Ricin is a potent A-B toxin that is transported from the cell surface to the cytosol where it inactivates ribosomes leading to cell death. Ricin enters cells via endocytosis where only a minute number of ricin molecules reach the ER (endoplasmic reticulum) lumen. Subsequently, the ricin A chain traverses the ER bilayer by a process referred to as dislocation or retrograde translocation to gain access to the cytosol. In order to study the molecular processes of ricin A chain dislocation, we have established for the first time a human cell system in which enzymatically attenuated ricin A chains (RTA E177D and RTA Δ) are expressed in the cell and directed to the ER. Using this human cell based system, ricin A chains were found to undergo a rapid dislocation event that was quite distinct than the dislocation of a canonical ER soluble misfolded protein, α1-antitrypsinHKnull. Remarkably, ricin A chain dislocation occurred via a membrane integrated intermediate and utilized the ER protein SEL1L (suppressor of lin-12-like) for transport across the ER bilayer to inhibit protein synthesis. The data support a model that ricin A chain dislocation occurs via a novel strategy of utilizing the hydrophobic nature of the ER membrane and selective ER components to gain access to the cytosol.

Ricin toxin is a toxic polypeptide found in castor beans (Ricinis communis) (1) comprised of a catalytically active polypeptide (A chain or RTA) covalently linked by a disulfide bond to a lectin binding B subunit (RTB). Like most A-B toxins, the specificity of the B chains for cell surface receptors dictates cell entry. In the case for ricin toxin, the galactose-specific lectin binding domain of the B chain interacts with cell surface glycolipids and glycoproteins allowing the toxin to enter the cell via clathrin-dependent and clathrin-independent endocytosis (2).

Following endocytosis, ricin toxin is transported in a retrograde manner through the Golgi, ER and into the cytosol by a similar manner as shiga toxin, cholera toxin, Pseudomonas exotoxin A, and pertussis toxin (3). These toxins are proposed to utilize protein kinases during the endocytosis step (4). In contrast, other AB toxins such as diphtheria toxin and anthrax toxin enter the cytoplasm following a low pH induced translocation event directly from endocytic vesicles (3).

The retrograde trafficking of ER-directed toxins requires numerous cellular factors to reach the ER lumen (4). Both ricin and Shiga toxin are dependent on Vps34, a PI3 kinase, and members of the sorting nexins family of proteins. In the case of ricin toxin, it is independent of Rab-9 and -11 (5) and dependent on dynamin (6) and cellular cholesterol levels (7). A recent high-throughput screen identified small molecule inhibitors that demonstrate the ability to protect cells from ricin toxicity by targeting endosome-to-Golgi retrieval factors, specifically syntaxin 5 and 6 (8).

Despite the lack of a KDEL sequence, the heterodimer is transported from the Golgi apparatus to the ER lumen. While in the ER, the ricin A chain disassociates from RTB and
is transported across the membrane by a process referred to as dislocation or retrograde translocation (9). Once in the cytosol, the A chain acts as an N-glycosidase which inactivates the ribosome and eventually causing cell death. However, ricin-induced cell death may also be due to inhibition of processes other than translation shut-down (10).

Ricin is a member of the A-B family of toxins (e.g. shiga, cholera, Pseudomonas exotoxin A, or pertussis toxin) that reach the ER lumen and are ultimately transported into the cytosol (3). Ricin takes advantage of the ER quality control machinery – a cellular system that normally scrutinizes nascent polypeptides and disposes of misfolded proteins by proteasome destruction (11). The ER chaperones PDI and calreticulin are proposed to play a role in ricin transport across the ER membrane (12,13). In addition, the ER degradation enhancing α-mannosidase I-like protein involved in degradation of misfolded ER proteins (14) also participates in ricin dislocation (15). Recently, ricin studies in yeast cells found that Hrd1p was implicated in ricin dislocation (16). The toxin is proposed to be extracted through the ER membrane via the Sec61 translocon (17), but a recent study suggests that the Hrd1p complex may act as the dislocon (18). Once dislocated, the Hsc70 cytosolic chaperone machinery protects ricin A chain from Hsp90 assisted cytosolic degradation (19). The mechanism of ricin dislocation in human cells has not been well defined despite the identification of cellular factors that ricin utilizes for dislocation.

The potency of ricin to inactivate 2,000 ribosomes/minute precludes the need for a large number of molecules to reach the cytoplasm (20,21). In order to characterize one of the rate limiting steps of ricin entry, dislocation, a human cell system has been created for the first time that expresses an enzymatic mutant of ricin A chain in the ER. Using this model system, ricin A chain dislocation occurs in a unique manner utilizing the ER membrane itself as well as ER proteins.

