Derlin-1 and the E3 Ubiquitin Ligases Hrd1 and gp78 Facilitate Cholera Toxin Retro-translocation

by

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Abstract

The endoplasmic reticulum (ER) is the site of folding and assembly for membrane and secretory proteins. When proteins fail to reach their proper conformations, the ER quality control system, ER-associated degradation (ERAD), is responsible for maintaining cell homeostasis via the identification, modification and transport (retro-translocation) of misfolded proteins from the ER to the cytosol for proteasomal degradation. As a model ERAD substrate, Cholera toxin (CT), produced by *Vibrio cholerae*, relies on its receptor-binding B subunit (CTB) to enter the ER of intestinal epithelial cells, where its catalytic A1 subunit (CTA1) hijacks the ERAD pathway to induce toxicity. This thesis focuses on understanding the role of membrane proteins in the retro-translocation of CTA1.

We found that two core components of the retro-translocon, Derlin-1 and the E3 ubiquitin ligases Hrd1 and gp78, facilitate the retro-translocation of CTA1. The expression of dominant negative variants of Derlin-1, Hrd1 and gp78 inhibits the ER-to-cytosol transport of CTA1. Derlin-1, Hrd1 and gp78 interact with CTB and CTA, as well as with the ER oxido-reductase PDI, a lumenal protein known for its role in unfolding the toxin prior to retro-translocation. Furthermore, we reveal a previously unknown role for CTB in the targeting of the toxin to this retro-translocation machinery in the ER membrane. Collectively, these findings suggest a model by which CTB targets the holotoxin to the retro-translocation machinery where PDI unfolds the toxin for retro-translocation through the ER membrane. The function of Hrd1 and gp78 in this model is perplexing, as CTA1 is presumed to be a non-ubiquitinated substrate. However, our data indicate that the catalytic activity of Hrd1, gp78 and an E2 ubiquitin conjugating enzyme dedicated to ERAD are essential for CTA1 transport. Thus, in addition to identifying novel ERAD components involved in CTA1 retro-translocation and elucidating a mechanism by which CT is targeted to the ERAD machinery, we demonstrate that similar to other known

ERAD substrates, an intact ubiquitination system is necessary for the ER-to-cytosol transport of CTA1. These data provide important insight into the general mechanism of ERAD and further our understanding of how ERAD substrates utilize the retro-translocation machinery.

Chapter 1

Introduction

The endoplasmic reticulum (ER) is the major site of folding and assembly for proteins destined for the secretory pathway. Nascent polypeptide chains are translocated into the endoplasmic reticulum (ER) through the Sec61 channel (reviewed in Rapoport, 2007) for folding and assembly. These substrates require the activity and coordination of multiple chaperones for efficient and correct processing to occur (reviewed in Buck et al., 2007). The ER quality control pathway known as ER associated degradation (ERAD) is responsible for disposing of newly synthesized proteins that fail to reach their proper conformations. Detailed steps of ERAD include the recognition and targeting of misfolded substrates to the retro-translocation machinery, transport into the cytosol and degradation by the ubiquitin-proteasome system (reviewed in Vembar and Brodsky, 2009). Defects in protein folding and ERAD can be detrimental to the cell, resulting in many diseases including, but not limited to: Cystic Fibrosis, Huntington's, Parkinson's, Alpha-1-Antitrypsin deficiency, and Alzheimer's. Moreover, ERAD is a popular pathway hijacked by various microbial virulence factors to cause disease (reviewed in Tsai et al., 2002). For example, Cholera toxin (CT), produced by ***Vibrio cholerae*** co-opts the ERAD pathway to cause debilitating and massive secretory diarrhea that results in death if treatment is not obtained (Sears and Kaper, 1996). Clearly, research focused on understanding the ER events that facilitate CT trafficking will help to elucidate the mechanism of ERAD and provide important details regarding associated diseases.

CT is an AB_5 toxin, consisting of a catalytic A subunit (CTA) and homopentameric receptor binding B subunit (CTB; Figure 1.1; Zhang et al., 1995). Upon secretion from

the bacterium, CTA is proteolytically nicked into CTA1 and CTA2 peptides that remain attached by a disulfide bond and noncovalent interactions (Spangler, 1992). While the holotoxin binds the ganglioside receptor GM1 at the plasma membrane of intestinal epithelial cells via CTB and enters as a holotoxin into the cell, it is only the CTA1 peptide that must reach the cytosol to induce toxicity. The arrival of CTA1 into the cytosol is preceded by endocytosis of the holotoxin and retrograde trafficking into the ER. CTB and CTA2 remain in the ER while CTA1 is targeted for ERAD and subsequently retro-translocated into the cytosol (Lencer and Tsai, 2003). CTA1 presumably avoids ubiquitination and thus proteasomal degradation, spontaneously refolds and is able to induce toxicity via the ADP-ribosylation of GαS which results in an increase in cAMP production, massive chloride secretion and subsequent diarrhea and dehydration (Figure 1.2; Spangler, 1992). While a great deal is known about the catalytic activity of CTA1 in the cytosol, much less is known about how the toxin co-opts the ERAD pathway.

The ER oxido-reductase protein disulfide isomerase (PDI) has been implicated in the unfolding of CTA1 which separates this peptide from the holotoxin and prepares it for retro-translocation (Tsai et al., 2001; Forster et al., 2006). The unfolding reaction occurs after the reduction of CTA to CTA1 by an unknown reductase (Majoul et al., 1996). As CTA1 must transport across the ER membrane to reach the cytosol, it is unclear whether PDI engages the toxin prior to its arrival at the ERAD membrane machinery or after. Because CTA1 refolds quickly upon dissociation from PDI (Rodighiero et al., 2002), it has been postulated that unfolding and retro-translocation are tightly coupled to guarantee successful transport of the toxin (Tsai et al., 2002). However, it remains unclear as to how the toxin is initially targeted to ERAD components and unknown which ERAD membrane complexes are involved in CTA1 retro-translocation. Furthermore, it is also unknown whether CTB plays any role in these ER events. As a response to these questions, this thesis provides insight into events occurring at the ER membrane, including targeting of the toxin to ERAD machinery and the identification of membrane complexes involved with the toxin's transport into the cytosol.

The composition and function of ERAD membrane complexes has been the subject of much debate (reviewed in Vembar and Brodsky, 2009). Specifically, the center of confusion lies in which membrane protein(s) constitute the retro-translocon and which others solely act as adaptor proteins, chaperones or E3 ubiquitin ligases. The debate intensifies upon discussion of what should actually be considered a critical channel component. For example, if an adaptor protein is required to induce formation of a channel, but it does not actually form the pore, is this considered a channel component? Certainly, further characterization of membrane proteins involved in ERAD is required before any definitive conclusions can be drawn in either argument. Currently, studies have focused attention on select proteins, namely the Sec61 complex and the Derlins, to assess their roles in ERAD and potential as channel components. Notably, as the indisputable recognition of a protein as the retro-translocon has yet to be accomplished, most studies implicating these membrane proteins in retro-translocation serve only to acknowledge that they play some role in ERAD, but not necessarily as a channel per se.

The Sec61 complex is mostly recognized for its role as the translocon through which nascent polypeptide chains enter the ER for folding (Rapoport, 2007). Studies in yeast and mammalian ERAD have also identified Sec61 to function as the retro-translocon due to its interactions with the proteasome (Kalies et al., 2005; Ng et al., 2007) and its ability to interact with various substrates and facilitate their degradation (Wiertz et al., 1996; Plemper et al., 1997; Pilon et al., 1997; Gillece et al., 2000; Pariyarath et al., 2001; Schmitz et al., 2004; Romisch, 2005; Scott et al., 2008; Willer et al., 2008). Interestingly, Sec61 has been implicated in the retro-translocation of CT (Schmitz et al., 2000). Unfortunately, many studies implicating Sec61 in ERAD are difficult to draw conclusions from due to the nonspecific effects that disrupting Sec61 function may have upon the forward translocation function of Sec61. Whether Sec61 serves as the channel for retro-translocation, or instead is an adaptor protein in this process, remains to be clarified. As Sec61 is not required for the retro-translocation of all tested substrates, it seems likely that additional channels may exist.

The Derlin family of proteins is required for the ERAD of a subset of misfolded substrates. In yeast, two Derlin proteins have been identified. Der1p interacts with lumenal and cytosolic ERAD components and is required for the degradation of a subset of proteins with lumenal lesions (Knop et al., 1996; Hitt and Wolf, 2004; Gauss et al., 2006; Carvalho et al., 2006; Denic et al., 2006). A role for Dfm1p, another Derlin protein, in ERAD has not been identified, but the finding that it its absence results in the ER stress induced unfolded protein response (UPR) suggests that it is important for maintaining ER homeostasis (Sato and Hampton, 2006). While Der1p and Dfm1p have a similar topology, form homo-oligomers, and share some similarities in the complexes they form, there are differences in their binding partners that may also suggest distinct functions (Hitt and Wolf, 2004; Schuberth and Buchberger, 2005; Goder et al., 2008). Additional evidence indicating diverse functions is the finding that Der1p and Dfm1p do not interact with one another (Goder et al., 2008), which is contrary to the mammalian Der1/Dfm1p homologues (Lilley and Ploegh, 2005). As the characterization of Der1p and Dfm1p is relatively recent, future studies will help to definitively address their roles in ERAD and/or other cellular processes.

