The VCP/p97 and YOD1 Proteins Have Different Substrate-dependent Activities in Endoplasmic Reticulum-associated Degradation (ERAD)*

Endoplasmic reticulum-associated degradation (ERAD) is an essential quality control mechanism of the folding state of proteins in the secretory pathway that targets unfolded/misfolded polypeptides for proteasomal degradation. The cytosolic p97/valosin-containing protein is an essential ATPase for degradation of ERAD substrates. It has been considered necessary during retro-translocation to extract proteins from the endoplasmic reticulum that are otherwise supposed to accumulate in the endoplasmic reticulum lumen. The activity of the p97-associated deubiquitinylase YOD1 is also required for substrate disposal. We used the in vivo biotinylation retro-translocation assay in mammalian cells under conditions of impaired p97 or YOD1 activity to directly discriminate their requirements and diverse functions in ERAD. Using different ERAD substrates, we found that both proteins participate in two distinct retro-translocation steps. For CD4 and MHC-Iα, which are induced to degradation by the HIV-1 protein Vpu and by the CMV immunoevasins US2 and US11, respectively, p97 and YOD1 have a retro-translocation-triggering role. In contrast, for three other spontaneous ERAD model substrates (NS1, NHK-α1AT, and Tetherin), p97 and YOD1 are required in the downstream events of substrate deglycosylation and proteasomal degradation.

Valosin-containing protein, p97 (VCP/p97, Cdc48 in yeast) is an abundant and conserved ATPase belonging to the type II ATPases family, associated with diverse cellular activities (1). p97 is organized into a homohexameric ring-shaped complex. Each protomer contains a flexible N-terminal domain and two ATPase domains (2). The N-terminal portion is involved in interactions with a large number of partners having distinct domains (i.e. UBX/UBX-like (ubiquitin regulatory X), UBD (ubiquitin D), PUB (PNGase/ubiquitin-associated), SHP box, PUL (PLAP (phospholipase A2-activating protein), Ufd3p, and Lub1p), VIM (VCP-interacting motif), VBM (VCP-binding motif) (3). Many of the various p97 functions are connected to the ubiquitin pathway (4–12).

Endoplasmic reticulum-associated degradation (ERAD) represents the main mechanism by which cells control the folding state of molecules within the secretory pathway. Several ER-resident proteins, including chaperones and lectins, participate in the recognition of misfolded or terminally unfolded molecules that are then targeted for proteasomal degradation (13, 14). A crucial step in ERAD, still poorly understood, is the retro-translocation from the ER lumen to the cytosol (15–21). Cytosolic p97 is a key player of ERAD in complex with the heterodimeric co-factor formed by ubiquitin fusion-degradation protein 1 (Ufd1) and nuclear protein localization protein 4 homolog (Npl4) (22, 23). The common view is that the p97-Ufd1-Npl4 complex is recruited to the ER membrane, where several different membrane-embedded ERAD protein components having p97-binding motifs reside (6, 24, 25). The precise mechanism and function of the p97 complex is not very clear. It is well established, however, that loss of p97 ATPase activity blocks the proteasomal degradation of several different ERAD substrates (26–29). These results have been generally interpreted as a stringent requirement of p97 activity in the retro-translocation step, therefore concluding that stabilization of the substrate protein occurs in the ER lumen or in partially

* The authors declare that they have no conflicts of interest with the contents of this article.
1 Both authors contributed equally to this work.
2 Supported by ICGEB predoctoral fellowships.
3 To whom correspondence may be addressed: CIBIO, University of Trento, Via delle Regole 101, 38123 Mattarello, Italy. Tel.: 39-461283282; Fax: 39-461283937; E-mail: gianluca.petris@unitn.it.
4 To whom correspondence may be addressed: Molecular Immunology Group, International Centre for Genetic Engineering and Biotechnology, Padriciano 99, 34149 Trieste, Italy. Tel.: 39-04037571; Fax: 39-040226555; E-mail: burrone@icgeb.org.
5 The abbreviations used are: ERAD, endoplasmic reticulum-associated degradation; ER, endoplasmic reticulum; BAP, biotin acceptor peptide; BIP, immunoglobulin binding protein; GPI, glycosylphosphatidylinositol; DBeqQ, N2,N4-dibenzylquinazoline-2,4-diamine; StrAv, streptavidin; NS1- STK, NS1 mutant which has all Ser, Thr, and Lys of the VL domain mutated to Ala for Ser and Thr) or Arg for (Lys); PNGase, peptide N-glycosidase; WB, Western blot; WB-ra, Western blot retardation assay(s); CHX, cycloheximide; OTU, ovarian tumor deubiquitination domain.
Different Activities of VCP/p97 and YOD1 in ERAD

dislocated forms not completely exposed to the cytosol. In this context, the ATPase activity of p97 is usually referred to as necessary for extraction of proteins from the ER lumen or membranes (4, 10, 23, 26, 28, 30–32). In addition, ubiquitylation and deubiquitylation cycles associated with the p97 complex have also been reported to be required for retro-translocation (4, 5). The p97 complex has also been reported to be involved in the extraction of multiquibquitinated proteins from complexes (33). It has been suggested previously that p97 could either mediate the actual movement across the membrane or, alternatively, remove ubiquinated substrates bound to the ERAD complex after retro-translocation (34). Therefore, the p97 complex might have diverse substrate-dependent activities in ERAD of luminal or membrane proteins.

YOD1 is a p97-associated deubiquitinylase shown recently to be a key player in ERAD (4, 5, 35, 36). The dominant negative mutant YOD1(C160S) has been shown to stabilize ERAD substrates mostly in a non-ubiquitylated and glycosylated form. As in the case of p97, these results have been interpreted as an accumulation of the substrate in the ER lumen as a consequence of stalling of molecules on the putative exit channel (4, 5). As for p97 ATPase, it has been concluded that YOD1 deubiquitinylase activity is also required for retro-translocation (4, 5, 35). By directly determining the extent of cytosolic exposure during retro-translocation of different ERAD substrates under conditions of compromised p97 and YOD1 activity, we demonstrated two different levels at which both proteins participate, depending on the substrate involved. A first level comprises the initial exposure of substrate luminal domains to the cytosol, as in the case of MHC-1α (induced by the CMV immunoevasins US2 and US11) and CD4 (induced by the HIV protein Vpu), whereas a second downstream level that makes substrates available for PNGase deglycosylation and proteasomal degradation was associated with three other substrates: the non-secretory Igκ light chain NS1 (NS1), the null Hong Kong mutant of α1-antitrypsin (NHK-α1AT), and BST-2/Tetherin (Tetherin).

