrotranslocation and degradation of ERAD substrates.

Mammalian p97/VCP1 and its yeast counterpart CDC48 play crucial roles in a number of cellular processes in conjunction with its cofactors, Ufd1, Npl4, and p47 (1). CDC48/p97/VCP is essential for cell cycle progression (2, 3), homotypic membrane fusion after mitosis, and disassembly of spindle at the end of mitosis (4–7), retrograde translocation of misfolded proteins from the ER (8–13), degradation of polyubiquitinated proteins by the proteasomes (14, 15), and activation of transcription factors (16, 17). Ubiquitin interaction with p97/VCP and its cofactors appears to play an important role in p97/VCP-regulated processes. For example, in order to target polyubiquitinated proteins for proteasomal degradation, p97/VCP must interact with polyubiquitin chains through its NDI domains and chaperone polyubiquitinated proteins to the proteasome (18). Interaction of ubiquitin with the UTD domain of Ufd1 enhances p97/VCP-polyubiquitin binding (19). This finding at least partially explains why the p97/VCP-Ufd1-Npl4 complex is required to dislodge ubiquitinated misfolded proteins from the ER to the cytosol for proteasomal degradation (20). Although Npl4 is required for p97/VCP function in retrotranslocation, its ubiquitin-interacting motif, the Npl4 zinc finger domain, is not involved in ERAD (19). This is supported by the fact that yeast Npl4 does not have an Npl4 zinc finger domain and is competent for retrotranslocation in ERAD. p47 interacts with ubiquitin via its ubiquitin-associated domain, which is required for membrane fusion during postmitotic reassembly of Golgi stacks (21). p47 also contains a Ubx domain. Recent studies indicate that a family of yeast Ubx domain-containing proteins, including p47, interact with CDC48 via their Ubx domain and are involved in proteasome-mediated proteolysis (22, 23). How Ubx domain-CDC48 interaction regulates the function of CDC48 as a chaperone for ubiquitin is unknown.

The ubiquitin binding in p97/VCP-Ufd1-Npl4- or p97/VCP-p47-regulated processes appears to be dynamic. A newly identified p97/VCP-interacting protein, VCIP135, is found to be a deubiquitinating enzyme (24, 25). p97/VCP-p47-mediated reassembly of Golgi stacks requires both VCIP135 and p47-ubiquitin interactions (21, 25). Thus, a cycle of ubiquitination and deubiquitination must play a role in the regulation of Golgi membrane dynamics during mitosis. Another p97/VCP-interacting protein is SVIP, which competes with p47 and Ufd1 for binding to the NDI domain of p97/VCP (26). SVIP may regulate p97/VCP complex binding to ubiquitin.

A recurrent role for CDC48/p97/VCP-Ufd1-Npl4 is disassembly of protein complexes through binding to protein(s) via ubiquitin-dependent and -independent mechanisms. Studies in Xenopus egg extracts have shown that CDC48/p97/VCP-Ufd1-Npl4 complex-mediated disassembly of mitotic spindles is through its interaction with XMAP215 and TPX2 and regulates their binding to microtubules at mitotic exit (6). This regulation is independent of XMAP215 and TPX2 degradation. XMAP215 and TPX2 are neither ubiquitinated nor degraded during in-
teraction with p97/VCP. A similar process occurs in yeast in which CDC48-Ufd1-Npl4 complex selectively binds to polyubiquitin on the activated transcription factor SPT23, separates it from its precursor, and enables it to enter the nucleus for gene transcription (16). It has been proposed that CDC48-Ufd1-Npl4 acts as a ubiquitin-selective segregase. In the case where proteolysis requires segregation of a proteolytic substrate from other proteins (e.g. in ERAD or in selective degradation of subunits from oligomeric complexes), the segregase and the proteasome may cooperate in degradation (16). In ERAD, a newly identified ER membrane protein, VIMP, appears to recruit p97/VCP and targets it to Derlin1, a proposed component of the retrotranslocation channel for selective dislodging of unwanted proteins from the ER (27, 28). In this scenario, p97/VCP interacts with ERAD substrates both before and after ubiquitination (19).

gp78 was originally identified as the receptor for the tumor autocrine motility factor that promotes tumor metastasis (29). We have recently established gp78 as a really interesting new gene (RING) finger-dependent E3 ubiquitin ligase involved in ubiquitinating ERAD substrates (30). Therefore, gp78 and p97/VCP functionally cooperate during ERAD. In this study, we demonstrate that gp78 physically interacts with p97/VCP. The interaction enhances p97/VCP complex-polyubiquitin binding and degradation of ERAD substrate under ER stress. These data suggest a novel mechanism by which gp78 physically interacts with p97/VCP in coupling ubiquitination, retrotranslocation, and proteasomal degradation during ERAD.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Plasmids**