**EXPERIMENTAL PROCEDURES**

**Cell lines and antibodies:** Human U373-MG astrocytoma expressing RTA polypeptides (U373<sub>RTAA</sub> and U373<sub>RTA-E177D</sub>) were generated and maintained as described (22). Anti-ricin toxin, anti-GAPDH, anti-α1antitrypsin, anti-SEL1L, anti-gamma tubulin, anti-GFP and anti-p97 antibodies were purchased from Biodesign International, Millipore Corporation, Chemicon International Inc., Sigma, Santa Cruz, and AbCam, respectively. Anti-HA (12CA5) antibodies were purified from hybridoma cells (23). The anti-calnexin monoclonal antibody (AF8) (Hochstenbach, et al., 1992) and anti-PDI antibody were gifts from Dr. Brenner (Harvard Medical School, Boston, MA) and Dr. Ploegh (Whitehead Institute, Cambridge, MA), respectively.

cDNA constructs: Ricin toxin mutant chimeras RTA<sub>E177D</sub> and RTA<sub>A177-181</sub> (RTA<sub>A</sub>) (24) were generated from PCR fragments (22) using full length wild type ricin toxin A chain (FL-RTAWT) cDNA as a template. The RTA<sub>E177D</sub> represents a site-directed point mutant in which residue 177 (glutamic acid, E) was changed to aspartic acid (D). The RTA<sub>A</sub> mutant is a deletion construct lacking residues 177-EAARF-181. Finally, the chimeras consisted of an amino terminal murine MHC class I heavy chain H2-K<sup>b</sup> signal peptide (K<sup>b</sup>) followed by the hemagglutinin (HA) epitope tag (AYPYDVPDYA), linker region (15 aa) and RTA<sub>WT</sub>, RTA<sub>E177D</sub> or RTA<sub>A</sub>. The Derlin-1-GFP construct was generated as a chimera by fusing the Derlin-1 cDNA to EGFP cDNA. The shRNA construct against SEL1L was a kind gift from Dr. Ploegh (Whitehead Institute). SEL1L knockdown in HeLa cells was performed using shRNAs from Santa Cruz Biotech.

**Cell lysis, Immunoprecipitation, SDS-PAGE, Endoglycosidase H and peptide N-glycanase assays:** Briefly, cells (1 X 10^6) were lysed in 0.5% Nonident P (NP)-40 lysis mix followed by incubation with the respective antibody and protein A agarose beads (22). The immunoprecipitates were subjected to immunoblot analysis using the respective immunoglobulin. Endoglycosidase H and
peptide N-glycanase (New England Biolabs) assays were performed at 37°C for 1.5 hrs (100 units enzyme/reaction).

**Pulse-chase analysis:** Briefly, cells were labeled with 35S-methionine and chased in cold methionine (25 mM) (22). RTA proteins were recovered from NP-40 cell lysates using anti-HA antibodies and resolved using SDS-PAGE (12.5%). The polyacrylamide gels were dried and exposed to autoradiography film. The polypeptide levels were quantified by densitometry analysis using an Alpha Imager 3400 (Alpha Innotech).

**Subcellular fractionation:** RTA expressing cells were mechanically homogenized using a 12 mm ball bearing homogenizer (12 passes) (Isobiotec, Heidelberg, Germany) in homogenization buffer (100 mM Tris, 150 mM NaCl, 250 mM sucrose, 1.5 µg/ml aprotinin, 1 µM leupeptin, and 200 mM phenylmethylsulfonyl fluoride) (25). Unbroken cells and other debris were pelleted at 15,000g (10 min @ 4°C) and the supernatants were untreated or treated with 4.5M urea (final) (10 min @ 4°C). Supernatants were centrifuged at 100,000g (1 h @ 4°C). 100,000g supernatants and pellets were resolved by SDS-PAGE (12.5%).

**Isoelectric focusing:** The RTA precipitates were incubated with isoelectric focusing sample buffer (57% (w/v) urea, 2% (v/v) NP-40, 0.02% ampholytes pH 3.5–10 (Amersham Biosciences), 0.025% 2-mercaptoethanol), and resolved on a 17-cm 1D-IEF gel (57% (w/v) urea, 2% (v/v) NP-40, 15.5% acrylamide (30/1.6 acrylamide/bisacrylamide), 4% ampholytes pH 5–7, 1% ampholytes pH 3.5–10, 0.4% ampholytes pH 7–9) for 14 hrs. After electrophoresis, the gel was soaked in 50% methanol, 1% (w/v) SDS, 5 mM Tris Cl, pH 8.0 for 2 hrs and subjected to immunoblot analysis (25).

**Ricin activity assays.** HEK-293T cells were co-transfected with RTA constructs and GFP-expression plasmid (pCAGGS(GFP)) using Lipofectamine 2000 (Invitrogen). Thirty hours post transfection, GFP positive cells were examined using a Beckman Coulter Cytomics FC 500 Flow Cytometer and the data was analyzed using FlowJo software. HeLa cell lines seeded in 96-well plates at 2x10^4/well were incubated with doubling dilutions of ricin in quadruplicate for various times. The cells were incubated with 35S-promix (Perkin Elmer). The amount of radioactivity incorporated into TCA-precipitable proteins was measured by scintillation counting in a Micro-Beta 1450 Trilux counter and IC_{50} values (representing the concentration of toxin reducing protein synthesis by 50% relative to untreated control cells) recorded.

**RESULTS**

**Ricin A chains directed to the ER block protein synthesis.**

The investigation of ricin toxin transport across the ER membrane has been hampered by the low number of toxin molecules that reach the ER lumen when added extracellularly (17). Hence, we aimed to create a human cell-based assay to specifically study RTA transport across the ER membrane. In line with this objective, we initially confirmed that RTA molecules directed to the ER (10,26) in human cells limited protein expression. Full length wild-type RTA (FL-RTA_{WT}) and wild-type mature RTA equipped with a murine MHC class I molecule (K^b) signal peptide and an HA epitope tag at its N-terminus (RTA_{WT}) were co-transfected with a GFP expression plasmid in HEK-293T cells and evaluated for GFP fluorescence using flow cytometry (Figure 1A-D). The lack of GFP fluorescent cells is indicative of functionally active RTA molecules. FL-RTA_{WT} and RTA_{WT} limited the expression of GFP in a significant percentage of cells (Figure 1C and D, 2% and 15%, respectively) when compared to cells transfected with GFP alone (Figure 1B, 85%). These data suggest that ER directed RTA molecules are dislocated across the ER and to the cytosol where they inhibit protein synthesis.

