A technique for delineating the unfolding requirements for substrate entry into retrotranslocons during endoplasmic reticulum–associated degradation

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ABSTRACT

The endoplasmic reticulum–associated degradation (ERAD)4 pathway mediates the ER-to-cytosol retrotranslocation of defective proteins through protein complexes called retrotranslocons. Defective proteins usually have complex conformations and topologies, and it is unclear how ERAD can thread these conformationally diverse protein substrates through the retrotranslocons. Here, we investigated the substrate conformation flexibility necessary for transport via retrotranslocons on the ERAD-L, ERAD-M, and HIV-encoded protein Vpu-hijacked ERAD branches. To this end, we appended various ERAD substrates with specific domains whose conformations were tunable by binding to appropriate ligands. With this technique, we could define the capacity of specific retrotranslocons in disentangling very tight, less tight but well-folded, and unstructured conformations. The Hrd1 complex, the retrotranslocon on the ERAD-L branch, permitted the passage of substrates with a proteinase K-resistant tight conformation, whereas the E3 ligase gp78–mediated ERAD-M allowed passage only of nearly completely disordered but not well-folded substrates, and thus may have the least unfoldase activity. Vpu-mediated ERAD, containing a potential retrotranslocon, could unfold well-folded substrates for successful retrotranslocation. However, substrate retrotranslocation in Vpu-mediated ERAD was blocked by enhanced conformational tightness of the substrate. On the basis of these findings, we propose a mechanism underlying polypeptide movement through the ER membrane. We anticipate that our biochemical system paves the way for identifying the factors necessary for the retrotranslocation of membrane proteins.

INTRODUCTION

Nearly one-third of human proteins are secretory and membrane proteins that are synthesized at the endoplasmic reticulum (ER). When misfolded or failed to be assembled into a complex, these proteins are retained within the ER and ultimately eliminated by a process termed ER-associated degradation (ERAD). ERAD is typically categorized into ERAD-L, ERAD-M and ERAD-C branches for their preference of substrates with lesions from ER
luminal, transmembrane and cytosolic domains, respectively (1,2). These branches are known to use different sets of factors to promote substrate destruction (3).

The mechanism of ERAD involves the retrotranslocation of substrates from the ER to the cytosol, where they are ubiquitinated, extracted from membrane by AAA+ ATPase p97, and degraded by 26S proteasome (4,5). Since the degradation machinery is mainly localized in the cytoplasm, all ERAD substrates must traverse the ER membrane to be relocated to the cytosol for their destruction. Therefore, a proteinaceous channel termed retrotranslocon should reside in the ER membrane to mediate retrotranslocation of soluble and membrane protein substrates into the cytosol (6). Many candidates were proposed to be the channels, including the transmembrane E3 ligase Hrd1. Recent studies have shown that misfolded CPY* can be retrotranslocated in a reconstituted liposome containing yeast Hrd1p (7,8). Cryo-EM structure revealed that the Hrd1p dimer shows a central cavity, likely the conduit for passing polypeptide (9). However, it is unclear whether Hrd1 is able to retrotranslocate the misfolded protein bearing a folded domain as these studies were performed using misfolded proteins. In mammalian cells, ubiquitin ligases Hrd1 and gp78 both show sequence homology to yeast Hrd1p within the transmembrane region. Hrd1 is strictly required for ERAD-L while gp78 is mainly responsible for ERAD-M, especially for polytopic membrane proteins (10). TRC8, an transmembrane E3 ligase required for cytomegalovirus-encoded protein US2-mediated degradation of MHC-I heavy chain (HC), also shows similar conserved characteristics to yeast Hrd1p (11). There are suggestions that some other (up to ~20) ER-resident ubiquitin ligases in mammals could serve as retrotranslocons for polypeptides movement through the membrane of respective subset of ERAD substrates on ERAD-L, -M, and -C branches of pathway (9,12).

Since folding is an error-prone process, most, if not all, newly synthesized secretory and membrane proteins have potential to become substrates of ERAD. Therefore, ERAD must recognize and ultimately degrade a highly diverse range of substrates: not only accept individual proteins with different sizes, but also handle potential diverse conformations when a protein is misfolded. Adding to the complexity is the fact that many substrates are modified with oligosaccharides in the ER lumen. It is largely unclear how limited number of potential retrotranslocons can handle substrates with potentially unlimited conformations. Specifically, the requirements on the size or conformation flexibility of substrates for threading through the pores of retrotranslocons in ERAD-L, -M and -C pathways are largely unknown.

A well-characterized strategy to study protein translocation takes advantage of a conformation-tunable cytosolic enzyme, dihydrofolate reductase (DHFR). When bound to folate analogs such as methotrexate (MTX) or the membrane-permeable derivative trimetrexate (TMX) (13), DHFR adopts a very tight conformation that becomes resistant to proteinase K digestion, which generates a 21-kDa proteinase K-resistant fragment. Therefore, ligand-stabilized DHFR can be employed as a testing substrate to evaluate the pore size or the unfolding activity of translocation machineries. Studies using DHFR fusion proteins showed that pre-unfolded conformation is necessary for protein import into the ER, mitochondria and lysosome (14-16), indicating that related translocation machineries lack the capability to unfold DHFR in tight conformation and only allow the passage of at least partially unfolded peptides. However, tightly folded DHFR/MTX complex can be imported across protein translocons present in the outer and inner chloroplast envelope membranes due to the large functional pore size with a diameter of 25.6 angstrom (17,18).

The destabilizing domains (DD) derived from FKBP12 is another example whose conformation can be tuned by ligand binding (19,20). Binding to its ligand Shield-1 stabilizes the structure of several FKBP12
mutants (i.e., the DDs) which would otherwise show highly flexible conformation (20). Although these nearly unstructured FKBP12 mutants can serve as a degron for ubiquitin-proteasome system (UPS) when present in the cytoplasm, they are very stable when confined within ER lumen (21). Thus, similar to DHFR/ligands, DD/Shield-1 pair could be used to study the conformation requirements for the entry into the pores of retrotranslocation machineries.

In order to get a comprehensive understanding as to how defective ER proteins with distinct conformations and topologies, such as soluble protein, and single- and multi-spanning membrane proteins, are retrotranslocated during ERAD, we harnessed both DHFR/TMX and DD/Shield-1 pairs to investigate the substrate unfolding requirements for threading through the retrotranslocons on ERAD-L, ERAD-M, and HIV-encoded protein Vpu-mediated ERAD pathways (22). This technique allows us to define the capacity of specific retrotranslocons in disentangling very tight, less tight but well folded, and unstructured conformations. Our results demonstrated that three retrotranslocon complexes showed distinct protein unfolding requirements for moving substrates across respective pores. Thus, different strategies may be used to accommodate or unfold substrates for entry into respective channels.

RESULTS
An experimental system to report unfolding requirement in cultured cells

DHFR, when fused in-frame to the MHC-I heavy chain (DHFR-HC), can fold properly in the lumen of the ER and bind TMX, a membrane-permeable folate analogue (23). We adopted a similar strategy to append DHFR to the ER luminal region of a serial of ERAD substrates that are degraded through ERAD-L or Vpu-hijacked ERAD pathways (Fig. 1A). These included null Hong Kong α1-antitrypsin (NHK) and a truncated version of CD4 (mini-CD4, mCD4) which is degraded from the ER by Vpu-hijacked ERAD (24,25). DHFR-fused GFPu containing a C-terminal CL1 degron was used as a control substrate in the cytoplasm (26).