Experimental Procedures

Constructs—The cyt-BirA and sec-BirA plasmids have been described previously (37). Plasmids encoding the N-terminal SV5-BAP tagged version of human MHC-1α and the same tag at the C-terminal domain of NHK-α1AT have been described previously (38). N terminus BAP-tagged CD4 has been described previously (39). US2 and US11 plasmids were provided by Domenico Tortorella. Human Tetherin was tagged with SV5 and BAP immediately upstream of the GPI anchor signal after Tyr-154 (Tetherin-BAP) or with BAP inserted after Ala-48 (12 amino acids distant from the transmembrane domain, BAP-Tetherin) as described previously (39). The CCHFV-L OTU (ovarian tumor deubiquitination domain, OTU, derived from the Crimean-Congo hemorrhagic fever virus protein L) plasmid was provided by Adolfo García-Sastre and the N-terminal FLAG-tagged human YOD1 and YOD1-C160S plasmids by Christian Schlieker. N- and C-terminal BAP-tagged NS1 and NS1-STK were generated by PCR amplification from the wild-type cDNA constructs (provided by Linda Hendershot) and cloned in pcDNA3 vectors containing a sequence encoding SV5-BAP. The His6-tagged rat p97 and p97QQ plasmids were also a gift from Linda Hendershot. No additional Ser, Thr, or Lys (excluding the Lys site for biotinylation) were introduced at the N terminus during cloning and tagging of NS1-STK. The BIP (immunoglobulin binding protein) sequence was amplified by RT-PCR from total RNA obtained from HEK-293T cells and cloned in pcDNA3. The BIP mutants T37G and G227D were obtained by site-directed mutagenesis. To BAP-tag BAP31, the cDNA sequence was cloned downstream of a fragment encoding the leader peptide (sec) SV5 and BAP in pcDNA3. The addition of the leader peptide was used to guarantee the correct insertion of the BAP tag into the ER lumen. BAP-Derlin1 was obtained by inserting the BAP sequence after Ala-45 into Derlin1 cDNA (in pcDNA3).

Cell Culture and Transfection—HEK293T cells were cultured in DMEM (Life Technologies) supplemented with 10% FCS (Life Technologies). Cells were transfected in 6-well plates (about 5 × 10⁵ cells/well) by the standard calcium phosphate technique. In all cases, unless indicated otherwise, transfections contained the cyt-BirA plasmid for biotinylation of BAP-tagged cytosolic proteins.

Approximately 18 h after transfection, the medium was discarded and replaced with 2 ml of medium supplemented with 0.1 mM biotin (Sigma), and cells were incubated further for at least 6–8 h. For biotin pulse, ~18 h after transfection, the medium was discarded and replaced with 2 ml of serum-free medium. After 24 h, the medium was discarded and replaced with 2 ml of medium supplemented with 0.15 mM biotin (Sigma), and cells were incubated further for 10 min or 2 h. When indicated, after 3–4 h of incubation with biotin, chemicals such as the proteasome inhibitor MG132 (Sigma) and DeBeQ (Sigma) were added at a concentration of 10–15 μM for 3–4 h.

For silencing experiments, HEK-293T cells were grown in 12-well plates. Irrelevant (UCGUCUCUACACGGUCU) and p97-specific (GAUAUGAGUGUGCUAGAU) siRNAs (Sigma) were transfected with Lipofectamine RNAiMax reagent (Life Technologies) according to the instructions of the manufacturer. 72 h after siRNA transfection, the medium was discarded, and cells were transfected with DNA plasmids by the standard calcium phosphate procedure and lysed 24 h post-transfection.

Cell Extract Preparation, Gel Retardation Assay, and Western Blotting—Transfected HEK293T cells were washed with 30 mM N-ethylmaleimide (Fluka) in PBS (pH 6.8) to remove free biotin and block BirA activity and then lysed immediately with 100 μl/well SDS lysis buffer (100 mM Tris-HCl (pH 6.8), 6% SDS, and 30 mM N-ethylmaleimide) and sonicated. These constituted total cell extracts. For Western blot retardation assays, samples were first boiled in PAGE loading buffer (25 mM Tris-HCl (pH 6.8), 6% SDS, and 10% glycerol, and 175 mM β-mercaptoethanol) and then incubated without or with 1 μg of streptavidin (StrAv) (Sigma) for 20 min at room temperature before loading. Gels were blotted onto PVDF membranes (Millipore) and reacted with anti-SV5 mAb, followed by incubation with an HRP-labeled anti-mouse whole IgG antibody (Jackson Immunoresearch Laboratories, Inc.) for ECL detection. Quantification of bands was performed using the image processing software ImageJ v1.43 (National Institutes of Health) or using the UVItect
Alliance detection system. Where indicated, cellular lysates were treated with peptide N-glycosidase F (PNGase-F) or endoglycosidase H (New England Biolabs) according to the instructions of the manufacturer. WBs of mAb 18/valosin-containing protein (mouse anti-valosin-containing protein/p97, BD Biosciences) and mAb 40/BiP (mouse anti-BiP, BD Biosciences) were used. In WBs of cell fractionation experiments, rabbit anti-actin (Sigma), mAb 37/calnexin (mouse anti-calnexin, BD Biosciences), and polyclonal rabbit anti-Derlin1 (Sigma) were used according to the instructions of the manufacturers.

Trypsin Sensitivity Assay and Cell Fractionation—Mechanical disruption of the plasma membrane was accomplished by resuspending cells in a fractionation buffer containing 20 mM Tris-HCl (pH 8), 250 mM sucrose, and 30 mM N-ethylmaleimide, followed by 12 passages through a 23-gauge needle and centrifugation at 100,000 × g for 5 min at 4 °C. The trypsin sensitivity assay, recovered post-nuclear supernatants were incubated with 1 μg of trypsin (Sigma) for 60–120 min at 37 °C. Where indicated, Nonidet P-40 was added at 1% final concentration. Cell fractionation was performed on post-nuclear supernatants by centrifugation at 100,000 × g for 1 h at 4 °C. Where indicated, Nonidet P-40 was added before ultracentrifugation. After a delicate wash in fractionation buffer (with 1% Nonidet P-40 for detergent-treated samples), pellets were resuspended in the same buffer containing 1.2% SDS.