In mammalian cells, Derlin-1, Derlin-2 and Derlin-3 have been implicated in ERAD, most notably for their potential roles as components of the retro-translocon (Ye et al., 2004; Lilley and Ploegh, 2004; Oda et al., 2006). Each mammalian Derlin protein interacts with a variety of ERAD factors in and across the ER membrane including lumenal chaperones, the cytosolic p97 which is responsible for extracting many ubiquitinated substrates from the ER membrane, and cytosolic and membrane associated E3 ubiquitin ligases (Ye et al., 2004; Ye et al., 2005; Lilley and Ploegh, 2005; Schulze et al., 2005; Katiyar et al., 2005; Younger et al., 2006; Wang et al., 2006; Wang et al., 2008). The aforementioned interactions, as well as the ability of each mammalian Derlin protein to form homo-oligomers and hetero-oligomers with one another, suggest the potential formation of a channel (Ye et al., 2005; Lilley and Ploegh, 2005). The number of ERAD substrates dependent on Derlin-mediated retro-translocation continues to grow. Interestingly, despite their shared topology, interactions and presumed similarities in function, the Derlin proteins require strict substrate specificity (Ye et al., 2004; Lilley and

Ploegh, 2004; Oda et al., 2006; Lilley et al., 2006; Sun et al., 2006; Younger et al., 2006; Schelhass et al., 2007; Okuda-Shimizu and Hendershot, 2007; Bernardi et al., 2008; Dixit et al., 2008; Gao et al., 2008; Schwieger et al., 2008; Wang et al., 2008; Rutledge et al., 2009; Jiang et al., 2009). These findings further suggest the strategic use of multiple channels in ERAD. Currently, Derlin-1 and Derlin-2 display the most substrate specificity, while Derlin-2 and Derlin-3 are redundant in substrate selection presumably due to the finding that they are 70% identical (Lilley and Ploegh, 2005; Oda et al., 2006). Therefore, most studies focus on the role of Derlin-1 and Derlin-2 in the retro-translocation of a particular substrate.

In an effort to identify new membrane proteins involved in the retro-translocation of CTA1, we performed functional and interaction studies with dominant negative versions of Derlin-1 and Derlin-2 to assess their potential roles in this process (Chapter 2). Using two functional assays, we found that Derlin-1, but not Derlin-2, facilitates the retro-translocation of CTA1. Binding studies isolated interactions between endogenous Derlin-1 and CTB, while the dominant negative Derlin-1 was able to bind CTB and CTA subunits (holotoxin), suggesting that it traps the substrate and prevents its retro-translocation. Additional binding studies identified an interaction between Derlin-1 and PDI, thereby emphasizing the coupling of ER lumenal and retro-translocation events. This study also highlights a novel role for CTB in CTA1 retro-translocation. CTB appears to target the toxin to the retro-translocation machinery at the ER membrane through its interaction with Derlin-1. The mechanism by which a substrate engages the retro-translocation machinery has remained elusive, but this study aids in elucidating the process while also characterizing the membrane complex that facilitates ER-to-cytosol transport of CTA1.

The fate of the toxin after its association with Derlin-1 remains unknown. Most ERAD substrates are ubiquitinated by E3 ubiquitin ligases at the ER membrane prior to their retro-translocation and subsequent degradation (Vembar and Brodsky, 2008; Hirsch et al., 2009). However, as CTA1 evades proteasomal degradation presumably by avoiding

ubiquitination of its two lysine residues and N-terminus (Rodighiero et al., 2002), it is unknown whether the ubiquitination system contributes to CTA1 retro-translocation.

To examine the potential effect of the ubiquitination pathway on CTA1 retro-translocation, we chose to study two E3 ubiquitin ligases, Hrd1 and gp78 due to their established interaction with Derlin-1 (Ye et al., 2005; Lilley and Ploegh, 2005; Schulze et al., 2005). Importantly, the yeast Der1 also exists in a complex with yeast Hrd1, drawing testament to the importance of this interaction as it has been preserved from yeast to mammalian systems (Gauss et al., 2006; Denic et al., 2006; Carvalho et al., 2006). Similar to their yeast counterpart, Hrd1 and gp78 are multi-spanning integral membrane E3 ligases that are dependent on their C-terminal cytosolic RING Finger domain for catalytic activity and degradation of substrates (Bays et al., 2001; Deak and Wolf, 2001; Fang et al., 2001; Kikkert et al., 2004; Chen et al., 2006). Regardless of the ability of Hrd1 and gp78 to hetero-oligomerize (Ye et al., 2005) and facilitate the ubiquitination of some shared substrates (Fang et al., 2001; Kikkert et al., 2004), they do retain specificity for various substrates (Song et al., 2005; Chen et al., 2006; Morito et al., 2008). How this specificity is achieved remains unknown.

To analyze whether Hrd1 and/or gp78 participated in CTA1 retro-translocation, we expressed dominant negative variants of each protein to assess their ability to inhibit CTA1 cytosolic entry (Chapter 3). Through functional and interaction studies, we determined that Hrd1 and gp78 are involved in CTA1 retro-translocation. Each E3 ligase interacts with CT (CTB and CTA subunits) as well as PDI. The Hrd1-CT interaction is downstream of the Derlin-1-CT interaction, providing evidence for sequential targeting of the substrate to various ERAD components. Additionally, we found that a dominant negative E2 conjugating enzyme dedicated to ERAD, Ube2g2, also decreased the retro-translocation of CTA1. These novel findings suggest the importance of an intact ubiquitination system in CTA1 transport and expand upon our initial discoveries identifying the Derlin-1 complex as necessary for CTA1 retro-translocation (Chapter 2).

This thesis describes key events occurring at the ER membrane that mediate the retro-translocation of CTA1, thereby providing insight into CT trafficking and the fundamental ERAD mechanism. Specifically, we provide evidence for the targeting of the substrate to the retro-translocation machinery, identify membrane components involved in the process and challenge the field to reevaluate its previous notions regarding the role of the ubiquitination machinery in CTA1 retro-translocation.

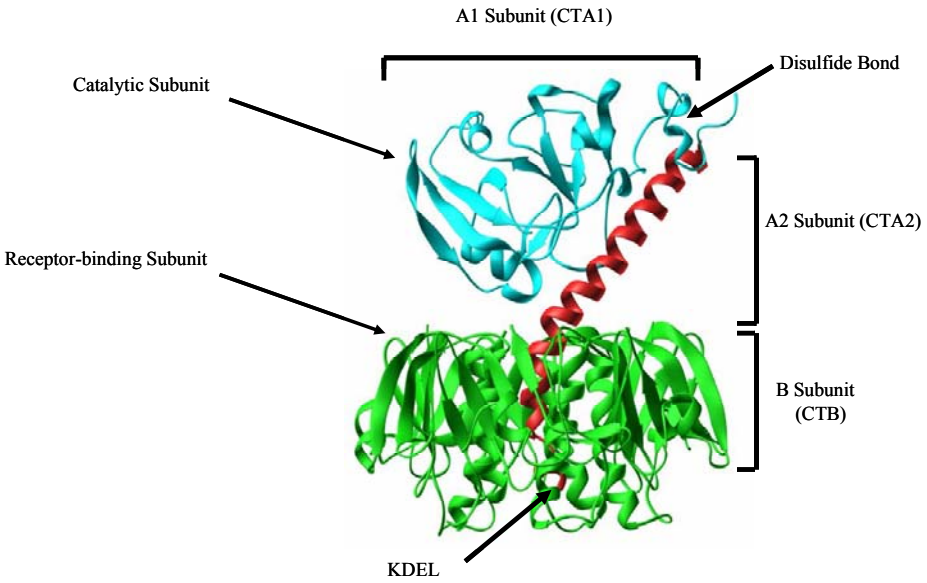


Figure 1.1. Crystal Structure of Cholera Toxin. Cholera toxin consists of a single catalytic A subunit (CTA) and a homopentameric receptor binding B subunit (CTB) attached by noncovalent interactions. Upon secretion from ***V. cholerae***, CTA is cleaved into A1 and A2 peptides that remain attached by a disulfide bond and noncovalent interactions. Figure adapted from Zhang et al. (1995).

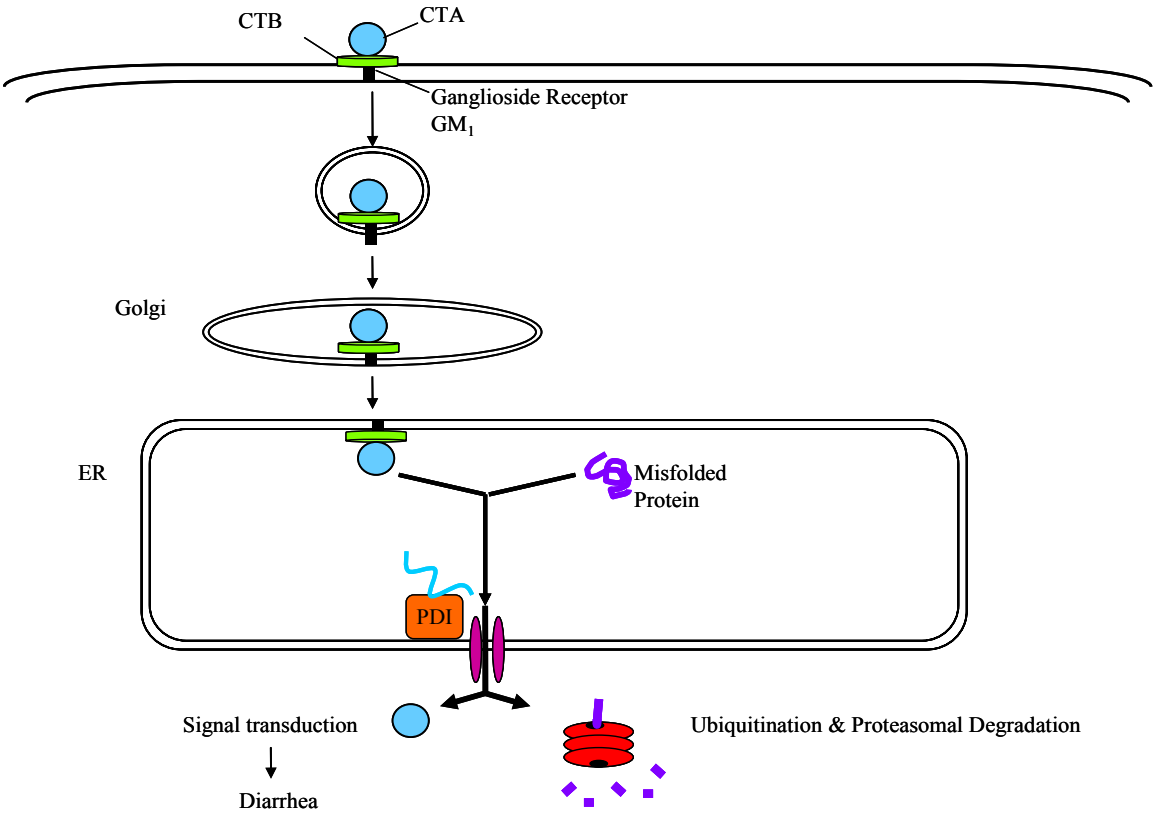


Figure 1.2. Intracellular Trafficking of Cholera Toxin. CTB binds the ganglioside receptor GM1 on the plasma membrane of an intestinal epithelial cell. The holotoxin is endocytosed and trafficked in a retrograde manner through the secretory pathway, eventually reaching the ER lumen. In the ER, CTA is reduced to generate the CTA1 peptide that is later unfolded by PDI and retro-translocated into the cytosol. CTA1 spontaneously refolds prior to inducing a signaling cascade that results in diarrhea, whereas a misfolded ERAD substrate is ubiquitinated and targeted for proteasomal degradation as it enters the cytosol.