**Ricin A chain expression in human cells**

Due to the toxic nature of wild type RTA, we cloned ricin A chain mutants (RTA_{E177D} and RTA_{A}), Experimental
Procedures) that are enzymatically attenuated to study A chain dislocation (27,28). RTA_E177D is a site-directed mutant with attenuated activity that is structurally similar to wild type (29), while RTAΔ has deletion of residues 177-181. These constructs like RTA_WT were also engineered with the N-terminal Kb signal peptide and HA epitope tag. An initial experiment was performed to determine whether RTA_E177D and RTAΔ inhibit protein synthesis. GFP positive cells were examined in HEK-293T cells co-transfected with RTA_E177D and RTAΔ and a GFP expression plasmid (Figure 1E-F). The RTA mutants, RTA_E177D and RTAΔ had a diminished ability to affect GFP expression (Figure 1E-F) with RTA_E177D retaining some capacity to inhibit GFP expression. These data demonstrate that ricin A chain mutants have limited capacity to prevent protein expression.

In order to study RTA dislocation, human U373 cells that stably express RTA_E177D and RTAΔ (Experimental Procedures) (Figure 2) were created. Our initial experiment examined the acquisition of an N-linked glycan onto RTA_E177D and RTAΔ to confirm their localization to the ER. RTA_E177D and RTAΔ molecules recovered from these cells were subjected to Endo H treatment followed by immunoblot analysis (Figure 2A). Endo H preferentially cleaves immature high mannose containing N-linked oligosaccharides characteristic of ER-resident molecules (30). RTA_E177D and RTAΔ polypeptides with the predicted molecular weight were recovered from cell lysates (Figure 2A, lanes 3 and 5). As expected, faster migrating RTA polypeptides were observed upon treatment with Endo H (Figure 2A, lanes 2, 4, and 6). The difference in the relative molecular weight of the two species (~3-4 kDa) confirmed that the RTA polypeptides acquired a single N-linked glycan and were retained in the ER. These data demonstrate that this human cell-based assay can be utilized to study ricin dislocation.

Ricin A chains are subjected to proteasome degradation.

We next investigated the stability of the RTA polypeptides in U373RTA_E177D and U373RTAΔ cells by pulse-chase analysis (Figure 2B and C). The cells were metabolically labeled with 35S-methionine for 15 min and chased up to 60 min. Strikingly, the half-life of RTA_E177D (~10 min) was less than RTAΔ (~20 min) (Figure 2B, lanes 1-8 and 2C). Given that ricin utilizes ER quality control to gain access to the cytosol (9,16), it is expected that a population of ricin would be degraded. However, the rapid half-life of RTA_E177D expressed in human cells when compared to yeast (16,26) and plant cells (31,32) suggests that RTA is efficiently transported out of the ER in human cells.

Are RTA polypeptides targeted for proteasome dependent degradation? To address this question, U373RTA_E177D and U373RTAΔ cells treated with or without proteasome inhibitor carboxybenzyl-leucyl-leucyl-leucyl vinyl sulfone (ZL_3VS) (25 µM, 1 hr) were subjected to pulse-chase analysis (Figure 3). As expected, RTA polypeptides decreased over the chase period (Figure 3A and B, lanes 1-3). Strikingly, the inclusion of proteasome inhibitor stabilized both RTA_E177D and RTAΔ molecules (Figure 3A and B, lanes 4-6). In addition, proteasome inhibition enabled the recovery of a faster migrating polypeptide intermediate (*) during the chase, most likely deglycosylated RTA species (Figure 3A and B, *, lanes 4-6). Collectively, the data demonstrate that ricin A chains are eventually degraded in a proteasome dependent manner.

Characterization of RTA_E177D and RTAΔ intermediates.

We next optimized conditions to observe the faster migrating RTA species. Total cell lysates from U373RTA_E177D and U373RTAΔ cells treated with proteasome inhibitor ZL_3VS (10 µM) for up to 5 hours were subjected to immunoblot analysis (Figure 4A). Strikingly, RTA_E177D proteins were almost completely converted to the faster migrating species after 2 hrs of ZL_3VS treatment (Figure 4A, lanes 9-12). In contrast, RTAΔ molecules displayed a biphasic
conversion in which an increase in glycosylated RTAΔ was observed during the early times of proteasome inhibition (Figure 4A, lanes 2-3) followed by an accumulation of glycosylated and faster migrating species during later times of ZL3VS treatment (Figure 4A, lanes 4-6). Interestingly, the ratio of glycosylated (RTA(+)CHO) to faster migrating species following proteasome treatment significantly varied between the RTA mutants (Figure 4A, lanes 1-6 and 7-12, respectively). These results suggest that RTAΔ and RTAE177D are likely processed differently due to the folding status of the polypeptide.