Results

The role of p97 and YOD1 in the ERAD retro-translocation step was investigated using our well established in vivo biotinylation retro-translocation assay, which is based on co-expression of the cytosolic biotin ligase BirA (cyt-BirA) with an ERAD substrate tagged with BAP in a luminal position (38–40). In this assay, molecules exposed to the cytosol during retro-translocation become biotinylated. Discrimination between the luminal and the cytosolic fractions was carried out by Western blot retardation assay (WB-ra), in which denatured samples incubated with StrAv produce a retarded band of biotinylated molecules (38).

p97 and YOD1 on MHC-Iα and CD4 Induced Retro-translocation—p97QQ is a p97 dominant negative mutant (Glu-305 and Glu-578 mutated into Gln) (23) with no ATPase activity. The effect of p97QQ and of the dominant negative deubiquitinylase YOD1(C160S) on retro-translocation of different ERAD model proteins was tested in mammalian cells (5).

First, retro-translocation was tested on two type I membrane proteins, MHC-Iα and CD4, BAP-tagged at the N terminus. ERAD of MHC-Iα was induced by co-expression with US2 (19, 38, 41). As reported previously, the biotinylated MHC-Iα fraction corresponds to molecules that have been exposed to the cytosol during retro-translocation (38). MHC-Iα was strongly stabilized when co-expressed with p97QQ regardless of MG132 treatment (Fig. 1A). The accumulated material under conditions of compromised p97 ATPase activity was mostly not biotinylated and glycosylated (with MG132, 13% with p97QQ versus 75% without; Fig. 1A, lanes 7 and 8 and 11 and 12), indicating almost no exposure of the MHC-Iα N terminus to the cytosol and, therefore, impaired retro-translocation at the initial steps. Similarly, YOD1(C160S) induced MHC-Iα accumulation that was not biotinylated, not ubiquitylated, and glycosylated and, therefore, entrapped in the ER lumen (Fig. 1B). US11-induced MHC-Iα degradation showed similar results (Fig. 1, C and D). CD4 degradation was induced by co-expression with the HIV-1 protein Vpu. MG132 treatment stabilized deglycosylated CD4 (Fig. 1, E and F, arrows) (39). In the presence of p97QQ (Fig. 1E) or YOD1(C160S) (Fig. 1F), a strong accumulation of CD4 was observed, mostly in a non-biotinylated (with MG132, 6% with p97QQ versus 37% without; Fig. 1E, lanes 7 and 8 and 11 and 12) and glycosylated form, also consistent with luminal ER localization. Therefore, p97QQ and YOD1(C160S) prevent exposure to the cytosol of MHC-Iα and CD4 luminal domains, blocking viral protein-induced ERAD before substrate retro-translocation. Consistently, cell fractionation of MHC-Iα and CD4 found them to be associated to the pelleted/microsomal fraction. In addition, the presence of small amounts of biotinylated material found with p97QQ remained associated to the pellet as we have demonstrated previously for CD4, which becomes soluble only after deglycosylation (39). In contrast, as shown below, three other different ERAD models, NS1, NHK-α1AT, and BST-2/Tetherin, follow retro-translocation pathways where p97 and YOD1 activities are dispensable for substrate exposure to the cytosolic environment.

p97QQ on NS1 Retro-translocation—NS1 is a soluble and non-glycosylated immunoglobulin κ light chain that is not secreted and proteasome-degraded in the absence of the Ig heavy chain (42). In cells treated with MG132, BAP-tagged NS1 accumulated in the biotinylated form, indicating cytosolic exposure as a consequence of retro-translocation (Fig. 2A, lanes 2 and 4). Co-expression of NS1 with p97QQ produced an increase in NS1 levels consistent with impaired ERAD (Fig. 2B, lanes 1 and 5). Most of NS1 was biotinylated (around 60–70%, Fig. 2B, lane 6), indicating that this fraction corresponded to molecules that were targeted to the cytosolic environment. In fact, the amount of non-biotinylated material was almost the same as the one in cells expressed without the mutant (Fig. 2, A, lanes 2 and 4, and B, lanes 2 and 6). Wild-type p97 caused some accumulation of NS1, which was mostly not biotinylated. Similar conclusions were obtained in cycloheximide (CHX)-treated cells (Fig. 2C, left panel). The non-biotinylated fraction present at the beginning of the CHX treatment continued to be biotinylated rapidly by cyt-BirA during the following 3 h both in the absence and presence of p97QQ, whereas degradation with p97QQ was blocked (Fig. 2C, right panel).

Therefore, in the presence of p97QQ, NS1 accumulates after having been targeted to the cytosol, indicating that it is the downstream proteasomal degradation step that is affected. Similar findings were obtained with the NS1-STK mutant, which has all Ser, Thr, and Lys of the VL domain mutated to Ala (for Ser and Thr) or Arg (for Lys) (Fig. 2, D and E). This mutant is poorly ubiquitylated, more stable than NS1, and retro-translocated less efficiently (43).

