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The E3 Ubiquitin Ligases Hrd1 and gp78 Bind to and Promote Cholera Toxin Retro-Translocation

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To cause disease, cholera toxin (CT) is transported from the cell surface to the endoplasmic reticulum (ER) lumen where the catalytic CTA1 subunit retro-translocates to the cytosol to induce pathological water secretion. Two retro-translocon components are the Derlins and ER-associated multi-spanning E3 ubiquitin ligases including Hrd1 and gp78. We demonstrated previously that Derlin-1 facilitates CTA1 retro-translocation. However, as CTA1 is neither ubiquitinated on lysines nor at its N-terminus, the role of E3 ligases in toxin retro-translocation is unclear. Here, we show that expression of mutant Hrd1 and gp78 and a mutant E2-conjugating enzyme dedicated to retro-translocation (Ube2g2) decrease CTA1 retro-translocation. Hrd1 knockdown also attenuated toxin retro-translocation. Binding studies demonstrate that Hrd1 and gp78 interact with CT and protein disulfide isomerase, an ER chaperone that unfolds CTA1 to initiate translocation. Moreover, we find that the toxin's association with Hrd1 and gp78 is blocked by dominant-negative Derlin-1, suggesting that CT is targeted initially to Derlin-1 and then transferred to Hrd1 and gp78. These data demonstrate a role of the E3 ubiquitin ligases in CTA1 retro-translocation, implicate a sequence of events experienced by the toxin on the ER membrane, and raise the possibility that ubiquitination is involved in the transport process.

INTRODUCTION

Cholera toxin (CT), the virulence factor produced by *Vibrio cholerae*, is responsible for inducing a signaling cascade in intestinal epithelial cells, resulting in massive secretory diarrhea and subsequent death if treatment is not obtained (Sears and Kaper, 1996). The toxin is equipped with a homopentameric B subunit (CTB) and a single A subunit (CTA; Spangler, 1992). To intoxicate cells, CTB binds the ganglioside receptor GM1 on the plasma membrane, and the holotoxin is endocytosed and transported into the endoplasmic reticulum (ER). In this compartment, CTA is reduced, and the resulting CTA1 peptide engages the ER-associated degradation (ERAD) machinery that normally retro-translocates misfolded proteins to the cytosol for ubiquitin-dependent proteasomal degradation (Tsai *et al.*, 2002; Vembar and Brodsky, 2008). On reaching the cytosol, CTA1 however escapes proteasomal degradation and triggers the inevitable opening of a chloride channel (Lencer and Tsai, 2003). The subsequent release of chloride ions and water from the cell ultimately results in diarrhea characterizing this disease.

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Abbreviations used: CT, cholera toxin; ER, endoplasmic reticulum; ERAD, ER-associated degradation; PDI, protein disulfide isomerase; TCRα, T-cell receptor α; YFP, yellow fluorescent protein.

The ER events that allow for the correct processing of the toxin and subsequent translocation of the catalytic subunit into the cytosol are poorly understood. Elucidating how the toxin utilizes the ERAD machinery and how it avoids proteasomal degradation is essential to illuminating both the toxin translocation process as well as the basic ERAD mechanism. We demonstrated previously that the ER lumenal oxido-reductase, protein disulfide isomerase (PDI), unfolds CTA1 to initiate toxin retro-translocation (Tsai *et al.*, 2001; Forster *et al.*, 2006). We then found that Derlin-1, a component of the retro-translocon on the ER membrane (Ye *et al.*, 2004; Lilley and Ploegh, 2004) binds to PDI and facilitates CTA1 retro-translocation (Bernardi *et al.*, 2008). That Derlin-1 mediates toxin translocation is supported by another study by Dixit *et al.* (2008). These findings link events within the ER lumen and membrane that act coordinately to propel the toxin into the cytosol. Specifically, these data depict a model in which CTB targets the holotoxin to Derlin-1, whereupon the Derlin-1–bound PDI unfolds the CTA1 chain, priming the toxin for translocation. However, how the toxin reaches the cytosol after engaging Derlin-1 is not known.

Normally, misfolded proteins emerging from the cytosol via the ERAD machinery are ubiquitinated by the ubiquitination machinery associated with the retro-translocon (Tsai *et al.*, 2002; Vembar and Brodsky, 2008; Hirsch *et al.*, 2009). This covalent modification tags a substrate for proteasomal destruction. As CT must evade proteasomal degradation to induce toxicity, the absence of ubiquitination on CTA1 due to the paucity of lysines on the toxin (which serves as the primary ubiquitination site) was hypothesized to be how the

toxin escapes ubiquitin-dependent proteasomal degradation (Hazes and Read, 1997). Two observations support this model. First, a mutant CT in which the two lysines on CTA1 were mutated and where the N-terminus was blocked chemically displayed activity similar to the wild-type (WT) toxin (Rodighiero *et al.*, 2002). Second, inhibition of the proteasome did not affect toxin action (Rodighiero *et al.*, 2002). These findings demonstrate that CTA1 is not ubiquitinated on lysines or at the N-terminus and that the toxin is not a substrate for the proteasome. Thus, whether the ubiquitination machinery associated with the retro-translocon plays any role in the toxin translocation process is not clear. The E3 ubiquitin ligases Hrd1 and gp78, along with the Derlin proteins, represent two core components of the retro-translocon (Ye *et al.*, 2005; Lilley and Ploegh, 2005; Schulze *et al.*, 2005). As Derlin-1 has been shown to mediate CTA1 retrotranslocation (Bernardi *et al.*, 2008; Dixit *et al.*, 2008), we sought to examine whether the E3 ligases Hrd1 and gp78 also play a role in this process.

Hrd1 and gp78 are responsible for ubiquitination of a subset of misfolded substrates before their extraction from the ER membrane, including the commonly studied T-cell receptor α (TCRα) and CD3δ (Fang *et al.*, 2001; Kikkert *et al.*, 2004; Chen *et al.*, 2006). As orthologues of the yeast Hrd1 protein, Hrd1 and gp78 each consist of multiple transmembrane domains and a C-terminal cytosolic RING-H2 motif that is essential for their catalytic function (Fang *et al.*, 2001; Kaneko *et al.*, 2002; Nadav *et al.*, 2003; Kikkert *et al.*, 2004; Chen *et al.*, 2006). Hrd1 and gp78 interact with an assortment of chaperones in the ER lumen and in the cytosol, and therefore are likely important factors that connect activities across the ER membrane (Ye *et al.*, 2005; Lilley and Ploegh, 2005; Hirsch *et al.*, 2009).

In this study, we found that expression of mutant Hrd1 and gp78, as well as a mutant E2-conjugating enzyme associated with ERAD, Ube2g2, decrease retro-translocation of CTA1. Knockdown of Hrd1 similarly led to a block in toxin translocation. Coimmunoprecipitation analyses demonstrate that Hrd1 and gp78 bind to CT and PDI. In addition, the binding studies also indicate sequential transfer of the toxin from Derlin-1 to Hrd1/gp78 before exiting the ER. This study thus identifies the E3 ligases Hrd1 and gp78 as components of the ER machinery that mediate CTA1 retrotranslocation and implicate a role of ubiquitination in this process.

MATERIALS AND METHODS

Materials

Polyclonal antibodies against PDI and Hsp90 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), the polyclonal green fluorescent protein (GFP) and CTB antibodies from Abcam (Cambridge, MA), the monoclonal BiP antibody from BD Biosciences (San Jose, CA), and the monoclonal and polyclonal FLAG antibodies and polyclonal Myc antibody from Sigma (St. Louis, MO). The polyclonal CTA antibody was produced against denatured CTA purchased from EMD Biosciences (San Diego, CA). The thiol-cleavable cross-linker dithiobis (succinimidyl propionate) (DSP) was purchased from Fisher Scientific (Pittsburgh, PA). The polyclonal Derlin-1 antibody was a gift from T. Rapoport (Harvard). The monoclonal Myc antibody and peYFP-N1 construct were gifts from K. Verhey (University of Michigan). Purified CT was purchased from EMD Biosciences. The Hrd1 Myc constructs (WT, C291A, TM1-6, and cyt) were from E. Wiertz (University Medical Center Utrecht), the FLAG Ube2g2 constructs (WT, delta loop, and H94K) from Y. Ye (National Institutes of Health), the gp78 Myc constructs (WT and C337/374S) from K. Nagata (Kyoto University); the PDI-FLAG construct was described previously (Bernardi *et al.*, 2008).