To address the N-linked glycosylation status of the RTAΔ and RTAE177D intermediates, we examined their sensitivity to in vitro glycosidase treatment (Figure 4B). RTA polypeptides recovered from U373RTAΔ and U373RTAE177D cells treated with proteasome inhibitor (2.5 µM, 10 hours) were undigested or digested with EndoH (EH) or peptide N-glycanase (PNGase or PN) followed by immunoblot analysis (Figure 4B). U373 cells were used as a negative control. As expected, two species of RTA polypeptides were observed exclusively from proteasome-inhibitor treated cells (Figure 4B, lanes 10 and 16). Moreover, only the slower migrating glycosylated species were sensitive to EndoH and PNGase digestion resulting in the faster migrating form of the RTA-reactive polypeptides (Figure 4B, lanes 11-12 and 17-18). Calnexin levels demonstrated equivalent protein loading (Figure 4B, lanes 19-36). These data suggest that the faster migrating RTA species are deglycosylated intermediates generated from the dislocation of ricin polypeptides.

In order to confirm that the N-terminal epitope tag does not affect the processing of the RTA polypeptides, we examined the accumulation of untagged RTA mutants upon proteasome inhibition (Supplemental Figure 1). Cell lysates from proteasome inhibitor treated U373, U373RTAΔ, U373RTAE177D, U373KRTAΔ, and U373KRTAΔΔ cells were subjected to immunoblot analysis. Two species of RTA molecules, glycosylated and deglycosylated, accumulated in proteasome inhibitor treated cells; a result demonstrating that the stability of both forms of RTA is independent of the N-terminus epitope tag.

To confirm that the faster migrating RTA species were indeed digested by cellular PNGase, the RTA polypeptides were resolved on a 1D-IEF gel. PNGase cleavage results in the conversion of asparagine to aspartic acid causing a more acidic species, thus changing the overall charge of the polypeptide (33). RTA polypeptides recovered from U373RTAΔ cells treated with proteasome inhibitor (2.5 µM, 10 hours) were untreated or treated with EndoH or PNGase, resolved on a 1D-IEF gel and subjected to immunoblot analysis (Figure 4C). RTAΔ and RTAE177D polypeptides from ZL3VS-treated cells migrated as two discrete bands likely corresponding to species with different charges (Figure 4C, lane 1, (* and **)). Strikingly, in vitro PNGase digestion of RTAE177D polypeptides caused the polypeptides to migrate at a similar position as the more acidic RTA molecules (Figure 4C, lane 2, **), while EndoH treatment did not alter the migration pattern of the RTAE177D polypeptides (Figure 4C, lane 3). The same result was observed for RTAΔ. These results demonstrate that RTA polypeptides are subjected to cellular deglycosylation during dislocation.

Ricin A chains utilize ER membrane factors for efficient dislocation.

To characterize the membrane topology of stabilized RTA polypeptides, we performed a subcellular fractionation protocol (Experimental Procedures). Membrane microsomes from U373RTAΔ and U373RTAΔ cells treated with proteasome inhibitor (2.5 mM, 10 hrs) were incubated with 4.5M urea and subjected to high-speed centrifugation (100,000g) to separate the membrane fraction from the supernatant fraction. The 100,000g supernatant, 100,000g pellet, and total cell lysates were subjected to immunoblot analysis for RTA mutants (Figure 5, lanes 1-10) and p97 (Figure 5, lanes 11-20). The p97 polypeptide is a soluble cytosolic protein that associates with the ER membrane (34) and as expected, was completely extracted from the
bilayer upon treatment with urea (Figure 5, lanes 12 and 14). As expected, some of the stabilized RTA_{E177D} and RTA_{Δ} molecules were found in the 100,000g supernatant (Figure 5, lanes 6 and 8). Strikingly, a significant population of RTA_{E177D} and RTA_{Δ} was observed with the membrane fraction (Figure 5, lanes 2 and 4) demonstrating that these RTA molecules are integrated into the bilayer. These results imply that ricin A chains are efficiently dislocated via membrane-integrated intermediates.

*Ricin A chains are dislocated utilizing selective components of ER quality control.*

To validate that RTA polypeptides were dislocated utilizing the ER quality control machinery, we examined the effect of a dislocation inhibitor, eeyarestatin I (35), on the stability of ricin A chains. U373_{RTA-E177D} and U373_{RTA_{Δ}} cells treated with or without eeyarestatin (5 µg/mL) or the proteasome inhibitor ZL3VS (2.5 µM) were subjected to immunoblot analysis (Figure 6A). PDI levels assured equivalent protein loading (Figure 6A, lanes 9-16). As expected, treatment with ZL3VS caused a significant increase in both RTA_{Δ} and RTA_{E177D} polypeptides (Figure 6A, lanes 5-8) with a difference between the levels of glycosylated (RTA(+CHO)) and deglycosylated (RTA(-CHO)) molecules. Interestingly, mostly glycosylated RTA_{Δ} and RTA_{E177D} polypeptides accumulated from cells treated with eeyarestatin I (Figure 6A, lanes 2 and 4). However, since eeyarestatin I attenuates the process of dislocation and not proteasome function (35), it was interesting that deglycosylated forms of the ricin A chain were observed (Figure 6A, lanes 2 and 4). Therefore, ricin A chain dislocation probably occurs using a distinct mechanism compared to ERAD substrates.