NS1 retro-translocation was also tested following co-expression with the cytosolic deubiquitinylase-like protein CCHFV-OTU. OTU deubiquitylates cytosolic poly-ubiquitylated proteins targeted to degradation, accumulating them in the cytosol.
Different Activities of VCP/p97 and YOD1 in ERAD

A. MHC-Ia + US2 + MG132

B. MHC-Ia + US2 + MG132

C. MHC-Ia + US11 + MG132

D. MHC-Ia + US11 + MG132

E. CD4 + Vpu + MG132

F. CD4 + Vpu + MG132

G. MHC-Ia + US2 + MG132

H. CD4 + Vpu + MG132
**FIGURE 1. The effect of p97QQ and YOD1(C160S) on MHC-I and CD4 induced retro-translocation.** Shown are WB-ra of total cell extracts from cells co-transfected with cyt-BirA and the indicated constructs. A, NS1-BAP in the absence or presence of MG132 (10 μM for 4 h). B, NS1-BAP with empty control vector, wild-type p97 (p97) or p97QQ. C, top panel, NS1-BAP with empty control vector or p97QQ in cells treated with CHX (100 μg/ml) before lysis for the indicated time periods. Bottom panel, quantification of results from three independent CHX experiments. D, NS1-STK-BAP with or without MG132 (10 μM for 4 h). E, NS1-STK-BAP with empty control vector, wild-type YOD1, or YOD1(C160S) (BAP-tagged MHC-I/H9251 and US2). F, NS1-STK-BAP with empty control vector, wild-type YOD1, or YOD1(C160S) (BAP-tagged MHC-I/H9251 and US2). G, BAP-NS1 or NS1-BAP with p97QQ. H, BAP-NS1-STK or NS1-STK-BAP with or without MG132 (10 μM for 4 h). I, left panel, NS1-BAP with empty control vector or the BIP mutants T37G or G227D with or without p97QQ. Right panel, NS1-BAP with empty control vector or WT BIP and p97QQ. J, NS1-BAP with BIP mutants and p97QQ with a secretory version of BirA (sec-BirA) instead of the cyt-BirA. NS1 and NS1-STK were detected by anti-SV5. Actin and p97 were used as controls of loading and overexpression, respectively. Where indicated, StrAv (+) was added before loading (1 μg).

**FIGURE 2. The effect of p97QQ on NS1 retro-translocation.** Shown are WB-ra of total cell extracts of cells co-transfected with cyt-BirA and the indicated constructs. A, NS1-BAP in the absence or presence of MG132 (10 μM for 4 h). B, NS1-BAP with empty control vector, wild-type p97 (p97) or p97QQ. C, top panel, NS1-BAP with empty control vector or p97QQ in cells treated with CHX (100 μg/ml) before lysis for the indicated time periods. Bottom panel, quantification of results from three independent CHX experiments. D, NS1-STK-BAP with or without MG132 (10 μM for 4 h). E, NS1-STK-BAP with empty control vector, wild-type p97, or p97QQ. F, NS1-STK-BAP with empty control vector, wild-type YOD1, or YOD1(C160S) (BAP-tagged MHC-I/H9251 and US2). G, BAP-NS1 or NS1-BAP with p97QQ. H, BAP-NS1-STK or NS1-STK-BAP with or without MG132 (10 μM for 4 h). I, left panel, NS1-BAP with empty control vector or the BiP mutants T37G or G227D with or without p97QQ. Right panel, NS1-BAP with empty control vector or WT BIP and p97QQ. J, NS1-BAP with BiP mutants and p97QQ with a secretory version of BirA (sec-BirA) instead of the cyt-BirA. NS1 and NS1-STK were detected by anti-SV5. Actin and p97 were used as controls of loading and overexpression, respectively. Where indicated, StrAv (+) was added before loading (1 μg).
Different Activities of VCP/p97 and YOD1 in ERAD

because they are unable to be engaged by the proteasome (44, 45). Biotinylated NS1 and NS1-STK accumulated in the presence of OTU and were not affected by p97QQ, demonstrating that p97 ATPase activity was not required for their cytosolic exposure (Fig. 2F). NS1 has been reported to become ubiquitylated mainly in the V_L domain and proposed to build up in a complex with only V_L exposed to the cytosol (28). Two different NS1 versions, BAP-tagged at the N- or C terminus, showed similar levels of biotinylation with p97QQ, indicating comparable cytosolic exposure of both termini (Fig. 2, G and H). Further demonstration that p97QQ induces cytosolically exposed NS1 stabilization was obtained by overexpressing two different dominant negative versions of the ER chaperone grp78/BiP, namely BiP(T37G) and BiP(G227D) (46). When co-expressed with NS1, both mutants stabilized non-biotinylated NS1, indicating ER lumen localization as reported previously (46) (Fig. 2F). Instead, overexpression of wild-type BiP and p97QQ did not prevent NS1 biotinylation (Fig. 2F, right panel). Two main conclusions can be drawn. A functional BiP is required for active NS1 targeting to the retro-translocation pathway, and, for NS1, p97 activity is involved in a step downstream of its cytosolic exposure during retro-translocation because the effect of BiP mutants was dominant over the one of p97QQ and consistent with stabilization in the ER lumen (Fig. 2F). In fact, NS1 was totally biotinylated when co-expressed with an ER-targeted BirA (sec-BirA) (37) (Fig. 2F).

The stabilization effect of p97QQ was specific for NS1. Two control ER proteins BAP-tagged on the luminal side, Derlin-1 and BCR associated protein 31 (BAP31) showed very different stabilization levels compared with NS1. Although NS1 was highly biotinylated and stabilized (up to 8-fold, Fig. 3A), Derlin-1 was almost unaffected (Fig. 3B), and BAP31 was only slightly affected (Fig. 3C).

Direct evidence that NS1 and MHC-IIa retro-translocate through pathways with distinct p97 requirements was obtained by co-expressing both substrates with US2 and with or without p97QQ. In the same cells expressing p97QQ, NS1 accumulated preferentially in the cytosol and MHC-IIa in the ER lumen. The level of biotinylation of each protein model expressed together was similar to the one observed when expressed alone (Fig. 4). With p97QQ, the NS1 retro-translocated fraction was 60% alone and 48% when co-expressed with MHC-IIa, whereas, for MHC-IIa, the corresponding values were 9% alone and 17% when co-expressed with NS1 (Fig. 4, right panel). Therefore, p97QQ caused NS1 accumulation after exposure to the cytosol, whereas MHC-IIa luminal domains were blocked in ER lumen.