Anti-green fluorescent protein (GFP) and anti-gp78 have been previously described (30). Anti-ubiquitin was acquired from StressGen and Santa Cruz Biotechnology, Inc. Anti-Myc was purchased from Sigma. Monoclonal antibody against p97/VCP was generated against a synthetic peptide derived from amino acids 792–806 of the murine p97/VCP (generously provided by Dr. Chou-Chi Li), and some were purchased from Research Diagnostics.

pCIneo-gp78, pCIneo-gp78C, pGEX-T2-gp78C (309–643), pGEX-gp78CB2m, pGEX-T1-gp78CΔRING, pGFP-N1-gp78, and pGFP-N1-gp78CΔRING have been previously described (30). gp78 fragment (amino acids 595–643) was cloned into a pGEX-4T3 vector for expressing glutathione S-transferase (GST) fusion gp78C49 protein. pCIneo-gp78C49 was constructed by site-directed mutagenesis (QuickChange mutagenesis kit, Stratagene), whereby a stop codon was introduced to truncate the C-terminal 49 amino acids. pGEX-T2-gp78CΔC65 and pGEX-T2-gp78CΔC135 were generated by introducing stop codons in pGEX-T2-gp78C. The cDNA for hHrd1 (KIAA1810) was pBluescript was obtained from KAZUSA DNA Research Institute, Japan, and was subcloned into pCIneo vector using Sall and NotI sites. The resulting pCIneo-hHrd1 was tagged with FLAG through site-directed mutagenesis.

**Determination of Protein Interactions**

**GST Pull-down Assay**—All GST fusion proteins were expressed as reported (30). Two micrograms of each GST fusion protein was immobilized on glutathione-Sepharose beads. For gp78 interactions with cellular p97/VCP and polyubiquitin, 293 cells were transfected with Myc-ubiquitin using LipofectAMINE 2000 (Invitrogen). Twenty hours post-transfection, cells were treated with MG132 at 30 μM for 4 h. Cells were then harvested and lysed in Triton X-100 lysis buffer (150 mM NaCl, 20 mM Tris/Cl, pH 7.5, 1 mM EDTA, 1% Triton X-100). Glutathione-Sepharose beads-immobilized GST-gp78C and its mutants were incubated with the lysates for 1 h at 4 °C. After washing with 1× phosphate-buffered saline containing 1% Triton X-100, bead-associated proteins were processed for immunoblotting (IB) for Myc-ubiquitin and p97/VCP. IB followed the previously published protocol (30).

**Analysis of p97/VCP Interaction with Polyubiquitin and CD3—**293 cells were transfected with the indicated plasmids using LipofectAMINE 2000. In some experiments, pCDNA3-Myc-ubiquitin was co-transfected for detection of polyubiquitin using anti-Myc antibody. Twenty-four hours after transfection, cells were lysed in radioimmune precipitation buffer (50 mM Tris/Cl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, and 1× protease inhibitor mixture from Sigma), and the lysates were processed for immunoprecipitation (IP) for p97/VCP or hemagglutinin (HA)-CD3. Co-immunoprecipitation of polyubiquitin or HA-CD3 with p97/VCP was examined by IB. For detecting multiple proteins on the same membrane, the membrane was stripped between each blotting. For IP and membrane stripping, previously reported procedures were used (30).

**RNAi**

A published RNAi procedure for expressing siRNA was adopted (31). Two regions from human gp78 cDNA, including hp 300–318 (for small interference RNA1 (siRNA1)) and 1318–1336 (for siRNA2), were cloned into the siRNA expression vector, pSuper (31). For determining the effectiveness of these siRNAs, 293 cells were co-transfected with 1 μg of pCIneo-gp78C with 3 μg of pSuper-siRNA1 or -siRNA2. Twenty-four hours after transfection, cells were processed for IB to detect gp78 and actin, respectively. To determine the effects of gp78 RNAi on the levels of total polyubiquitinated proteins, 293 cells were transfected with 3 μg of pSuper-siRNA2. Transfected cells were analyzed by IB for ubiquitin and p97/VCP, respectively.

**RESULTS**

**gp78 Interacts with p97/VCP**—The functional cooperation between gp78 and p97/VCP during ERAD prompted us to look for a possible physical association between these two proteins. In a GST pull-down assay, GST fusions of gp78 cytosolic tail (gp78C; amino acids 309–643) and its variants (see the schematic representation of the mutants in Fig. 1A, middle) were incubated with 293 cell lysates, and the associated p97/VCP was identified by IB. The result shows that gp78C indeed binds to p97/VCP (Fig. 1A, left, lane 3). Mutation or deletion of the RING finger (gp78CR2m; C341G and C356G mutations or gp78CΔRING) did not affect p97/VCP binding (Fig. 1A, left, lanes 4 and 5), indicating that the RING finger is not required for binding. Truncations of the C-terminal 135 and 65 amino acids of gp78 (gp78ΔC135 and gp78ΔC65) led to loss of p97/VCP binding (Fig. 1A, left, lanes 7 and 8), suggesting that the C-terminal region interacts with p97/VCP. Consistently, the C-terminal 49 amino acids of gp78 (gp78C49) were sufficient for p97/VCP interaction (Fig. 1A, left, lane 9). Therefore, the p97/VCP-interacting domain resides within the C-terminal 49 amino acids of gp78.

