The Role of a Novel p97/Valosin-containing Protein-interacting Motif of gp78 in Endoplasmic Reticulum-associated Degradation

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Petek Ballar†§1, Yuxian Shen†, Hui Yang‡ and Shengyun Fang†§2

From the †Medical Biotechnology Center, University of Maryland Biotechnology Institute, Baltimore, Maryland 21201, §The Institute of Clinical Pharmacology, Anhui Medical University, Hefei, P. R. China, and the ‡Program in Molecular Medicine, University of Maryland School of Medicine, Baltimore, Maryland 21201

Improperly folded proteins in the endoplasmic reticulum (ER) are eliminated via ER-associated degradation, a process that dislocates misfolded proteins from the ER membrane into the cytosol, where they undergo proteasomal degradation. Dislocation requires a subclass of ubiquitin ligases that includes gp78 in addition to the AAA ATPase p97/VCP and its cofactor, the Ufd1-Npl4 dimer. We have previously reported that gp78 interacts directly with p97/VCP. Here, we identify a novel p97/VCP-interacting motif (VIM) within gp78 that mediates this interaction. We demonstrate that the VIM of gp78 recruits p97/VCP to the ER, but has no effect on Ufd1 localization. We also show that gp78 VIM interacts with the ND1 domain of p97/VCP that was shown previously to be the binding site for Ufd1. To evaluate the role of Ufd1 in gp78-p97/VCP-mediated degradation of CD3δ, a known substrate of gp78, RNA interference was used to silence the expression of Ufd1 and p97/VCP. Inhibition of p97/VCP, but not Ufd1, stabilized CD3δ in cells that overexpress gp78. However, both p97/VCP and Ufd1 appear to be required for CD3δ degradation in cells expressing physiological levels of gp78. These results raise the possibility that Ufd1 and gp78 may bind p97/VCP in a mutually exclusive manner and suggest that gp78 might act in a Ufd1-independent degradation pathway for misfolded ER proteins, which operates in parallel with the previously established p97/VCP-Ufd1-Npl4-mediated mechanism.

The endoplasmic reticulum (ER) is the first compartment in the secretory pathway (1, 2). Proteins destined for the secretory pathway are translocated into the lumen or inserted into the membrane of the ER where they are properly folded and modified before being delivered to their functional destinations (1, 2). However, protein folding in the secretory pathway is an imperfect process that generates misfolded products (3, 4). These misfolded proteins are retained in the ER by a quality-control mechanism and eliminated through a process known as ER-associated degradation (ERAD) (3–6). ERAD requires retrotranslocation, in which misfolded proteins are dislocated from the ER membrane into the cytosol where they are degraded by the proteasomes (7). Genetic evidence from yeast indicates that the ATPase CDC48 in combination with its cofactors, the Ufd1-Npl4 dimer, are essential for retrotranslocation (8). The mammalian homolog of CDC48, p97/VCP, in concert with Ufd1-Npl4 dimer, appears to play a similar role in mammalian cells (9, 10).

p97/VCP, Ufd1, and Npl4 are cytosolic proteins. They must be recruited to the cytosolic surface of the ER to carry out the retrotranslocation process. In yeast, an ER membrane-anchored protein, Ubx2, plays a central role in coupling ubiquitination with retrotranslocation. Ubx2 recruits CDC48 along with the Ufd1-Npl4 dimer using its Ubx domain (11, 12), thereby linking the CDC48-Ufd1-Npl4 complex to ER-anchored ERAD E3s, that include Hrd1 and Doa10, as well as ERAD substrates (see a diagrammatic illustration of this complex in Fig. 1a) (11, 12). Recent studies suggest that more complicated mechanisms exist for coupling ubiquitination with retrotranslocation in higher eukaryotes (Fig. 1b) (13–15). In mammalian systems, p97/VCP interacts with a protein complex that contains at least four ER membrane-anchored proteins, the p97/VCP-interacting membrane protein (VIMP), Derlin-1, Derlin-2, and ERAD E3 Hrd1. The Ufd1-Npl4 dimer is also recruited along with p97/VCP by the four-protein complex (Fig. 1b) (16). This is consistent with previous reports that the p97/VCP-Ufd1-Npl4 complex is functionally required for ERAD in mammalian cells (9, 10). gp78, a multimembrane-spanning ERAD E3, also interacts with p97/VCP (13, 17–19). Functionally, the gp78-p97/VCP complex enhances the binding of p97/VCP to polyubiquitinated proteins, as well as degradation of ERAD substrates, including CD3δ, apolipoprotein B100, and 3-hydroxy-3-methylglutaryl-CoA reductase (18–20). However, the role of p97/VCP cofactors, Ufd1 and Npl4, in gp78-mediated ERAD is yet to be determined.
gp78-mediated ERAD