Construction of TM1-6 Hrd1 Myc and cyt Hrd1 Myc

To generate TM1-6 Hrd1 Myc, the following primers were used: forward: GGC CAA GCT TGA ATT CAC CAT GTT CCG CAC GGC AGT G; reverse: GG GTAC CGG TGT GCA CCT TGA TCA TGA TG. The resulting PCR product was cloned into pcDNA3.1-GFP-TOPO resulting in pcDNA3.1-Hrd1-GFP. TM1-6 Hrd1 was digested from this vector with EcoRI and KpnI and cloned in frame with the tags into pcDNA3.1-MycHis A(−). To generate cyt Hrd1 Myc, the following primers were used: forward: CAG TGA ATT CAC CAT GGT CTG CAT CAT CTG CCG AGA AG; reverse: CCG GCT CGA GGT ACC GTG GGC AAC AGG AGA CTC CAG C. PCR products were digested with EcoRI and KpnI and cloned into pcDNA3.1-MycHisA(−). All vectors were sequenced before experimentations.

Construction of Hrd1-FLAG

WT Hrd1 FLAG was generated by excising the Hrd1 coding sequence from the WT Hrd1 Myc construct and ligating it into a pcDNA3.1 FLAG vector.

Assays Used

The following assays were used: the retro-translocation assay as described previously in Bernardi *et al.* (2008); the cAMP assay as described previously in Forster *et al.* (2006); and the in vitro ubiquitination assay as described previously in Li *et al.* (2007).

Cell Transfection

293T or HeLa cells were grown to 30% confluency on a 10-cm dish before transfection with the Effectene system (Qiagen, Chatsworth, CA). The cells were grown for an additional 48 h before experimentation.

siRNA Knockdown of Hrd1 and XBP1 Splicing

Duplex siRNA (200 nM) corresponding to a segment of human Hrd1 (5′-GGA GAC TGC CAC TAC AGT TGT-3′; Invitrogen, Carlsbad, CA) was transfected into 293T cells for 48 h according to the manufacturer's protocol. XBP1 splicing was done as described previously in Uemura *et al.* (2009).

Immunoprecipitation

293T or HeLa cells were incubated with or without CT (10 or 100 nM) for 90 min. Cells were harvested, lysed in buffer containing KOAc (150 mM), Tris, pH 7.5 (30 mM), $MgCl_2$ (4 mM), NEM (10 mM), and protease inhibitors with either 1% Triton X-100 or 1% deoxyBigChap for 30 min on ice. Cells were centrifuged at 16,000 $\times$ *g* for 15 min, and the supernatant was used for immunoprecipitation experiments. Coimmunoprecipitation experiments between PDI-FLAG and Hrd1 Myc/gp78 Myc were performed using a lysis buffer containing 1% Triton X-100 after the addition of the cross-linker DSP (2 mM) for 30 min at room temperature. Where indicated, monoclonal Myc or monoclonal FLAG antibodies were added to the lysate and incubated overnight at 4°C. The immune complex was captured by the addition of protein A agarose beads (Invitrogen), washed, and subjected to SDS-PAGE followed by immunoblotting with the appropriate antibody.

Alkali Extraction

293T cells were harvested from a confluent 10-cm dish, and 25% of the cells was resuspended in 150 μl $NaCO_3$ (0.1 M, pH 11.6). Cells remained on ice for 30 min. Fifty microliters of each sample was subjected to centrifugation in an ultracentrifuge using the TLA100 Rotor (Beckman, Fullerton, CA) at 100,000 $\times$ *g* for 30 min at 4°C. Supernatant and pellet fractions were harvested and subjected to reducing SDS-PAGE and immunoblot analysis.

Chemical Cross-Linking

DSP was dissolved in DMSO (10 mg/ml). DSP, 800 μl, was further diluted with 9.2 ml of PBS. 293T cells from a confluent 10-cm dish were harvested and resuspended in 1.4 ml of the DSP in PBS and incubated at room temperature for 30 min. Cells were pelleted and the DSP was removed. After washing with PBS, cells were lysed in a buffer containing 1% Triton X-100 and subjected to immunoprecipitation described above.

RESULTS

Expression of Hrd1 Mutants Decreases Retro-Translocation of CTA1

We first expressed WT Hrd1 and mutant variants of Hrd1, each containing a C-terminal Myc tag, in 293T cells to examine whether they act as dominant-negative factors during retro-translocation of CTA1. In this study, we chose to utilize the well-characterized Hrd1 mutant in which the cysteine 291 in the RING finger domain is mutated to alanine (C291A Hrd1 Myc) because expression of this inactive ligase mutant inhibits the degradation of a variety of misfolded substrates (Kikkert *et al.*, 2004; Yang *et al.*, 2007; Okuda-Shimizu and Hendershot, 2007). Additionally, we generated