Chapter 2

Derlin-1 Facilitates the Retro-Translocation of Cholera Toxin

Introduction

Cholera toxin (CT), a virulence factor produced by ***Vibrio cholerae***, consists of six subunits that are essential for its activity: a homopentameric receptor binding B subunit (CTB) and a single catalytic A subunit (CTA). Upon secretion from the bacterium, CTA is proteolytically nicked to produce two domains, A1 and A2, which remain linked by a disulfide bond and noncovalent interactions (Spangler, 1992). It is the CTA1 domain that is toxic and must reach the cytosol of the mammalian intestinal epithelial cell to advance its pathogenesis (Spangler, 1992). Its cytosolic entry allows for the initiation of a signaling cascade that leads to the dangerous opening of a chloride channel, whereby the significant loss of chloride ions and water result in the deadly diarrhea that is characteristic of this disease (Lencer and Tsai, 2003).

While the cytosolic signaling events depicting CT activity are well understood, less is known about how the toxin gains entry into the cytosol. CT begins its journey through host cells when CTB binds the ganglioside receptor GM1 on the plasma membrane, allowing for endocytosis of the holotoxin and eventual retrograde movement into the endoplasmic reticulum (ER; Fujinaga et al., 2003). As the ER is the final cellular location of the toxin prior to its residence in the cytosol, understanding ER events that lead to the transfer of the toxin into the cytosol is an area of significant interest. Recent studies have clarified an essential role for the ER oxido-reductase protein disulfide isomerase (PDI) in the binding, unfolding and disassembly of the CTA1 chain prior to its movement into the cytosol (Tsai et al., 2001; Forster et al., 2006). However, little is known as to how CTA1 is able to move across the ER membrane. In vitro

experiments with PDI and toxin have suggested that the unfolding activity of PDI and the transport of CTA1 into the cytosol may be a tightly coupled process, as the CTA1 chain quickly refolds when released from PDI and this is unfavorable for transport across the ER membrane (Rodighiero et al., 2002; Tsai et al., 2002). How this mechanistic detail is achieved remains to be understood, as does whether CTB has any role in this part of the trafficking process.

PDI is part of the quality control pathway ER associated degradation (ERAD; Gillece at al., 1999; Molinari et al., 2002; Wahlman et al., 2007), through which misfolded proteins originally destined for the secretory pathway are transported into the cytosol and targeted for proteasomal degradation. As CT exploits this process for its own cytosolic entry by masquerading as misfolded substrate (Hazes and Read, 1997), clarifying the mechanism by which CTA1 is retro-translocated will also provide insight into this fundamental pathway and the degradation of substrates inherent to the host cell.

In this study we identify Derlin-1, an ER membrane protein postulated to be the retro-translocon, as a key factor in the retro-translocation of CT. Overexpression of a dominant negative Derlin-1 protein decreased the transport of CTA1 into the cytosol. This inhibitory activity was potentially exerted by the ability of the dominant negative to bind and sequester holotoxin from endogenous Derlin-1 while also imparting conformational changes to the endogenous protein that may render it structurally defective to some extent. Co-immunoprecipitation studies demonstrated that Derlin-1 interacts with both CTB and PDI. The significance of the CTB interaction was further clarified by the finding that CTB enhances the interaction between CTA1 and the ER membrane. Additionally, CTB was able to inhibit the degradation of the cystic fibrosis transmembrane conductance regulator (CFTR), a Derlin-1-dependent substrate. These findings strongly suggest an efficient model wherein CTB targets the holotoxin to the site of retro-translocation, initiating binding of the toxin to Derlin-1, thereby allowing bound PDI to unfold CTA1 and prepare it for transport.

Results

Derlin-1 Facilitates the Retro-Translocation of CT

We previously described a cell based retro-translocation assay in HeLa cells that allows one to analyze the amount of CTA1 that has entered the cytosol of host cells under varying conditions (i.e. protein expression, temperature) (Forster et al., 2006). As 293T cells granted us the high transfection efficiency needed for this study, we first established the retro-translocation assay in this cell line. Cells were intoxicated with CT, harvested and treated with a low concentration of digitonin to selectively permeabilize only the plasma membrane, and then fractionated by centrifugation to separate supernatant and pellet fractions. The supernatant contains cytosolic proteins such as Hsp90 (Figure 2.1A, fourth panel, lanes 1 and 3) while the pellet contains proteins enclosed in intracellular membranes, such as the ER lumenal protein PDI which is notably excluded from the supernatant (Figure 2.1A, third panel, compare lanes 2 and 4 to lanes 1 and 3). These controls indicate that the cytosol was separated from all intracellular compartments, including the ER and that all organelles remained intact throughout the assay.

When cells were intoxicated with CT at 37°C, CTA1 was detected in the supernatant fraction, suggesting CT had retro-translocated into the cytosol (Figure 2.1A, top panel, lanes 1 and 2). The B subunit, which remains in the ER and is not retro-translocated, was found in the pellet fractions as expected (Figure 2.1A, second panel, lanes 1 and 2). To confirm that the presence of CTA1 in the cytosol was due to a retro-translocation event, cells were treated with CT at 37°C in conjunction with brefeldin A (BFA), a drug that inhibits the trafficking of the toxin to the ER (Fujinaga et al., 2003). As expected, CTA1 was not in the supernatant of cells under these conditions and instead, all toxin was found in the pellet (Figure 2.1A, top panel, compare lane 3 to lanes 1 and 4). Thus, the successful separation of cellular components and the ability to monitor the retro-translocation event of CT in 293T cells validated the use of this assay in this study.

Derlin-1 and Derlin-2 are ER membrane proteins involved in the ERAD of misfolded proteins by facilitating their transport form the ER to the cytosol (Ye et al., 2004; Lilley and Ploegh, 2004; Oda et al., 2006). To assess their potential roles in CT retro-translocation, we tested the previously characterized dominant negative variant of each

protein, Derlin-1-GFP and Derlin-2-GFP (Lilley and Ploegh, 2004) in the retro-translocation assay. (This current study utilizes YFP instead of GFP). 293T cells robustly expressed YFP, Derlin-1-YFP and Derlin-2-YFP during transient transfection experiments (Figure 2.1B, lanes 1-3). When these cells were intoxicated with CT for 45 minutes, less CTA1 was retro-translocated in cells expressing Derlin-1-YFP compared to cells expressing YFP or Derlin-2-YFP, as depicted by the decreased amount of CTA1 in the supernatant fraction (Figure 2.1C, top panel, compare lane 2 to lanes 1 and 3, quantified in the bottom graph). A similar result was obtained when cells were treated with toxin for 90 minutes (Figure 2.1C, top panel, compare lane 5 to lanes 4 and 6, quantified in the bottom graph). Furthermore, co-expression of Derlin-1-YFP and Derlin-2-YFP resulted in a similar decrease in the amount of CTA1 retro-translocated as seen in cells expressing Derlin-1-YFP alone (Figure 2.1C, quantified in the bottom graph), thereby attributing this inhibitory effect solely to the dominant negative Derlin-1-YFP. When the catalytic CTA1 reaches the cytosol, it induces a signaling cascade via the ADP ribosylation of GαS protein and subsequent activation of adenylate cyclase that results in the production of cAMP. Therefore, an additional functional assay was conducted in which we measured the amount of cAMP produced in cells treated with toxin and expressing either YFP or Derlin-1-YFP. We found that in cells expressing Derlin-1-YFP, the CT induced cAMP response was reduced compared to YFP expressing cells (Figure 2.1D). These findings coincide with results obtained from the retro-translocation assay and suggest a role for Derlin-1 in the retro-translocation of CT.

CTB Associates with Derlin-1 and Enhances Transfer of CTA1 to the ER Membrane

We next asked whether the toxin is able to physically interact with Derlin-1. 293T cells were incubated with or without CT (30nM) for 90 minutes, lysed in buffer containing 1% Triton X-100 and subjected to immunoprecipitation to isolate endogenous Derlin-1 and Derlin-2 proteins. We found that CTB co-immunoprecipitated with Derlin-1, but not Derlin-2, ERp29 (an ER lumenal protein) or a nonspecific IgG (Figure 2.2A, fifth panel, compare lane 3 to lanes 4-6). Additionally, CTB was not found in immunoprecipitates of cells not treated with toxin (Figure 2.2A, fifth panel, lanes 1 and 2). CTA and CTA1 subunits were not detected in any immunoprecipitate from this experiment (Figure 2.2A,

fourth panel). Cells intoxicated with a lower concentration of CT (10 nM) displayed similar results, as Derlin-1, but not Derlin-2 or a nonspecific IgG was able to co-immunoprecipitate CTB (Figure 2.2B, third panel, compare lane 5 to lanes 6 and 8). CTB was found in a very low amount of immunoprecipitate obtained from an antibody targeting the Sec61 ER membrane translocation channel (Figure 2.2B, third panel, lane 7). Interestingly, CTB was able to co-immunoprecipitate with Derlin-1 in cells treated with CTB alone (Figure 2.2C, lane1), suggesting that the CTA subunit is not necessary for this interaction to occur. However, it does not exclude the possibility that the ganglioside GM1 helps to mediate the interaction, as CTB remains bound to GM1 in the ER membrane (Fujinaga et al., 2003). The interaction between CTB and Derlin-1, but not Derlin-2 is consistent with the functional data presented in this study that depicts a role for Derlin-1, but not Derlin-2 in the retro-translocation of CT and it raises important questions as to the significance of CTB binding.