In this study, we have identified a novel p97/VCP-interacting motif (VIM) in the cytosolic tail of gp78 and in the N-terminal region of the small p97/VCP-interacting protein (SVIP) (21). The VIM of gp78 interacts with the ND1 domain of p97/VCP, the reported binding site for SVIP, Ufd1, and p47, another p97/VCP cofactor required for Golgi and ER membrane fusion (22, 23). SVIP, Ufd1, and p47 are known to bind p97/VCP in a mutually exclusive manner. In the current study, our data suggest that gp78 and Ufd1 also bind p97/VCP in a mutually exclusive fashion. Functionally, gp78-mediated ERAD apparently requires p97/VCP but not Ufd1. We have also confirmed the presence of an Ufd1-dependent ERAD pathway. Collectively, these data suggest that gp78 may mediate an Ufd1-independent degradation pathway for misfolded ER proteins. This pathway appears to operate in parallel with the previously established p97/VCP-Ufd1-Npl4-mediated mechanism.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Antibodies**—Mouse Ufd1 with an N-terminal FLAG tag was cloned into the pcDNA3.1 vector to generate pcDNA3-FLAG-Ufd1. To construct pFLAG-His-Npl4, His-Npl4 cDNA was excised from pQE9-Npl4 (generously provided by Dr. Chou-chi Li, NCI-Frederick) and then cloned into the pFLAG-CMV vector. The pFLAG-CMV-N (aa 1–198), ND1 backbone was assembled into the pCD48 vector (pCD48-ND1) and then cloned into the pcDNA3.1 vector to generate pcDNA3-FLAG-Ufd1. To construct pFLAG-His-Npl4, His- Npl4 cDNA was excised from pQE9-Npl4 (generously provided by Dr. Hemmo Meyer (Swiss Federal School of Technology, Switzerland) and purified by BD Biosciences. Monoclonal anti-gp78 antibody (clone 2G5) was generated using recombinant GST-gp78C as an antigen. 2G5 recognizes the epitope located between amino acids 497–578 of gp78.

**Fractionation, Immunoprecipitation, and Immunoblotting**—For subcellular fractionation, 293 cells were transfected as indicated in the figure legends. Cell fractionation was carried out by homogenization in fractionation buffer containing 50 mM Tris-HCl (pH 8), 1 mM 2-mercaptoethanol, 1 mM EDTA, 10 mM triethanolamine, and 0.32 M sucrose via passage through a 27-gauge syringe 20 times. Homogenates were centrifuged at 1000 × g to sediment unbroken cells, cell debris, and nuclei (pellet fraction (p)). The remaining supernatant was separated by centrifugation at 105,000 × g for 60 min. The resulting pellet contained the microsomal fraction (m) and the supernatant contained the cytosolic fraction (c). All three fractions (m, c, and p) were further processed for immunoblotting. Immunoprecipitation and immunoblotting were carried out following previously published protocols (17).

**Interaction between GST-VIM and Purified p97/VCP—**His-tagged p97/VCP, Ufd1, and Npl4 were expressed in *E. coli* after induction with isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested and lysed by sonication in buffer containing 500 mM KCl, 100 mM Tris/HCl (pH 7.4), 5 mM MgCl<sub>2</sub>, 1 mM ATP, 4 mM dithiothreitol, 20 mM imidazole. His-tagged p97/VCP, Ufd1, and Npl4 in lysates were affinity-purified using nickel-nitrilotriacetic acid-agarose beads (Qiagen) and eluted with lysis buffer containing 300 mM imidazole. Imidazole was removed by dialysis against 1× phosphate-buffered saline containing 1 mM dithiothreitol. The in vitro binding assay was carried out essentially as previously reported (25). Briefly, 3 μg of GST-VIM immobilized and purified on glutathione agarose beads was incubated with His-tagged p97/VCP, Ufd1, and Npl4 proteins (2 μg each) for 1 h at 4°C in binding buffer containing 25 mM Tris/HCl (pH 8.0), 200 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM ATP, 1 mM dithiothreitol, 5% glycerol, and 1% Triton X-100. After washing, GST-VIM-bound proteins were analyzed by SDS-PAGE followed by Coomassie Blue staining.

**Fluorescence Microscopy—**U2OS cells were cultured on coverslips in 6-well dishes and transfected with the indicated plasmids using Lipofectamine 2000. For double immunofluorescence staining, gp78 and endogenous p97/VCP were labeled using rabbit anti-gp78 and mouse anti-p97/VCP antibodies in conjunction with Alexa Fluor-594 and Alexa Fluor-488. A similar procedure was used to label gp78 and Ufd1, as well as Hrd1/Der1 plus VIM and p97/VCP. Staining was performed as described previously (17). Fluorescent microscopy was performed using a Zeiss Axiovert 200M fluorescent microscope.