Recently, the cue domains of yeast Vps9 and cue2 have been shown to be a ubiquitin-interacting motif (32–35). By analogy, we speculated that the cue domain of gp78 might serve a similar role. Therefore, we examined the association of ubiquitin in the GST pull-down assay. In the experiments shown in Fig. 1A, all of gp78C and its mutants with the cue domain and the C-terminal 49 amino acids preserved binding to both polyubiquitin and p97/VCP (left, lanes 2–5). Further, deletion of the cue domain abolishes polyubiquitin binding (Fig. 1A, left, lanes 6 and 9), whereas deletion of gp78C49 abrogates the p97/VCP interaction (Fig. 1A, left, lanes 6–8). When both the cue domain and gp78C49 were removed, neither polyubiquitin nor p97/VCP binding were detected (Fig. 1A, left, lane 6; see a summary of these bindings in the right panel of Fig. 1A). Variable amounts of polyubiquitin binding between gp78C and its cue domain-containing mutants suggest that the cue-flanking regions affect ubiquitin binding. We also noticed that gp78-bound p97/VCP migrate slower than those in input used for the GST pull-down assay. This more slowly migrating form may represent a modified p97/VCP that binds to gp78 or an artifact.
caused by the SDS-PAGE itself. Nonetheless, these results indicate that the cue domain of gp78 binds polyubiquitin, whereas the C-terminal 49 amino acids contain the p97/VCP-interacting domain in vitro.

To determine whether the same interactions occur in vivo, 293 cells were transfected either with WT gp78 or with a deletion mutant (amino acids 309–643; gp78/H9004C-GFP) that lacks its cytosolic tail and carries a C-terminal GFP tag (refer to schematic representations of these proteins in Fig. 1A). Transfected cells were then processed for IP and IB. As predicted, p97/VCP co-immunoprecipitated with gp78-GFP (Fig. 1C, lane 2) but not with gp78C-GFP or GFP alone (Fig. 1C, lanes 3 and 1, respectively). The smeared appearance of both gp78-GFP and gp78AC-GFP in the blot is due to their transmembrane domains as has been reported previously (30). As with our in vitro studies, the C-terminal 49 amino acids of gp78 are required for p97/VCP but not for polyubiquitin interaction in 293 cells. 293 cells were transfected with gp78 and gp78AC49. Left, transfected cells were processed for IP with anti-gp78 (for both gp78 and gp78AC49). Associated p97/VCP and polyubiquitin were examined by IB. Right, expression of related proteins were determined by IB in whole cell lysates used for IP.