To further explore the involvement of dislocation-associated cellular proteins in the transport of ricin toxin across the ER membrane, we investigated the role of SEL1L and Derlin-1 in the dislocation of RTA_{E177D} and RTA_{Δ} polypeptides. SEL1L functions as part of the mammalian Hrd1 ligase complex and is proposed to be involved in the recognition of ER degradation substrates (36,37). Derlin-1 plays a role in the extraction of certain misfolded ER proteins and a dominant negative form, Derlin-1-gfp can impede substrate dislocation (38-40). U373_{RTA-E177D} and U373_{RTA_{Δ}} transduced with Derlin-1-gfp and shRNA against SEL1L were untreated or treated with ZL3VS (2.5 µM, 10 hrs) and subjected to immunoblot analysis (Figure 6B). The expression of the constructs was confirmed using anti-SEL1L (Figure 6B, lanes 13-24) and anti-GFP (Figure 6B, lanes 25-36) immunoblots. Derlin-1-gfp expression did not alter the levels of RTA polypeptides regardless of proteasome inhibition (Figure 6B, lanes 1-2, 4-5, 7-8, and 10-11). These results are consistent with published findings implicating that ricin toxin transport across the ER membrane is Derlin-1 independent (15). On the other hand, silencing of SEL1L resulted in a substantial increase of RTA polypeptides in both the presence and absence of proteasome inhibition (Figure 6B, lanes 3, 6, 9, 12). Anti-GAPDH immunoblot indicates equal protein loading (Figure 6B, lanes 37-48). These results imply that ricin can utilize specific components of ER quality control machinery to be dislocated across the ER membrane.

We next examined whether deficiency in SEL1L levels affects ricin toxicity (Figure 7). Ricin holotoxin was exogenously administered to HeLa cells stably expressing shRNA against SEL1L or a control shRNA (Figure 7A). A control cell line (C1), a cell line revealing no SEL1L knockdown (120% of control; S8) and a cell line showing 53% knockdown (S24) were utilized in subsequent cytotoxicity experiments (Figure 7A). After inclusion of ricin holotoxin, the incorporation of radiolabeled amino acids into nascent polypeptides was measured by scintillation counting. Protection from toxin challenge would be demonstrated if the IC_{50} ratio of SEL1L knockdown cells to control cells is greater than 1. The ricin IC_{50} for S8 cells was equivalent to the C1 cell line (Figure 7B). In contrast, a significant protection from ricin was observed in the SEL1L knockdown cell line S24, ranging from 1.6-1.4 fold during the time course of ricin challenge (Figure 7C).
Collectively, ricin requires SEL1L for the dislocation of its A chain from the ER to the cytosol and subsequently induce toxicity.

The ricin A chain is not dislocated as a canonical soluble misfolded ER protein.

We compared the degradation intermediates of RTA and the canonical soluble ER degradation substrate, α1-antitrypsin-Hong Kong null (α1-ATHKnull) variant (41,42). The total cell lysates of U373, U373RTA-E177D, and U373RTAΔ cells, and U373 cells that stably express the α1-ATHKnull variant (U373α1-AT-HKnull) were untreated or treated with ZL3VS (2.5 µM, 10 hrs) and subjected to immunoblot analysis (Figure 8). PDI levels confirmed equal protein loading (Figure 8, lanes 17-24). As expected, both glycosylated and deglycosylated RTA E177D and RTAΔ molecules accumulated upon inclusion of proteasome inhibitor (Figure 8, lanes 4 and 6). In sharp contrast, only an increase in glycosylated α1-ATHKnull species was observed in ZL3VS-treated cells (Figure 8, lane 16). These results indicate that even though both RTA and α1-ATHKnull polypeptides represent soluble substrates of dislocation, RTA extraction across the ER membrane probably occurs in a directed manner to increase the likelihood of gaining access to the cytosol.

DISCUSSION

Ricin toxin co-opts the trafficking machinery and ER quality control to gain access to the cytosol. The current study has established a human cell based assay to study ricin dislocation by generating for the first time human cells that stably express enzymatic RTA mutants in the ER (Figure 2). Using this human cell based system, we demonstrated that ricin A chains were rapidly extracted from the ER in a directed manner utilizing specific ER factors to enhance its dislocation efficiency. These ER factors include the ER membrane itself (Figure 5) as well as SEL1L, a component associated with the degradation of ER proteins (Figures 6-7). These data support a model that RTA is transported out of the ER by a novel strategy of co-opting specific ER proteins and the ER bilayer to gain access to the cytosol.

Ricin toxin has evolved to selectively utilize cellular proteins to promote its dislocation across the membrane bilayer. The toxin likely undergoes a conformational change induced by the interaction with ER chaperones such as calreticulin and PDI (12,13), thus allowing its engagement with specific factors of the dislocation apparatus, SEL1L (Figures 6-7) and probably Hrd1 (16). Yet, this process seems to act independent of Derlin-1 (Figure 6 and (15)), which is unlike cholera toxin whose dependence on Derlin-1 for dislocation remains in question (43). SEL1L is as an auxiliary protein for E3-ubiquitin ligase hHrd1 (37) and the dependency of RTA dislocation on SEL1L would typically suggest that RTA is ubiquitylated prior to dislocation. Despite the low number of lysines within ricin as observed for other toxins (44), ubiquitin conjugation to ricin A chains would be selective for the purposes of dislocation (3,45). Therefore, ricin can utilize SEL1L for dislocation and yet a population of the A chains can escape degradation to block protein synthesis (Figure 1).