**Establishment of 293 Cell Lines That Stably Express HA-CD38 or HA-CD38 and gp78—**293 cells were transfected with plasmids encoding HA-CD38 or HA-CD38 and gp78. pBABE vector conferring puromycin-resistance was co-trans-
Identification of a Novel p97/VIM in gp78 and SVIP—Many p97/VCP-interacting proteins have been reported (26–28). In search of a common p97/VCP-interacting domain that might be present in different p97/VCP-interacting proteins, we identified a conserved amino acid sequence within the C-terminal portion of gp78 and the N-terminal region of SVIP, both of which have previously been found to bind P97/VCP (18, 21). We named this sequence the p97/VCP-interacting motif (VIM). VIM comprises the C-terminal 30 amino acids of gp78 and is well conserved in vertebrates (examples are given in Fig. 2a). However, it is not present in the yeast Cee1p protein or Caenorhabditis elegans gp78. Consistently, Cee1p and C. elegans gp78 do not interact with p97/VCP (supplemental Fig. S1).

Bioinformatic analysis predicts a helix formed by 17 amino acids (aa 625–641 in gp78 and aa 20–35 in SVIP, underscored in Fig. 2a) within the VIM of human gp78 and SVIP (29). To ascertain whether the VIM of gp78 is sufficient to bind p97/VCP, we assessed its ability to recruit p97/VCP to microsomal membranes. 293 cells were transfected with wt gp78 or its VIM deletion mutant (gp78ΔVIM) (Fig. 2b). Transfected cells were fractionated into cytosolic (c) and ER-containing microsomal (m) fractions where gp78 is generally localized, which we have previously reported (17). Wild-type (wt) gp78 efficiently moves P97/VCP from the cytosol to the microsomes as determined by immunoblotting (Fig. 2, b and c, lanes 4 and 5 versus lanes 1 and 2). Deletion of VIM from gp78 (gp78ΔVIM) largely diminishes this effect (Fig. 2b, lanes 10 and 11 versus lanes 4 and 5). To investigate the role of the E3 activity of gp78 in relocalization of p97/VCP, we examined the effects of

**RESULTS**

**Identification of a p97/VCP-interacting motif (VIM) in gp78 and SVIP.** a, alignments of the VIM of gp78 and SVIP. Hs, human; Rn, rat; Mm, mouse; aa, amino acid. Underlined is the predicted sequence that forms a helix. b, 293 cells were transfected with plasmids encoding wt gp78, or gp78ΔVIM, or gp78R2m. Empty vector was transfected as a negative control. Eighteen hours after transfection, cells were fractionated into membrane (m) and cytosol (c). The initial pellet (p) obtained from clear homogenates was retained to monitor total levels of proteins. Endogenous p97/VCP was revealed by immunoblotting using monoclonal antibody. p97/VCP in the microsome (m) and cytosolic (c) fractions are framed for easy comparison. Transfected gp78 was detected by immunoblotting using polyclonal anti-gp78 antibodies that do not recognize endogenous gp78 as previously reported (17). gp78 tends to form high molecular weight smears (arrow) due to oligomerization and autoubiquitination (17), *, residual signal of p97/VCP from the first blotting for p97/VCP. Calnexin was blotted as an ER marker. c, 293 cells were transfected with plasmids encoding wt gp78 or empty vector as control. Transfected cells were analyzed as in b. Endogenous p97/VCP and Ufd1 were blotted using monoclonal antibodies.

**Pulse-chase Analysis**—Pulse-chase analysis was performed essentially as we have previously described (17). CD3δ clone-5 and CD3δ/gp78 clone-20 cells with a knockdown of either p97/VCP or Ufd1 were pulse-labeled with a mixture of L-[35S]methionine and L-[35S]cysteine (Redivue™ Pro-mix L-[35S]) in vitro cell labeling mix, GE HealthCare) at a concentration of 200 μCi/ml for 30 min. After labeling, unincorporated L-[35S]methionine and L-[35S]cysteine were removed and chased for 0, 2, 4, and 6 h. Labeled cells were processed for immunoprecipitation of HA-tagged CD3δ followed by SDS-PAGE. Radioactive CD3δ was detected by a Typhoon scanner (Amersham Biosciences) and quantified by ImageQuaNT (Molecular Dynamics).