We then confirmed that the TMX was able to bind the DHFR moiety of substrates in cultured cells using the protease K digestion assay (23) (Fig. 1B). Treating total cells lysates with protease K results in the generation of a ~21-25 kDa DHFR fragment, indicating that the DHFR portions of these substrates bind the TMX and show tightly folded conformation (Figs. 1C-1E). No post-lysis binding of TMX to DHFR was observed in control experiments (Figs. 1B-1E; sample No. 5). This result is consistent with a previous report (23), indicating that the TMX is able to form complex with the DHFR moiety fused with various substrates localized in cytoplasm as well as ER lumen. Quantification of these results indicates that at least 90% of DHFR-fused substrates were bound with TMX in cultured cells (Fig. 1F). Thus, TMX binding to DHFR significantly improved substrate solubility in buffers containing mild detergents (Fig. 1G).

UPS is able to eliminate DHFR-GFPu bound with folate analogs
We made HEK293T cells stably expressing DHFR-GFPu and asked whether DHFR-GFPu could be degraded by UPS in the presence of folate analogs. Significant accumulation of DHFR-GFPu in cultured cells was observed when the cells were treated with proteasome or p97 inhibitors (MG132 and CB5083) (27,28), while the addition of TMX or MTX, had little effect (Fig. 2A). In addition, the translational arrest by cycloheximide resulted in degradation of DHFR-GFPu that could be impeded by the treatment of either MG132 or CB5083, but not folate analogues (Fig. 2B). These results revealed that DHFR bound with folate analogs, although resistant to protease K, could be degraded by protease activities of 20S proteasome, and suggested that the AAA-ATPase activity in the 19S proteasome might disentangle the tightly folded conformation of DHFR charged with folate analogs (29).
It has been reported that in the presence of MTX, CL1 degron appended DHFR (DHFR*) is not able to be degraded by purified proteasome or in crude rabbit reticulocyte lysate (30,31) due to lack of flexible region of peptide chain required for a “tight binding” to proteasome (32). Here, the additional GFP moiety besides DHFR could provide sufficient flexible regions for the recognition of DHFR portion by the 19S proteasome. Thus, we concluded that once retrotranslocated from the ER, DHFR-linked ERAD substrates can be degraded even in the presence of folate analogues, unless the proteasome activity is blocked.

The ERAD-L pathway is compatible for the retrotranslocation of substrates with a folded domain

To test if TMX-occupied DHFR could traverse the ER membrane through ERAD-L pathway, we compare the turnover rate of DHFR-NHK in the absence or the presence of TMX. DHFR-NHK were rapidly degraded after blocking ribosomal translation by CHX (Figs. 2C and 2D). However, TMX did not slow down degradation speed of substrates from the ER. In contrast, MG132 and CB5083 significantly extended the half-lives of substrates, suggesting that the degradation of these ERAD-L substrates depends on both p97 ATPase and proteasome. Notably, TMX somehow enhanced turnover rate of glycosylated DHFR-NHK, while cotreatment with MG132 and CHX led to the accumulation of a deglycosylated DHFR-NHK, which represents retrotranslocated substrates due to the removal of glycan by cytosolic N-glycanase (33) (Fig. 2C). These results demonstrated that TMX-charged DHFR is able to be retrotranslocated across ER membrane via the ERAD-L pathway, and ultimately degraded by the proteasome in the cytosol.

We then asked if retrotranslocated DHFR in cytoplasm maintained in the native-like conformation. We used a deubiquitinase from the Ebola virus (EBV-DUB), which could inhibit proteasomal degradation while permit efficient retrotranslocation when expressed in cultured cells (34). The expression of EBV-DUB led to stabilization of the retrotranslocated DHFR-NHK in the cytoplasm, as shown by the appearance of the deglycosylated band (Fig. 2E; lanes 3 and 5; lanes 7 and 9). Proteinase K digestion for DHFR-NHK and DHFR-GFP* showed that MTX only bound with DHFR localized within the cytosol, but not that of ER lumen (Fig. 2E; compare the proteinase K resistant DHFR in lanes 2 and 4). However, the proteinase K resistant DHFR band became evident concomitant with the appearance of retrotranslocated and deglycosylated DHFR-NHK (Fig. 2E; lanes 5 and 6). This result indicates that the retrotranslocated DHFR when in binding with MTX may still maintain the native-like conformation.

Our results on the retrotranslocation of DHFR-NHK are consistent with that of DHFR-HC in the presence of US2, a CMV encoded adaptor protein for mediating MHC-I heavy chain ubiquitination and retrotranslocation (23). The degradation of DHFR-NHK strictly depends on the Hrd1 E3 ligase activity (Figs. S1A and S1B) (35), a highly potential retrotranslocon in mammalian cells as its yeast counterpart, Hrd1p, has been shown to be a retrotranslocation channel (7-9). Since the misfolded NHK functions as a degradation signal, the retrotranslocation should be initiated on at least partially unfolded structure of NHK moiety. The capability of TMX-charged DHFR to transverse the membrane suggests that once the Hrd1 retrotranslocon is opened for initiating the passage of the leading NHK moiety, the trailing 21 kDa TMX-bound DHFR is able to enter the channel and finally cross the membrane. However, according to the cryo-EM structure, yeast Hrd1p shows a narrow central channel which is closed at the luminal side (9). It is possible that the opened Hrd1 channel and/or Hrd1-associated factors such as Derlins could be dynamic to accommodate 21 kDa DHFR for successful retrotranslocation of ERAD-L substrates. After retrotranslocation, p97 might unfold and extract DHFR from the rigid DHFR-TMX complex, thread the flexible DHFR peptide
through the p97’s central pore, and release it into the cytosol (36,37). The DHFR polypeptide could be partially renatured to restore its native-like conformation before the degradation by proteasome.

**Unfolding of luminal domain is a prerequisite for Vpu-mediated CD4 degradation**

In order to investigate how ER luminal domain would affect retrotranslocation of an ERAD-C substrate, we utilized the Vpu-mediated ERAD system (22). When inserted into the ER membrane of host cells, Vpu is phosphorylated and associates with E3 ligase SCF$$^\text{BTrCP}$$ to form a tightly bound complex anchored on ER membrane (Fig. S2A). The Vpu-ligase complex mediates degradation of many newly synthesized membrane proteins, including BST-2, PSGL-1, and CD4, by an ERAD-like pathway (Fig. S2B) (38-41).

Vpu-ligase recognizes CD4 primarily via CD4 cytosolic tail and catalyzes polyubiquitination on the lysine residues in the cytosolic tail (25,42,43). Thus, CD4 can be considered as an ERAD-C substrate. In accordance with this, we observed that Vpu induces highly efficient degradation of various luminal domain mutated CD4, including DHFR-mCD4 in which DHFR was substituted for D1-D3 domains of CD4 (Figs. 3A, and S2C-S2F). Notably, CD4 degradation is independent of Hrd1 and gp78, as evidenced by efficient retrotranslocation and degradation of CD4 variants in Hrd1 and gp78 double KO cells (Figs. S2B, S2E, and S2G).