FIG. 1. gp78 interacts with p97/VCP and polyubiquitinated proteins in vitro and in cells. A, middle, schematic representation of gp78 and its mutants. TM, transmembrane domain; R2m, C341G and C356G mutations in the RING. Left, in vitro interaction. GST fusions of gp78C and its various mutants were incubated with lysates of 293 cells that were transfected with Myc-ubiquitin. Associated polyubiquitin and p97/VCP were examined by IB. A summary of the results is displayed on the right. B, schematic representation of gp78 and its mutant used in experiments in C and D. C, the cytosolic tail of gp78 is required for p97/VCP interaction. 293 cells were transfected with the indicated plasmids. Cells were then processed for IP with anti-GFP for gp78-GFP and gp78AC-GFP. Co-immunoprecipitated p97/VCP was examined by IB. D, the C-terminal 49 amino acids of gp78 are required for p97/VCP but not for polyubiquitin interaction in 293 cells. 293 cells were transfected with gp78 and gp78AC49. Left, transfected cells were processed for IP with anti-gp78 (for both gp78 and gp78AC49). Associated p97/VCP and polyubiquitin were examined by IB. Right, expression of related proteins were determined by IB in whole cell lysates used for IP.
gp78 Enhances p97/VCP-Polyubiquitin Interaction—Like Ufd1 and Npl4, gp78 also interacts with both p97/VCP and polyubiquitin. It has been shown that Ufd1-Npl4 dimer association increases p97/VCP complex-ubiquitin interaction (19). This increase may be due to the additional ubiquitin-binding sites provided to the p97/VCP-Ufd1-Npl4 complex by Ufd1 and Npl4. The double \( \psi \) barrel folds that are present in both the UT3 domain of Ufd1 and the N domain of p97/VCP simultaneously interact with ubiquitin, which could also increase the affinity toward ubiquitin (19). Based on this evidence, we speculated that as a ubiquitin-interacting protein, gp78 might enhance p97/VCP interaction with polyubiquitin. To test this hypothesis, we examined the effects of gp78 on p97/VCP-ubiquitin interaction. WT gp78, gp78ΔC49, or gp78CΔRING (see schematic representation of these proteins in Fig. 1, A and B) were co-transfected with Myc-tagged ubiquitin into 293 cells. Transfected cells were lysed in radiolabeled precipitation buffer, and p97/VCP was immunoprecipitated with monoclonal anti-p97/VCP antibody. Associated polyubiquitin was determined by IB. The results show that WT gp78 and gp78CΔRING markedly enhance polyubiquitin co-immunoprecipitation with p97/VCP (Fig. 2A, upper left, lanes 2 and 4). Co-immunoprecipitated gp78CΔRING was also evident (Fig. 2A, lower left, lane 4). However, there was no detectable level of co-immunoprecipitated gp78 (Fig. 2A, lower left, lane 2), although it was readily detected in the whole cell lysate used for IP (Fig. 2A, lower right, lane 2). Our inability to detect co-immunoprecipitated gp78 could be due to lower levels of co-immunoprecipitated gp78 and/or low sensitivity of the anti-gp78 antibodies. However, these experiments indeed show that enhanced polyubiquitin-p97/VCP co-immunoprecipitation requires gp78 interaction with p97/VCP, since gp78CΔ49 without the p97/VCP-interacting domain fails to increase p97/VCP and polyubiquitin co-immunoprecipitation (Fig. 2A, lower left, lane 3). Further, increased p97/VCP-polyubiquitin binding does not appear to be due to increases in total polyubiquitinated proteins. This observation is supported by the fact that gp78CΔ49 increases total polyubiquitinated proteins to the same levels as does WT gp78 and gp78CΔRING (Fig. 2A, upper right, lanes 2 and 3), but only the WT gp78 and gp78CΔRING enhance p97/VCP-polyubiquitin co-immunoprecipitation.

Next we asked whether the enhanced p97/VCP-ubiquitin interaction is specific for gp78. To approach this question, we examined the effect of hHrd1 (36–38), another E3 for ERAD that does not interact with p97/VCP,2 on p97/VCP-polyubiquitin interactions. When hHrd1 was transfected into 293 cells, hHrd1 increased the levels of total polyubiquitinated proteins, as did WT gp78, but did not enhance p97/VCP-polyubiquitin co-immunoprecipitation (Fig. 2B, lane 5). These data indicate that gp78 interaction enhances p97/VCP-polyubiquitin binding, which is not dependent on the levels of total polyubiquitinated proteins. The enhanced binding could be due to the cue domain of gp78 that provides an additional ubiquitin-binding site when gp78 complexes with p97/VCP, as does Ufd1, or due to other unknown mechanisms.

Enhanced p97/VCP-Polyubiquitin Binding Regulates Degradation of Ubiquitinated Proteins—Based on the roles of p97/VCP in targeting polyubiquitinated proteins for degradation, enhanced p97/VCP-polyubiquitin binding is predicted to increase degradation of polyubiquitinated ERAD substrates. However, we observed that both gp78 and gp78CΔRING increased total polyubiquitin (Fig. 2, A and B). Since ERAD occurs under conditions of ER stress, we speculated that ER stress might facilitate degradation of these p97/VCP-bound ubiquitinated proteins. To test this possibility, we examined levels of polyubiquitinated proteins in cells transfected with wild type and gp78CΔRING in the presence and absence of tunicamycin, an inhibitor of N-linked glycosylation. Levels of polyubiquitinated proteins in gp78 and gp78CΔRING-transfected cells were compared with empty vector-transfected cells treated with tunicamycin for 0, 4, and 8 h. Tunicamycin induced a time-dependent increase in polyubiquitinated proteins in empty vector-transfected cells (Fig. 3A, top, lanes 1–3). As previously demonstrated (Fig. 2, A and B), gp78 and gp78CΔRING increased total polyubiquitin proteins (Fig. 3A, top, lanes 4 and 7). Importantly, tunicamycin induced time-dependent decreases of total polyubiquitinated proteins in gp78- and gp78CΔRING-transfected cells (Fig. 3A, top, lanes 4–9). To determine whether this decrease in ER stress-induced polyubiquitinated proteins was due to enhanced p97/VCP-polyubiquitin binding, gp78CΔ49 was transfected into 293 cells, and the levels of ER stress-induced polyubiquitinated proteins were assessed. As predicted, gp78CΔ49, a mutant that does not enhance p97/VCP-polyubiquitin association (Fig. 2, A and B), failed to decrease tunicamycin-induced polyubiquiti-
nated proteins (Fig. 3B, lane 6). In the same experiment, WT gp78 markedly decreases the level of polyubiquitinated protein after treatment with tunicamycin (Fig. 3B, lanes 3 and 4). These data suggest that gp78-p97/VCP interactions facilitate the removal of ER stress-induced polyubiquitinated proteins.