**FIGURE 2. Identification of a p97/VCP-interacting motif (VIM) in gp78 and SVIP.** a, alignments of the VIM of gp78 and SVIP. Hs, human; Rn, rat; Mm, mouse; aa, amino acid. Underlined is the predicted sequence that forms a helix. b, 293 cells were transfected with plasmids encoding wt gp78, or gp78ΔVIM, or gp78R2m. Empty vector was transfected as a negative control. Eighteen hours after transfection, cells were fractionated into membrane (m) and cytosol (c). The initial pellet (p) obtained from clear homogenates was retained to monitor total levels of proteins. Endogenous p97/VCP was revealed by immunoblotting using monoclonal antibody. p97/VCP in the microsome (m) and cytosolic (c) fractions are framed for easy comparison. Transfected gp78 was detected by immunoblotting using polyclonal anti-gp78 antibodies that do not recognize endogenous gp78 as previously reported (17). gp78 tends to form high molecular weight smears (arrow) due to oligomerization and autoubiquitination (17), *, residual signal of p97/VCP from the first blotting for p97/VCP. Calnexin was blotted as an ER marker. c, 293 cells were transfected with plasmids encoding wt gp78 or empty vector as control. Transfected cells were analyzed as in b. Endogenous p97/VCP and Ufd1 were blotted using monoclonal antibodies.
gp78R2m on p97/VCP localization in cytosol and microsomal fractions. gp78R2m is an E3-inactive mutant form of gp78 with two defective zinc ligand residues in the RING finger (17). The results showed that gp78R2m recruits p97/VCP from the cytosol to the microsomes (Fig. 2b, lanes 7 and 8 versus lanes 1 and 2) as efficiently as wt gp78 (Fig. 2b, lanes 4 and 5 versus lanes 7 and 8). These results strongly suggest that the VIM of gp78 interacts with and recruits p97/VCP from the cytosol to the ER and gp78 E3 activity is not required for p97/VCP relocalization.

A previous study has shown that VIMP, another ER-anchored p97/VCP-interacting membrane protein, co-localizes with p97/VCP in the ER (16). Importantly, VIMP interacts with p97/VCP along with its cofactor, the Ufd1-Npl4 dimer (16). To determine whether gp78 could recruit p97/VCP along with Ufd1, a similar fractionation experiment to that shown in Fig. 2b was performed and the effects of gp78 on localization of endogenous Ufd1 were examined by immunoblotting. According to this data, gp78 has no apparent effect on Ufd1 localization despite the fact that p97/VCP is relocated to the microsomes (Fig. 2c, lanes 1 and 2 versus lanes 3 and 4). These results suggest that gp78 interacts only with p97/VCP and that Ufd1 is not a part of the gp78-p97/VCP complex (Fig. 2c, lanes 1 and 2 versus lanes 4 and 5).

Based on previous studies showing that SVIP, Ufd1, and p47 bind to the ND1 domain of p97/VCP in a mutually exclusive manner (21), we reasoned that gp78 might also bind to the p97/VCP ND1 domain. A GST pull-down assay with GST fusion of the VIM of gp78 (GST-VIM) was designed to test this hypothesis. Various truncation mutants of p97/VCP (N (aa 1–198), ND1 (aa 199–470), D1 (aa 471–806) domain) tagged with FLAG were generated based on structural studies that pinpointed key structural domains of p97/VCP (Fig. 3a) (24). In pull-down assays with 293 cell lysates, the VIM of gp78 interacted only with proteins containing the ND1 domain of p97/VCP (Fig. 3b, lanes 1 and 2). Therefore, like Ufd1, p47, and SVIP, gp78 also binds to the ND1 domain of p97/VCP.

**GST-VIM Interacts with p97/VCP but Not the p97/VCP-Ufd1-Npl4 Complex**—The interaction of gp78 with the ND1 domain of p97/VCP suggests that gp78 and Ufd1 might form mutually exclusive complexes with p97/VCP. To test this possibility, GST-VIM pull-down assays of lysates from 293 cells transfected with FLAG-tagged p97/VCP or FLAG-tagged N, ND1, D1, or D2 domain of p97/VCP were used for pull-down assays with GST-VIM from gp78. Only the full-length p97/VCP and its ND1 domain were pulled down by GST-VIM (lanes 1 and 2). Whole cell lysates (WCL) used for pull-down are shown in lanes 6–10. The lower panel shows Coomassie Blue staining of GST-VIM purified on glutathione agarose beads used in the pull-down assay.

Analysis of the precipitates by immunoblotting indicates that, indeed, p97/VCP, Ufd1, and Npl4, appear to be capable of forming a complex (Fig. 4b, lane 8) under conditions used for the GST-pull-down assay (Fig. 4a). Therefore, it is plausible that GST-VIM forms a complex with p97/VCP and excludes Ufd1-Npl4 from the complex.