We next tested whether the CTB-Derlin-1 interaction could be replicated in vitro. Purified CT was incubated in the absence or presence of proteoliposomes which contain basically all ER membrane proteins in randomized orientation. This allows CTB to interact with the lumenal domain of Derlin-1. Once solubilized, Derlin-1 and Derlin-2 were immunoprecipitated and the precipitated complexes probed for the presence of CTB. We found that Derlin-1 was able to co-immunoprecipitate CTB while only a very low amount of CTB immunoprecipitated with Derlin-2 (Figure 2.2D, compare lane 3 to 4). Similar to the cell based interaction studies described in Figure 2.2A and 2.2B, no CTA was found in these precipitates (data not shown). As a control, CT was incubated with microsomes stripped of ribosomes (PK-RM) which do not have randomized orientation of membrane proteins, in an effort to determine if the in vitro binding of CTB to Derlin-1 is artificial. The CTB-Derlin-1 interaction was hardly detected when these microsomes were used compared to proteoliposomes (Figure 2.2D compare lane 6 to lane 5), suggesting that the CTB-Derlin-1 interaction described in proteoliposomes is valid. We additionally examined the ability of the Sec61 antibody to immunoprecipitate CTB in this in vitro study. Similar to the cell based immunoprecipitation experiments (Figure 2.2B), much less CTB co-immunoprecipitated with Sec61 antibody compared to the

Derlin-1 antibody (Figure 2.2E, compare lane 2 to lane 1). Taken together, these findings further support the conclusion that CTB specifically interacts with Derlin-1, but leaves open the question as to whether or not this interaction has any function.

To assess whether the CTB-Derlin-1 interaction has functional significance, we utilized a membrane-pelleting assay that previously demonstrated the ability of the PDI-unfolded CTA1 chain to bind an unidentified ER membrane protein (Tsai and Rapoport, 2003). We wanted to test if in this assay, CTB is able to stimulate the CTA1 chain to bind the membrane protein. ER proteoliposomes were incubated with either the holotoxin CT or the CTA subunit in the presence of PDI. We then compared the level of CTA1 that was bound to the ER membrane (pellet fraction) in each condition. More CTA1 chain was found at the ER membrane when CT was present as opposed to CTA alone (Figure 2.2F, compare lane 4 to lane 2). As we know that the PDI unfolded CTA1 chain is released from CTB (Tsai et al., 2001), we are confident the amount of CTA1 at the membrane is due to the capture of the A1 peptide by an ER membrane protein, rather than a result of CTB binding to Derlin-1 in the ER membrane. This data suggests that CTB may help to transfer the A1 peptide to the ER membrane, perhaps by initially binding Derlin-1 and thereby targeting the holotoxin to the retro-translocation machinery.

Derlin-1-YFP Binds to CT and Imparts a Conformational Change on Derlin-1

As this study suggests a role for Derlin-1 in the retro-translocation of CT, we became interested in examining how the Derlin-1-YFP dominant negative may exert its inhibitory effects demonstrated in the retro-translocation assay (Figure 2.1C). We reasoned that Derlin-1-YFP may interact with the toxin and thereby compete with endogenous Derlin-1 for substrate binding. To test this hypothesis, 293T cells expressing YFP, Derlin-1-YFP or Derlin-2-YFP were intoxicated with CT, lysed and subjected to immunoprecipitation with GFP antibody. Consistent with our observation that endogenous Derlin-1, but not Derlin-2, interacts with CTB (Figure 2.2), we found CTB associated with Derlin-1-YFP but not YFP or Derlin-2-YFP (Figure 2.3A, third panel, compare lane 2 to lanes 1 and 3). Furthermore, CTA was found in a complex with Derlin-1-YFP (Figure 2.3A, middle panel, lane 2). As we were unable to obtain an interaction between CTA and endogenous

Derlin-1 (Figure 2.2), this data suggests that Derlin-1-YFP may inhibit retro-translocation by trapping the substrate and preventing its modification to its CTA1 form. This CTA-Derlin-1-YFP interaction is probably mediated by CTB, as CTB alone was found to bind to Derlin-1-YFP (Figure 2.3B, lane 2). Therefore, it seems that CTB may target the holotoxin to Derlin-1-YFP, which is able to inhibit retro-translocation by titrating substrate away from endogenous Derlin-1.

In addition to the model presented above, another possible way to explain Derlin-1-YFP dominant negative activity is that it may disrupt the function of endogenous Derlin-1 by altering its conformation. To examine this idea, we utilized limited proteolysis to determine if Derlin-1-YFP was able to induce structural changes to endogenous Derlin-1 protein. In the absence of Derlin-1-YFP, endogenous Derlin-1 in 293T cell lysates was sensitive to proteinase K digestion at both a low (0.1mg/ml) and high (1mg/ml) concentration (Figure 2.3C, top panel, compare lanes 2 and 3 to lane 1). However, when Derlin-1-YFP was present in cell lysates, endogenous Derlin-1 became more resistant to digestion at the low concentration while maintaining its sensitivity at the high concentration (Figure 3C top panel, compare lane 5 to lanes 4 and 6). To ensure that the observed resistance of endogenous Derlin-1 to proteinase K digestion was due to the presence of a dominant negative Derlin-1-YFP and not simply due to the abundance of overexpressed protein, lysates from cells expressing the wild-type Derlin-1-HA protein were also assessed. Consistent with the inability of Derlin-1-HA to act as a dominant negative factor (data not shown), endogenous Derlin-1 was not resistant to low levels of proteinase K in the presence of Derlin-1-HA (Figure 2.3C, top panel, compare lane 8 to lane 7). Moreover, Derlin-1-YFP was not able to challenge the structural integrity of endogenous Derlin-2 protein (Figure 2.3D, compare lane 5 to lane 4). These findings confirm the specific ability of Derlin-1-YFP to induce conformational changes to Derlin-1, which is consistent with previous studies describing a preference of Derlin-1-GFP to bind Derlin-1 rather than Derlin-2 (Lilley and Ploegh, 2005). Collectively these data suggest that in addition to Derlin-1-YFP's ability to titrate holotoxin away from endogenous protein, it may also exert dominant negative behavior by inducing structural

changes to endogenous Derlin-1 that render it incapable of fulfilling its role in retro-translocation.

Intoxication by CTB Stabilizes a Derlin-1-dependent Substrate

As our data suggest that CTB may target the holotoxin to the retro-translocation machinery containing Derlin-1, we reasoned that CTB may be able to compete with a Derlin-1-dependent substrate, thereby inhibiting the substrate's degradation. To test this idea, we examined the degradation of the cystic fibrosis transmembrane conductance regulator (CFTR), which has been identified as a Derlin-1 substrate (Sun et al., 2006; Younger et al., 2006). Cells expressing CFTR were intoxicated with a high concentration of CTB (100nM) for 3 hours, labeled with [^{35}S] methionine for 30 minutes and chased for up to 2 hours. Cell lysates were then subjected to immunoprecipitation with CFTR antibody and the samples analyzed by SDS-PAGE followed by autoradiography. We found that CTB stabilized the immature (nonglycosylated) B form of CFTR and was also able to increase the level of the mature C form (Figure 2.4A, compare lanes 5 and 6 to lanes 2 and 3; quantified in graph below). Of note, CTB did increase the total amount of CFTR (both B and C forms) at time = 0 by approximately 10%. It is possible that CTB was able to stabilize CFTR during the 30 minute labeling of cells.

Semi-quantitative RT-PCR confirmed that the increased level of CFTR found in cells was due to the ability of CTB to stabilize the protein and not due to a transcriptional event, as CTB did not affect the level of CFTR mRNA (Figure 2.4B, compare lane 2 to lane 1). CTB treatment also did not increase the expression of ER-resident proteins and chaperones PDI, ERp72 or Derlin-1 (Figure 2.4C, compare lane 2 to lane 1). Additionally, CTB had no affect on the degradation of the Derlin-2-dependent substrate, the null Hong Kong (NHK) mutant of α1-antitrypsin (Oda et al., 2006; Figure 2.4D, compare lane 2 to lane 1). Together, these results support the finding that CTB is able to attenuate the degradation of CFTR via the Derlin-1 pathway and further support the hypothesis that Derlin-1 interacts with the holotoxin via CTB.

Interaction of PDI with the Derlin Family Proteins

PDI was previously found to prepare the CTA1 peptide for retro-translocation via an unfolding reaction (Tsai et al., 2001; Forster et al., 2006). It is currently unknown whether PDI unfolds CTA1 and then brings it to the ER membrane or if the toxin is first targeted to the retro-translocation machinery at the ER membrane prior to its modification by PDI. Currently our data support the latter of the two possibilities, as CT seems to be targeted to Derlin-1 by CTB prior to the reduction and unfolding of the catalytic subunit. In this model, we would expect to find an interaction between Derlin-1 and PDI. Indeed, when cell lysates obtained from the gentle solubilization of 293T cells were subjected to immunoprecipitation, a portion of PDI was found associated with Derlin-1 and Derlin-2 (Figure 2.5A, top panel, lanes 1 and 2). Notably, heterodimerization of Derlin-1 and Derlin-2 was demonstrated under this condition, as each of the two proteins was found in the immunoprecipitate of the other (Figure 2.5A, second and third panels, lanes 1 and 2). This heterodimerization of Derlin-1 and Derlin-2 has been previously reported (Lilley and Ploegh, 2005). The Derlin-1 and Derlin-2 interactions with PDI was also found in HeLa cells (Figure 2.5B, lanes 1 and 2), suggesting that this complex exists in multiple cell lines. Similar to its endogenous counterpart, Derlin-1-YFP was also able to bind PDI (Figure 2.5C, lane 2). We next asked whether the PDI-Derlin-1 interaction is due to Derlin-1 acting as a substrate of PDI. A mutation at residue 272 from isoleucine to tryptophan (I272) of human PDI inhibits substrate binding (Pirneskoski et al., 2004). We generated a FLAG-tagged mouse PDI with the equivalent mutation (I272W) and expressed either the mutant PDI or wild-type PDI in 293T cells. We found that wild-type and mutant PDI equally bound Derlin-1 (Figure 2.5D, compare lane 1 and lane 2), therefore making it unlikely that Derlin-1 is a substrate of the ER oxido-reductase PDI. We therefore conclude that PDI and Derlin-1 interact to form a functional retro-translocation complex whereby after the toxin reaches Derlin-1, PDI is able to efficiently unfold and dissociate the A1 chain from CTB.