Interestingly, tunicamycin induces time-dependent stabilization of both WT gp78 (Fig. 3, A, lanes 4–6, and B, lanes 3 and 4) and gp78ΔC49 (Fig. 3B, lanes 5 and 6) but not gp78CÆRING (Fig. 3A, lanes 7–9). Previous studies have shown that ER stress up-regulates expression of ubiquitination machinery for ERAD at the level of mRNA (39, 40). This result reveals an additional level of regulation of ERAD via stabilization of E3 protein. It is worth noting that stabilization of gp78ΔC49 did not lead to decrease of ER stress-induced polyubiquitinated proteins. This is consistent with our notion that gp78-p97/VCP interaction is essential for degradation of polyubiquitinated proteins under ER stress.

To determine the effects of endogenous gp78 on polyubiquitinated protein degradation, gp78RNAi was employed. Since the available anti-gp78 antibody is not sensitive enough to detect endogenous gp78, the effectiveness of RNAi was determined by co-transfection of gp78 with two different siRNAs. The results show that both siRNAs efficiently inhibited gp78 expression (>90% inhibition at the protein level) and have no effect on actin levels (Fig. 3C), indicating that siRNAs are specific toward gp78. As predicted, polyubiquitinated proteins were markedly stabilized in gp78 siRNA-expressing cells (Fig. 3D, lane 3), as did inhibition of proteasome activity by lactacystin (Fig. 3D, lane 2). As shown in Fig. 2, overexpression of gp78 and hHrd1 increased total polyubiquitin (Fig. 3D, lanes 4 and 5). These data suggest that endogenous gp78 is involved in degradation of polyubiquitinated proteins, probably through enhancement of p97/VCP-polyubiquitin binding.

**gp78 Regulates the Levels of CD3δ and p97/VCP-CD3δ Interaction**—To further study the role of gp78-enhanced p97/VCP-polyubiquitin binding in ERAD, we analyzed CD3δ, a well-characterized ERAD substrate (30, 41, 42). We assessed the levels of CD3δ and p97/VCP-CD3δ binding in cells in which endogenous gp78 expression was inhibited by RNAi and in cells that overexpressed WT gp78 or gp78ΔC49. 293 cells transfected with plasmids encoding gp78 siRNA, WT gp78, or gp78ΔC49 were processed for IF for CD3δ. Under these conditions, gp78RNAi dramatically stabilized CD3δ, whereas overexpression of WT gp78 markedly decreased CD3δ protein (Fig. 4A, middle, lanes 2 and 3). gp78 lacking a p97/VCP-interacting domain (gp78ΔC49) failed to suppress CD3δ (Fig. 4A, middle, lane 4). Importantly, this decrease in CD3δ protein levels is associated with enhanced p97/VCP-CD3δ binding (Fig. 4A, top, lane 3). However, in contrast to our expectations, there appears to be elevated p97/VCP-CD3δ binding in gp78 siRNA-transfected cells compared with that observed in empty vector-transfected cells (Fig. 4A, top, lanes 1 and 2). Two possible explanations for this discrepancy exist. First, p97/VCP-bound CD3δ was efficiently targeted for degradation in vector-transfected cells, leading to undetectable p97/VCP-CD3δ interactions (Fig. 4A, lane 1). Second, the elevated levels of CD3δ in gp78 siRNA-transfected cells (Fig. 4A, lane 3) might have caused its interaction with p97/VCP, since p97/VCP is an abundant chaperone that tends to bind misfolded proteins directly (1, 43). To distinguish between these two possibilities, we used proteasome inhibitor lactacystin to block p97/VCP-mediated degradation of CD3δ. A duplicate set of cells as in Fig. 4A, lanes 1–4, were treated with lactacystin. The treatment increased p97/VCP-CD3δ binding in vector-transfected cells (Fig. 4A, top, lanes 1 and 5). Binding was further enhanced in WT gp78-transfected cells (Fig. 4A, top, lanes 3 and 7). Importantly, lactacystin...
treatment failed to increase p97/VCP-CD3δ interaction in gp78 siRNA-transfected cells (Fig. 4A, middle, compare lanes 2 and 6), suggesting that p97/VCP-CD3δ binding in these cells is not competent for targeting CD3δ for degradation. This finding supports the notion that high levels of accumulated CD3δ have caused p97/VCP-CD3δ interaction. This interaction is different from gp78-enhanced binding. There was no interaction between p97/VCP and CD3δ in gp78C49-transfected cells even in the presence of lactacystin (Fig. 4A, top, lanes 4 and 8), suggesting that the p97/VCP-interacting domain of gp78 is required for enhancing p97/VCP-CD3δ binding. When the immunoblots in the left panel of Fig. 4A were quantified and compared with that in vector-transfected cells (lane 5), p97/VCP-CD3δ binding shows an 88% decrease by gp78RNAi and a 804% increase by gp78 overexpression in lactacystin-treated cells (Fig. 4B, right). These data indicate that gp78 enhances p97/VCP-CD3δ binding and suppresses levels of CD3δ, whereas gp78RNAi abrogates p97/VCP-CD3δ interaction and stabilizes CD3δ. However, the failure of gp78ΔC49 to facilitate CD3δ degradation could be due to insufficient ubiquitination of CD3δ. To address this possibility, we examined the extent of ubiquitination of CD3δ in WT gp78- and gp78ΔC49-transfected cells by IP for HA for CD3δ followed by IB for ubiquitin. The results show that CD3δ immunoprecipitated from gp78ΔC49-transfected cells are heavily ubiquitinated (Fig. 4C, right). Thus, the failure to decrease the levels of CD3δ by gp78ΔC49 is not due to failure in CD3δ ubiquitination but rather to degradation after CD3δ is ubiquitinated.