Once targeted for extraction out of the ER, we propose that ricin A chains utilize the hydrophobic nature of the bilayer to catalyze its dislocation (Figure 5). The recovery of membrane integrated glycosylated A chains suggests that RTA is inserted into the bilayer due to a conformational change induced by its engagement with ER factors. In fact, RTA molecules associated with ER microsomes upon an increase in temperature suggests that extrinsic factors can induce a partial conformational change in ricin A chains (46). The ability of ricin to insert into the bilayer would afford ricin dislocation to be less dependent on cellular proteins. Yeast cell studies demonstrate that wild type RTA dislocation is independent of the p97/Npl4/Ufd1 complex (16). Therefore, membrane integration of RTA may provide some of the driving force for extraction out of the ER. Alternatively, ricin A chains may be extracted from the ER as a protein/lipid micelle (47). In either case, RTA has evolved
a unique strategy to efficiently cross the ER bilayer.

Ricin A chain contains only two lysines as a possible strategy to limit ubiquitin-dependent degradation (45). Despite the low lysine number and the use of the protective nature of Hsc70, a population of ricin A chain is degraded by the proteasome (Figures 2-4 and (26)). However, there are distinct differences between ricin A chain and misfolded ER proteins that demonstrate ricin co-opts select components of ER quality control to gain access to the cytosol. A major difference is observed in the dislocation kinetics of ricin as evaluated by the stability of glycosylated proteins and the accumulation of deglycosylated intermediate. This is apparent when compared to the levels of the canonical soluble misfolded ERAD substrate α1-ATHKnull in proteasome inhibitor treated cells (Figure 8). Another factor may be its ability to integrate into the bilayer. Membrane integration may allow limited exposure to the degradation machinery and permit RTA to escape destruction for a period of time to inhibit protein synthesis (Figure 1). Thus, the ricin has evolved strategies inherent to its sequence and structure to bypass ERAD to reach the cytosol and inhibit protein synthesis.

Our human cell based ricin dislocation assay is a powerful system to elucidate the molecular details of how ricin co-opts ER quality control, bypassing the ubiquitin-proteasome degradation process and gaining access to the cytosol. Our future objectives will be focused on delineating the components of the ER that ricin toxin co-opts to gain access to the cytosol.

Footnotes

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1 α1-ATHKnull, alpha 1-antitrypsin Hong Kong null variant; CHO, N-linked glycans; Derlin1, Der1-like family member 1; EDEM1, ER degradation enhancing a-mannosidase I-like protein; EndoH, Endoglycosidase H; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Kb, murine MHC class I heavy chain H2-Kb signal peptide; NP-40, Nonident P-40; PDI, protein disulflde-isomerase; PNGase, peptide N-glycanase; RTA, ricin toxin A chain; SEL1L, suppressor of lin-12-like; HA, hemagglutinin; Hsc70, Heat shock protein 70; Hsp90, Heat shock protein 90; Hrd1, Hmg-CoA Reductase Degradation-1; TCA, Trichloroacetic acid; ZL3VS= carboxybenzyl-leucyl-leucyl-leucyl vinyl sulfone; 1D-IEF, one dimensional isolectric focusing;

2 Unpublished Data

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**FIGURE LEGENDS**

**Figure 1.** *RTA inhibits protein expression in human cells.* HEK-293T cells transfected with empty plasmid (Control, A) or a GFP-expressing plasmid (B) with full length wild type RTA (FL-RTA<sub>WT</sub>) (C), RTA<sub>WT</sub> (D), RTA<sub>Δ</sub> (E), and RTA<sub>E177D</sub> (F) were analyzed for GFP expression using flow cytometry. The values in the quadrants represent percentage of total cells.

**Figure 2.** *RTA polypeptides are unstable ER proteins.* (A) U373, U373<sub>RTA-E177D</sub>, and U373<sub>RTAΔ</sub> cell lysates were incubated with anti-HA antibodies. The recovered precipitates were subjected to endoglycosidase H (Endo H) digestion and analyzed by immunoblot analysis. (B) U373<sub>RTA-E177D</sub> and U373<sub>RTAΔ</sub> cells were pulsed with <sup>35</sup>S-methionine for 15 min and chased for up to 60 min. The recovered RTA polypeptides were resolved by SDS-PAGE and detected by autoradiography. (C) Levels of RTA polypeptides were quantified by densitometry and plotted as a percentage to the 0 min chase point (100%). RTA polypeptides and molecular weight markers are indicated.

**Figure 3.** *RTA polypeptides are stabilized in the presence of proteasome inhibitor.* U373<sub>RTAΔ</sub> (A) and U373<sub>RTA-E177D</sub> (B) cells treated with or without proteasome inhibitor (ZL<sub>3VS</sub>) were pulsed with <sup>35</sup>S-methionine for 15 min and chased for up to 40 min. RTA polypeptides were recovered with an anti-HA antibody, resolved by SDS-PAGE, and detected by autoradiography. RTA polypeptides and molecular weight markers are indicated. * Indicates faster migratory species.