p97QQ on NHK-a1AT Retro-translocation—NHK-a1AT is a natural truncated version of the wild-type α1 antitrypsin glycoprotein that is not secreted and is a well known ERAD substrate (47). As for NS1, cytosolic exposure of NHK-a1AT was p97-independent. NHK-a1AT co-expressed with p97QQ showed impaired degradation with a large accumulation of biotinylated material (53% of the total) (Fig. 5A, lanes 5 and 6). The non-biotinylated fraction was at the same level as in control cells (Fig. 5A, lanes 2 and 6). With WT p97, the accumulated NHK-a1AT was not biotinylated. Upon proteasome inhibition, deglycosylated NHK-a1AT, which was totally biotinylated, was detected in the control and WT p97 samples. Also, a fraction of glycosylated molecules was biotinylated (1:1 ratio between glycosylated and deglycosylated). The glycosylation status was established by PNGase treatment (Fig. 5A, right panel). In contrast, when co-expressed with p97QQ, a large proportion of biotinylated NHK-a1AT (90%) was glycosylated, and only a minor part was deglycosylated (Fig. 5A, lanes 11 and 12). Experiments in CHX treated-cells further confirmed ongoing NHK-a1AT biotinylation in the presence of p97QQ (Fig. 5B). Therefore, although p97 ATPase activity is not required for NHK-a1AT to initiate retro-translocation to the cytosol, it is necessary for effective deglycosylation and proteasome degra-
dation, indicating a downstream role in rendering the ERAD substrate accessible to both PNGase and the proteasome. Further evidence was obtained by co-expression with OTU. As expected, cytosolic OTU largely stabilized (mainly deglycosylated) NHK-α1AT, which was totally biotinylated (Fig. 5C). A partially degraded, deglycosylated, biotinylated fragment was also apparent (38). Notably, upon co-expression with both p97QQ and OTU, the profile of biotinylated material was similar to the one obtained with OTU alone, indicating that p97QQ did not impair cytosolic exposure and that PNGase recruitment to the dislocation complex was facilitated by OTU activity. These results indicate that, for NHK-α1AT, p97 ATPase activity is also not required for triggering retro-translocation but, rather, for downstream steps that involve deglycosylation and proteasomal degradation.

p97QQ on BST-2/Tetherin Retro-translocation—BST-2/Tetherin is a type-II transmembrane glycoprotein with viral restriction factor activity (48–51). It has a GPI anchor at its C terminus and forms covalent dimers by three interchain disulfide bridges between two parallel monomers (52, 53). Tetherin is targeted to degradation by the HIV-1 accessory protein Vpu. It has, however, an intrinsic capacity to be degraded through ERAD when expressed in the absence of Vpu (39).

Tetherin retro-translocates as a glycosylated dimer that then is deglycosylated, reduced, and degraded by the proteasome (39). Tetherin was BAP-tagged in two different ER luminal positions: at the C-terminal part, just upstream of the GPI anchor signal (Tetherin-BAP), or at the N-terminal part, proximal to the trans-membrane domain (BAP-Tetherin) (Fig. 6A). SV5 was present upstream of the GPI anchor signal. p97QQ did not prevent Tetherin-BAP reaching the cytosol (Fig. 6B). In contrast to wild-type p97, p97QQ caused strong protein stabilization with most of the accumulated material exposed to cytosolic biotinylation, both in cells treated and not treated with MG132 (Fig. 6B, lanes 2 and 6 and 8 and 12). In MG132-treated cells, impaired deglycosylation of the biotinylated fraction with p97QQ was also apparent. The bands corresponding to deglycosylated molecules were assigned following endoglycosidase H digestion (Fig. 6C). Kinetics experiments in CHX-treated cells (Fig. 6D) confirmed Tetherin exposure to the cytosol in a p97-independent manner.

Co-expression with OTU, alone or in combination with p97QQ, produced a large accumulation of biotinylated Tetherin (Fig. 6E). As demonstrated previously (39), the biotinylated Tetherin was mostly dimeric (Fig. 6F). The phenotype with OTU was dominant over the one with p97QQ. Biotinylation of the membrane-proximal BAP-tagged Tetherin (BAP-Tetherin) upon co-expression with p97QQ was comparable with the one with the tag in a membrane-distal position (Tetherin-BAP) (Fig. 6G), indicating that impaired p97 activity does not preclude exposure of the luminal domain to the cytosolic side.

Silencing and Inhibition of p97—We next blocked p97 activity using a p97-specific siRNA and the chemical inhibitor

![FIGURE 5. The effect of p97QQ on NHK-α1AT retro-translocation. Shown are WB-ra of total cell extracts of cells co-transfected with cyt-BirA and the indicated constructs. A, left panel, NHK-α1AT-BAP with empty control vector (ctrl), wild-type p97 (p97), or p97QQ in the absence or presence of MG132 (10 μM for 4 h). Right panel, PNGase treatment of NHK-α1AT-BAP co-expressed with p97QQ. B, NHK-α1AT-BAP with empty control vector or p97QQ in cells treated with CHX (100 μg/ml) before lysis for the indicated time periods. C, NHK-α1AT-BAP with empty control vector, OTU, p97QQ, or both OTU and p97QQ. Open and filled arrowheads indicate deglycosylated non-shifted and shifted NHK-α1AT, respectively. Open and filled arrows indicate a deglycosylated degradation fragment non-shifted and shifted, respectively. NHK-α1AT was detected by anti-SV5. Actin was used as a loading control and p97 as a control of overexpression. Where indicated, StrAv (+) was added before loading (1 μg).](image-url)
DBeQ. In contrast to MHC-I and CD4, which, despite large accumulation upon p97 silencing, were barely biotinylated (no more than 1–2%), NS1, NHK-α1AT, and Tetherin were largely exposed cytosolically, as revealed by the increase in biotinylated forms (NS1 from 8% to 35%, NHK-α1AT from 0% to 49%, and tetherin from 4% to 16%) (Fig. 7A). Proteasome inhibition fur-
ther confirmed the requirement of p97 for deglycosylation of the two glycoproteins NHK-α1AT and Tetherin, although it was less pronounced for Tetherin (Fig. 7B).

A short (3-h) treatment with DBeQ, a p97 ATPase inhibitor (54), produced phenotypes similar to p97QQ and p97 siRNA. DBeQ alone or in combination with MG132 caused accumula-
tion of biotinylated NS1 (Fig. 7C). For the glycoproteins NHK-α1AT and Tetherin, DBEQ did not inhibit their biotinylation, whereas the effect on the impaired deglycosylation was evident (Fig. 7D). In a representative experiment, the relative level of deglycosylated biotinylated NHK/H92511AT changed from 50% in the presence of MG132 to 17% in the presence of both MG132 and DBEQ, whereas, for Tetherin, it changed from 75% with MG132 to 45% with DBEQ and MG132 (Fig. 7D, bottom panel).