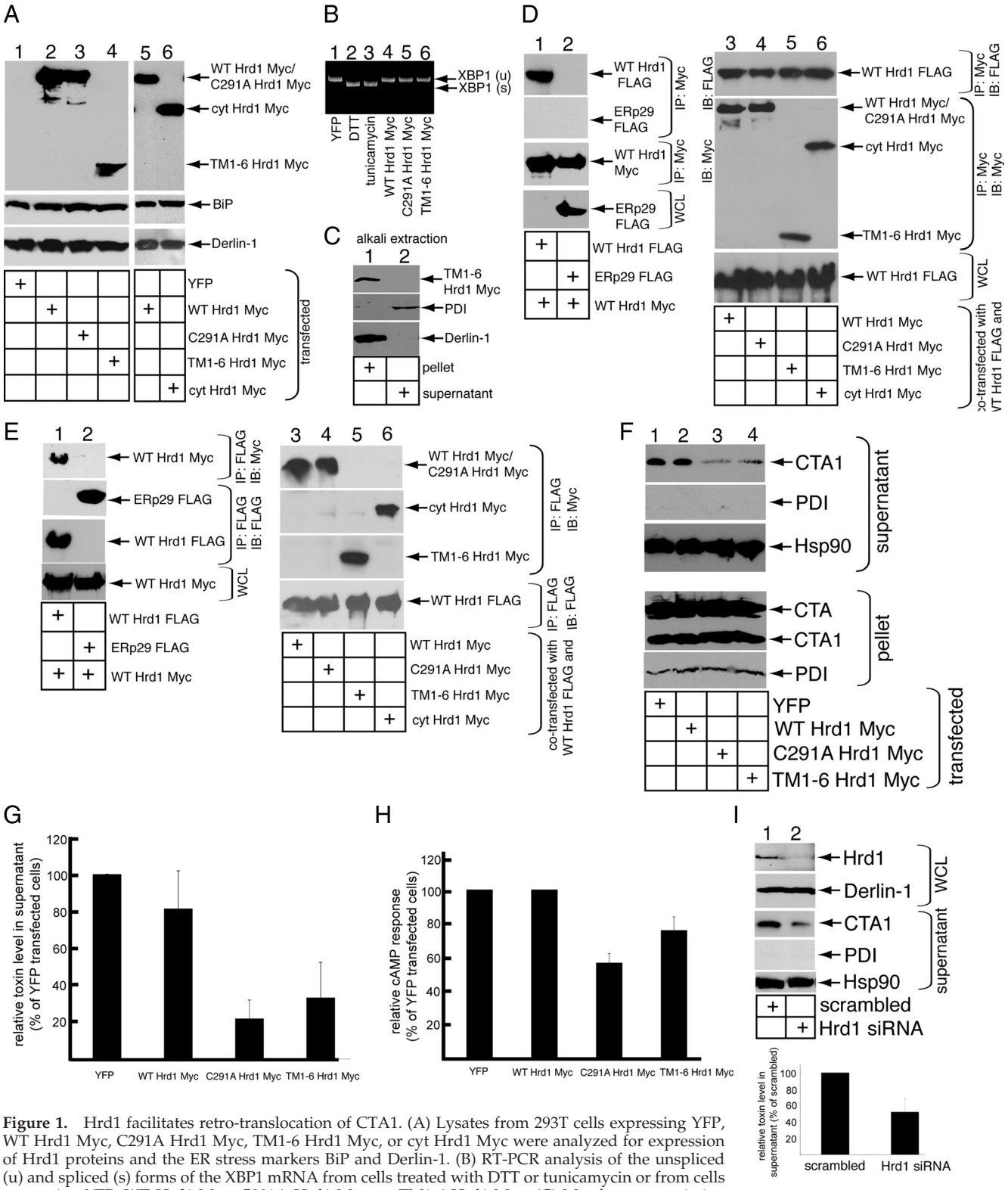

Figure 1. Hrd1 facilitates retro-translocation of CTA1. (A) Lysates from 293T cells expressing YFP, WT Hrd1 Myc, C291A Hrd1 Myc, TM1-6 Hrd1 Myc, or cyt Hrd1 Myc were analyzed for expression of Hrd1 proteins and the ER stress markers BiP and Derlin-1. (B) RT-PCR analysis of the unspliced (u) and spliced (s) forms of the XBP1 mRNA from cells treated with DTT or tunicamycin or from cells expressing YFP, WT Hrd1 Myc, C291A Hrd1 Myc, or TM1-6 Hrd1 Myc. (C) Membrane association of TM1-6 Hrd1 Myc. Cells expressing TM1-6 Hrd1 Myc were subjected to alkali extraction and pelleted, and the supernatant and pellet fractions were separated. Samples were immunoblotted with an Myc antibody, a PDI antibody (soluble marker), and a Derlin-1 antibody (membrane marker). (D and E) 293T cells expressing the indicated combinations of WT Hrd1 FLAG, ERp29 FLAG, WT Hrd1 Myc, C291A Hrd1 Myc, TM1-6 Hrd1 Myc, and cyt Hrd1 Myc were harvested and lysed in a 1% Triton X-100 buffer. Lysates were subjected to immunoprecipitation with the indicated antibodies. The immunoprecipitation complexes were analyzed by SDS-PAGE and immunoblotted with polyclonal antibodies to either FLAG or Myc. (F) Cells expressing YFP, WT Hrd1 Myc, C291A Hrd1 Myc, or TM1-6 Hrd1 Myc were treated with 10 nM CT and subjected to the retro-translocation assay. Supernatant and pellet fractions were analyzed by nonreducing SDS-PAGE, followed by immunoblotting with indicated antibodies. CTA is 28 kDa and CTA1 is 22 kDa. (G) The intensity of the CTA1 band

a construct consisting of only the six transmembrane domains of Hrd1 (TM1-6 Hrd1 Myc) and a construct containing only the cytosolic portion of Hrd1 (cyt Hrd1 Myc) to assess if they affect toxin transport. We found that WT Hrd1 Myc, C291A Hrd1 Myc, TM1-6 Hrd1 Myc, and cyt Hrd1 Myc were all expressed robustly in 293T cells (Figure 1A, top, lanes 2–6). Importantly, overexpression of the Hrd1 proteins did not induce the up-regulation of the known unfolded protein response (UPR) markers BiP and Derlin-1 (Oda *et al.,* 2006; Figure 1A, second and third panels). Additionally, in contrast to incubating cells with the known ER stress inducers dithiothreitol (DTT) and tunicamycin, overexpressing the Hrd1 proteins also did not trigger splicing of the XBP1 transcription factor mRNA (Figure 1B, cf. lanes 2 and 3 with lanes 4–6). We conclude that massive ER stress was not stimulated by the overexpression of the Hrd1 proteins. To test whether TM1-6 Hrd1 Myc is integrated properly into the ER membrane, cells expressing this protein were subjected to alkali extraction. Essentially all of TM1-6 Hrd1 was found in the pellet fraction, similar to endogenous Derlin-1 (Figure 1C, top and bottom panels, cf. lanes 1 and 2). This finding demonstrates that TM1-6 behaves as an integrated protein. In addition, we found that TM1-6 Hrd1 Myc colocalizes with the ER marker calnexin using fluorescence microscopy (data not shown), indicating that TM1-6 Hrd1 is targeted to the appropriate organelle.

We further characterized the TM1-6 Hrd1 Myc and cyt Hrd1 Myc mutants before assessing their potential effect on toxin retro-translocation. Specifically, we asked whether these Hrd1 mutants bind individually to WT Hrd1. To this end, we generated an additional construct in which a FLAG-tag is appended to the C-terminus of WT Hrd1 (WT Hrd1 FLAG). In cells expressing WT Hrd1 Myc and either WT Hrd1 FLAG or the control protein ERp29 FLAG, immunoprecipitation of WT Hrd1 Myc coprecipitated WT Hrd1 FLAG but not ERp29 FLAG (Figure 1D, first and second panels, cf. lanes 1 and 2), indicating that WT Hrd1 FLAG binds to WT Hrd1 Myc specifically. Importantly, in cells cotransfected with WT Hrd1 FLAG and either WT Hrd1 Myc, C291A Hrd1 Myc, TM1-6 Hrd1 Myc, or cyt Hrd1 Myc, we found that WT Hrd1 FLAG coprecipitated with Myc-tagged WT Hrd1, C291A Hrd1, TM1-6 Hrd1, and cyt Hrd1 (Figure 1D, top panel, lanes 3–6). A similar result was observed when the immunoprecipitation was performed in reverse. Immunoprecipitation of WT Hrd1 FLAG but not ERp29 FLAG coprecipitated WT Hrd1 Myc (Figure 1E, top panel, cf. lanes 1 and 2), demonstrating the specificity of the Hrd1 FLAG-Hrd1 Myc interaction. In this context, WT Hrd1 Myc, C291A Hrd1 Myc, TM1-6 Hrd1 Myc, and cyt Hrd1Myc all coprecipitated with WT Hrd1 FLAG (Figure 1E, top three panels, lanes 3–6). As TM1-6 Hrd1 and cyt Hrd1 bind to WT Hrd1 individually, we conclude that two distinct binding sites mediate Hrd1 dimerization or oligomerization, one located within the transmembrane region and another within the cytosolic domain. It should be noted that dimerization of Hrd1 has been implicated previously (Schulze *et al.,* 2005). Interestingly, a recent finding demonstrates that oligomerization of the related E3 ligase gp78 was mediated by two distinct sites as well, one in the transmembrane region and the other in its cytosolic domain (Li *et al.,* 2009).

To determine whether expression of the WT Hrd1, C291A Hrd1, TM1-6 Hrd1, or cyt Hrd1 Myc-tagged proteins affect retro-translocation of CTA1, cells were transfected with the different Hrd1 constructs and subjected to an ER-to-cytosol retro-translocation assay established previously in our laboratory (Forster *et al.,* 2006; Bernardi *et al.,* 2008). Briefly, cells were intoxicated with CT (10 nM) for 90 min, harvested, and treated with a low concentration of digitonin to gently permeabilize the plasma membrane, allowing for the release of only the cytosolic content while leaving intracellular membranes intact. Cells were then subjected to fractionation by centrifugation to separate cytosolic (supernatant) and membrane (pellet) fractions. As expected, Hsp90, a cytosolic protein, is present in the supernatant (Figure 1F, third panel). Importantly, the presence of the ER lumenal protein PDI in the pellet but not the supernatant fraction (Figure 1F, cf. fifth and second panels) demonstrates that the ER membrane was intact. Thus any CTA1 that appears in the supernatant is not due to leakage but instead represents retro-translocated toxin. As controls, we found previously that CTA1 does not appear in the supernatant in cells treated with brefeldin A (which blocks COPI-dependent retrograde transport to the ER) or in cells incubated at 4°C (which blocks endocytosis; Forster *et al.,* 2006; Bernardi *et al.,* 2008). In addition, a mutant CT that is predicted to not undergo retro-translocation does not appear in the supernatant (Forster *et al.,* 2006). And finally, inactivation of different ER factors that blocked CT-induced cAMP synthesis also caused a decrease in CTA1 level in the supernatant (Forster *et al.,* 2006; Bernardi *et al.,* 2008).

Thus, using this cell-based assay, we asked if expression of a mutant Hrd1 affects the toxin level in the supernatant fraction to monitor retro-translocation of CTA1. We found that the level of CTA1 in the supernatant was similar in cells transfected with yellow fluorescent protein (YFP) or WT Hrd1 Myc (Figure 1F, top panel, cf. lanes 1 and 2; quantified in G). However, the CTA1 level in the supernatant decreased in cells expressing C291A Hrd1 Myc and TM1-6 Hrd1 Myc when compared with cells expressing YFP or WT Hrd1 (Figure 1F, top panel, cf. lanes 3 and 4 with lanes 1 and 2; quantified in G). Expression of cyt Hrd1 Myc did not affect the amount of CTA1 in the supernatant (not shown). We conclude that expression of C291A Hrd1 Myc and TM1-6 Hrd1 Myc inhibits retro-translocation of CTA1.

When CTA1 reaches the cytosol, it induces a signal transduction cascade that results in an increase in cAMP production. We therefore measured the amount of cAMP produced in cells expressing C291A Hrd1 Myc or TM1-6 Hrd1 Myc and found that the cAMP level was decreased in these cells when compared with YFP or WT Hrd1 Myc-expressing cells (Figure 1H). These findings are consistent with the results of the retro-translocation assay (Figure 1, F and G) and implicate a role for Hrd1 in CTA1 retro-translocation.