Discussion

Elucidating the mechanism by which a misfolded substrate is targeted to the ER membrane for retro-translocation and the characterization of the membrane components

involved in this process are important discoveries that will enhance our understanding of the ERAD pathway. While recent advances in the field have highlighted the complexity of the retro-translocation machinery (Carvalho et al., 2006; Denic et al., 2006; Gauss et al., 2006), much remains to be discovered.

This study sought to examine the role of the Derlin proteins, members of the retro-translocation machinery at the ER membrane, in the ER-to-cytosol transport of CT. We found that expression of the dominant negative Derlin-1-YFP protein attenuated the retro-translocation of CTA1, likely through its ability to titrate toxin away from endogenous Derlin-1 and/or because Derlin-1-YFP induced conformational changes on endogenous Derlin-1 that affected its function. Co-immunoprecipitation studies identified two novel interactions important for CT retro-translocation. First, Derlin-1 associates with CTB in the presence or absence of CTA. Whether this interaction is mediated by the ganglioside receptor GM1 remains to be examined. Previous literature detailed a role for CTB in carrying the holotoxin from the plasma membrane to the ER (Fujinaga et al., 2003), but any additional role of CTB in CT retro-translocation was not known. In this study, we provide evidence supporting a role for CTB in targeting the toxin to the retro-translocation machinery. An in vitro membrane pelleting assay demonstrated that CTB stimulates the binding of CTA1 to the ER membrane. Furthermore, treatment of CTB stabilized the Derlin-1-dependent substrate CFTR. The hypothesis that targeting of the holotoxin to Derlin-1 via CTB is an early event in the ER trafficking of CT is supported by the finding that CTA, not CTA1 associates with Derlin-1-YFP. This suggests that binding to Derlin-1 at the ER membrane precedes reduction and unfolding of the CTA peptide to CTA1, modifications that are inhibited by the Derlin-1-YFP dominant negative protein. The targeting of CTA1 to Derlin-1 by CTB mirrors the mechanism by which the US11 viral protein encoded by human cytomegalovirus, targets MHC class I molecules to Derlin-1 for retro-translocation (Ye et al., 2004; Lilley and Ploegh, 2004).

The second novel interaction conveyed in the study is the association of PDI with Derlin-1 and Derlin-2. As PDI is a soluble ER protein, the finding that only a small amount

associated with the Derlins at the ER membrane is not surprising. In the context of our substrate of interest, the interaction between PDI and Derlin-1 provides crucial mechanistic detail as to how the retro-translocation of CT occurs. PDI was previously found to unfold CTA1, which is a necessary step for its retro-translocation (Tsai et al., 2002; Forster et al., 2006). As it was demonstrated that upon release from PDI, the toxin is able to spontaneously refold (Rodighiero et al., 2002), it was hypothesized that the unfolding of CTA1 would occur close to the retro-translocon to ensure efficient and successful transfer of the toxin to the cytosol (Tsai et al., 2002). The finding that PDI associates with Derlin-1 supports this hypothesis and helps to complete the model depicted in Figure 2.5E: upon delivery of the holotoxin to Derlin-1 via CTB (step 1), CTA is reduced to CTA1 (by an unidentified reductase) and subsequently unfolded by the Derlin-1 bound PDI (step 2), thereby providing an organized mechanism for transfer of the toxin to the cytosol by coupling unfolding and retro-translocation events. As Derlin-1-YFP retained the ability to bind PDI as its endogenous counterpart does, it is unlikely that its ability to behave as a dominant negative is due to a changed interaction with PDI. However, it is possible that Derlin-1-YFP fails to interact with other unidentified proteins important for transport of the toxin which may contribute to its dominant negative behavior.

It is important to note that while Derlin-1 is likely a key component of the retro-translocation channel, the Sec61 translocon has previously been implicated in the retro-translocation of CT (Schmitz et al., 2000). Our current study neither supports nor excludes a role for Sec61 in this process. It is possible that after its interaction with Derlin-1, the toxin is transferred to the Sec61 channel. Future studies should address this possibility.

The mechanistic detail provided in this study regarding the early stages of CT retro-translocation will likely enhance our understanding of how misfolded ER substrates also engage the ERAD membrane machinery. Analogous to CT, ERAD substrates may be targeted to the Derlins, modified by membrane associated chaperones, such as PDI, Bip and Yos9 (Carvalho et al., 2006; Denic et al., 2006; Gauss et al., 2006) and subsequently

retro-translocated into the cytosol. The localization of chaperones to the ER membrane is an effective way to provide efficient transport after substrate modification. Interestingly, in addition to CT, PDI and Derlin-1 are responsible for the degradation of prepro-α-factor (Gillece et al., 1999; Wahlman et al., 2007). However, the ER-to-cytosol transport of murine polyomavirus does not require Derlin-1, but is dependent on PDI and Derlin-2 (Gilbert et al., 2006; Lilley et al., 2006). Furthermore, not all toxins that utilize PDI for retro-translocation also use Derlin-1, as seen by the inability of the dominant negative Derlin-1 to inhibit the retro-translocation of the plant toxin ricin (Spooner et al., 2004; Slominska-Wojewodzka et al., 2006). These findings raise important questions regarding the determinants of substrate specificity. Whether the Derlins are also necessary for the retro-translocation of other ERAD substrates shown to require PDI, such as the human β-secretase variant BACE457 (Molinari et al., 2002), remains to be determined.

Experimental Methods

Materials

Polyclonal antibodies against PDI and Hsp90 were purchased from Santa Cruz, the polyclonal and monoclonal GFP antibodies and the monoclonal PDI antibody from Abcam, the polyclonal antibody against α1–antitrypsin and the mouse antibody against FLAG from Sigma, the monoclonal CFTR (M3A7) antibody from Upstate, and the polyclonal calnexin antibody from Stressgen. The CTA and CTB antibodies were provided by the W. Lencer (Harvard). The polyclonal and monoclonal ERp29 antibodies were generous gifts from S. Mkrtchian (Karolinska Intitutet). The polyclonal Derlin-1 and Sec61 beta antibodies were gifts from T. Rapoport (Harvard). The polyclonal Derlin-2 antibody and the HA-tagged Derlin-1 construct were gifts from Y. Ye (NIH). Purified CT and CTB were purchased from Calbiochem/EMB Biosciences. The pcDNA3.1 containing YFP construct and the monoclonal antibody against HA were gifts from K. Verhey (University of Michigan). The CFTR construct was a gift from R. Frizzell (University of Pittsburgh). The pcDNA3.1 construct containing the FLAG-tag was a gift from E. Wiertz (Leiden University Medical Center). A HeLa cell line stably expressing

the NHK mutant of α1-proteinase inhibitor was a gift from C. Wojcik (Indiana University).

Construction of the Derlin-1-YFP, Derlin-2-YFP, wild-type mouse PDI FLAG-tagged, and the I272W mouse PDI FLAG-tagged constructs

The Derlin-1-YFP construct was generated by PCR amplification of the Derlin-1 coding sequence using a HA-tagged Derlin-1 construct as the template (Ye et al., 2004), while the Derlin-2-YFP construct was generated by PCR amplification of the Derlin-2 coding sequence using a HeLa cell cDNA library as the template. The respective PCR amplified fragments were subsequently ligated into a pcDNA3.1 vector containing YFP, with the YFP attached to the C-terminus of the protein. Wild-type mouse PDI-FLAG was generated by PCR amplification of the PDI coding sequence using a plasmid containing mouse PDI as the template. The PCR product was ligated into pcDNA3.1 containing the FLAG tag. Mutagenesis to obtain the I272W PDI-FLAG construct was conducted using the Stratagene QuikChange II Site-directed Mutagenesis Kit (LaJolla, CA) and the wild-type PDI-FLAG plasmid as the template. The mutant PDI construct was confirmed by sequencing.

Retro-translocation assay

293T cells were incubated with CT (10 nM) in Hanks' balanced salt solution (HBSS) at 37°C for 45 min or 90 min. For permeabilization, 2 x 10^6 cells were resuspended in 100 µl of 0.02% digitonin in HCN buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 2 mM $CaCl_2$, and 10 mM N-ethyl maleimide (NEM), and protease inhibitors), incubated on ice for 10 min and centrifuged at 16,000 g for 10 min. The supernatant was removed and the pellet washed with PBS and resuspended in 100 µl of the original buffer. Fractions were analyzed by non-reducing SDS-PAGE and immunoblot analysis.

cAMP assay

A cAMP enzyme immunoassay system (GE Healthcare) was used to quantify cAMP synthesis induced by 10 nM CT or 50 µM forskolin in HBSS. Samples were assayed in

triplicate, and the mean optical density obtained to calculate the cAMP level/well. The cAMP response was determined by dividing the cAMP level in CT- or forskolin -treated cells by the cAMP level in untreated cells. Reported results are a percentage of the wild-type CT-induced cAMP response normalized to the forskolin-induced cAMP response. Adapted from Forster et al., 2006.

Cell transfection

YFP, Derlin-1-YFP, Derlin-2-YFP, CFTR, mouse wild-type PDI FLAG-tagged, or mouse I274W PDI FLAG-tagged was transfected into 30% confluent 293T cells in a 10 cm dish using the Effectene system (Qiagen), and the cells grown for an additional 48 hours prior to experimentation.