The p97-associated YOD1 Deubiquitinylase—Because NS1, NHK, and Tetherin follow a retro-translocation pathway that is p97-independent, we tested the effect of the dominant negative mutant YOD1(C160S). Biotinylated NS1, NHK-1AT, and Tetherin accumulated when co-expressed with YOD1(C160S), indicating that the three substrates were still able to initiate retro-translocation but not to be degraded (Fig. 8, A–C). In addition, the two glycoproteins were found predominantly in the deglycosylated forms (Fig. 8, B and C). This was clearly observed in MG132-treated cells (Fig. 8, B and C), indicating that YOD1 was required to allow deglycosylation downstream of retro-translocation. Co-expression of the active deubiquitinylase OTU with YOD1(C160S) caused accumulation of deglycosylated (and biotinylated) NHK-α1AT (Fig. 8B, lanes 11 and 12, and 7C, lanes 5 and 6, respectively). This further demonstrated that, for the two glycoproteins, YOD1 was not needed to reach the cytosol but was required for targeting to PNGase and the proteasome. Therefore, NS1, NHK-1AT, and tetherin reach the cytosolic environment from the ER lumen independently from p97 and YOD1 activities, but through pathways that entail their functions in downstream ERAD steps.

The Dislocated Substrate—ERAD substrates are believed to form dynamic protein complexes that become stacked when p97 activity is blocked (4, 25, 55). We then searched for the sensitivity to trypsin and solubility properties of the biotinylated Tetherin accumulated in the presence of p97QQ. As shown in Fig. 9A, after mechanical disruption of the plasma membrane, most of the biotinylated material was accessible to trypsin digestion, indicating cytosolic localization. This was more evident for newly biotinylated molecules (from 33% to 2% after trypsin treatment), whereas, for longer labeling periods (2 h), sensitivity was lower (from 55% to 18%), suggesting localization of a fraction of the biotinylated material in a somehow protective complex/structure (Fig. 9A, left panel, lanes 3, 4, 9, and 10).
Also, for NS1 and NHK-α1AT, a large fraction of the biotinylated material accumulated upon co-expression with p97QQ was trypsin-sensitive (from 77% to 20% after trypsin for NS1 and from 43% to 12% for NHK-α1AT). Following Nonidet P-40 treatment, digestion was complete in both cases (Fig. 9A, center and right panels). In p97QQ-expressing cells, the complex formed by Tetherin was stabilized. When post-nuclear supernatants of cellular extracts derived from control and p97QQ-expressing cells, obtained by mechanical disruption, were fractionated by ultracentrifugation, all Tetherin in both control and p97QQ extracts was found in the pellet regardless of biotinylation status (Fig. 9B, left panel, lanes 5 and 6, and right panel, lanes 15 and 16). In extracts in which membranes were solubilized with Nonidet P-40, Tetherin became soluble under control conditions (Fig. 9B, left panel, lanes 7 and 8), whereas, in the presence of p97QQ, all material recovered in the pellet was biotinylated (Fig. 9B, right panel, lanes 19 and 20), representing more than 80% of the total biotinylated fraction. The non-biotinylated fraction was, instead, soluble and recovered in the supernatant (Fig. 9B, right panel, lanes 17 and 18). These results indicated that, in the presence of p97QQ, the cytosolically exposed (biotinylated) material was initially in a complex sensitive to trypsin that pellets down upon ultracentrifugation even after detergent treatment, most likely as part of a large complex.

**Discussion**

The involvement of p97 in ERAD has been documented widely. p97 is believed to provide the primary driving force for retro-translocation and extraction of substrates from the ER membrane by actively pulling the substrate after at least one round of ubiquitylation (4, 10, 23, 27, 32, 56–58). Requirement of a deubiquitylation step by YOD1 upstream of p97-mediated extraction from the ER has also been reported (4). Following impairment of p97 or YOD1 activities, ERAD substrates accumulate in a non-ubiquitylated form with folded disulfide bridges (for instance, Tetherin) and, in the case of glycoproteins, still glycosylated. Under these conditions, only a minor fraction contains markers of cytosolic localization like ubiquitylation, deglycosylation, or interaction with cytosolic proteins such as p97 (4). For these reasons, they have often been considered as partially dislocated substrates stalled within the ER membrane in a putative retro-translocon rather than intermediates exposed to the cytosol (4, 5, 23, 31, 43, 57). Other criteria, such as association of the accumulated material (even when ubiquitylated) to the pellet (microsomal/ER-containing fraction) in cell fractionation experiments and partial resistance to protease digestion, have led to the same conclusions. Moreover, down-regulation or sequestration of p97 induces the unfolded protein response, suggesting that unfolded protein response activation is the consequence of substrate accumula-

---

**FIGURE 9. The dislocated substrate.** A, trypsin sensitivity assay of Tetherin, NS1, and NHK-α1AT. Shown are WB-ra of samples from cells co-expressing Tetherin-BAP, NS1-BAP or NHK-α1AT-BAP, and p97QQ incubated with biotin for 10 min and/or 120 min and disrupted mechanically before treatment with trypsin and with and without Nonidet P-40 (1%), as indicated. To facilitate comparison of NS1 and NHK-α1AT, the samples corresponding to lanes 3–6 were balanced to lane 1. B, WB-ra of cell fractionation samples obtained by ultracentrifugation of post-nuclear supernatants derived from cells transfected with Tetherin-BAP with (right panel) or without (left panel) p97QQ. Cyt-BirA was included in all transfections. Where indicated, Nonidet P-40 (1% final concentration) was added before centrifugation. SN, supernatants. Blots were developed with anti-SV5, anti-actin, and anti-calnexin. Where indicated, StrAv (+) was added before loading (1 μg).
Different Activities of VCP/p97 and YOD1 in ERAD

...in the ER lumen (31, 59). Nevertheless, whether and how p97 acts on substrates by “pulling” molecules already exposed to the cytosol or on subunits of the dislocation complex to trigger ERAD substrate movement to the cytosol is not clear (14).