**FIGURE 3. The VIM of gp78 interacts with the ND1 domain of p97/VCP.** a, diagrammatic representation of p97/VCP and its mutants with N-terminal FLAG (FL) tag. b, GST-VIM interacts with the ND1 domain of p97/VCP. Lysates from 293 cells transfected with FLAG-tagged p97/VCP or FLAG-tagged N, ND1, D1, or D2 domain of p97/VCP were used for pull-down assays with GST-VIM from gp78. Only the full-length p97/VCP and its ND1 domain were pulled down by GST-VIM (lanes 1 and 2). Whole cell lysates (WCL) used for pull-down are shown in lanes 6–10. The lower panel shows Coomassie Blue staining of GST-VIM purified on glutathione agarose beads used in the pull-down assay.
gp78-mediated ERAD

To test this possibility further, we

generated a construct that expresses

a fusion protein of VIM to the C ter-

minus of Hrd1, another ER-an-

chored E3. Double immunofluores-

cent staining revealed that Hrd1

alone has minimal effects on the

localization of p97/VCP (Fig. 5d).

However, fusion of VIM to Hrd1

(Hrd1 plus VIM) dramatically

increases co-localization of p97/

VCP with Hrd1 plus VIM (Fig. 5e).

Interestingly, the co-localized p97/

VCP and Hrd1 plus VIM exhibit a

punctate pattern in the cytoplasm

of the cells. Because Hrd1 is an ER

protein, the punctate structures

are likely to be derived from the ER.

These results further indicate that

the VIM of gp78 mediates the

recruitment of p97/VCP from the
cytosol to the ER.

gp78 recruits p97/VCP but does

not appear to recruit Ufd1 to the

microsomes by fractionation assay

(Fig. 2c). To confirm this unex-

pected result, immunofluorescent

staining was utilized to further test

the effect of gp78 on Ufd1 localiza-

tion in cells. We co-transfected

U2OS cells with plasmids that

encoded FLAG-tagged Ufd1 and wt

gp78. Transfected cells were doubly

labeled with monoclonal anti-FLAG

antibody for Ufd1 and polyclonal

anti-gp78 antibodies. Consistent

with the fractionation results (Fig.

2c), we found that gp78 does not affect

Ufd1 localization in cells

(Fig. 5f). Together, these results suggest that gp78 recruits p97/

VCP but not the p97/VCP-Ufd1-Npl4 complex to the ER.

Ufd1 Is Dispensable in gp78-mediated Degradation of CD3β—

To analyze the involvement of p97/VCP and its cofactor Ufd1-

Npl4 dimer in the gp78-mediated ERAD of CD3β, we

established 293 cell lines that stably express CD3β and gp78

(generating CD3β/gp78 clones, Fig. 6, lanes 7–12) or CD3β

alone (our control: CD3β clones, Fig. 6, lanes 1–6). In both cell

lines, CD3β was rapidly degraded as revealed by CHX chase

analysis, a technique widely used in determining degradation of

ERAD substrates in both yeast and mammalian cells (31–35).

Steady-state levels of CD3β were lower in CD3β/gp78 clones

(Fig. 6, lanes 1–6 versus 7–12), which may be due to enhanced
degradation of CD3β associated with gp78 overexpression. The
level of gp78 is approximately four times higher in CD3β/gp78
clones than that in CD3β clones (Fig. 6, lanes 7–12 versus 1–6).
gp78 levels also decrease during a CHX chase (Fig. 6). This is
consistent with our previous report that gp78 is an unstable
protein degraded through ERAD (17).

FIGURE 4. GST-VIM interacts with p97/VCP, but not the p97/VCP-Ufd1-Npl4 complex. a, GST-VIM pulls down p97/VCP, but not p97/VCP-Ufd1-Npl4 complex. Lysates prepared from 293 cells transfected with FLAG-Ufd1 and FLAG-6His-Npl4 were used for GST-VIM pull-down. p97/VCP, but not Ufd1 and Npl4, were pulled down by GST-VIM (lanes 9–12). GST alone pulls down neither p97/VCP nor Ufd1 or Npl4 (lanes 5–8). Whole cell lysates (WCL) used for the pull-down assay are shown in lanes 1–4. b, p97/VCP, FLAG-Ufd1, and FLAG-6His-Npl4 form complex in cells. 293 cells transfected as in lanes 1–4 were lysed and used for nickel-nitrilotriacetic acid-agarose-bead pull-down of FLAG-6His-Npl4. p97/VCP and FLAG-Ufd1 are associated with FLAG-6His-Npl4 (lane 8). WCL used for pull-down is shown in lanes 1–4. c, GST-VIM of gp78 interacts with purified recombinant p97/VCP. Two micrograms each of purified His-tagged p97/VCP, Ufd1, and Npl4 were incubated with 3 μg of GST-VIM or GST immobilized on beads. After washing, GST-VIM-bound proteins were detected by Coomassie Blue staining. Lanes 1, molecular weight marker; 2, 6His-p97/VCP; 3, 6His-Ufd1; 4, 6His-Npl4; 5, GST-VIM alone; 6, GST-VIM plus 6His-p97/VCP plus 6His-Ufd1 plus 6His-Npl4; 7, GST-VIM plus 6His-p97/VCP plus 6His-Ufd1 plus 6His-Npl4, d, schematic illustration of results from a to c. p97/VCP forms a mutually exclusive complex with GST-VIM and Ufd1-Npl4 dimer.
gp78-mediated ERAD