We then asked how conformational change would affect substrate retrotranslocation and degradation. To address this, we enhanced conformational rigidity of DHFR portion of DHFR-mCD4 by charging it with TMX. The CHX chase analysis revealed a rapid degradation of DHFR-mCD4, while treatment with the p97 inhibitor CB5083 blocked substrate turnover from the ER membrane, and that of MG132 stabilized deglycosylated retrotranslocated intermediate within the cytoplasm (Figs. 3B and 3C). We then found that addition of TMX, similar to that of CB5083, significantly slowed down turnover rate of glycosylated DHFR-mCD4, resulting in stabilization of the glycosylated form of DHFR-mCD4 (Figs. 3D, S2F and S2H). These results implied that binding of TMX with DHFR inhibited the retrotranslocation of the DHFR moiety. Furthermore, cells treated with both TMX and MG132 showed significantly decreased amount of retrotranslocated substrate compared with MG132-treatment alone (Fig. 3D; compare lanes 3 and 6), strongly suggesting that TMX, similar to that of CB5083, can block the retrotranslocation of CD4 (Fig. S2H; compare lanes 3 and 4). Consistent with this, membrane protection experiment showed that the DHFR portion indeed tightly bound to TMX and resided within ER lumen (Fig. 3E).

Additional co-immunoprecipitation result showed that DHFR-mCD4 maintains interaction with Vpu and its phosphorylation mutant Vpu-SN in the presence of TMX (Fig. 3F). Accordingly, TMX treatment allowed Vpu-mediated ubiquitination of DHFR-mCD4 (Fig. 3G). These results clearly showed that TMX did not impair substrate interaction with E3 or substrate ubiquitination. Thus, it was the retrotranslocation process that was blocked by the rigid structure of DHFR formed upon binding to TMX.

Our results demonstrate the conformation-dependent retrotranslocation and suggest that Vpu-mediated retrotranslocation route is able to fully unfold the ligand-free DHFR, but not the TMX-occupied DHFR. Although both NHK and CD4 degradation depend on the activity of p97 ATPase which is the only known source of mechanical force for extracting substrate from the ER, the two substrates show totally different behaviors during retrotranslocation, suggesting that different substrates use distinct retrotranslocons and/or strategies for moving substrates across membrane (44). This was supported by the fact that NHK degradation is strictly dependent on Hrd1, a protein-conducting channel in mammalian cells, while Vpu-mediated CD4 degradation...
remains efficient in Hrd1/gp78 DKO cells (Figs. S1A and S2B).

**TMX entraps the retrotranslocating intermediate of DHFR-mCD4 in the ER membrane**

Since TMX only binds to the DHFR portion fused with mCD4, we reasoned that the D4 domain could be allowed for retrotranslocation in the presence of TMX (Fig. 4A). To detect the retrotranslocated species, a BioTag (45) was inserted into a loop within the D4 domain (Figs. 4A and S3A). We predicted that expression of a cytosolic BirA (BirA<sup>cyto</sup>) would lead to biotinylation of the BioTag transported into cytosolic compartment (Fig. 4A).

Indeed, we observed biotinylated DHFR-Bio-mCD4 in the crude cell lysate when cells coexpressing BirA<sup>cyto</sup>, Vpu and DHFR-mCD4 was treated by TMX (Fig. 4B). The biotinylated species could be captured by denaturing immunoprecipitation for substrate, suggesting the biotin was indeed covalently conjugated onto the DHFR-mCD4 per se (Fig. 4B). The appearance of biotinylated substrate was strictly dependent on TMX treatment and expression of BirA<sup>cyto</sup>, suggesting that the BioTag on the luminal D4 domain was transported across the ER membrane and reached the cytoplasm. In addition, protease digestion of microsomes containing biotinylated substrates showed that membrane-protected fragment was not modified with biotin, confirming that the biotinylation only occurred in the cytoplasm where BirA<sup>cyto</sup> was expressed (Fig. 4C).

Previous work showed that inhibition of proteasome by MG132 stabilized retrotranslocated CD4 (46,47), which migrated faster on SDS-PAGE due to glycan removal by cytosolic N-glycanase (Figs. 3B and 3D). As expected, the retrotranslocated deglycosylated substrate was biotinylated by the cytosolic BirA when cells were treated with MG132 (Fig. 4D).

To test whether a BioTag before DHFR can be biotinylated, we inserted the BioTag into DHFR-mCD4 between the FLAG tag and DHFR to make Bio-DHFR-mCD4 (Fig. 4E). We found that the BioTag cannot be modified with biotin in the presence of TMX (Fig. 4F), suggesting that the BioTag and the TMX-occupied DHFR domain may not be retrotranslocated out of the membrane. In contrast, inhibition of the proteasome by MG132 leads to accumulation of biotinylated Bio-DHFR-mCD4 which is also deglycosylated, suggesting that retrotranslocated substrate species is stabilized in the cytosol and thus modified with biotin by the BirA<sup>cyto</sup>.

We noticed that, in the presence of TMX, the majority of biotinylated substrate fully retained the oligosaccharide chain (Figs. 4D and 4G). These results suggested that although the BioTag adjacent to the TMD has been exported out of the membrane, the majority of the glycan moiety has not yet reached the cytosol. It was probably still retained within the ER lumen as depicted in the Fig. 4A, or inserted into the lipid bilayer. The width between both phosphate heads of a typical lipid bilayer is around 5 nm, while the distance from last amino acid of DHFR to the α-carbon of the Asn-325 may be not sufficient to span the membrane (Fig. S3A). Alternatively, it is also possible that the oligosaccharide chain does reach the cytosol but not far enough from the membrane surface to be access by the N-glycanase, which is known to associate with p97 complex (48). Nevertheless, our results provide a snapshot catching the moment that DHFR-mCD4 was trapped in a retrotranslocating channel in the presence of TMX.

To determine if the partially retrotranslocated species were integral within membrane, we isolated crude microsomes from TMX treated cells, and performed the alkaline wash assay. The membrane-associated p97 and luminal chaperone calreticulin were removed from microsomes by the alkaline treatment, while the biotinylated substrate, similar to Calnexin, still remained associated with the membrane (Fig. 4H). Thus, the biotinylated and glycosylated DHFR-mCD4 is likely spanning the membrane lipid bilayer with the BioTag.
and the glycan moieties divided by the membrane, representing a \textit{bona fide} intermediate undergoing retrotranslocation. The residues in the D4 domain are rather hydrophilic and could not favor the hydrophobic environment within lipid bilayer (Fig. S3B). Thus, we propose that the intermediate may be integrated into the ER membrane by forming a complex with retrotranslocation machineries. In fact, TMX induced massive accumulation of ubiquitinated DHFR-mCD4 on the ER membrane (Fig. 3G; lane 3), which is probably due to the blockade of potential retrotranslocon by the stalled translocating intermediate.

Results in Figs. 2, 3 and 4 revealed that the mechanisms of the Hrd1- and Vpu-mediated retrotranslocation routes are distinct, because TMX-occupied DHFR is able to move through the former but not the latter.