Since gp78CARING appears to enhance p97/VCP-polyubiquitin co-immunoprecipitation (Fig. 3, A and B), we wondered whether it could also enhance p97/VCP-CD3δ binding. To test this possibility, gp78CARING, WT gp78, or gp78ΔC49 were co-transfected with HA-CD3δ into 293 cells. HA-CD3δ was co-immunoprecipitated with gp78ΔC49-transfected cells, whereas WT gp78 decreased the levels of CD3δ. These effects were confirmed when the levels of CD3δ were determined in total cell lysates by IB for HA tag in a parallel experiment.
experiment with the same transfection (Fig. 5B). Indeed, gp78CΔRING failed to decrease the level of CD3δ (Fig. 5B, lane 5). As positive controls, gp78 siRNA markedly stabilized CD3δ, whereas WT gp78 decreased CD3δ levels (Fig. 5B, lanes 2 and 3). gp78CΔ49 failed to decrease the levels of CD3δ (Fig. 5B, lane 4). These results indicate that gp78CΔRING enhances p97/VCP-CD3δ binding but does not decrease the levels of CD3δ. This is consistent with the effects of gp78CΔRING on total polyubiquitinated proteins (Fig. 2A, lane 4, right and left). Importantly, both gp78RNAi and gp78CΔ49 increased slowly migrating CD3δ species that are ubiquitinated (data not shown), indicating that a defect in the ability to decrease CD3δ levels is not due to lack of ubiquitination. This is also consistent with our notion that p97/VCP fails to bind and deliver the ubiquitinated CD3δ for degradation in those cells.

To assess whether p97/VCP is indeed involved in regulating CD3δ levels, we studied the effects of a dominant negative mutant p97/VCP, p97/VCPQQ (K251Q/K524Q), on gp78-mediated decrease of CD3δ. The results show that p97/VCPQQ expression inhibits gp78-induced decreases in CD3δ level (Fig. 5C, lanes 3 and 4). On the other hand, p97/VCPQQ-mediated increase of CD3δ was partially reversed by overexpression of gp78 (Fig. 5C, compare lane 2 with lane 4), suggesting that gp78-enhanced p97/VCP-CD3δ binding may have overcome the inhibitory effects of p97/VCPQQ. These data suggest that gp78-p97/VCP interaction is involved in degradation of CD3δ.