The requirement of p97/VCP and the Ufd1-Npl4 dimer in degradation of CD3δ were investigated in CD3δ/gp78 and CD3δ clones via RNA interference (RNAi)-mediated silencing of p97/VCP and Ufd1 expression. RNAi was first carried out in cells derived from CD3δ/gp78 clone-20, a randomly selected clone (Fig. 6, lanes 10–12). More than 90% reduction in the levels of p97/VCP and Ufd1 proteins was achieved by transfection with their specific siRNAs (Fig. 7a). p97/VCP knockdown leads to a dramatic stabilization of CD3δ (Fig. 7a, lanes 2–5), whereas inhibition of Ufd1 expression results in minimal effects (Fig. 7a, lanes 6 and 7). To confirm these results, we performed CHX chase analysis. As shown (Fig. 7b), degradation of CD3δ is largely diminished in p97/VCP knockdown cells (lanes 5–8 versus 1–4), whereas Ufd1 knockdown leads to stabilization of only a small amount of CD3δ that can be rapidly degraded (lanes 9–12 versus 1–4). Using a co-immunoprecipitation assay, we found that p97/VCP but not Ufd1 co-precipitates with gp78 in CD3δ/gp78 clone-20 cells (Fig. 7c, lane 4). However, when p97/VCP was immunoprecipitated from the same cells, Ufd1 was readily detected in precipitates (Fig. 7c, lane 5) supporting the notion that p97/VCP forms mutually exclusive complexes with either gp78 or Ufd1 in CD3δ/gp78 cells (Fig. 2c, lane 4).

To examine the requirement for Ufd1 in CD3δ degradation in CD3δ clone-5 cells, we silenced Ufd1 or p97/VCP in these cells. As in CD3δ/gp78 clone-20 cells, p97/VCP siRNAs markedly stabilizes CD3δ in CD3δ clone-5 cells (Fig. 8, a (lanes 3–6) and b (lanes 5–8)). Although Ufd1 siRNA has minimal effects on CD3δ degradation in CD3δ/gp78 clone-20 cells (Fig. 7, a (lanes 7 and 8) and b (lanes 9–12)), it significantly stabilizes CD3δ in CD3δ clone-5 cells (Fig. 8, a (lanes 7 and 8) and b (lanes 9–12)), suggesting that Ufd1 participates in CD3δ degradation in CD3δ clone-5 cells.

We next asked whether the difference in the steady-state levels of CD3δ between CD3δ/gp78 clone-20 and CD3δ clone-5 cells.
However, knockdown of p97/VCP leads to marked stabilization of CD3ε in cells overexpressing gp78. Effects of knockdown of p97/VCP and Ufd1 on the levels of CD3ε in these cells were evaluated by immunoblotting. The results show that, although the steady-state levels of CD3ε are lower, knockdown of Ufd1 produces significant stabilization of CD3ε in cells with physiological levels of gp78 and higher levels of CD3ε in cells overexpressing gp78. Effects of knockdown of p97/VCP and Ufd1 on the levels of CD3ε in these cells were evaluated by immunoblotting. The results show that, although the steady-state levels of CD3ε are lower, knockdown of Ufd1 produces significant stabilization of CD3ε in cells with physiological levels of gp78 (Fig. 8c, lanes 1 and 3). Little effect on CD3ε levels is observed in cells that overexpress gp78 (Fig. 8c, lanes 4 and 6). However, knockdown of p97/VCP leads to marked stabilization of CD3ε in both cell types (Fig. 8c, lanes 2 and 5). These results are identical to those for CD3ε/gp78 clone-20 (Fig. 7a) and CD3ε clone-5 (Fig. 8a). Therefore, we conclude that any differences we observed in responses to Ufd1 knockdown in CD3ε/gp78 clone-20 and CD3ε clone-5 are not due to differences in their steady-state levels of CD3ε. Rather, the p97/VCP-Ufd1-Npl4 complex is involved in CD3ε degradation in CD3ε clone-5 cells, whereas Ufd1 is dispensable for CD3ε degradation in clone-20 cells that overexpress gp78. To determine if this Ufd1-independent degradation is true for other gp78 substrate, we examined the effects of p97/VCP and Ufd1 knockdown on the levels of the Z variant of α-1-antitrypsin, a newly identified gp78 substrate.4 The knockdown was carried out in 293 cells that stably express the Z variant of α-1-antitrypsin. The results showed that silencing p97/VCP but not Ufd1 stabilizes the Z variant of α-1-antitrypsin (supplemental Fig. S2), which further supports the possibility that gp78 mediates a Ufd1-independent ERAD pathway.