**Well-folded structure within ER lumen impairs multi-spanning membrane protein retrotranslocation and degradation**

To test how the folding status of ER luminal region affects retrotranslocation and degradation of multi-spanning membrane proteins, we used a truncated TAP2 (mini-TAP2, mTAP2) that contains N-terminal four transmembrane segments from polytopic membrane protein TAP2 (Fig. 5A) (49). Thus, mTAP2 has an aberrant transmembrane region and can be considered as an ERAD-M substrate. When expressed in cells, its turnover is predominantly dependent on gp78 E3 ligase (Fig. S4A). The DHFR and a destabilizing domain (DD) of FKBP12 mutant V4A/I7V/F36V (20) were added to the N-termini to make DHFR-mTAP2 and DD-mTAP2, respectively (Fig. 5A).

To our surprise, the expression of DHFR-mTAP2 led to massive accumulation of ubiquitinated species with a much slower turnover rate than that of mTAP2 (Figs. 5B and 5C). This result clearly demonstrates that the well-folded DHFR domain appended to the luminal region of mTAP2 impairs retrotranslocation and degradation.

The FKBP12-derived DD is intrinsically flexible, but binding to its ligand Shield-1 induces formation of a compact and at least partially folded structure (20). A control experiment showed that the DD with a C-terminal ER-retaining motif -KDEL (DD-KDEL) was stable when expressed in ER (Fig. 5D), consistent with previous report that targeting DD to the ER lumen leads to accumulation even in the absence of Shield-1 (21). These results indicated that ER luminal DD itself may not act as a degron. We then found by CHX chase that DD-mTAP2 was rapidly degraded when expressed in WT 293T cells, but significantly stabilized in gp78KO cells (Fig. 5E). Incubation with either MG132, CB5083 or Shield-1 stabilized DD-mTAP2 as well (Figs. 5F, 5G and S4B). Incubation with Shield-1 did not change ubiquitination level of DD-mTAP2 (Fig. 5H), suggesting that Shield-1 may impair post-ubiquitination steps of ERAD such as retrotranslocation. Consistently, treatment with CB5083 or Shield-1 inhibited MG132-induced deglycosylation of DD-mTAP2, a hallmark of retrotranslocation (Fig. 5I). Therefore, Shield-1 slowed down substrate turnover mostly by inducing formation of a folded structure in DD, which in turn impairs mTAP2 retrotranslocation. This result implicates that unfolding of the luminal part on multi-spanning membrane protein might be necessary for the retrotranslocation.

**DISCUSSION**

Although many factors required for ERAD have been identified, substrate configuration before and during movement through the pore of a retrotranslocon remains largely obscure. In the current study, we systematically measured the capacity of three ERAD pathways in unfolding substrates for successful ER-to-cytosol transport. The most interesting finding lies in a gradually decreases in the capacities of ERAD-L, Vpu-hijacked ERAD-C, and ERAD-M in unfolding of respective substrates for retrotranslocation (Fig. 6).
The Hrd1-mediated retrotranslocation route in the ERAD-L allows the passage of 21 kDa TMX-charged DHFR. Similar results have been described for protein import through membrane of chloroplast envelop (17), and US2-mediated MHC-I heavy chain retrotranslocation, for which TRC8 E3 ligase was shown to be required (11,23). These suggested that the pore size of related translocation machineries (e.g., Hrd1 and Derlins) was large enough to accommodate the DHFR in complex with stabilizing ligand. However, cryo-EM structure showed a narrow pore within Hrd1 transmembrane region (9), suggesting that substrate unfolding might be required for threading; this led us to propose that Hrd1, TRC8 and/or its cofactors in ERAD-L pathway might have strong unfoldase activity to disentangle the TMX-charged DHFR to thread the polypeptide into the channel. It is conceivable that the unfolding steps require energy and the Derlins and associated p97 ATPase could be involved. Mechanism on the substrate unfolding before entering channel requires further investigations.

Contrary to ERAD-L substrates in which retrotranslocation initiates before ubiquitination, ERAD-M and -C substrates, such as mTAP2 and CD4 in this study, are believed to be ubiquitinated on cytosolic lysine residues before being extracted from the membrane. Retrotranslocation factors in the Vpu-hijacked pathway, however, could unfold well-folded domains such as D1-D4 domains of CD4, superfolder GFP, and the ligand-free DHFR, but not the TMX-occupied DHFR. In fact, TMX-stabilized DHFR inhibits retrotranslocation probably by docking on the entrance of potential retrotranslocon and causing steric hindrance, as evidenced by the retrotranslocating intermediate integrated into lipid bilayer (Fig. 4H). We surmise that the peptide sequence of the intermediate trapped within lipid bilayer might bind to the potential retrotranslocon stoichiometrically. This potential channel is not Hrd1 or gp78 as they are not required for Vpu-mediated CD4 retrotranslocation and degradation (Figs. S2B, S2E, and S2G).

From current and earlier studies (23), we conclude that there may be three types of retrotranslocation channels for ERAD substrates with distinct properties (Fig. 6). The first type includes Hrd1/Derlins complex and probably TRC8 that function in ERAD-L and mediate retrotranslocation of NHK and MHC-I heavy chain, respectively (11). The channels could either be very dynamic to accommodate a 21 kDa globular protein or have strong unfoldase activity capable to disentangle a tightly-folded structure like TMX-bound DHFR. The second type of channel, hijacked by Vpu, permits retrotranslocation of well-folded structure, such as superfolder GFP, DHFR and luminal domain of CD4, but does not allow structurally tight DHFR to enter. The threshold of substrate size for entering the channel is currently unknown. The third type of channel, specifically targeting multi-spanning membrane proteins is very stringent in terms of substrate flexibility, because even well folded domain, such as the DHFR and the DD bound with Shield-1, inhibits retrotranslocation. Only disordered peptides such as ligand-free DD are allowed to cross membrane. These results indicate that at least partial unfolding of luminal domain is required for entering and traversing the last two types of channels. To what extent the luminal part should be unfolded is currently unknown, and fully understanding the question awaits the advent of more quantitative method to precisely measure the extent of protein folding and maturation within ER lumen. Nevertheless, we speculated that an unfoldase might be necessary for ERAD-M substrate retrotranslocation, not only for CD4, but also for polytopic membrane proteins such as TAP2. Prominent candidates include Torsin/Lul1 that is an ER-localized AAA+ ATPase complex and has been shown to be involved in CFTR degradation (50). Whether and how Torsins and Torsin-interacting proteins are involved in ERAD-M of other substrates requires future studies.

For polytopic membrane protein like mTAP2, our results suggest that multiple
transmembrane helices are likely segregated into individual segments or sub-groups and then threaded through the channel in a sequential order. It is very unlikely that the channel allows en bloc extraction of a whole bundle of transmembrane helices, because only unstructured DD enters the retrotranslocation route. The FKBP12-derived DD used here contains 107 amino acids, which might show a small globular shape when fixed by Shield-1 according to the structure of the FKBP12-rapamycin complex (51).

In summary, our results provide insights into various routes for substrate retrotranslocation, and point out that substrates with diverse sizes or conformational properties may require different types of channel and modes of actions for successful ER-to-cytosol transport. Our biochemical system to capture the retrotranslocating intermediate paves the way to purify and identify the retrotranslocation channel and other factors in the HIV-mediated ERAD pathway.