To further establish a role of Hrd1 in toxin retro-translocation, endogenous Hrd1 was down-regulated using the siRNA-mediated approach (Figure 1I, top panel, cf. lane 2 with lane 1). Under this condition, using the retro-translocation assay, we found that the CTA1 level in the supernatant was decreased in the Hrd1-knockdown cells when compared with cells incubated with a scrambled siRNA (Figure

Figure 1 (cont). generated in E was quantified with ImageJ (http://rsb.info.nih.gov/ij/). Mean of five independent experiments; error bars, ± SD. (H) 293T cells expressing YFP, WT Hrd1 Myc, C291A Hrd1 Myc, or TM1-6 Hrd1 Myc were incubated with CT for 90 min, and the cAMP level was measured with a cAMP Biotrak Enzyme Immunoassay System (GE Healthcare, Waukesha, WI). Data were normalized against the forskolin-induced cAMP level, as demonstrated previously (Forster *et al.,* 2006). (I) Cells transfected with a scrambled or a Hrd1-specific siRNA were either lysed and the lysates immunoblotted with the indicated antibodies or were subjected to the retro-translocation assay as described in F. Quantification of the CTA1 band intensity as in G is shown below.

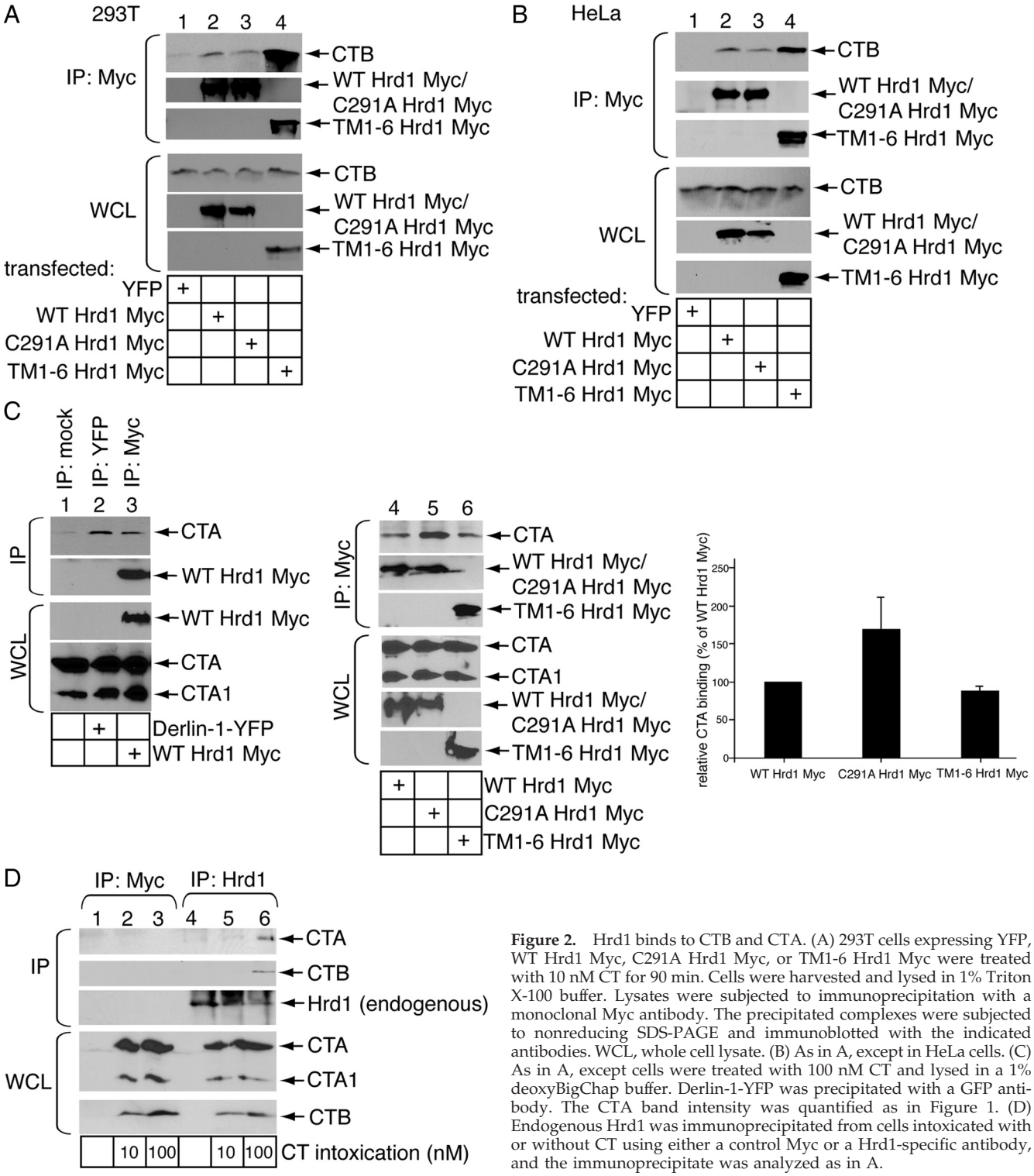

Figure 2. Hrd1 binds to CTB and CTA. (A) 293T cells expressing YFP, WT Hrd1 Myc, C291A Hrd1 Myc, or TM1-6 Hrd1 Myc were treated with 10 nM CT for 90 min. Cells were harvested and lysed in 1% Triton X-100 buffer. Lysates were subjected to immunoprecipitation with a monoclonal Myc antibody. The precipitated complexes were subjected to nonreducing SDS-PAGE and immunoblotted with the indicated antibodies. WCL, whole cell lysate. (B) As in A, except in HeLa cells. (C) As in A, except cells were treated with 100 nM CT and lysed in a 1% deoxyBigChap buffer. Derlin-1-YFP was precipitated with a GFP antibody. The CTA band intensity was quantified as in Figure 1. (D) Endogenous Hrd1 was immunoprecipitated from cells intoxicated with or without CT using either a control Myc or a Hrd1-specific antibody, and the immunoprecipitate was analyzed as in A.

1I, third panel, cf. lane 2 with lane 1; quantified below). This finding is consistent with the overexpression studies and demonstrates that Hrd1 plays an important function during CTA1 retro-translocation.

Hrd1 Binds to CTB and CTA

To examine if Hrd1 physically interacts with the toxin, 293T cells transfected with YFP, WT Hrd1 Myc, C291A Hrd1 Myc, or TM1-6 Hrd1 Myc were incubated with CT (10 nM) for 90 min, and lysed with a 1% Triton X-100–containing buffer, and the lysate was subjected to immunoprecipitation with an Myc antibody. The immunoprecipitation complexes were resolved by SDS-PAGE and probed for the presence of CTB. We found that in cells expressing YFP, the Myc antibody did not appreciably precipitate CTB, as expected (Figure 2A, top, IP: Myc, lane 1). In contrast, immunoprecipitation of WT and

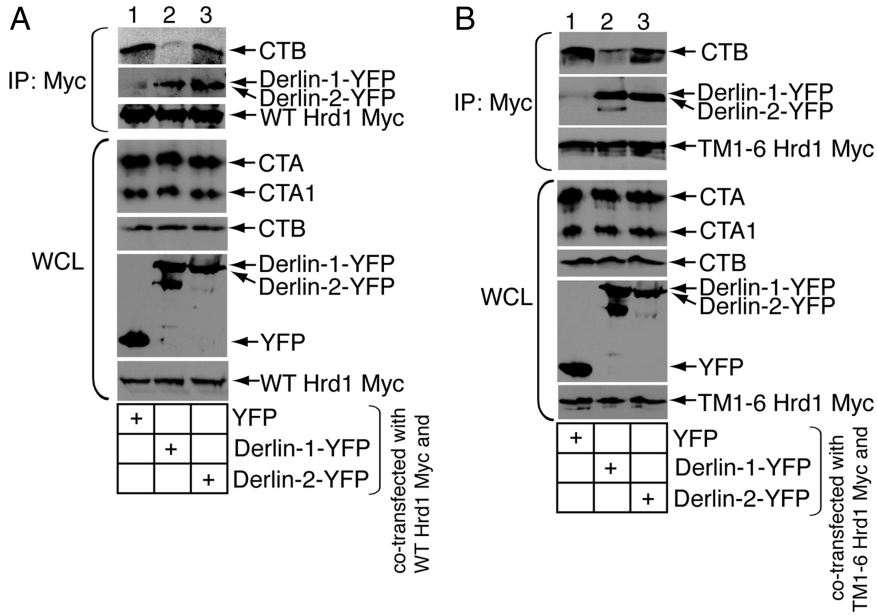

Figure 3. Expression of Derlin-1-YFP blocks the interaction between WT Hrd1/TM1-6 Hrd1 and CTB. (A) 293T cells expressing WT Hrd1 Myc and either YFP, Derlin-1-YFP or Derlin-2-YFP were treated with 10 nM CT for 90 min, harvested, and lysed in a 1% Triton X-100 buffer. Lysates were subjected to immunoprecipitation with monoclonal Myc antibody. Precipitated complexes were analyzed by nonreducing SDS-PAGE and immunoblotted with the indicated antibodies. (B) As in A, except TM1-6 Hrd1 Myc was expressed.