To investigate whether gp78 is involved in CD3ε degradation in CD3ε clone-5 cells, gp78 expression was inhibited by RNAi, and degradation of CD3ε was evaluated by CHX chase. Our results show that silencing of gp78 expression significantly stabilizes CD3ε (Fig. 8d, lanes 5–8 versus 1–4), indicating that gp78 facilitates CD3ε degradation in CD3ε clone-5 cells.

We then examined the Ufd1 dependence of the gp78-mediated ERAD in CD3ε clone-5 cells. The Ufd1 dependence was determined by comparing the stabilization effects on CD3ε in gp78 or Ufd1 single knockdown and gp78-Ufd1 double knock-

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4 Yang Shen, Pallav Baller, and Shao Dong Fang, unpublished observation.
If gp78 ubiquititates CD3/H9254, and then the p97/VCP-Ufd1-Npl4 complex retrotranslocates the ubiquitinated CD3/H9254 from the ER for degradation, we would expect little or no additive inhibitory effects on CD3/H9254 degradation by a double knockdown of gp78 and Ufd1. However, the results from our CHX chase experiments show that the double knockdown of Ufd1 and gp78 produces a significant additive effect in the inhibition of CD3/H9254 degradation (Fig. 8e, lanes 9–12). In addition, individual knockdown of gp78 or Ufd1 resulted in similar inhibitory effects (Fig. 8e, lanes 1–4 versus 5–8) suggesting that both gp78- and Ufd1-mediated pathways may contribute significantly to CD3/H9254 degradation in CD3/H9254 clone-5 cells. Interestingly, knockdown of Ufd1 appears to stabilize gp78 (Fig. 8e, lanes 1–4). This stabilization effect may represent a compensatory mechanism to ensure ERAD in the shortage of Ufd1.

To assess the involvement of p97/VCP and Ufd1 in CD3/H9254 degradation in a more quantitative way, we utilized pulse-chase analysis of CD3/H9254 degradation determined by pulse-chase analysis. a, CD3δ clone-5 and CD3δ/gp78 clone-20 cells with knockdown of p97/VCP or Ufd1 were analyzed by pulse-chase. C: control siRNA; V1: VCP siRNA1; U: Ufd1 siRNA. b and c, densitometry quantification of CD3δ degradation from three independent pulse-chase experiments. The data were analyzed by ImageQuaNT and was expressed as mean ± S.D.
CD3δ degradation in an Ufd1-independent manner. In addition, the results help explaining why the steady-state levels of CD3δ are lower in clone-16 and -20 cells (Fig. 6). Collectively, our results suggest the presence of at least two pathways for CD3δ degradation in cells with physiological levels of ERAD machinery: the p97/VCP-Ufd1-Npl4 complex-mediated pathway and the gp78-mediated and Ufd1-independent pathway.

DISCUSSION

Studies of both yeast and higher eukaryotes have indicated that the Ufd1-Npl4 dimer is indispensable for CDC48/p97/VCP-mediated retrotranslocation of ubiquitinated ER proteins (7, 8). Here, we provide evidence to suggest a novel mechanism by which gp78-mediated ubiquitination is coupled with p97/VCP-mediated retrotranslocation without requiring Ufd1 (see a diagrammatic illustration of this model in Fig. 10). The molecular mechanism underlying this Ufd1-independent pathway is mediated through a novel p97/VCP-interacting motif, i.e. VIM of gp78. The VIM of gp78 interacts with the ND1 domain of p97/VCP, which is also the binding site for Ufd1 (36, 37). Sharing of the interaction site leads gp78 and Ufd1 to form mutually exclusive complexes with p97/VCP (Fig. 4d). Therefore, gp78 physically excludes Ufd1 from the gp78-p97/VCP complex, thereby functionally excluding Ufd1 from gp78-mediated ERAD. However, our data does not exclude the possibility that other p97/VCP cofactor(s) might be involved in gp78-p97/VCP-mediated ERAD (Fig. 10).