Immunoprecipitation

293T cells were incubated with or without CT (10 nM or 30 nM) or CTB (10 nM) for 90 min. Cells were harvested, lysed in the buffer containing KOAc (150 mM), Tris pH7.5 (30 mM), $MgCl_2$ (4 mM), NEM (10 mM) with either 1% Triton X-100 or 1% deoxyBigChap, centrifuged at 16,000 g for 10 min, and the supernatant used for immunoprecipitation experiments. Co-immunoprecipitation experiments between PDI-FLAG and Derlin-1 were performed using a lysis buffer containing 1% Tween 20. Where indicated, polyclonal Derlin-1, polyclonal Derlin-2, monoclonal ERp29, polyclonal Sec61 beta, or polyclonal calnexin 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 non-reducing SDS-PAGE followed by immunoblotting with the appropriate antibody.

Toxin membrane pelleting assay

CTA subunit (160 nM) or CT (160 nM) was incubated with proteoliposomes, PDI (2.3 μM), GSH (3 mM) for 60 min at 37°C. Samples were sedimented for 20 min at 40,000 rpm in a tabletop ultracentrifuge using a TLA 100.4 rotor. The supernatant and pellet fractions were analyzed by SDS-PAGE followed by immunoblotting.

Proteinase K digestion

293T cells non-transfected or over-expressing Derlin-1-YFP or Derlin-1-HA were incubated with 0.04% digitonin for 5 min at 4°C, centrifuged at 14,000 rpm for 10 min, and the pellet resuspended in a buffer containing HEPES (50 mM, pH 7.4), KOAc (150 mM), sucrose (250 mM), $MgCl_2$ (4 mM). The samples were incubated with 0.1 mg/ml or 1 mg/ml Proteinase K for 30 min at 4°C, subjected to SDS-PAGE, and immunoblotted with an antibody against Derlin-1, Derlin-2, GFP, or HA.

Pulse-chase analysis of CFTR

Cells were pretreated with CTB (100 nM) for 3 hrs prior to the experiment. HEK293T cells were starved in methionine- and cysteine-free Dulbecco's modified Eagle's medium for 30 min, and then metabolically labeled with ^{35}S L-Methionine (100 μCi/ml; Fisher Scientific) for 30 min at 37°C. Cells were washed twice with phosphate-buffered saline and lysed immediately or incubated in complete Dulbecco's modified Eagle's medium for the indicated chase periods. Lysates were subjected to immunoprecipitation with CFTR antibody (M3A7). Immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Adapted from Zhang et al., 2002.

RNA isolation and mRNA analysis by semi-quantitative RT-PCR

RNA extractions were carried out with the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. $1x10^6$ cells were used for the experiment. Samples were applied to the QIA shredder homogenizers (Qiagen). RNA was subjected to the RNase-Free DNase Set (Qiagen), and reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol. Two μl of cDNA products were amplified using the Expand High Fidelity PCR system (Roche) in the $MgCl_2$-free buffer in the presence of specific primers for CFTR or GAPDH used at a concentration of 0.1 μM each. $MgCl_2$ was added to the reaction at a concentration of 1mM. Reactions were carried out in the Eppendorf Mastercycler Personal. A first cycle of 10 minutes at 95°C, 45 seconds at 65°C and 1 minute at 72°C was followed by 45 seconds at 95°C, 45 seconds at 65°C and 1 minute at 72°C for 30 cycles. Each set of reactions contained an RNA negative control to rule out genomic DNA contamination. The following primers

were used: CFTR 5’-CAGCTGGAGAGGAGGAAGGGAG-3’ and 5’ GAGGGTCTGCA GGCAGGCAGTG-3’; GAPDH 5’-ACCACAGTCCATGCCATCACTGCC-3’ and 5’-TC CACCACCCTGTTGCTGTAGCC-3’. CFTR yielded an amplification product of 765 bp and GAPDH of 453 bp, which were resolved by an agarose gel.

Figure 2.1. Derlin-1 Facilitates the Retro-translocation of CT. A. 293T cells were incubated with CT (10 nM) for 90 min at 37°C with or without BFA. Cells were permeabilized, centrifuged, the supernatant and pellet fractions separated, subjected to non-reducing SDS-PAGE, and immunoblotted with the indicated antibodies. CTA, CTA1, and CTB are 28 kDa, 22 kDa, and 11 kDa respectively. B. 293T cells expressing YFP, Derlin-1-YFP, or Derlin-2-YFP were harvested, lysed, and the lysates subjected to SDS-PAGE and immunoblotted with a GFP antibody. C. 293T cells expressing either YFP, Derlin-1-YFP, or Derlin-2-YFP were incubated with CT (10 nM) for 45 min or 90 min, the cells permeabilized and centrifuged, and the supernatant fraction analyzed as in A. The intensity of the CTA1 band generated from cells treated with CT for 90 min was quantified with ImageJ. Mean +/- SD (error bars) of two to five independent experiments are shown. D. 293T cells expressing YFP or Derlin-1-YFP were incubated with CT for 90 min and the cAMP level was measured by a cAMP Biotrak Enzyme Immunoassay System (GE Healthcare). Data were normalized against the forskolin-induced cAMP level, as demonstrated previously (Forster et al., 2006).

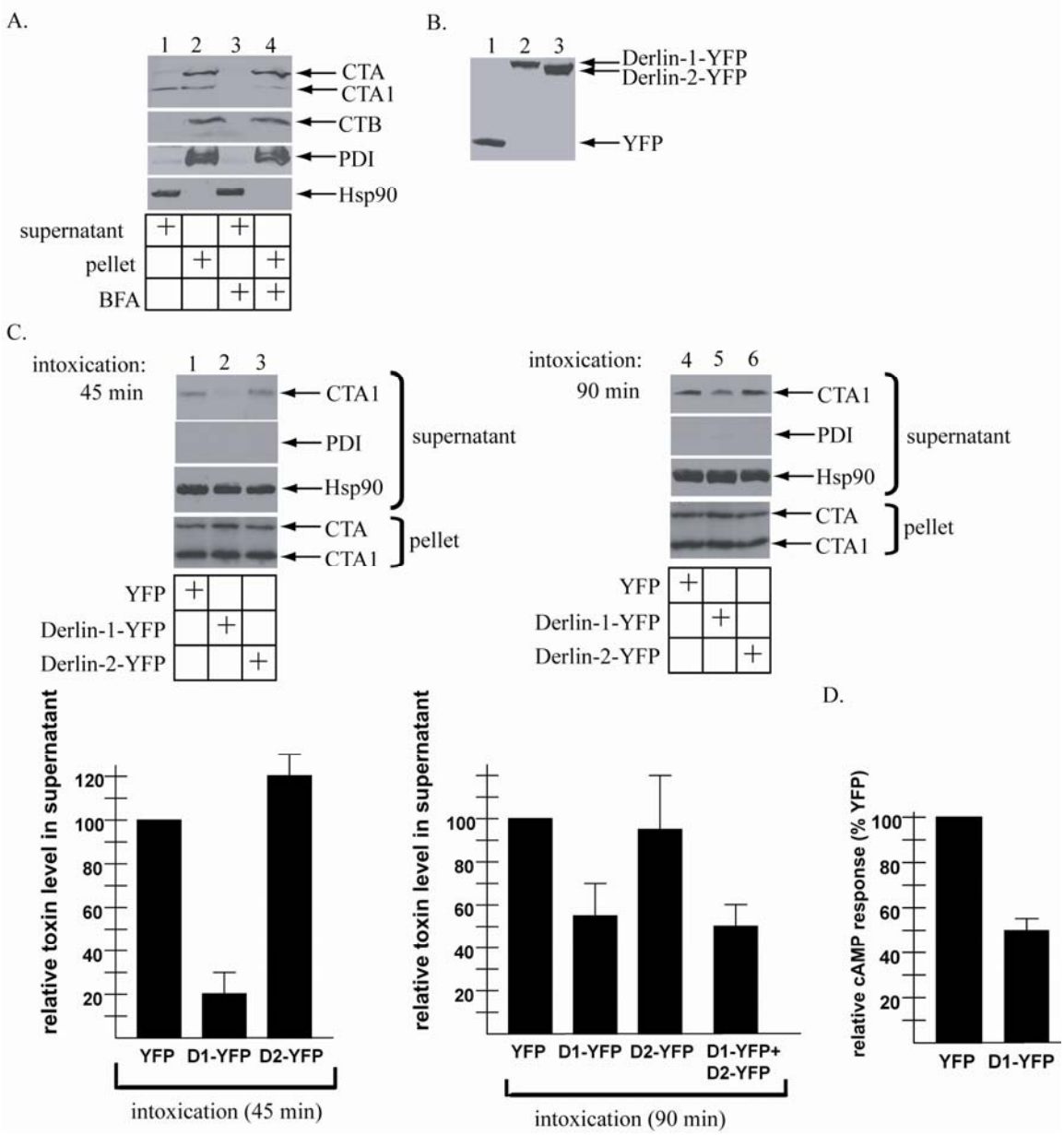
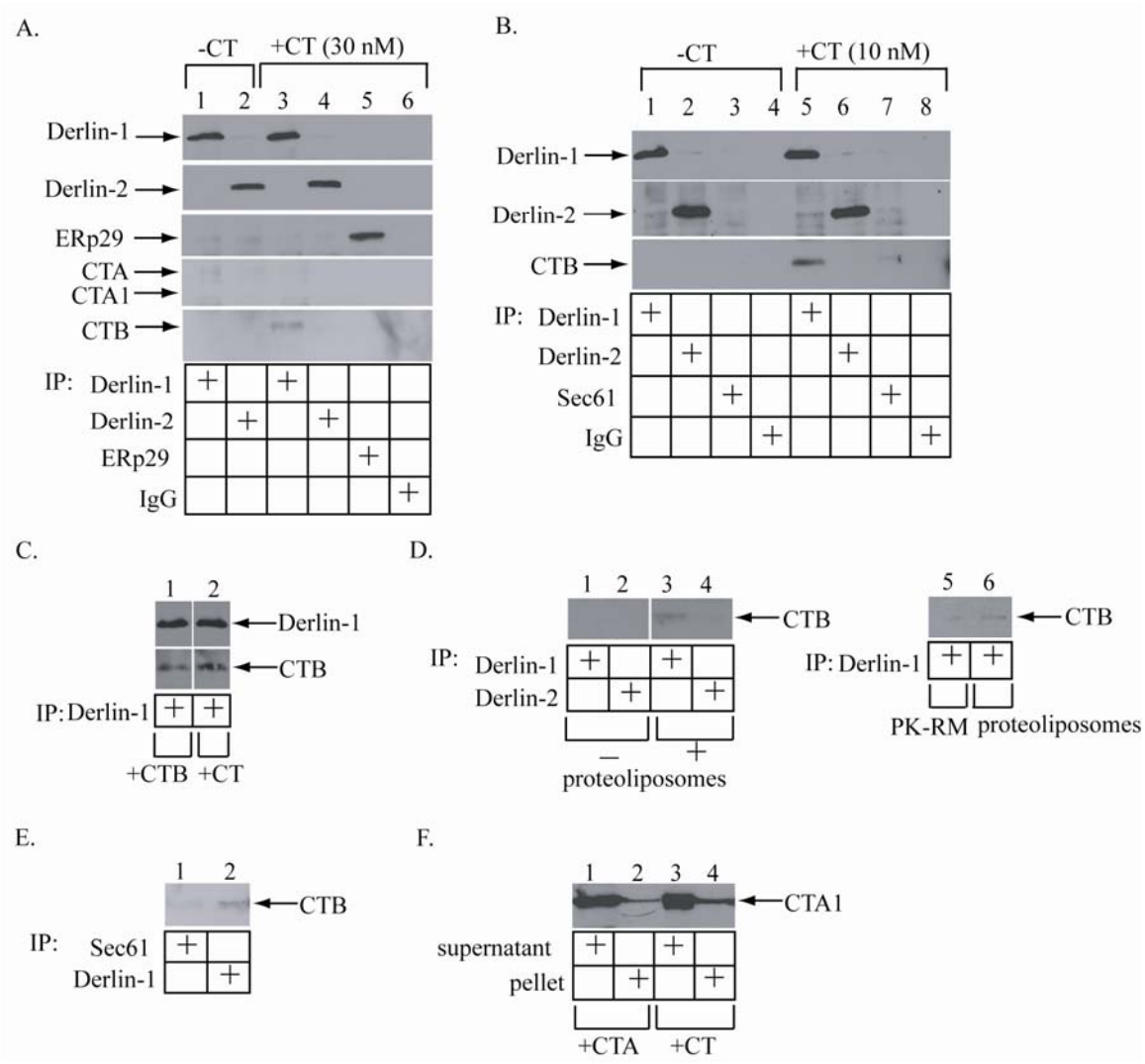