mutant Hrd1 with the Myc antibody pulled down different levels of CTB (Figure 2A, top, lanes 2–4). Interestingly, TM1-6 Hrd1 precipitated a significantly higher level of CTB when compared with WT or C291A Hrd1 (Figure 2A, top, cf. lane 4 with lanes 2 and 3). This pattern of Hrd1-CTB interaction was also observed in HeLa cells (Figure 2B, top, IP: Myc, lanes 1–4). Thus, we conclude that CTB binds to Hrd1 in different cell types, as it does to Derlin-1 (Bernardi *et al.*, 2008).

Under this condition, we were unable to detect Hrd1-CTA interaction. We reasoned that CTA must interact with the retro-translocon components weakly and transiently so that it can be released into the cytosol. Thus, to capture a potential interaction between Hrd1 and CTA, cells expressing the different Hrd1 proteins were incubated with a higher concentration of CT (100 nM) and lysed with the gentle detergent deoxyBigChap (1%). Cells expressing Derlin-1-YFP were used as a positive control in this experiment as Derlin-1-YFP was shown previously to bind to CTA (Bernardi *et al.*, 2008). Using this modified condition, we found that Derlin-1-YFP and WT Hrd1 both coprecipitated CTA (Figure 2C, top, IP, lanes 2 and 3), indicating that CTA associates with Hrd1, similar to CTB. Interestingly, whereas CTA coprecipitated with TM1-6 Hrd1 to a similar extent as WT Hrd1, the toxin bound to C291A Hrd1 more efficiently (Figure 2C, top, cf. lanes 4 and 6 with lane 5; quantified on the right graph). This finding suggests that the molecular basis by which the C291A Hrd1 mutant blocks CTA1 retro-translocation (Figure 1) is likely due to its ability to trap the substrate. Such an enhanced interaction between a substrate and the inactive Hrd1 has been demonstrated with the Hrd1-dependent substrate CD3δ (Kikkert *et al.*, 2004).

To assess whether endogenous Hrd1 associates with CT, cells were intoxicated with (10 or 100 nM) or without CT, and the cell lysate was subjected to immunoprecipitation using the control Myc or a Hrd1-specific antibody. We found that CTA and CTB specifically coprecipitated with Hrd1 when cells were intoxicated with the higher CT concentration (100 nM; Figure 2D, first and second panels, lane 6). This result demonstrates that, similar to transfected Hrd1, endogenous Hrd1 also binds to CT.

Derlin-1-YFP Blocks the Interaction between WT Hrd1/TM1-6 Hrd1 and CTB

Next we sought to establish the temporal order of CTA1 retro-translocation. In the current model, CTB targets the holotoxin to Derlin-1 (Bernardi *et al.*, 2008). Our data demonstrate that CT also binds to Hrd1 (Figure 2); thus, does the holotoxin first target to Derlin-1 (via interaction with CTB) and then to Hrd1, or vice versa? Expression of Derlin-1-YFP was shown previously to inhibit CTA1 retro-translocation by binding to and trapping the holotoxin (Bernardi *et al.*, 2008), so we asked whether its expression blocks CT-Hrd1 interaction. We reasoned that should the dominant-negative Derlin-1-YFP protein block the CT-Hrd1 interaction, the CT-Hrd1 interaction likely occurs downstream of the CT-Derlin-1 interaction. Cells expressing WT Hrd1 Myc were cotransfected with YFP (as control), Derlin-1-YFP, or Derlin-2-YFP (as an additional control). These cells were intoxicated with CT (10 nM) for 90 min and lysed, and the resulting lysate was subjected to immunoprecipitation using an Myc antibody; the sample then was analyzed by SDS-PAGE and immunoblotted with the indicated antibody. We found that binding of CTB to WT Hrd1 is essentially blocked in the presence of Derlin-1-YFP but not YFP or Derlin-2-YFP (Figure 3A, top, IP: Myc, cf. lane 2 with lanes 1 and 3). Moreover, TM1-6 Hrd1's interaction with CTB also decreased in the presence of Derlin-1-YFP, but not YFP or Derlin-2-YFP (Figure 3B, top, IP: Myc, cf. lane 2 with lanes 1 and 3). These findings indicate that the CT-Hrd1 interaction occurs downstream of the CT-Derlin-1 interaction.

Expression of an Enzymatic-inactive gp78 Mutant Decreases CTA1 Retro-Translocation

Similar to mammalian Hrd1, the E3 ubiquitin ligase gp78 is also an ortholog of the yeast Hrd1. gp78 possesses a cytosolic RING-H2 domain and demonstrates a membrane topology similar to that of Hrd1 (Fang *et al.*, 2001; Chen *et al.*, 2006). Does it also play a role in CTA1 retro-translocation? Myc-tagged WT gp78 and a catalytically inactive gp78 mutant in which cysteine 337 and 374 in the RING domain are mutated to serines (C337S:C374S gp78 Myc) were expressed in 293T cells (Figure 4A, top panel, lanes 2 and 3). Expression