**EXPERIMENTAL PROCEDURES**

### Plasmid, antibody and other materials

Constructs encoding Vpu, CD4, BirA<sup>cyto</sup>, and EBV-DUB have been described (34,45,46). Coding sequence for DHFR and mTAP (amino acid 1-187 of TAP2) was amplified from pCB268 (Addgene plasmid # 53363, a gift from Dr. Alexander Varshavsky) and pCDNA5/FRT/TO-TAP2-HA (a gift from Dr. Mals Mariappan), respectively. DNA sequence encoding FKBP12 was amplified from RT-PCR products of total RNA extracted from 293T cells, and cloned into pCDNA5/FRT/TO vector carrying a C-terminal either FLAG-tag. Coding region of null Hong Kong α1-antitrypsin (NHK) were amplified from pCDNA-A1AT-NHK-Venus (a gift from Dr. Mals Mariappan), and cloned into pCDNA5/FRT/TO vector, with a C-terminal FLAG-tag. Destabilizing domain (20), and superfolder GFP (52) were made by site-directed mutagenesis using FKBP12-FLAG, NHK-FLAG, and EGFP sequences as templates, respectively. For chimeric constructs shown in Figs. 1A and 5A, Gibson assembly was used to fuse linearized pCDNA5/FRT/TO, Prolactin signal sequence, DHFR or DD, and corresponding DNA sequences amplified as described above. The Prolactin signal peptide at the N-terminus directs these molecules to the ER. Plasmid expressing HA-Ub was kindly provided by Dr. Yanfen Liu. All constructs were verified by DNA sequencing.

Antibodies were from the following sources: anti-HA (Covance), anti-FLAG M2 (Sigma-aldrich), anti-FLAG polyclonal antibody (Proteintech), anti-FLAG agarose affinity gel (Sigma-aldrich), anti-CD4 (Leica), anti-Hrd1 (Cell Signaling), anti-gp78 (Cell Signaling), anti-Calnexin (Proteintech), anti-Calreticulin (Proteintech), anti-p97 (Abclonal), anti-Tubulin (Proteintech), anti-GAPDH (Proteintech), and anti-DHFR (Abcam). Antibodies against BAG6 and GFP are previously described (53). The anti-Vpu antibody was purified from serum generated by immunizing rabbits with a bacterially expressed cytosolic fragment of HIV-1 Vpu. HRP-conjugated Avidin and secondary antibodies were from the Jackson ImmunoResearch.

Small molecule compounds are as following: cycloheximide (Sigma-aldrich), MTX (MCE), TMX (MCE), CB5083 (MCE), Shield-1 (TaKaRa), and MG132 (Boston Biochem).

### Cell culture and transfection

293T and HEK293T Flp-In (Invitrogen) cell lines were cultured in high glucose DMEM (Thermo Fisher Scientific) containing 10% FBS (Hyclone, GE healthcare) at 5% CO2. Transfections with plasmids were performed with Lipofectamine 2000 according to the manufacturer’s suggestions. HEK293T Flp-In cell line stably expressing DHFR-GFP<sup>a</sup> were made by cotransfection of pCDNA/FRT/TO-DHFR-GFP<sup>a</sup> and pOG44 vector (Invitrogen), followed by screening using hygromycin (100 μg/ml) until colonies appeared.

**In vivo TMX binding**
To measure the extent to which ligands (TMX or MTX) bind to DHFR appended to various protein substrates in cultured cells, we used a reported method with modifications (Fig. 1B) (23). Typically, cells expressing substrates were treated with 100 μM ligand for 4 hours and then washed with cold PBS twice before harvesting. The cells were lysed with buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.5% Triton X-100. The lysate was clarified by centrifugation, and the supernatant was added by either proteinase K or water (as a control) and incubated for 1 hour on ice. To measure maximum binding, clarified cell lysate was incubated with 100 μM TMX 10 minutes before digestion with the proteinase K. The reactions were terminated by 2 mM PMSF and subsequently added into boiling sample buffer with continued incubation at 95 °C for 3 min. To measure post-lysis binding of DHFR with TMX, cells expressing empty vector were treated with TMX, while those expressing substrates were treated with DMSO. The cells were then washed with cold PBS for three times. Equal amounts of cells from two treatments were combined, lysed, and clarified as mentioned. The resulting supernatant was treated directly with either 0.5 mg/ml proteinase K or water (control), as described above.

Cycloheximide chase
To measure substrate turnover rate, we performed translational shut-off assay by treating cell with cycloheximide (CHX). Except DHFR-GFP that was stably expressed in HEK293 Flp-In cell line, most degradation substrates were expressed by transfection in 293T cell for 24 hours, unless otherwise indicated. Cells were then incubated with 100 μg/ml CHX for various times as shown in the figures. At each time point after adding CHX, cells were directly harvested with 2x SDS-PAGE sample buffer and boiled before loading. To measure how small molecule inhibitors impinge upon protein degradation, cells were pre-incubated with individual compound for initial 1 hour, unless otherwise indicated, followed by CHX and compound cotreatment for indicated times. DMSO was used as a control.

Immunoprecipitation (IP)
Native immunoprecipitations in Fig. 3F were performed as described (46) with some modifications. Basically, 24-48 hours after transfection, cells expressing indicated proteins were harvested and incubated in 1 ml IP buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, and protease inhibitor cocktail. The lysate was then centrifuged at 21,000 x g for 20 min. The resulting supernatant was incubated with 10 μl agarose conjugated with anti-HA antibody for 2 hours. The beads were washed three times with IP buffer, and eluted with 50 μl 2x SDS-PAGE sample buffer.

For analysis of biotinylated and ubiquitinated products in Figs. 3, 4 and 5, cells were lyzed in denaturing buffer. Briefly, cell expressing indicated constructs for 24-48 hours were washed with cold PBS, lyzed by addition of 100 μl lysis buffer containing 100 mM Tris-HCl, pH 8.0, and 1% SDS, and immediately denatured by boiling. The resulting lysate was diluted with 1 ml IP buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% Triton X-100. The diluted lysate was spun down to remove aggregates and the supernatant was subjected to IP as described above.

Microsome preparation and alkaline treatment
Crude microsome was isolated from cultured cells as described (46). Briefly, cells expressing BirA<sup>cyto</sup>, Vpu, and DHFR-Bio-mCD4 for 24 hours were washed once on 10-cm dish and then collected by pipetting in cold PBS. Cell pellet was resuspended in 1 ml hypotonic buffer (10 mM HEPES, pH 7.4, 250 mM Sucrose, 2 mM MgCl<sub>2</sub>, plus 1x protease inhibitor cocktail) and homogenized by repeated passage through a 23-gauge needle. Nuclei and unbroken cells were pelleted by centrifugation at 2000 x g for 20 minutes. The supernatant was then collected and centrifuged again to remove nuclei thoroughly. The resulting supernatant
was centrifuged at 100,000 \( \times \) g for 60 min at 4 °C. The pellet was resuspended and gently homogenized in the hypotonic buffer, 0.5 M NaCl, 0.1 M NaHCO\(_3\), pH 11, or 1% SDS, as shown in Fig. 4H. The mixtures were kept on ice for 20 min and then spun again at 200,000 x g for 60 min. The resulting supernatant and pellet were diluted and solubilized in 1.5 x SDS-PAGE sample buffer, respectively.