**Figure 4.** *RTA molecules accumulate as deglycosylated intermediates.* (A) U373<sub>RTAΔ</sub> and U373<sub>RTA-E177D</sub> cells treated with ZL<sub>3VS</sub> for up to 5 hrs were subjected to immunoblot analysis for RTA (lanes 1-12) and PDI (lanes 13-24). (B) Total cell lysates from U373, U373<sub>RTA-E177D</sub>, and U373<sub>RTAΔ</sub> cells untreated or treated with proteasome inhibitor were subjected to endoglycosidase H (EH), or peptide N-glycanase (PN). Immunoblot analysis was performed for RTA (lanes 1-18) and calnexin (lanes 19-36) proteins. (C) RTA polypeptides recovered from U373<sub>RTA-E177D</sub> cells treated with ZL<sub>3VS</sub> were subjected to PN or EH treatment. Subsequently, the samples were resolved by one-dimensional isoelectric focusing (1D-IEF) and subjected to immunoblot analysis.
The respective polypeptides and molecular weight markers are indicated. *Indicates faster migratory species.

**Figure 5. RTA dislocation utilizes membrane components.** Microsomal preparation from subcellular homogenates of U373<sub>RTA</sub> and U373<sub>RTA-E177D</sub> cells were treated with or without 4.5 M urea and centrifuged at 100,000g. Total cell lysates, 100,000g pellets, and the 100,000g supernatant were subjected to immunoblot analysis against RTA (lanes 1-10) and p97 (lanes 11-20). The respective polypeptides and molecular weight markers are indicated.

**Figure 6. RTA is dislocated utilizing selective ER proteins.** (A) U373<sub>RTA</sub> and U373<sub>RTA-E177D</sub> cells untreated or treated with eyarestatin I and ZL3VS were subjected to immunoblot analysis for RTA (lanes 1-8) and PDI (lanes 9-16) polypeptides. (B) Total cell lysates from U373<sub>RTA</sub> and U373<sub>RTA-E177D</sub> cells knocked-down for SEL1L or over-expressing derlin-1-gfp, untreated or treated with proteasome inhibitor, were subjected to immunoblot analysis for RTA (lanes 1-12), SEL1L (lanes 13-24), GFP (lanes 25-36), and GAPDH (lanes 37-48). The respective polypeptides and molecular weight markers are indicated.

**Figure 7. SEL1L knockdown protects cells from ricin.** HeLa cells were transfected with shRNA targeted against SEL1L and a control shRNA. (A) SEL1L levels from lysates of cells expressing a control shRNA (C1) or shRNAs targeting SEL1L (S8 and S24) were analyzed by immunoblot analysis. SEL1L and gamma tubulin were quantified to assess levels of SEL1L knockdown (n=8). (B and C) The cytotoxicity of ricin against the stable cell lines was measured and the mean IC<sub>50</sub> values for different times of toxin incubation were plotted (ng/ml) (n=2). The respective polypeptides and molecular weight markers are indicated.

**Figure 8. RTA is not degraded as a canonical misfolded ER protein.** Total cell lysates from U373, U373<sub>RTA</sub>, U373<sub>RTA-E177D</sub>, and U373<sub>α1-AT-HKnull</sub> cells treated with ZL3VS were subjected to immunoblot analysis for RTA (lanes 1-8), α<sub>1</sub>-AT<sub>HKnull</sub> (lanes 9-16), and PDI (lanes 17-24). The respective polypeptides and molecular weight markers are indicated.
Figure 1

- A: Control
- B: GFP
- C: FL-RTA<sub>WT</sub>
- D: RTA<sub>WT</sub>
- E: RTA<sub>Δ</sub>
- F: RTA<sub>E177D</sub>

Side scatter vs. GFP fluorescence graph.
Figure 2

A

| Cells          | U373           | U373<sub>RTA-E177D</sub> | U373<sub>RTA Δ</sub> |
|----------------|----------------|---------------------------|-----------------------|
| Endo H         | -              | +                         | -                     |
| 35kDa          | Lane 1         | 2                         | 3                     |
|                | Immunoprecipitation: anti-HA | Immunoblot: anti-HA       |                       |

B

| Cells          | U373<sub>RTA-E177D</sub> | U373<sub>RTA Δ</sub> |
|----------------|---------------------------|-----------------------|
| Chase (min)    | 0 15 30 60               | 0 15 30 60            |
| 35kDa          | Lane 1 2 3 4             | 5 6 7 8               |

Pulse: 15 min
Immunoprecipitation: anti-HA

C

![Graph showing RTA protein change over time]
Figure 3

### A

| ZL3VS | U373<sub>RTAΔ</sub> cells |
|-------|---------------------------|
| -     |                           |
| +     |                           |

| Chase (min) | 0 | 20 | 40 |
|-------------|---|----|----|
|             |   |    |    |

35kDa

| Lane | 1 | 2 | 3 | 4 | 5 | 6 |
|------|---|---|---|---|---|---|
|      |   |   |   |   |   |   |

Pulse: 15 min
Immunoprecipitation: anti-HA

### B

| ZL3VS | U373<sub>RTA-E177D</sub> cells |
|-------|---------------------------------|
| -     |                                 |
| +     |                                 |

| Chase (min) | 0 | 20 | 40 |
|-------------|---|----|----|
|             |   |    |    |

35kDa

| Lane | 1 | 2 | 3 | 4 | 5 | 6 |
|------|---|---|---|---|---|---|
|      |   |   |   |   |   |   |