A.
1 2 3 4
CTA
CTA1
CTB
PDI
Hsp90
supernatant
pellet
BFA
B.
1 2 3
Derlin-1-YFP
Derlin-2-YFP
YFP
C.
intoxication:
45 min
1 2 3
CTA1
PDI
Hsp90
supernatant
CTA
CTA1
pellet
YFP
Derlin-1-YFP
Derlin-2-YFP
intoxication:
90 min
4 5 6
CTA1
PDI
Hsp90
supernatant
CTA
CTA1
pellet
YFP
Derlin-1-YFP
Derlin-2-YFP
relative toxin level in supernatant
120
100
80
60
40
20
YFP D1-YFP D2-YFP
intoxication (45 min)
relative toxin level in supernatant
100
80
60
40
20
YFP D1-YFP D2-YFP D1-YFP+ D2-YFP
intoxication (90 min)
D.
relative cAMP response (% YFP)
100
80
60
40
20
YFP D1-YFP

Figure 2.2. CTB Interacts with Derlin-1 and Enhances the Transfer of CTA1 to the ER Membrane. A. 293T cells were incubated with or without CT (30 nM) for 90 min, the cells harvested and lysed in a buffer containing 1% Triton X-100. The lysates were subjected to immunoprecipitation using the indicated antibodies. The precipitated samples were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. B. As in A, except cells were incubated with CT (10 nM), and where indicated, the Sec61 antibody was used instead of the ERp29 antibody. C. As in A, except cells were incubated with either CT (10nM) or CTB (10 nM). D.CT (200 nM) and PDI (2 μg/ml) were incubated with or without proteoliposomes or with PK-RM, and the samples solubilized and precipitated using the indicated antibodies. The samples were analyzed as in A. E. As in D, except the samples were precipitated using Sec61 or Derlin-1 antibodies. F. CTA or CT was incubated with proteoliposomes, PDI, and GSH. Samples were sedimented and the supernatant and pellet fractions analyzed in SDS-PAGE followed by immunoblotting with an antibody against CTA.


A.
-CT
+CT (30 nM)
1 2 3 4 5 6
Derlin-1
Derlin-2
ERp29
CTA
CTA1
CTB
IP: Derlin-1
Derlin-2
ERp29
IgG
B.
-CT
+CT (10 nM)
1 2 3 4 5 6 7 8
Derlin-1
Derlin-2
CTB
IP: Derlin-1
Derlin-2
Sec61
IgG
C.
1 2
Derlin-1
CTB
IP: Derlin-1
+CTB +CT
D.
1 2 3 4
CTB
IP:
Derlin-1
Derlin-2
proteoliposomes
5 6
CTB
IP: Derlin-1
PK-RM proteoliposomes
E.
1 2
CTB
IP:
Sec61
Derlin-1
F.
1 2 3 4
CTA1
supernatant
pellet
+CTA +CT

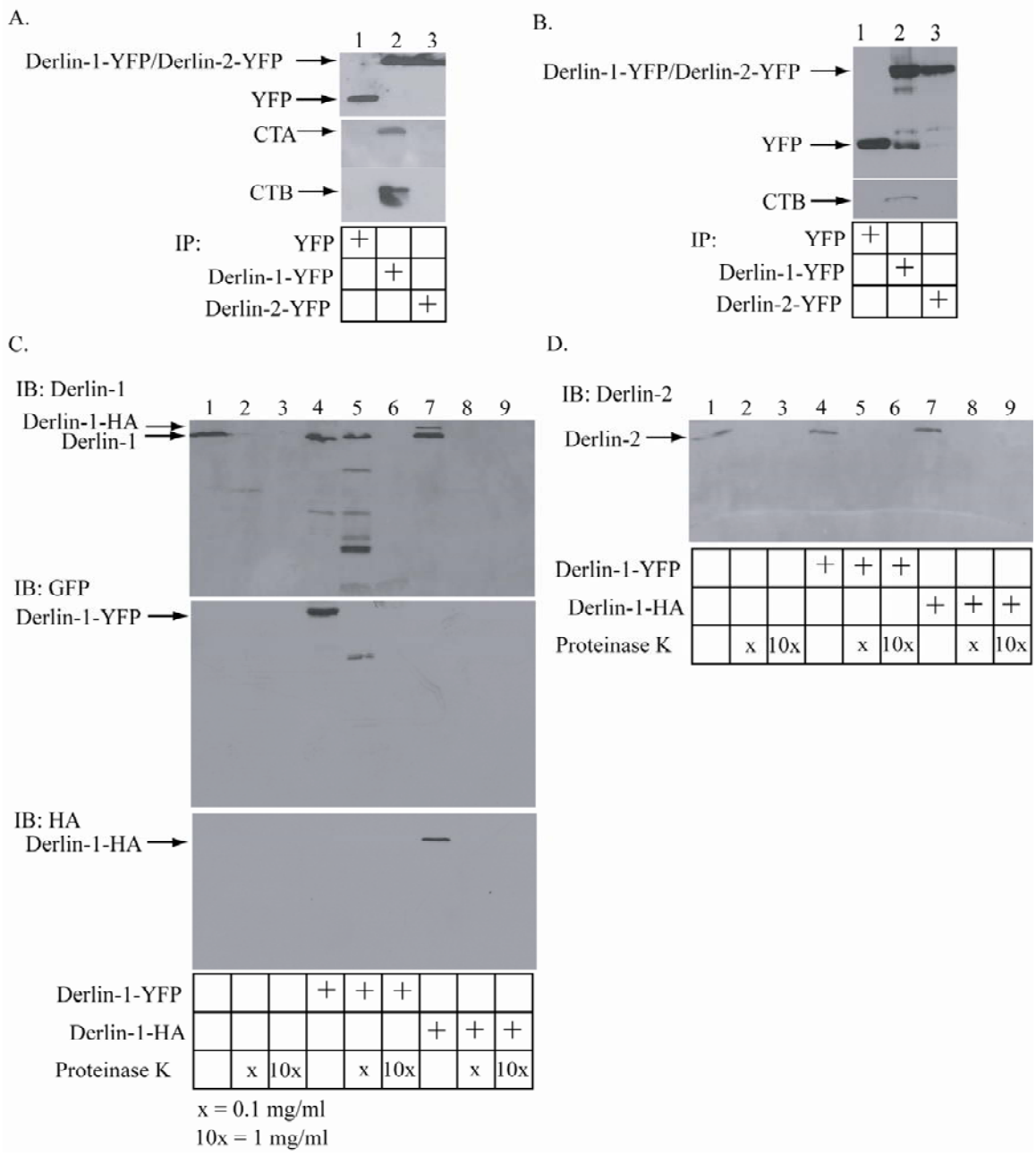


Figure 2.3. Derlin-1-YFP Binds to CT and Imparts a Structural Change on Derlin-1. A. 293T cells expressing YFP, Derlin-1-YFP, or Derlin-2-YFP were intoxicated with CT (10 nM), the cells lysed in a buffer containing 1% Triton X-100, and the lysate subjected to immunoprecipitation as in Figure 2A. B. As in A, except cells were intoxicated with CTB (10 nM). C. Digitonin-treated 293T cells or cells over-expressing Derlin-1-YFP or Derlin-1-HA were incubated with 0.1 mg/ml or 1 mg/ml Proteinase K, subjected to SDS-PAGE, and immunoblotted with an antibody against Derlin-1, GFP, or HA. D. As in C, except samples were immunoblotted with a Derlin-2 antibody.