**Membrane protection experiment**

Cells coexpressing Vpu and DHFR-mCD4 (Fig. 3E) or Vpu, DHFR-Bio-mCD4 and BirA\(_{\text{Cys}}\) (Fig. 4C) was treated with TMX for 4 hours before harvesting. Crude microsome was isolated as described above. The crude microsomes were homogenized in a hypotonic buffer containing 10 mM HEPES, pH 7.4, 2 mM MgCl\(_2\), and 250 mM Sucrose. The resuspended microsomes were then digested with 0.5 mg/ml proteinase K in the absence or presence of detergent. The digesting reaction was stopped by PMSF and subsequently added into boiling denaturing buffer containing 1% SDS. The samples were either directly diluted with SDS-PAGE sample buffer for loading (Fig. 3E) or subjected to denaturing IP (Fig. 4C) as described above.

**Deglycosylation assay**

Samples were digested with Endo H or PNGase F (NEB) at 37 °C for 2 hours according to the manufacturer’s instructions.

**Construction of knock-out cell line using CRISPR/Cas9 technology**

Hrd1\(_{\text{KO}}\) and gp78\(_{\text{KO}}\) cells were made as described with slight modification (54). The targeting sequence to the first coding exon of Hrd1 (3′-GTGCCGTCACCTACTACC-CG-5′) and gp78 (3′-CCGCCTCGCCCTGGTCGATTG-5′) were cloned into the vector pSpCas9(BB)-2A-Puro (55) which expresses both gRNA and Cas9 nuclease simultaneously. 293T cells were transfected with the plasmid with Lipofectamine 2000 and cultured for 24 hours for expression of Cas9 and gRNA, which was then selected for by treating cell with puromycin for 3 days. The cells were then cultured in non-selecting media for additional 3 days, plated at 0.33 cells/well in 96 well plates, and expanded for 3 weeks. Individual clones were examined for Hrd1 or gp78 knock out by western blot to test expression of endogenous Hrd1 and gp78. Hrd1/gp78DKO (DKO) cells were made by transfecting plasmid expressing gRNA targeting Hrd1 exon1 into gp78\(_{\text{KO}}\) cells. The rest of the steps are the same as above described.

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**CONFLICT OF INTEREST**

The authors declare that they have no conflicts of interest with the contents of this article.

**AUTHOR CONTRIBUTIONS**

J. Shi, X. Hu, Y. Guo, L. Wang, J. Ji, and J. Li performed the experiments. All authors analyzed the results. Z.-R. Zhang supervised the project and wrote the manuscript with input from all authors.
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FOOTNOTES

3These authors contributed equally to this work.

4The abbreviations used are: ERAD, endoplasmic reticulum-associated degradation; ER, endoplasmic reticulum; DHFR, dihydrofolate reductase; DD, destabilizing domain; TMX, trimetrexate; MTX, methotrexate; HC, MHC-I heavy chain.

FIGURE LEGEND

Figure 1. TMX binds to the DHFR moiety of various substrates within the ER lumen.

(A) Schematic representation of ERAD substrates fused with DHFR used in this study. NHK is an ERAD-L substrate. Newly synthesized CD4 can be regarded as an ERAD-C substrate in the presence of HIV-encoded protein Vpu. Cytosolic UPS substrate DHFR-GFPa is used as a control. (B) Schematic representation of the protocol used to detect TMX-bound DHFR. Cells expressing DHFR-fused substrates (DHFR-sub.) were incubated with TMX or DMSO for 4 h, lyzed and treated with the proteinase K as indicated by the protocol. The indicated samples were analyzed by western blot. (C-E) Analyze binding of TMX to the DHFR moieties of DHFR-GFPa (C), DHFR-NHK (D), and DHFR-mCD4 (E) by immunoblotting with anti-DHFR antibody. Note that endogenous DHFR (endo.) is detected, while the migration of proteinase K-resistant DHFR (PK-res.) is slightly slower than that of endogenous DHFR on the gel. The experiments were performed three times independently with similar results. (F) Quantify the binding efficiency of TMX to DHFR portion in various ERAD substrates in cells. The bands in samples No. 1 and No. 2 in (C-E) were quantified by densitometry. Percent of the DHFR that binds to TMX was calculated by dividing the intensity of the 21-kDa proteinase K resistant fragment in sample No. 2 by the intensity of the non-digested protein in sample No. 1. The errors stand for S.E. of the mean from three independent experiments. (G) Analyze solubility of various substrates in the presence or absence of TMX. Cells expressing indicated substrates were treated with or without TMX and then lyzed with buffers containing sodium deoxycholate (RIPA), Triton X-100 (TX100), or SDS. After centrifugation to remove insoluble proteins, clarified lysates were subjected to immunoblotting analysis for levels of indicated proteins. Endogenous BAG6 was blotted as a loading control. The asterisk in the top panel indicates non-specific bands detected by the α-GFP antibody. Each substrate was quantified by densitometry and normalized to BAG6 levels.

Figure 2. DHFR-appended GFPα and ERAD-L substrates are degraded by proteasome in the presence of folate analogues.

(A) Cells stably expressing DHFR-GFPα were treated with DMSO, TMX, MTX, CB5083 or MG132 for indicated times before harvesting. Cell lysates were subjected to immunoblot analysis for the level of indicated proteins. This experiment was performed four times independently with similar results. Arrowheads indicate DHFR-GFPα in cells with 4 h treatment of chemicals. These bands were quantified, summarized and plotted (bottom). The error bars stand for the S.E. from four independent experiments. (B) Cells stably expressing DHFR-GFPα were treated with CHX plus DMSO (as a control) or indicated chemical compounds. At indicated time points, cell lysates were analyzed by
immunoblotting for levels of DHFR-GFPu and BAG6 (loading control). This experiment was performed three times independently with similar results. The asterisks denote non-specific bands reacting with α-GFP antibody. Bottom panel: quantification of DHFR-GFPu in the presence of indicated compounds. DHFR-GFPu level in cells without treatment was set as 100% at 0 h. The asterisks denote non-specific bands reacting with α-GFP antibody. Bottom panel: quantification of DHFR-GFPu in the presence of indicated compounds. DHFR-GFPu level in cells without treatment was set as 100% at 0 h. The errors are the S.E. of the mean from three independent experiments. (C) 293T cells expressing DHFR-NHK were incubated with CHX plus TMX, MG132, CB5083 or DMSO where indicated. At indicated time points, cell lysates were analyzed by immunoblotting for levels of indicated proteins. Arrowhead in a long exposure image indicates deglycosylated DHFR-NHK, a hallmark of retrotranslocation. (D) Quantification of DHFR-NHK in the presence of indicated compounds shown in (C). The errors are the S.E. of the mean from three independent experiments. (E) Analyze binding of MTX to retrotranslocated DHFR-NHK in the cytoplasm. Cells were transfected with DHFR-GFPu or DHFR-NHK. Where indicated, deubiquitinase EBV-DUB (or empty vector as a control) was co-transfected with DHFR-NHK. These proteins were expressed for 24 hours in cells, and then either MTX or TMX was added to incubate for 4 hours before harvesting using a buffer containing 1% Triton X-100. The clarified detergent lysates were then either left untreated or treated with proteinase K, followed by immunoblot analysis with α-DHFR antibody. Black arrowheads denote deglycosylated retrotranslocated substrate, while the red one represents proteinase-K resistant DHFR bound with MTX.