The Ufd1-independent degradation of CD3δ becomes dominant in cells that overexpress gp78 as demonstrated by our RNAi experiments in CD3δ/gp78 clone-20 cells. Our data also support the presence of this pathway in cells that express endogenous levels of gp78. First, knockdown of gp78 stabilizes CD3δ in CD3δ clone-5 cells, indicating that endogenous gp78 is involved in ERAD (Fig. 8c). Second, although knockdown of Ufd1 accumulates CD3δ, CD3δ degradation still occurs although at a slower rate (Figs. 8b, 9a, and 9b). This suggests that an Ufd1-independent pathway exists in CD3δ clone-5 cells. Third, simultaneous knockdown of gp78 and Ufd1 results in a significant additive effect on CD3δ stabilization in CD3δ clone-5 cells (Fig. 8d). When expression of both gp78 and Ufd1 are almost abolished (>90% reduction), this additive inhibitory effect would only be possible if gp78 and Ufd1 act in parallel independent pathways. If gp78 and Ufd1 work sequentially, that is, gp78 mediates ubiquitination of CD3δ and then Ufd1 in conjunction with p97/VCP and Npl4 retrotranslocates the ubiquitinated CD3δ for degradation, knockdown of Ufd1 would have little effect when the function of gp78 is diminished. If gp78 and Ufd1 work in parallel in different degradation pathways, that is, they act in independent pathways on CD3δ degradation, simultaneous knockdown would block both pathways and produce significant additive effects of inhibition, which is what we have demonstrated. In contrast, p97/VCP is essential to both gp78 and Ufd1-mediated pathways. p97/VCP knockdown significantly stabilizes CD3δ regardless the levels of gp78 expression (Figs. 7, 8a, 8b, 9a, 9b, and 9c). This suggests that both Ufd1-dependent and -independent pathways require the function of p97/VCP.

Studies have suggested that the p97/VCP-Ufd1-Npl4 complex is recruited to the ER by an ER-localized multiprotein complex through multiple interactions (13, 14, 16, 38) (Fig. 1b). In addition, other cytosolic ERAD components are also recruited to the ER by the multiprotein complex. For examples, both Derlin-1 and p97/VCP interact with the peptide:N-glycanase, a key enzyme that removes N-glycan from misfolded ER proteins before proteasomal degradation (39, 40). Peptide:N-glycanase also interacts with hHR23B, a polyubiquitin-binding protein that chaperones polyubiquitinated ERAD substrates to the proteasomes for degradation (39, 41). HERP, an ER stress-inducible protein, has recently been identified as a new member of the ERAD complex (14). However, how HERP regulates ERAD remains unknown. Nevertheless, there appears to be a large protein complex in the ER, which integrates recruitment, ubiquitination, retrotranslocation, deglycosylation, and proteasomal targeting of misfolded ER substrates during ERAD. The functional details and the dynamics of the complex are yet to be determined.

This report and recent studies by others suggest that gp78 may be the central player in another ERAD pathway. gp78 acts not only as an ERAD E3, but also as a scaffold protein to assemble a complex that couples ubiquitination, retrotranslocation, and deglycosylation. gp78 has a multifunctional cytosolic tail that conveys E3 activity, binds polyubiquitin, and recruits Ubc7 and p97/VCP (17, 18, 35). Recent evidence indicates that p97/VCP recruited by gp78 is associated with peptide:N-glycanase (39, 42). We have now demonstrated that gp78 has a p97/VIM responsible for recruiting p97/VCP to the ER. Because gp78 and Ufd1 compete for binding to p97/VCP, gp78 recruits only p97/VCP but not the p97/VCP-Ufd1-Npl4 complex to the ER. Consistent with the absence of Ufd1 in the gp78-p97/VCP complex, our evidence also suggests that Ufd1 is dispensable in gp78-mediated CD3δ degradation. Other p97/VCP cofactor(s), however, may be involved in gp78-mediated ERAD (Fig. 10).

Ufd1-independent ERAD may not be limited to gp78. VIM is also present in SVIP, another ER-membrane localized p97/VCP-binding protein (21).5 SVIP, p47, and Ufd1 form mutually exclusive complexes with p97/VCP (21). It is likely that the VIM of SVIP interferes with the binding of p47 or Ufd1 to p97/VCP, as does the VIM of gp78. Therefore, SVIP may cooperate with other ERAD E3 to mediate Ufd1-independent ERAD. Thus, Ufd1-independent ERAD could represent one of the major pathways for disposal of misfolded ER proteins. In addition, many additional CDC48/p97/VCP cofactors with ubiquitin-binding capability have been identified (28, 43). Once the functions of the cofactors are fully uncovered, other mechanisms of coupling ubiquitination with retrotranslocation and degradation may be discovered. The presence of multiple ERAD pathways may be required to degrade different substrates and to facilitate efficient removal of misfolded proteins under different circumstances.

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REFERENCES

1. Federovitch, C. M., Ron, D., and Hampton, R. Y. (2005) Curr. Opin. Cell Biol. 17, 409–414

5 P. Ballar and S. Fang, unpublished observation.