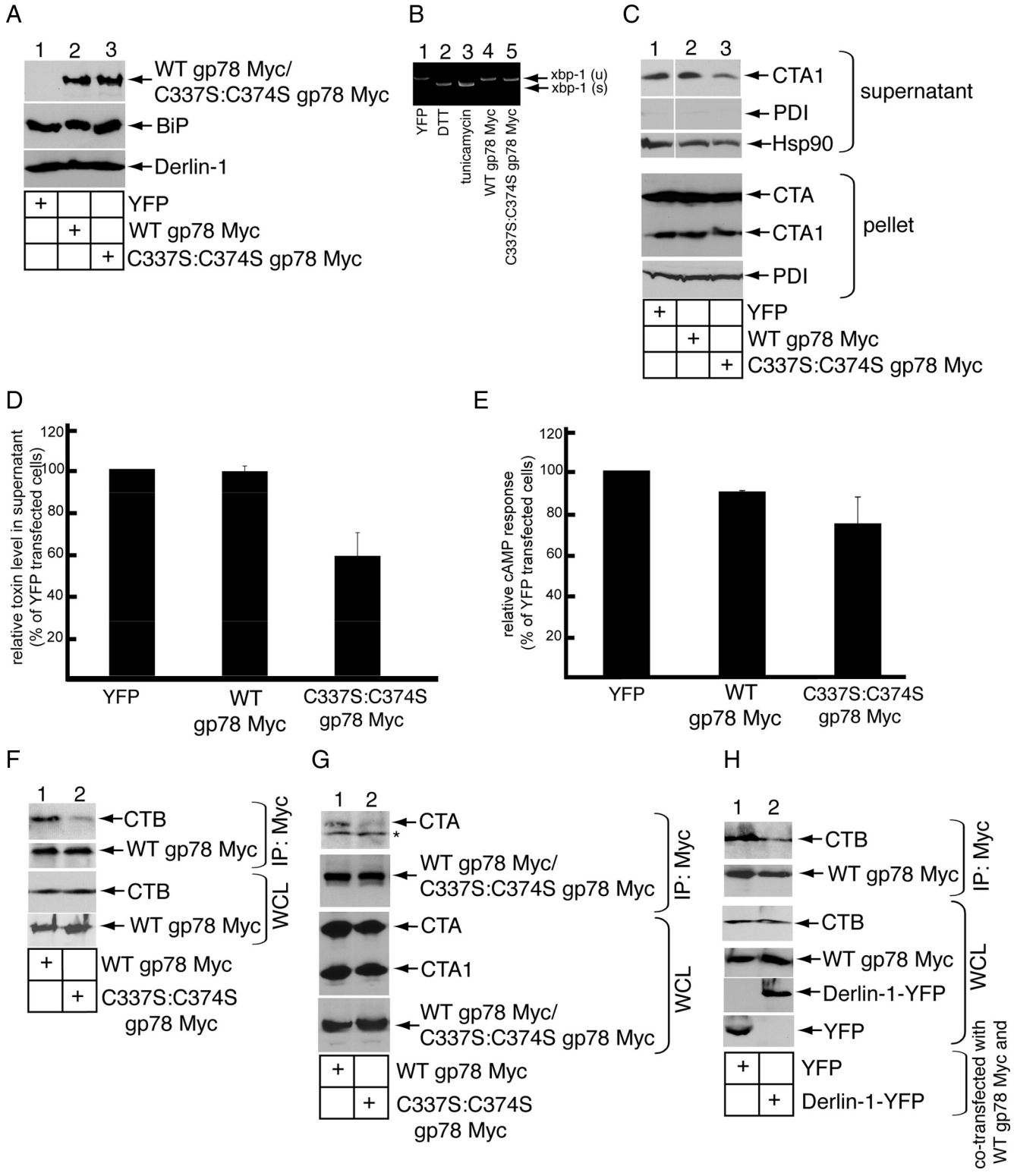

Figure 4. Expression of a catalytic-inactive gp78 mutant decreases CTA1 retro-translocation. (A) 293T cells expressing YFP, WT gp78 Myc, or C337S:C374S gp78 Myc were harvested and lysed, and the lysates were subjected to SDS-PAGE and analyzed with the indicated antibodies. (B–E) As in Figure 1, except gp78 constructs were used. (F and G) As in Figure 2, except gp78 constructs were used. Asterisk denotes unidentified protein. (H) As in Figure 3, except WT gp78 Myc was cotransfected with either YFP or Derlin-1-YFP.

of C337S:C374S gp78 was shown previously to block degradation of the CFTR deltaF508 substrate (Morito *et al.*, 2008). The expression of WT and C337S:C374S gp78 neither caused the up-regulation of BiP and Derlin-1 (Figure 4A, second and third panels, cf. lanes 2 and 3 with lane 1) nor triggered the splicing of the XBP1 mRNA (Figure 4B, cf. lanes 4 and 5

with lanes 2 and 3). Thus, similar to overexpression of the mutant Hrd1 proteins (Figure 1), overexpressing the gp78 proteins also did not induce ER stress significantly.

When cells expressing YFP, WT gp78 Myc and C337S:C374S gp78 Myc were incubated with CT (10 nM) for 90 min and the cells were subjected to the retro-translocation assay described in Figure 1, we found less CTA1 in the supernatant fractions of cells expressing C337S:C374S gp78 Myc when compared with YFP or WT gp78 Myc-expressing cells (Figure 4C, top panel, cf. lane 3 with lanes 1 and 2; quantified in D). Furthermore, compared with cells transfected with YFP and (to a lesser extent) WT gp78 Myc, cells expressing C337S:C374S gp78 Myc also exhibited a decrease in cAMP production (Figure 4E). These data implicate a role of gp78 in the retro-translocation of CTA1.

We then analyzed whether gp78 interacts with CT. To assess CTB-gp78 interaction, cells expressing WT gp78 Myc or C337S:C374S gp78 Myc were treated with CT (10 nM) for 90 min and lysed in a 1% Triton X-100–containing buffer. Complexes were immunoprecipitated with an Myc antibody and probed for CTB by immunoblot analysis. Our results showed that CTB interacted with the mutant gp78 to a lesser extent than with WT gp78 (Figure 4F, top panel, cf. lane 2 with lane 1). To detect a potential CTA-gp78 interaction, this same experiment was conducted with 100 nM CT and with a 1% deoxyBigChap lysis buffer. We found that CTA bound to mutant gp78 less strongly than to WT gp78 (Figure 4G, top panel, cf. lane 2 with lane 1), consistent with the CTB binding pattern. We conclude that the enzymatic-inactive gp78 does not associate with CT efficiently. Thus the decrease in CTA1 retro-translocation in the presence of the mutant gp78 could therefore be attributed to its inability to engage the toxin, contrary to the behavior of the catalytic-inactive Hrd1 mutant which appears to bind the toxin more strongly than WT Hrd1 (Figure 2C).

As expression of Derlin-1-YFP blocks CTB-Hrd1 interaction (Figure 3), we asked whether Derlin-1-YFP would also decrease the CTB-gp78 interaction and found that it did (Figure 4H, top panel, cf. lane 2 with lane 1). This finding suggests that, similar to the CT-Hrd1 interaction, the CT-gp78 interaction occurs after the CT-Derlin-1 binding event.

Expression of Ube2g2 Mutants Decrease CTA1 Retro-Translocation

That the ubiquitin ligase activity of Hrd1 and gp78 are important for retro-translocation of CTA1 suggests a previously unpredicted role for ubiquitination in this essential step of CTA1 trafficking. To further assess if ubiquitination is involved in CTA1 retro-translocation, we examined whether defects in an additional component of the ubiquitination machinery could alter CTA1 transport into the cytosol.

Ube2g2 is an E2-conjugating enzyme utilized by both Hrd1 and gp78 during retro-translocation of misfolded proteins (Fang *et al.*, 2001; Kikkert *et al.*, 2004). A recent report showed that the Ube2g2/gp78-dependent polyubiquitination reaction involves the preassembly of ubiquitin chains on the catalytic cysteine residue of Ube2g2 (Li *et al.*, 2007). The preassembled ubiquitin chain is then transferred to a substrate in a gp78-dependent manner. The initial growth of the ubiquitin chains relies on a transfer reaction between two Ube2g2 molecules. Mutants of Ube2g2 that fail to catalyze this transfer reaction thus prevent the formation of polyubiquitinated chains anchored to Ube2g2. A simple version of this reaction can be recapitulated in vitro using purified recombinant Ube2g2 and the cytosolic portion of gp78 (gp78c; Li *et al.*, 2007; Figure 5A). In the presence of both WT Ube2g2 and gp78c, polyubiquitinated Ube2g2 can

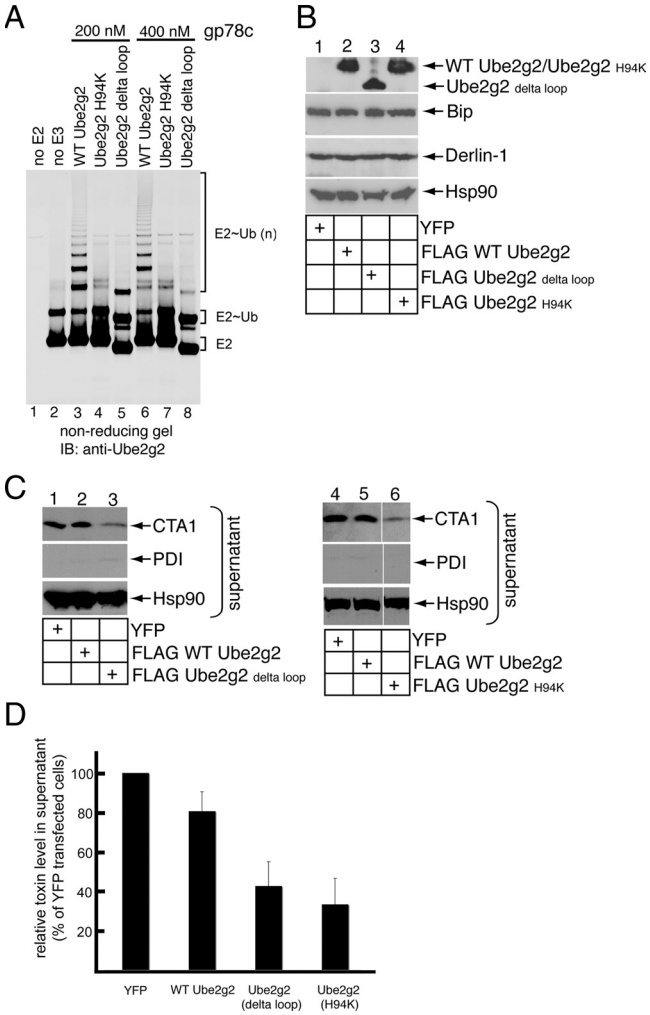

Figure 5. Expression of Ube2g2 mutants decreases CTA1 retro-translocation. (A) Purified recombinant WT Ube2g2 (400 nM), Ube2g2 delta loop (400 nM), and Ube2g2 H94K (400 nM) were incubated with or without the cytosolic domain of gp78 (gp78c, 200 or 400 nM) for 12 min at 37°C. The samples were subjected to nonreducing SDS-PAGE and immunoblotted with an Ube2g2 antibody. E2~Ub, one ubiquitin molecule anchored to E2. E2~Ub(n), multiple ubiquitin molecules anchored to E2. (B) 293T cells expressing YFP, WT FLAG Ube2g2, FLAG $Ube2g2_{delta\ loop}$, or FLAG $Ube2g2_{H94K}$ were harvested and lysed, and the lysates were subjected to immunoblot analysis with the indicated antibodies. (C) 293T cells expressing YFP, WT FLAG Ube2g2, FLAG $Ube2g2_{delta\ loop}$, or FLAG $Ube2g2_{H94K}$ were treated with 10 nM CT for 90 min and subjected to the retro-translocation assay described in Figure 1. The supernatant fraction is shown. (D) The intensity of the CTA1 band in the supernatant fraction was quantified with ImageJ. Mean of three independent experiments; error bars, ± SD.