Figure 3. TMX inhibits Vpu-mediated DHFR-mCD4 retrotranslocation and degradation.

(A) Schematic diagram of factors involved in Vpu-mediated ERAD of DHFR-mCD4, which represents the DHFR fused mini-CD4 (mCD4). (B) A CHX chase experiment to analyze DHFR-mCD4 degradation. Cells cotransfected with plasmids encoding Vpu and DHFR-mCD4 were treated with TMX, MG132, or CB5083 for 1 hour before co-incubation with CHX for indicated times. Cell lysates were analyzed by immunoblotting for the levels of DHFR-mCD4, Vpu, and BAG6 (loading control). Upon MG132 treatment, faster migration species were observed and indicated as deglycosylated substrate. Note that Vpu is phosphorylated, evidenced by its slower migration on the gel. The result is the representative of three independent experiments. (C) Quantification of results in (B). The errors are the S.E. of the mean from three independent experiments. (D) DHFR-mCD4 retrotranslocation in cells. DHFR-mCD4 and wild-type Vpu (WT) or Vpu-S52N/S56N phosphorylation mutant (SN) were expressed in 293T cells for 24 hours. Cells were then treated with MG132 (4 hours), CB5083 (4 hours) or TMX (5 hours). For TMX and MG132 co-treatment, cells were pre-treated with TMX for 1 h before co-incubation with additional MG132 for 4 hours. Cell lysates were analyzed by immunoblotting for levels of indicated proteins. Retrotranslocated DHFR-mCD4 appeared as a deglycosylated species upon MG132 treatment. This experiment was performed three times independently with similar results. (E) Analyze topology of DHFR-mCD4 after TMX treatment. Cells expressing Vpu and DHFR-mCD4 were treated with TMX for 4 hours before harvesting and microsome preparation. Crude microsomes were resuspended in a buffer with or without Triton X-100. They were then either left untreated or treated with proteinase K before denaturation with boiling SDS sample buffer. Samples were analyzed by immunoblotting with α-DHFR antibody. (F) DHFR-mCD4 was coexpressed with HA-tagged WT or SN Vpu, treated with TMX for 4 hours, and analyzed by immunoblotting directly (top) or after immunoprecipitation (IP) with α-HA antibodies (bottom). (G) DHFR-CD4 ubiquitination in cells. DHFR-mCD4 (FLAG tag) and HA-ubiquitin were cotransfected with or without Vpu, treated with indicated combinations of chemicals for 4 hours, subjected to denaturing IP with α-FLAG, and analyzed by immunoblotting for HA-ubiquitin and DHFR-mCD4. The numbers of HA-ubiquitins attached on FLAG tagged DHFR-mCD4 are indicated. This experiment was performed twice independently with similar results.

Figure 4. Characterization of a trapped retrotranslocating intermediate of DHFR-mCD4.
(A) Diagram of three potential forms of substrate DHFR-Bio-mCD4, with the glycan indicated in blue, and the BioTag in red. The BioTag is inserted into the D4 domain, as indicated. The substrate become biotinylated once the BioTag traverses the ER membrane and reaches the cytosol where BirA<sup>c</sup> is expressed. (B) Analyze biotinylation of DHFR-Bio-mCD4 in the absence or presence of TMX. 293T cells coexpressing Vpu and FLAG tagged DHFR-Bio-mCD4 with or without BirA<sup>c</sup> were either left untreated or treated with TMX before harvesting. The cell lysates were analyzed by immunoblotting directly or after denaturing IP with α-FLAG antibody. The asterisk indicates non-specific bands that are used as a loading control. This experiment was performed three times independently with similar results. (C) Analyze biotinylation status of full-length (FL) DHFR-Bio-mCD4 and the membrane-protected fragment (PF). Crude microsomes were isolated from 293T cells coexpressing Vpu, DHFR-Bio-mCD4, and BirA<sup>c</sup> with TMX treatment for 4 hours, and were subjected to digestion with proteinase K (PK) in the absence or presence of detergent (Deter.). The products were immunoprecipitated using antibodies against N-terminal FLAG tag and analyzed by blot with α-FLAG antibody and HRP-conjugated avidin. The asterisks indicate non-specific bands. (D) Cells coexpressing Vpu, DHFR-Bio-mCD4 and BirA<sup>c</sup> were treated with TMX, MG132 or DMSO (control). Cell lysates were analyzed as in (B). The asterisk indicates non-specific bands that are used as a loading control. This experiment was performed three times independently with similar results. (E) Diagram of potential forms of Bio-DHFR-mCD4 in which the BioTag is inserted between FLAG tag and the DHFR moiety of the substrate. (F) Analyze biotinylation of Bio-DHFR-mCD4 with indicated treatment. Cell lysates were analyzed by western blot. The experiment was repeated for two times with the same result. (G) Analyze glycosylation status of biotinylated DHFR-Bio-mCD4. Crude microsomes prepared in (C) were solubilized with detergent and subjected to digestion with Endo H or PNGase F. The resulting materials were analyzed by western blot with indicated reagents. (H) Alkaline wash of microsome containing biotinylated DHFR-mCD4. Microsomes prepared in (C) were resuspended in low salt buffer, 0.5 M NaCl, 0.1 M NaHCO<sub>3</sub> (pH 11), or 1% SDS. The mixtures were subjected to ultracentrifugation and the supernatant (S) and pellet (P) fractions were analyzed by blot with indicated antibodies or HRP-conjugated avidin. The total lysate from the cells coexpressing Vpu, DHFR-Bio-mCD4 and BirA<sup>c</sup> and treated with MG132 was loaded in the last lane to show the position of deglycosylated DHFR-Bio-mCD4 on the gel. This experiment was performed three times independently with similar results.

Figure 5. Well-folded domains impair retrotranslocation of multi-pass membrane protein from the ER.