Using our sensitive cyt-BirA retro-translocation reporter system (in vivo biotinylation retro-translocation assay), which directly biotin labels cytosolically exposed proteins just after retro-translocation (38), we demonstrate that p97 and YOD1 have distinct retro-translocation and post-retro-translocation activities in ERAD depending on the substrate. In the viral protein-induced ERAD pathways followed by MHC-Iα or CD4, we found accumulation in the ER lumen in a glycosylated, non-ubiquitylated, and non-biotinylated form upon impairment of p97 or YOD1 activities, consistent with several reports (35, 60, 61). In these cases, p97 was required to trigger retro-translocation of the luminal domains. A cytosolic tail is present in both CD4 and MHC-Iα proteins. In the case of CD4, this domain is known to be ubiquitylated in Lys, Ser, and Thr residues during ERAD by the Vpu-recruited SCF^{Ticp} complex (62). Similarly, the cytosolic tail of MHC-Iα is required for its dislocation by US2 and US11 (63). However, Lys residues in the MHC-Iα tail seem to be not essential for US2 mediated dislocation (64), and Lys in the full-length MHC-Iα is partially dispensable for US11 dislocation despite the process still being ubiquitin-dependent (65). As in the case of CD4, ubiquitination may be targeted to non-lysine residues, as in MHC-Iα down-regulation by the viral γ-herpesvirus protein E3 ligase mk3 (66). Alternatively, requirement of ubiquitylation may involve proteins of the dislocation complex (65).

Because YOD1 catalytic activity was required as much as the one of p97 for MHC-Iα- and CD4-induced retro-translocation, at least one round of ubiquitylation and deubiquitylation is, in these cases, needed to expose their luminal domains. Interestingly, it has been shown recently that YOD1 activity was required for retro-translocation of non-ubiquitylated substrates, indicating that it was acting exclusively on components of the ERAD machinery rather than on the substrate (36).

In contrast to MHC-Iα and CD4, three different spontaneous ERAD substrates, the non-glycosylated NS1, the soluble and glycosylated NHK-α1AT, and the type II membrane-bound and GPI-anchored Tetherin, upon impairment of p97 or YOD1, retro-translocated in a form accessible to cyt-BirA biotinylation in the cytosol, revealing a post-retro-translocation requirement of both proteins. This was also the case in cells co-expressing MHC-Iα and NS1, in which the two p97 requirements were apparent. In the case of Tetherin, we further investigated the characteristics and the fate of the retro-translocated and non-degraded molecules after p97 inhibition, finding that the biotinylated fraction was largely trypsin-sensitive, confirming its cytosolic localization. However, a fraction of the biotinylated material becomes protected over time, suggesting retargeting to a different cellular compartment. This may be a cellular response to potentially toxic ERAD substrates when the cell is unable to degrade them. Cellular compartments like insoluble protein deposits or autophagosomes (67) might be the possible destination when retro-translocation and degradation are uncoupled artificially.

We found the fraction of biotinylated Tetherin accumulated after co-expression with p97QQ was not soluble because its large majority (80–90%) was able to be pelleted down upon ultracentrifugation in the presence of Nonidet P-40. Therefore, the function of p97 in this pathway resembles the role proposed in mediating protein extraction from chromatin (68) and segregation of polypeptides from large protein complexes and aggregates.

Large protein complexes are formed during ERAD (10, 55), and some of them have been shown to localize to the cytosolic side of the ER, interacting with membrane-bound proteins (4, 5, 16, 17, 55) and likely containing the cytosolic dislocated fraction (4, 5, 30, 69, 70). Therefore, according to our data, at least for some substrates, the widely reported requirement of p97 ATPase activity for protein retro-translocation (4, 10, 23, 30, 71) could represent the need of ATP hydrolysis to release substrates from the dislocation complex (already exposed to the cytosol) rather than a halt at the retro-translocation step, a possibility suggested previously (34).

In this scenario, p97 and YOD1 might participate in reorganizing the ERAD machinery after retro-translocation to favor recruitment of PNGase (as in the case of NHK-α1AT and Tetherin) and to consent to delivering to the proteasome. Indeed, expression of OTU, similar to what has already been reported for the EBV derived deubiquitinylase (4), was capable to rescue the effect of the YOD1 dominant negative mutant, allowing efficient deglycosylation of the NHK-α1AT and Tetherin but impairing their degradation. On the basis of our findings and described roles of p97 and YOD1, two main levels for their involvement in ERAD could be envisaged. One is a retro-translocation-triggering activity comprising the pathway followed by CD4 and MHC-Iα upon induced degradation, where p97 and YOD1 are crucial for exposing luminal domains to the cytosol. The second is a post-retro-translocation function required by diverse ERAD substrates such as NS1, NHK-α1AT, and Tetherin, where p97 and YOD1 are dispensable for the cytosolic exposure, but needed for the downstream deglycosylation and proteasomal degradation steps. p97 and YOD1 are not necessarily required equally because ubiquitin-independent substrates such as pre-pro α factor (72) and the cholera toxin A1 chain (27) appear to also be independent of p97 (58) but still regulated by YOD1 (36). In conclusion, by exploiting the unique efficiency of the in vivo biotinylation retro-translocation assay system we were able to demonstrate the existence of two different levels at which p97 and YOD1 participate in ERAD depending on the involved substrates.

Author Contributions—L. S., G. P., F. C., and O. R. B. conceived and designed the experiments, analyzed the data, and wrote the paper. L. S., G. P., and F. C. performed the experiments. O. R. B. contributed reagents, materials, and analysis tools.