gp78-p97/VCP Interaction Regulates CD3δ Degradation—To determine whether changes in levels of CD3δ under the influences of gp78RNAi, WT gp78, and its mutants as well as VCPQQ (Figs. 4 and 5) are due to changes in rates of CD3δ degradation, we utilized cycloheximide chase analysis. CD3δ was co-expressed with siRNA for gp78, WT gp78, gp78CΔ49, gp78CΔRING, or p97/VCPQQ. Degradation of CD3δ was examined by IB after chase for 4 and 8 h. The results showed that WT gp78 facilitated CD3δ degradation (Fig. 6, A (lanes 7–9) and B), gp78RNAi (Fig. 6, A (lanes 4–6) and B) and p97/VCPQQ (Fig. 6, A (lanes 16–18) and B) markedly inhibited CD3δ degradation. This is consistent with the roles for gp78 and VCP in ERAD of CD3δ. gp78CΔ49 expression also inhibited CD3δ degradation (Fig. 6, A (lanes 10–12) and B), supporting the hypothesis that gp78-p97/VCP interaction is involved in CD3δ degradation. Although gp78CΔRING interacts with p97/VCP and enhances CD3δ-p97/VCP binding (Fig. 5, A and B), it failed to decrease CD3δ levels (Fig. 5, A and B) and also inhibited CD3δ degradation (Fig. 6, A (lanes 13–15) and B). We noticed that there was no difference in degradation between WT gp78 and pCNeo vector-transfected cells in this experiment. We reasoned that their differences occur within 4 h of chase. To test this possibility, we performed a similar experiment to that in Fig. 6A but used shorter chase times. Indeed, WT gp78 increases, whereas gp78CΔ49 inhibits, CD3δ degradation compared with that in pCNeo vector-transfected cells (Fig. 6C). These data indicate that gp78-p97/VCP interaction is required for facilitating CD3δ degradation. Based on the fact that gp78CΔRING increases p97/VCP-CD3δ binding but failed to facilitate CD3δ degradation, we propose that a WT gp78 is required for targeting of p97/VCP-bound CD3δ for degradation.

gp78 and p97/VCP Interaction Facilitates Removal of CD3δ from the ER—Because gp78-p97/VCP interaction is likely to enhance p97/VCP-mediated retrotranslocation, we attempted a previously reported retrotranslocation procedure in which proteasomes are inhibited (8). This would allow us to see accumulation of retrotranslocated CD3δ in the cytosol. However, we found that inhibition of proteasome activity also inhibits retrotranslocation of CD3δ in cells and in vitro.3 These results are consistent with previous reports that proteasome activity is coupled with retrotranslocation during ERAD, at least for some ERAD substrates (44, 45). Unfortunately, this coupling prevents us from directly assessing the role of gp78-p97/VCP interaction in retrotranslocation. Therefore, we compared the removal of CD3δ from the ER under the influence of gp78, gp78RNAi, gp78CΔ49, or gp78CΔRING. The results show that WT gp78 facilitates removal of CD3δ from the ER, whereas gp78RNAi, gp78CΔ49, and gp78CΔRING inhibit this effect (Fig. 7, A and B). The changes in removal of CD3δ from the ER correlate well with the ability of gp78 and its mutants to enhance p97/VCP-polysubiquitin binding and degradation. Therefore, in conjunction with the abilities of gp78 to increase

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![Figure 6](http://www.jbc.org/) Effects of gp78 WT, gp78 mutants, gp78 siRNA, and VCPQQ on CD3δ degradation. A, HA-CD3δ was co-transfected into 293 cells with pCNeo, gp78, gp78CΔ49, gp78CΔRING, gp78 siRNA, or VCPQQ. Sixteen hours after transfection, transfected cells were treated with cycloheximide (CHX, 50 μg/ml) for the indicated time followed by evaluation of lysates by IB for HA-CD3δ. Endogenous calnexin was determined by IB in the same lysates. B, graphic presentation of HA-CD3δ degradation from A. C, enhanced degradation of HA-CD3δ by WT gp78 but not gp78CΔ49. 293 cells were transfected as indicated. Cycloheximide chase was performed as in A, but the chase time points are 0.5, 1, and 2 h.
Calnexin was blotted as an ER marker. The removal of CD3 provides additional ubiquitin binding sites for p97/VCP and p47, bind ubiquitin (19). gp78 also binds ubiquitin through its cue domain. One possibility is that when complexed with p97/VCP, gp78 provides additional ubiquitin binding sites for p97/VCP and enhances p97/VCP-polyubiquitin association, as does the Ufd1-Npl4 dimer. Another possibility is that gp78 interaction with p97/VCP enhances the assembly of the p97/VCP-Ufd1-Npl4 complex, resulting in more efficient binding of polyubiquitin. The enhanced polyubiquitin-p97/VCP binding is not due to increases in total polyubiquitinated proteins, since another ERAD E3, hHrd1, also increases total polyubiquitinated protein but does not increase p97/VCP-polyubiquitin association. Moreover, deletion of the p97/VCP-interacting domain preserves the ability of gp78 to increase total polyubiquitin but does not enhance p97/VCP-polyubiquitin binding. Regardless of the mechanism, the observed p97/VCP-polyubiquitin co-immunoprecipitation probably represents polyubiquitin association with p97/VCP complex, not p97/VCP alone.