be observed (Figure 5A, lane 3). In contrast, when two mutant versions of Ube2g2, H94K ($Ube2g2_{H94K}$) and delta loop ($Ube2g2_{delta\ loop}$), were individually incubated with gp78c, the formation of a polyubiquitinated chain anchored to the catalytic cysteine on Ube2g2 was severely attenuated (Figure 5A, cf. lanes 4 and 5 with lane 3). The Ube2g2 loop is located near the catalytic cysteine consisting of several acidic residues and is critical for assembling ubiquitin chains on the E2 active site. Similar results were observed when a higher concentration of gp78c was used (Figure 5A, lanes 6–8). Thus, the $Ube2g2_{H94K}$ and $Ube2g2_{delta\ loop}$ mutants fail

to stimulate growth of ubiquitin chain on Ube2g2 and thus are not expected to promote the subsequent E3-dependent polyubiquitination of substrates.

On the basis of this in vitro observation, we reasoned that expression of $Ube2g2_{H94K}$ and $Ube2g2_{delta\ loop}$ in cells may act as dominant-negative factors and block the endogenous Ube2g2 activity required during the Hrd1/gp78-dependent polyubiquitination reactions associated with ERAD. Accordingly, N-terminal FLAG-tagged $Ube2g2_{H94K}$ and $Ube2g2_{delta\ loop}$, along with N-terminal FLAG-tagged WT Ube2g2 and YFP, were expressed separately in cells (Figure 5B, top panel, lanes 2–4). As before, the levels of BiP and Derlin-1 were not up-regulated (Figure 5B, second and third panels, lanes 1–4), indicating that ER stress was not significantly stimulated under these conditions. The transfected cells were treated with CT (10 nM) for 90 min, the cells were subjected to the retro-translocation assay as before, and the level of CTA1 in the supernatant was quantified. We found that expression of $Ube2g2_{H94K}$ and $Ube2g2_{delta\ loop}$ decreased the transport of CTA1 into the cytosol when compared with the toxin level in the cytosol of cells expressing WT Ube2g2 and YFP (Figure 5C, top panels, cf. lane 3 with lanes 2 and 1, and lane 6 with lanes 5 and 6; quantified in D). Thus, an E2 enzyme associated with ERAD also plays a crucial role in facilitating toxin retro-translocation. In conjunction with our studies using the E3 mutants, these data suggest that ubiquitination plays a key role during retro-translocation of CTA1, revealing a novel and unexpected finding.

Hrd1 and gp78 Bind to PDI

As Hrd1 and gp78 mediate retro-translocation of CTA1, we sought to demonstrate their physical interaction with PDI, an ER lumenal chaperone that unfolds the CTA1 chain before its exit into the cytosol (Tsai *et al.*, 2001; Forster *et al.*, 2006). To explore this potential interaction, 293T cells expressing mouse WT PDI FLAG were cotransfected with or without WT Hrd1 Myc. The thiol cleavable cross-linker DSP was added to cells before lysis to stabilize any weak protein–protein interactions. Lysates were subjected to immunoprecipitation with an Myc antibody to isolate the Hrd1 Myc complex. The immunoprecipitated samples were subjected to reducing SDS-PAGE, followed by immunoblotting with the indicated antibodies. We found that PDI FLAG was present in the Myc pulldown from cells expressing WT Hrd1 Myc and not from cells lacking WT Hrd1 Myc (Figure 6A, top panel, cf. lane 2 with lane 1), demonstrating that PDI interacts with Hrd1 specifically. We then assessed whether Hrd1 is a PDI substrate or a stable binding partner. Previous analysis showed that mutation of the isoleucine residue at position 272 to tryptophan (I272W) in human PDI generated a substrate-binding mutant of PDI (Pirneskoski *et al.*, 2004). Thus, in cells expressing WT Hrd1 Myc, we cotransfected a mouse FLAG-tagged PDI with the corresponding mutation (I272W PDI FLAG) or WT PDI FLAG and found that the mutant and WT PDI bound to Hrd1 with equal efficiency (Figure 6A, top panel, cf. lane 4 with lane 3). This finding indicates that Hrd1 is unlikely a PDI substrate but a binding partner instead.

When the same approach was applied to examine gp78-PDI interaction, we found that gp78 associates with PDI specifically (Figure 6B, top panel, cf. lane 2 with lane 1), and that it binds to WT and mutant PDI with a similar efficiency (Figure 6B, top panel, cf. lane 4 with lane 3). These results implicate gp78 as a binding partner of PDI. Thus, the physical link between PDI and Hrd1/gp78 functionally couples

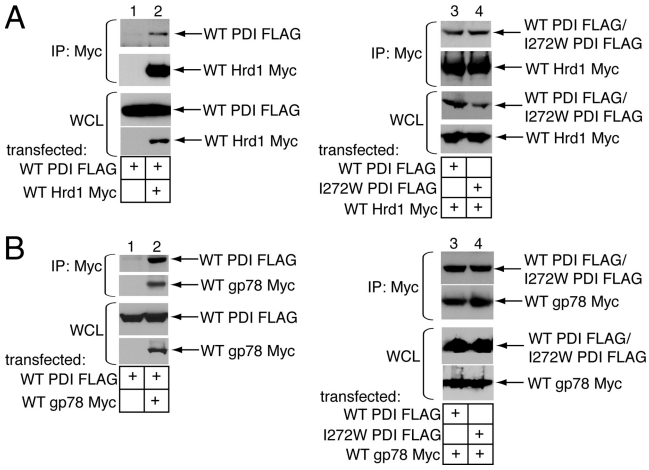

Figure 6. Hrd1 and gp78 bind to PDI. (A) 293T cells were transfected with WT PDI FLAG (lane 1) or cotransfected with WT PDI FLAG and WT Hrd1 Myc (lane 2). In lanes 3–4, cells were transfected with WT Hrd1 Myc and either WT PDI FLAG or I272W FLAG. Cells were harvested, treated with DSP for 30 min, and then lysed in a 1% Triton X-100 buffer. Lysates were subjected to immunoprecipitation with a monoclonal Myc antibody. Complexes were subjected to reducing SDS-PAGE and immunoblotted with the indicated antibodies. WCL, whole cell lysate. (B) As in A, except WT gp78 Myc was expressed.

events in the ER lumen and membrane that act coordinately to drive the toxin into the cytosol.

DISCUSSION

When CT reaches the ER from the cell surface, it coopts the ERAD quality control machinery, gains access to the cytosol, and evades proteasomal degradation. How these distinct steps are achieved remains under intense investigation. In this study, we identify new components of the ERAD machinery that mediate retro-translocation of CTA1. We demonstrated previously that the ER membrane protein, Derlin-1, facilitates retro-translocation of CTA1 (Bernardi *et al.*, 2008), similar to a subsequent report (Dixit *et al.*, 2008). As E3 ubiquitin ligases Hrd1 and gp78 form complexes with Derlin-1 (Ye *et al.*, 2005, Lilley and Ploegh, 2005) and are responsible for the degradation of a variety of misfolded substrates (Hirsch *et al.*, 2009), we tested the possibility that this ubiquitination machinery may similarly regulate toxin retro-translocation, despite the observation that the CTA1 chain is neither ubiquitinated on its two lysines nor at its N-terminus when the toxin reaches the cytosol (Rodighiero *et al.*, 2002).