(A) Schematic representation of mTAP2 fused with DHFR or destabilizing domain (DD). mTAP2 is an ERAD-M substrate. Note that the DD-mTAP2 contains a glycosylation site at the C-terminal (indicated as -CHO). (B) A CHX chase experiment to analyze turnover rate of mTAP2 and DHFR-mTAP2 in the absence or presence of TMX. Cell lysates were analyzed by immunoblotting for levels of indicated proteins. Modified substrates, most likely by ubiquitination, were observed after long exposure. The positions of substrates containing one, two, three, or four ubiquitins were indicated. The result is a representative of three independent experiments. (C) Relevant bands in (B) are quantified, and the percentage of remaining nonubiquitinated (top) and ubiquitinated (bottom) substrates are plotted. (D) Analyze turnover rate of the DD expressed in the ER lumen. DD-KDEL containing a cleavable Prolactin signal peptide and a C-terminal KDEL motif was expressed in cells for 24 hours. Cells were then treated with Shield-1 or DMSO (control) for an initial period of 5 hours, followed by CHX and Shield-1 (or DMSO) co-incubation for the indicated times before lysis. The cell lysates were then analyzed for levels of DD and BAG6. The experiment was performed two times independently with similar results. (E) A CHX chase experiment to analyze turnover rate of DD-mTAP2 in WT and gp78<sup>K/O</sup> cells. A part of DD-mTAP2 is unglycosylated. Quantification of the result is shown in the bottom panel. The errors are the S.E. of the mean from three independent
experiments. (F) Analysis of DD-mTAP2 degradation in the presence of Shield-1 or MG132 by CHX chase. Where indicated, cells were pre-incubated with either Shield-1 for 5 hours or MG132 for 1 hour before co-incubation with CHX. The experiment was repeated for three times independently with similar results. (G) Quantification of results in (F). The errors are the S.E. of the mean from three independent experiments. (H) Ubiquitination of DD-mTAP2 in the presence or absence of Shield-1. The result is a representative of two independent experiments. (I) Analyze retrotranslocation of DD-mTAP2 in the presence of Shield-1, CB5083 or DMSO (control). The relevant bands were quantified, and the percentage of unglycosylated substrate was plotted and shown in the histogram (bottom). The error bars shown represent the S.E. from four independent experiments. P values determined by Student’s t test are indicated.

Figure 6. Proposed model of three potential retrotranslocation pathways in mammalian ERAD.

NHK follows Hrd1-mediated retrotranslocation pathway (ERAD-L). A strong unfoldase activity might disentangle tightly-folded structure in substrates (e.g., TMX-occupied DHFR). Alternatively, the pore of retrotranslocation complex could be large enough to accommodate TMX-bound DHFR. Vpu-mediated protein degradation exploits a currently unidentified retrotranslocation channel (Vpu-hijacked ERAD-C). For moving substrate through membrane, the channel and/or cofactors are able to unfold the DHFR fused to the substrate, but not the TMX-bound DHFR. Luminal region of polytopic membrane proteins must be in a flexible conformation in order to be threaded through the potential channel; the channel or channel-associated cofactors may not disentangle well-folded domains such as DHFR or Shield-1-occupied destabilizing domain (ERAD-M).
Figure 1

A

B

C

D

E

F

G

DHFR-GFP

ER lumen

Cytosol

ERAD-L

DHFR-NHK

DHFR-mCD4

Vpu

ERAD-C (Vpu-hijacked)

DHFR-sub. TMX

Vector

DHFR-sub. TMX

Vector

DMSO

DMSO

TMX

Wash

Mix

Wash

Mix

Lysis

Lysis

Mock

Real binding

Maximal binding

Mock

Carry over

No.: 1 2 3 4 5

IB: α-DHFR

No.: 1 2 3 4 5

IB: α-DHFR

No.: 1 2 3 4 5

IB: α-DHFR

No.: 1 2 3 4 5

IB: α-DHFR

% of DHFR charged with TMX

DHFR-GFP

DHFR-NHK

DHFR-mCD4

Net

23 38 16 89 73 100

24 68 13 61 85 100

18 53 14 54 86 100

55

180

130

55

180

130

lysis buffer

RIPA

TX100

SDS

TMX

- + - - +

DHFR-GFP

DHFR-NHK

DHFR-mCD4

BAG6

BAG6

BAG6
Figure 3

(A) Diagram illustrating the pathways of DHFR-mCD4 retrotranslocation and proteasomal degradation.

(B) Western blot analysis of DHFR-mCD4, Vpu, and BAG6 with various inhibitors.

(C) Graph showing the percentage of DHFR-mCD4 remaining over time with different inhibitors.

(D) Table showing the effect of different inhibitors on DHFR-mCD4, Vpu, and Tubulin.

(E) Western blot for IB: α-DHFR with Detergent and Microsome.

(F) Western blot for IB: α-DHFR with Detergent and Microsome.

(G) Western blot for α-FLAG IPs with Cell lysate and α-HA (Ub) with Ub5, Ub4, Ub3, Ub2, Ub1.
Figure 4

A

Retrotrans-
located

B

Retrotrans-
located

Cytosol

ER lumen

D

Total lysate

α-FLAG IPs

(denaturing)

TMX

MG132

Blot:

α-FLAG

55

glyc.

derglyc.

Blot:

Avidin

55

55

40

DHFR-Bio-mCD4

E

Blot:

α-FLAG

55

55

40

DHFR-Bio-mCD4

F

Total lysate

Vpu

Bio-DHFR-mCD4

BirA<sup>Δ</sup>

TMX

MG132

Blot:

α-FLAG

55

55

40

DHFR-Bio-mCD4

G

untr eat

Ench

Endo

DHFR-Bio-mCD4

H

Microsome

Buffer

NaCl

pH11

SDS

MG132

long exposure

Blot:

α-FLAG

Avidin

α-Calnexin

α-p97

α-Calreticulin

55

55

100

55

100

55

35

70

100

100

100

55

35

70

100

100

100

55

40

55

40

55

40

55

40
Figure 5

A) Schematic diagram showing the movement of mTAP2, DHFR-mTAP2, and DD-mTAP2 between the cytosol and ER lumen.

B) Western blot analysis showing the effect of CHX treatment on the levels of mTAP2, DHFR-mTAP2, and Tubulin. The blots are shown for both TMX conditions: -TMX and +TMX.

C) Graphs showing the percentage of nonubiquitinated and ubiquitinated substrates (%) remaining at different CHX (h) concentrations for mTAP2, DHFR-mTAP2 (-TMX), and DHFR-mTAP2 (+TMX).

D) Western blot analysis comparing the effect of DMSO and Shield-1 on the levels of DD-KDEL and BAG6 under CHX treatment.

E) Western blot analysis showing the effect of CHX treatment on the levels of WT and gp78KO FLAG-DD-mTAP2. The blots are shown for both glyc. and unglyc. conditions.

F) Western blot analysis showing the effect of DMSO, Shield-1, and MG132 on the levels of DD-mTAP2, DD-mTAP2 (Dark), and BAG6.

G) Graph showing the percentage of DD-mTAP2 remaining at different CHX (h) concentrations for DMSO, Shield-1, and MG132.

H) Western blot analysis showing the effect of Shield-1 and α-FLAG IPs on the levels of Cell lysate and FLAG-DD-mTAP2 (FLAG tag). The blots are shown for both glyc. and unglyc. conditions.

I) Western blot analysis comparing the effect of DMSO, Shield-1, and CB5083 on the levels of DD-mTAP2, BAG6, and Retrotrans. under MG132 treatment.
Figure 6

Unfolding capacity (or pore size) of retrotranslocation complex

High (large)  
ERAD-L  
Vpu-hijacked ERAD-C  
ERAD-M  
Low (small)

Cytosol  
Hrd1 complex  
Unknown channel  
High  
Low (large)  
Low (small)

ER lumen  
+TMX  
DHFR-CD4  
DHFR-mTAP2  
DD-mTAP2  
high  
Conformational flexibility  
High  
Low
A technique for delineating the unfolding requirements for substrate entry into retrotranslocons during endoplasmic reticulum–associated degradation
Junfen Shi, Xianyan Hu, Yuan Guo, Linhan Wang, Jia Ji, Jiqiang Li and Zai-Rong Zhang

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