Functionally, enhancing p97/VCP-polyubiquitin binding suggests that gp78 also facilitates protein degradation after ubiquitination in addition to its role in increasing ubiquitination as an ERAD E3. However, while enhancing p97/VCP-polyubiquitin interactions, gp78, in fact, increases total polyubiquitinated proteins. This result suggests that gp78-enhanced p97/VCP-polyubiquitin binding is not coupled to proteasomal degradation, and an additional factor(s) is required for facilitating degradation of p97/VCP-bound proteins. In agreement with this, we found that gp78, but not its p97/VCP-interacting domain deletion mutant gp78AC49, markedly decreases the levels of polyubiquitinated proteins under ER stress. We speculate that ER stress may induce the additional factor(s) to facilitate degradation of p97/VCP-bound proteins. Indeed, ER stress is known to induce expression of an array of genes, including chaperones for protein folding and ubiquitinating machinery for protein degradation (39, 40). However, in the case of CD3, gp78 enhances CD3-p97/VCP binding and degradation without imposing ER stress. The underlying mechanism for this difference is not known. It is possible that overexpression of CD3 may be sufficient to induce ER stress or the unknown factor(s), which along with gp78, increases CD3 degradation. Another intriguing observation is that ER stress stabilizes gp78 (Fig. 3) and hHrd1.4 This stabilization does not depend on p97/VCP interaction, since gp78AC49 was also stabilized by ER stress. This is surprising in that gp78 itself is an ERAD substrate (30). However, while increasing ubiquitination and degradation of other ERAD substrate, gp78 itself is stabilized under ER stress. Therefore, ER stress not only up-regulates mRNA expression of ERAD E3s as previously reported (36, 39, 40) but may also directly stabilize proteins of these E3s.

gp78-p97/VCP interaction and enhanced p97/VCP-polyubiquitin binding are likely to represent one way to couple ubiquitination with retrotranslocation during ERAD. At least for some ERAD substrates, gp78 performs two roles: ubiquitinating substrates and recruiting p97/VCP to the ER to interact with ubiquitinated substrates. This is supported by the fact that gp78 lacking the p97/VCP-interaction domain fails to enhance p97/VCP-polyubiquitin binding and also fails to increase degradation of the ERAD substrate CD3 and ER stress-induced polyubiquitinated proteins. Moreover, CD3 is highly ubiquitinated in gp78AC49-transfected cells, suggesting an absence of the necessary step to target ubiquitinated CD3 for degradation. It is known that multiple pathways operate to degrade unwanted proteins from the ER (45, 47–50). In agreement with this, a recent study describes the discovery of a novel ER membrane-anchored protein VIMP that interacts with p97/VCP and recruits p97/VCP to Derlin1.

FIG. 7. Removal of CD3 from the ER. A, 293 cells were transfected as indicated. Sixteen hours after transfection, cells were fractionated into microsomes (m) and cytosol (c). CD3 was determined by IB. Calnexin was blotted as an ER marker. B, quantitative data of CD3 remaining in the ER.

CD3 degradation and CD3-p97/VCP binding, the enhanced removal of CD3 from the ER by gp78 supports the hypothesis that gp78-p97/VCP interaction couples ubiquitination with retrotranslocation and proteasomal degradation during ERAD. However, further study is required to establish a retrotranslocation assay to elucidate the exact mechanism.

DISCUSSION

In this study, we demonstrated that gp78, a previously identified ERAD E3 and the receptor for the tumor autocrine motility factor, interacts with p97/VCP and ubiquitin. The interaction with p97/VCP enhances p97/VCP-polyubiquitin association. The enhanced association decreases the levels of ER stress-induced polyubiquitinated proteins. By analyzing the ERAD substrate CD3, we demonstrated that gp78-p97/VCP interactions enhance p97/VCP-CD3 binding and facilitate CD3 degradation. The results suggest that gp78-p97/VCP interaction may represent one mechanism for coupling ubiquitination, retrotranslocation, and proteasomal degradation during ERAD.

How gp78 enhances p97/VCP-polyubiquitin association is not known. It is likely that polyubiquitin may not directly bind to p97/VCP but rather mediates the interaction through a complex of p97/VCP-interacting proteins. There have been several reports that p97/VCP by itself interacts weakly with polyubiquitin (19, 21, 46). p97/VCP-associated proteins enhance its affinities for ubiquitin (19). All three of its cofactors, Ufd1, Npl4, and p47, bind ubiquitin (19, 21). It has been demonstrated that association of Ufd1-Npl4 dimer enhances p97/VCP-polyubiquitin binding by providing ubiquitin-binding sites for p97/VCP. As a novel p97/VCP-interacting protein, gp78 also binds ubiquitin through its cue domain. One possibility is that when complexed with p97/VCP, gp78 provides additional ubiquitin binding sites for p97/VCP and

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a component of a proposed channel for retrotranslocation during ERAD (27). This intriguing proposal also leads us to speculate that as multiple membrane-spanning protein, gp78 may also form a channel for retrotranslocation. Thus, gp78 ubiquitinates ERAD substrates and provides a channel and recruits p97/VCP for retrotranslocation during ERAD.

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