Using siRNA knockdown and dominant-negative approaches, we demonstrate that Hrd1 is involved in the ER-to-cytosol transport of CTA1. Specifically, down-regulation of Hrd1, as well as expression of the enzymatically inactive C291A Hrd1 and the truncated TM1-6 Hrd1 mutants, inhibited CTA1 retro-translocation. In the case of the dominant-negative approach, a trivial explanation to account for this block in toxin retro-translocation is that expression of the Hrd1 mutants causes the buildup of endogenous misfolded substrates at the retro-translocation sites, potentially inducing ER stress and thereby preventing CT from engaging this machinery to reach the cytosol. However, we found that expressing the Hrd1 mutants did not induce massive ER stress, and CTA bound more strongly with C291A Hrd1 than with WT Hrd1.

These findings therefore indicate that expression of the Hrd1 mutants interferes with a specific step in the retro-translocation of CTA1. Although the ligase activity of this E3 is not required to bind initially the CTA substrate, it appears to be necessary for the subsequent transport and release of the toxin into the cytosol. Similar to CTA, the Hrd1-dependent substrate CD3δ displayed a more stable interaction with C291A Hrd1 than with WT Hrd1 (Kikkert *et al.,* 2004), suggesting that the mechanistic basis of Hrd1's role in CD3δ degradation and CTA1 retro-translocation may be similar.

Expression of TM1-6 Hrd1 decreased CTA1 retro-translocation. This finding demonstrates that the cytosolic domain of Hrd1 is essential for transport. That CTB binds more strongly to TM1-6 Hrd1 than WT Hrd1 suggests that CTB normally undergoes cycles of binding and release from Hrd1 that is disrupted in the absence of its cytosolic domain. This observation supports the view that active communication between the Hrd1 transmembrane and cytosolic domains plays a crucial role in transferring a substrate from the ER into the cytosol.

What might be the functions of the Hrd1 transmembrane and cytosolic domains in the toxin translocation process? The Hrd1 cytosolic domain contains the active site responsible for ubiquitination. Recent findings indicate that the Hrd1 transmembrane domain transfers a membrane substrate from the ER into the cytosol (Omura *et al.,* 2008) and senses the misfolded state of a membrane protein (Sato *et al.,* 2009). Thus, the different Hrd1 domains have distinct roles. A potential scenario of how Hrd1 might eject the toxin into the cytosol could be envisioned as follows: the Hrd1 lumenal domain first engages CT, followed by the Hrd1 transmembrane domain assisting in the transfer of CTA1 into the cytosol. Finally, the Hrd1 cytosolic domain promotes ubiquitination of CTA1 (on nonlysine residues, see below) to allow the toxin to be extracted. Alternatively, the Hrd1 cytosolic domain ubiquitinates a cellular factor that releases the toxin from the ER membrane. Expression of the Hrd1 cytosolic domain did not affect CTA1 retro-translocation (not shown), perhaps because this domain neither binds to CT nor disrupts any cytosolic components required for extracting the toxin into the cytosol.

The repertoire of E3 ligases involved in ERAD continues to expand (Vembar and Brodsky, 2008; Hirsch *et al.,* 2009). We therefore asked whether CT utilizes other E3 ligases in addition to Hrd1. As Hrd1 hetero-dimerizes with the E3 ligase gp78 (Ye *et al.,* 2005), we tested whether gp78 plays any role in the ER-to-cytosol transport of CTA1. Our functional and interaction analyses demonstrate that the gp78 E3 ligase regulates CTA1 retro-translocation, suggesting that there is conservation in function among the E3 ligases to enable CT to utilize these proteins interchangeably. This finding is similar to the retro-translocation of certain misfolded substrates such as TCRα and CD3δ (Fang *et al.,* 2001; Kikkert *et al.,* 2004), but different from that of other substrates (such as CFTR and HMG CoA reductase) that show preference for a specific E3 ligase (Song *et al.,* 2005; Chen *et al.,* 2006; Morito *et al.,* 2008). Thus the selection of an E3 ligase during retro-translocation appears to be substrate-dependent.

We found that in contrast to the enzymatic-inactive Hrd1, the inactive gp78 mutant binds less efficiently to CT than WT gp78. Because expression of this mutant gp78 did not induce significant ER stress, we suspect that the mutant gp78's decreased affinity for the toxin is unlikely due to a build-up of misfolded proteins that would preclude its interaction with CT. Instead, potential structural changes imparted on the mutant gp78 might prevent its interaction with CT directly or disrupt the E3 ligase's interaction with other cellular components used to recruit CT to gp78. Further experiments are required to clarify these possibilities.

How a particular substrate engages sequentially the various ERAD components is not clear. In the case of CT, we found that dominant-negative Derlin-1 (i.e., Derlin-1-YFP) blocks the CT-Hrd1 and -gp78 interactions, suggesting that the toxin is first recruited to Derlin-1 and then transferred to Hrd1/gp78. Derlin-1-YFP's inhibitory effect is likely imparted within the ER lumen because CTB never reaches the cytosol. Moreover, we showed previously that Derlin-1-YFP exerts its dominant-negative effect by titrating CT away from endogenous Derlin-1 (Bernardi *et al.,* 2008). Thus, by preventing endogenous Derlin-1 from engaging the toxin, Derlin-1-YFP is also expected to block interaction between CT and cellular components that normally interacts with the toxin after Derlin-1, such as Hrd1 and gp78. This sequential transfer mechanism depicted for CT has also been proposed in the Der1/Hrd1-dependent degradation of the yeast ERAD substrate CPY* (Gauss *et al.,* 2006). Clearly, elucidating the fate of the toxin after it is released from Hrd1 will be important to understand the entire CTA1 retro-translocation pathway.

Our previous results showed that the ER lumenal factor PDI unfolds CTA1 to prime the toxin for transport across the ER membrane (Tsai *et al.,* 2001; Forster *et al.,* 2006). We then determined that PDI interacts with the Derlin-1 membrane protein (Bernardi *et al.,* 2008), suggesting that events within the ER lumen and on the membrane that control toxin retro-translocation are coupled. That PDI also associates with the membrane proteins Hrd1 and gp78 further supports this view. As the holotoxin is transferred from Derlin-1 to Hrd1, we believe that the Hrd1-bound PDI unfolds CTA1 once the toxin reaches Hrd1.

The observations that expression of two enzymatic defective E3 ligases and a catalytic-inactive E2 enzyme dedicated to ERAD decreased the ER-to-cytosol transport of CTA1 led us to conclude that a functional ubiquitin system is required for toxin retro-translocation. This possibility was surprising because CT has been considered to be a nonubiquitinated ERAD substrate (Rodighiero *et al.,* 2002; Kothe *et al.,* 2005), a conclusion based on the finding that a CTA1 variant in which the two lysines are mutated, and where the N-terminus was blocked chemically, displayed an activity similar to WT toxin. However, as recent studies have identified nonlysine ubiquitination sites on substrates, including serine, threonine, and cysteine residues (Cadwell and Coscoy, 2005; Wang *et al.,* 2007), ubiquitination of CTA1 (which contains numerous serines/threonines and a single cysteine) remains a formal possibility. In this context, we have not observed any higher molecular weight CTA1 in our experiments that might correspond to ubiquitinated CTA1. This may be because ubiquitinated CTA1 is deubiquitinated rapidly or that CTA1 is not ubiquitinated. Interestingly, although degradation of TCRα requires a functional ubiquitination pathway (Yu and Kopito, 1999), ubiquitination of TCRα on lysine residues is not required for its degradation (Yu *et al.,* 1997).

If CTA1 is not ubiquitinated, ubiquitination of a cellular factor may be required for toxin transport. For example, it has been postulated that ubiquitination of adapter proteins may be required to recruit cellular components to the retro-translocation complex to properly execute the US11-mediated retro-translocation of MHC class I molecules, as retro-translocation does not require ubiquitination of the substrate itself (Hassink *et al.,* 2006), but an intact ubiquitination system is necessary (Shamu *et al.,* 1999; Kikkert *et al.,* 2001). These findings further support the contention that ubiquiti-

nation of cellular components other than the substrate regulates retro-translocation. We suspect that a similar mechanism is operating in the ubiquitin-dependent ER-to-cytosol transport of CTA1. Perhaps auto-ubiquitination of Hrd1/gp78 (Fang *et al.*, 2001; Kaneko *et al.*, 2002; Nadav *et al.*, 2003; Kikkert *et al.*, 2004) recruits adaptor proteins that facilitate CTA1 release into the cytosol. Alternatively, it is also conceivable that CTA1 "shuttles" on a misfolded substrate that is ubiquitinated upon entry into the cytosol. Clearly, understanding how CT utilizes the ubiquitination machinery during retro-translocation will illuminate a critical step in CT's intoxication process as well as the fundamental mechanics of ERAD.

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