Acknowledgments—We thank Linda Hendershot (St. Jude Children’s Research Hospital) for the US2 and US11 plasmids as well as the p97 and p97QQ plasmids; Adolfo García-Sastre (Mount Sinai Hospital) and Christian Schlieker (Yale University), respectively, for the CHFV-L OTU and YOD1 plasmids; and Domenico Tortorella (Mount Sinai Hospital) for the US2 and US11 plasmids.
Different Activities of VCP/p97 and YOD1 in ERAD

globulin light chains as a prerequisite for secretion. A model for oligomerization-dependent subunit folding. J. Biol. Chem. 272, 3117–3123.
43. Shimizu, Y., Okuda-Shimizu, Y., and Hendershot, L. M. (2010) Ubiquitylation of an ERAD substrate occurs on multiple types of amino acids. Mol. Cell 40, 917–926.
44. Frias-Staheli, N., Giannakopoulos, N. V., Kikkert, M., Taylor, S. L., Bridgen, A., Paragas, J., Richt, J. A., Rowland, R. R., Schmaljohn, C. S., Lenschow, D. J., Snijder, E. J., García-Sastre, A., and Virgin, H. W., 4th (2007) Ovarian tumor domain-containing viral proteases evade ubiquitin- and ISG15-dependent innate immune responses. Cell Host Microbe 2, 404–416.
45. Ashour, J., Laurent-Rolle, M., Shi, P. Y., and García-Sastre, A. (2009) NS5 of dengue virus mediates STAT2 binding and degradation. J. Virol. 83, 5408–5418.
46. Hendershot, L., Wei, J., Gaut, J., Melnick, J., Aviel, S., and Argon, Y. (1996) Inhibition of immunoglobulin folding and secretion by dominant negative BiP ATPase mutants. Proc. Natl. Acad. Sci. U.S.A. 93, 5269–5274.
47. Sifers, R. N., Brashears-Macatee, S., Kidd, V. J., Muensch, H., and Woo, S. L. (1988) A frameshift mutation results in a truncated α 1-antitrypsin that is retained within the rough endoplasmic reticulum. J. Biol. Chem. 263, 7330–7335.
48. Nakatsukasa, K., and Brodsky, J. L. (2008) The recognition and retro-translocation of misfolded proteins from the endoplasmic reticulum: ubiquitin in charge. Nat. Struct. Mol. Biol. 15, 1055–1061.
49. Christianson, J. C., Olzmann, J. A., Shaler, T. A., Sowa, M. E., Bennett, E. J., Richter, C. M., Tyler, R. E., Greenblatt, E. J., Harper, J. W., and Kopito, R. R. (2012) Defining human ERAD networks through an integrative mapping strategy. Nat. Cell Biol. 14, 93–105.
50. Nakatsukasa, K., and Brodsky, J. I. (2008) The recognition and retro-translocation of misfolded proteins from the endoplasmic reticulum. Traffic 9, 861–870.
51. Elkebaz, Y., Shapira, I., Rabinovich, E., and Bar-Nun, S. (2004) Distinct steps in dislocation of luminal endoplasmic reticulum-associated degradation substrates: roles of endoplasmic reticulum-bound p97/Cdc48p and proteasome. J. Biol. Chem. 279, 3980–3989.
52. Christianson, J. C., and Ye, Y. (2014) Cleaning up in the endoplasmic reticulum: ubiquitin in charge. Nat. Struct. Mol. Biol. 21, 325–335.
53. Leitman, J., Ulrich Hartl, F., and Lederkremer, G. Z. (2013) Soluble forms of polyQ-expanded huntingtin rather than large aggregates cause endoplasmic reticulum stress. Nat. Commun. 4, 2753.
54. Mueller, B., Klemm, E. J., Spooner, E., Claessen, J. H., and Ploegh, H. L. (2008) SEL1L nucleates a protein complex required for dislocation of misfolded glycoproteins. Proc. Natl. Acad. Sci. U.S.A. 105, 12325–12330.
55. Christianson, J. C., Olzmann, J. A., Shaler, T. A., Sowa, M. E., Bennett, E. J., Harpa, J. W., and Kopito, R. R. (2011) Multilayered mechanism of CD4 downregulation by HIV-1 Vpu involving distinct ER retention and ERAD targeting steps. PLoS Pathog. 6, e1000869.
56. Story, C. M., Furman, M. H., and Ploegh, H. L. (1999) The cytosolic tail of class I MHC heavy chain is required for its dislocation by the human cytomegalovirus US2 and US11 gene products. Proc. Natl. Acad. Sci. U.S.A. 96, 8516–8521.
57. Furman, M. H., Loureiro, J., Ploegh, H. L., and Tortorella, D. (2003) Ubiquitinylation of the cytosolic domain of a type I membrane protein is not required to initiate its dislocation from the endoplasmic reticulum. J. Biol. Chem. 278, 34804–34811.
58. Hassink, G. C., Barel, M. T., Van Voorsten, S. B., Kikkert, M., and Wiertz, E. J. (2006) Ubiquitination of MHC class I heavy chains is essential for dislocation by human cytomegalovirus-encoded US2 but not US11. J. Biol. Chem. 281, 30063–30071.
59. Wang, X., Herr, R. A., Chua, W. J., Lybarger, L., Wiertz, E. J., and Hansen, T. H. (2007) Ubiquitination of serine, threonine, or lysine residues on the cytoplasmic tail can induce ERAD of MHC-I by viral E3 ligase mK3. J. Cell Biol. 177, 613–624.
60. Wolff, S., Weissman, J. S., and Dillin, A. (2014) Differential scales of protein quality control. Cell 157, 52–64.
61. Dantuma, N. P., and Hoppe, T. (2012) Growing sphere of influence: Cdc48/p97 orchestrates ubiquitin-dependent extraction from chromatin. Trends Cell Biol. 22, 483–491.
62. Greenblatt, E. J., Olzmann, J. A., and Kopito, R. R. (2011) Derlin-1 is a rhomboid pseudoprotease required for the dislocation of mutant α1 antitrypsin from the endoplasmic reticulum. Nat. Struct. Mol. Biol. 18, 1147–1152.
63. Baker, B. M., and Tortorella, D. (2007) Dislocation of an endoplasmic reticulum membrane glycoprotein involves the formation of partially dislocated ubiquitinated polypeptides. J. Biol. Chem. 282, 26845–26856.
64. Flierman, D., Ye, Y., Dai, M., Chua, V., and Rapoport, T. A. (2003) Polyubiquitin serves as a recognition signal, rather than a ratcheting molecule, during retro-translocation of proteins across the endoplasmic reticulum membrane. J. Biol. Chem. 278, 34774–34782.
65. Lee, R. J., Liu, C. W., Hartly, C., McCracken, A. A., Latterich, M., Römisch, K., DeMartino, G. N., Thomas, P. J., and Brodsky, J. L. (2004) Uncoupling retro-translocation and degradation in the ER-associated degradation of a soluble protein. EMBO J. 23, 2206–2215.