Pulse: 15 min
Immunoprecipitation: anti-HA
Figure 4

A Cells | U373_{RTA△} | U373_{RTA-E177D}

| ZL3VS (hrs) | 0 | 1 | 2 | 3 | 4 | 5 | 0 | 1 | 2 | 3 | 4 | 5 |
|-------------|---|---|---|---|---|---|---|---|---|---|---|---|
| Lane Immunoblot: anti-HA | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 35kDa RTA (+)CHO | | | | | | | | | | | | |
| Lane Immunoblot: anti-PDI | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
| 55kDa PDI | | | | | | | | | | | | |

B Cells | U373 | U373_{RTA△} | U373_{RTA-E177D}

| ZL3VS Enzyme | - | + | - | + | - | + |
|-------------|---|---|---|---|---|---|
| Lane Immunoblot: anti-HA | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
| 34kDa RTA (+)CHO | | | | | | | | | | | | | | | | | | |
| Lane Immunoblot: anti-calnexin | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 |
| 95kDa calnexin | | | | | | | | | | | | | | | | | | |

C Cells | U373_{RTA-E177D}

| Enzyme | - | PN | EH |
|--------|---|----|----|
| Lane Immunoprecipitation: anti-HA | 1 | 2 | 3 |
| Lane Immunoblot: anti-HA | | | |
Figure 5

| U373 cells | RTAΔ | RTA<sub>E177D</sub> | RTAΔ | RTA<sub>E177D</sub> | RTAΔ | RTA<sub>E177D</sub> |
|------------|------|---------------------|------|---------------------|------|---------------------|
| Urea       | -    | +                   | -    | +                   | -    | +                   |
| 35kDa      | 1    | 2                   | 3    | 4                   | 5    | 6                   |
| Lane       | 1    | 2                   | 3    | 4                   | 5    | 6                   |

Immunoblot: anti-HA

- RTA (+)CHO
- RTA (-)CHO

100kDa

Lane 11 12 13 14 15 16 17 18 19 20

Immunoblot: anti-p97

- p97

100,000g pellet

100,000g supernatant

Total cell lysates
Figure 6

A

Cells

| Cells | U373<sup>RTAΔ</sup> | U373<sup>RTA-E177D</sup> |
|-------|---------------------|--------------------------|
| Eeyarestatin | - | + | - | + |
| ZL<sub>3</sub>VS | - | + | - | + |

Immunoblot: anti-HA

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|------|---|---|---|---|---|---|---|---|
| 35kDa | - | + | - | + | RTA (+)CHO | RTA (-)CHO |

Immunoblot: anti-calnexin

| Lane | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|------|---|---|---|---|---|---|---|---|
| 50kDa | - | + | - | + | PD1 |

Total cell lysates

B

Cells

| Cells | U373<sup>RTAΔ</sup> | U373<sup>RTA-E177D</sup> |
|-------|---------------------|--------------------------|
| ZL<sub>3</sub>VS | - | + | - | + |

DNA construct

| DNA construct | Der-gfp | sh-SEL1L | - | + |
|----------------|--------|----------|---|---|

Immunoblot: anti-HA

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|------|---|---|---|---|---|---|---|---|---|----|----|----|
| 35kDa | - | + | - | + | RTA (+)CHO | RTA (-)CHO |

Immunoblot: anti-SEL1L

| Lane | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|------|----|----|----|----|----|----|----|----|----|----|----|----|
| 100kDa | - | + | - | + | SEL1L |

Immunoblot: anti-GFP

| Lane | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 |
|------|----|----|----|----|----|----|----|----|----|----|----|----|
| 40kDa | - | + | - | + | Derlin-1-GFP |

Immunoblot: anti-GAPDH

| Lane | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 |
|------|----|----|----|----|----|----|----|----|----|----|----|----|
| 35kDa | - | + | - | + | GAPDH |

Total cell lysates
Figure 7

A

S8 C1 C1 S24
98kDa
64kDa
50kDa
Lane 1 2 3 4
SEL1L
gamma tubulin

B

IC50 (ng/ml ricin)

15
10
5
0

0.6
0.3
0.0

C1 2hr
S8

C1 4hr
S8

time

C

IC50 (ng/ml ricin)

300
150
0

4

0.70
0.35
0.00

C1 1hr
S24

C1 2hr
S24

C1 4hr
S24

time
### Figure 8

| Cells | U373 | U373<sub>RTA</sub> | U373<sub>RTA-E177D</sub> | U373<sub>α<sub>1</sub>-AT-HKnull</sub> |
|-------|------|-------------------|---------------------------|----------------------------------|
| ZL3VS | -    | +                 | -                         | +                                |

#### Immunoblot: anti-HA

- 35kDa

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|------|---|---|---|---|---|---|---|---|
|      |  |   |   |   |   |   |   |   |

- RTA (+)CHO
- RTA (-)CHO

#### Immunoblot: anti-α<sub>1</sub>-AT

- 43kDa

| Lane | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|------|---|----|----|----|----|----|----|----|
|      |   |    |    |    |    |    |    |    |

- α<sub>1</sub>-AT<sub>HKnull</sub>

#### Immunoblot: anti-PDI

- 56kDa

| Lane | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|------|----|----|----|----|----|----|----|----|
|      |    |    |    |    |    |    |    |    |

- PDI

### Total cell lysates
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and Domenico Tortorella

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