Figure 2.4. CTB Stabilizes the Derlin-1-dependent Retro-translocation Substrate CFTR. A. 293T cells transiently expressing CFTR were incubated with CTB (100 nM), labeled with ^{35}S-methionine, harvested at the indicated chase times, and the resulting cell lysate used for CFTR immunoprecipitation. Signals were detected by autoradiography. Bottom panel: quantification of the intensity of Bands B and C from three independent experiments; values expressed as a percentage of the total intensity of Band B and Band C at chase time = 0. Bars represent standard deviation. Band B refers to the immature core-glycosylated form of CFTR, and Band C the mature complex-glycosylated form. B. RNA isolated from cells incubated with or without CTB (100 nM) was subjected to semi-quantitative RT-PCR analysis. Shown is the result of 30 cycles of amplifications. A similar result was obtained with 27 cycles of amplifications. C. The same lysates in B were analyzed for the presence of PDI, ERp72, and Derlin-1. D. HeLa cells stably expressing NHK were incubated with CTB (100 nM), and the lysate subjected to SDS-PAGE and immunoblotted with an antibody against NHK.

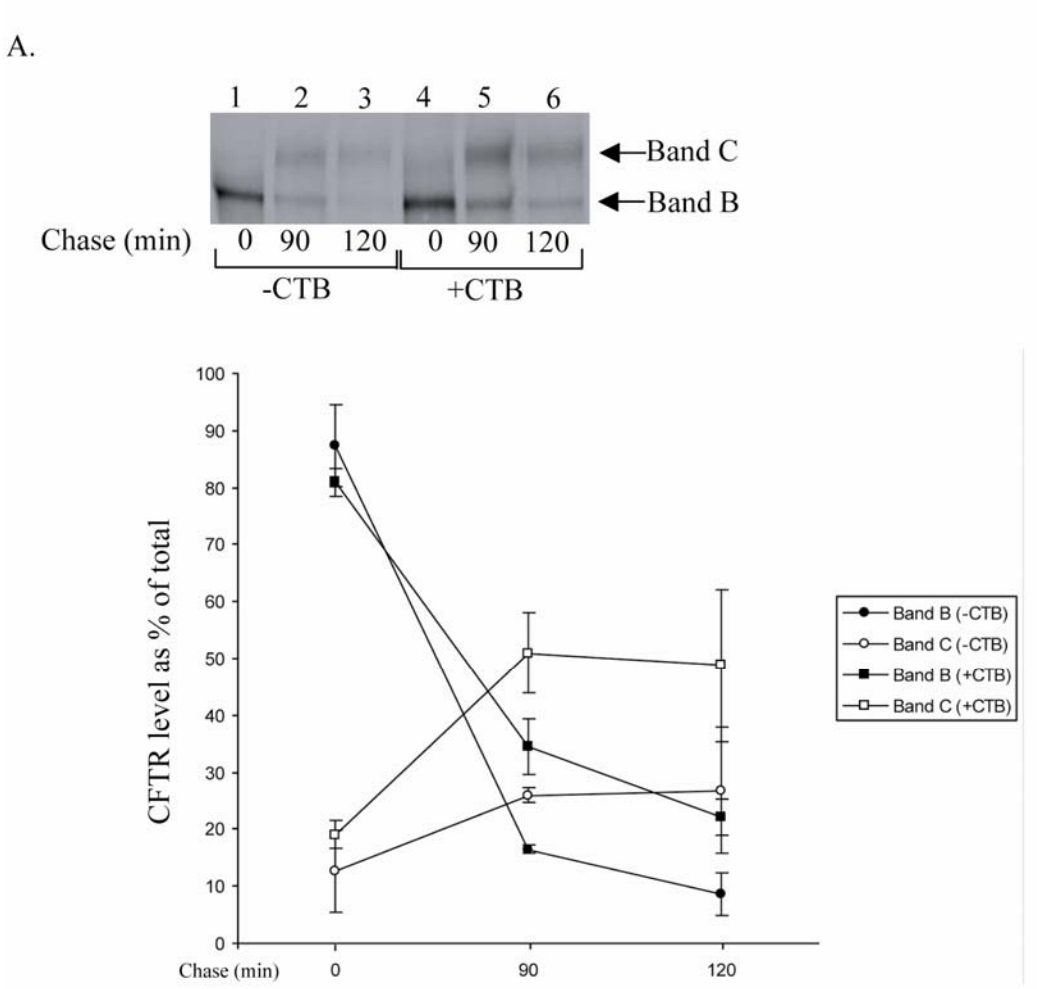
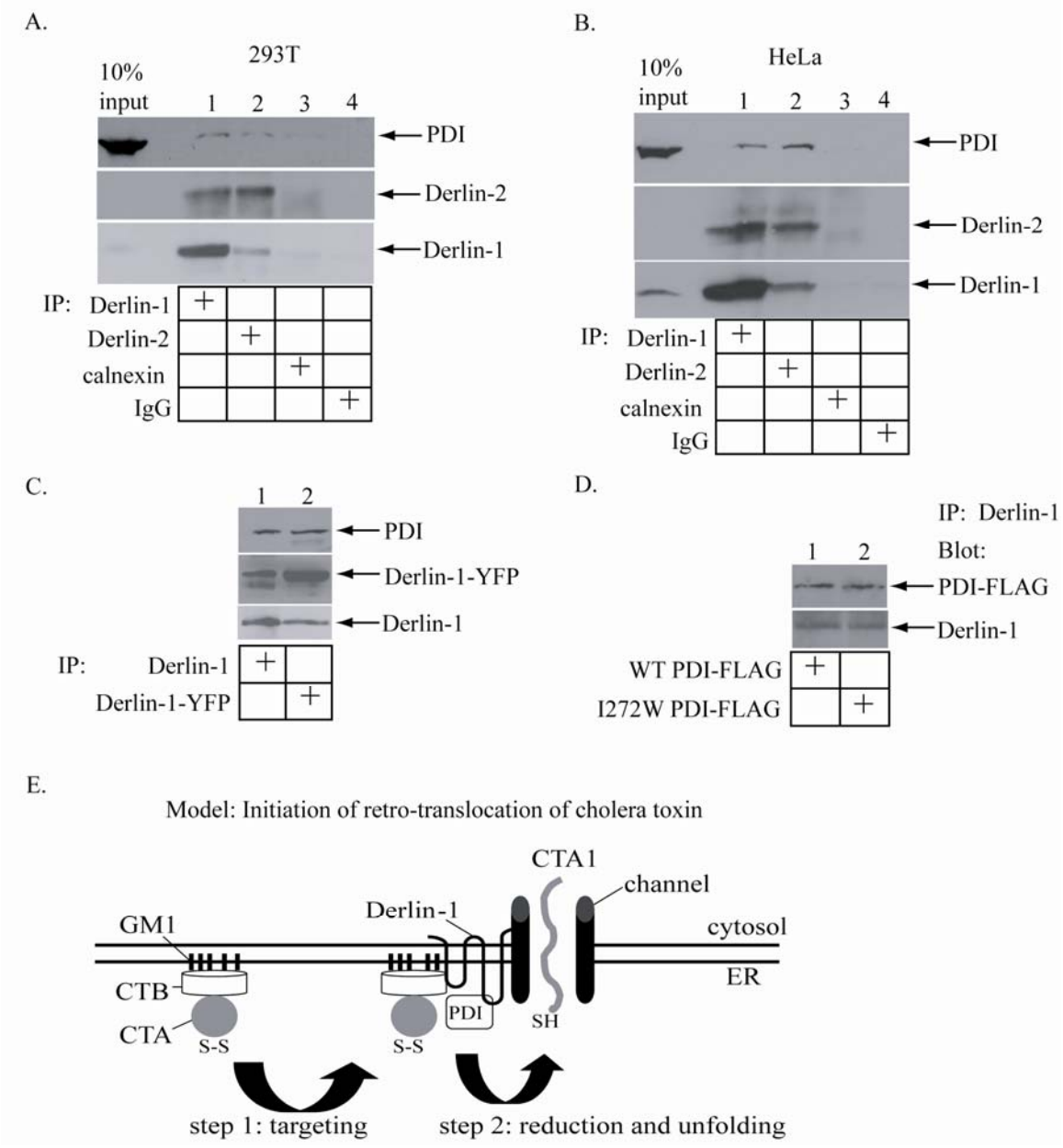

A.
1 2 3 4 5 6
Band C
Band B
Chase (min)
0 90 120 0 90 120
-CTB
+CTB
CFTR level as % of total
Chase (min)
0
90
120
Band B (-CTB)
Band C (-CTB)
Band B (+CTB)
Band C (+CTB)
B.
1 2
CFTR
GAPDH
CTB
C.
1 2
PDI
ERp72
Derlin-1
CTB
D.
Derlin-2-dependent substrate
1 2
NHK
Derlin-1
CTB

Figure 2.5. Association of PDI with Derlins. A. 293T cells were lysed in a buffer containing 1% deoxyBigChap, and the lysates subjected to immunoprecipitation as in Figure 2A. B. As in A, except HeLa cells were used. C. As in A, except 293T cells expressing Derlin-1-YFP were used. D. As in A, except 293T cells expressing either wild-type mouse PDI-FLAG or the substrate-binding I272W mutant PDI-FLAG were lysed in a buffer containing 1% Tween 20. E. Model of the initiation of retro-translocation of cholera toxin. See text for discussion.


A.
293T
10%
input
1
2
3
4
PDI
Derlin-2
Derlin-1
IP:
Derlin-1
Derlin-2
calnexin
IgG
B.
HeLa
10%
input
1
2
3
4
PDI
Derlin-2
Derlin-1
IP:
Derlin-1
Derlin-2
calnexin
IgG
C.
1
2
PDI
Derlin-1-YFP
Derlin-1
IP:
Derlin-1
Derlin-1-YFP
D.
IP: Derlin-1
Blot:
1
2
PDI-FLAG
Derlin-1
WT PDI-FLAG
I272W PDI-FLAG
E.
Model: Initiation of retro-translocation of cholera toxin
CTA1
channel
Derlin-1
GM1
cytosol
ER
CTB
CTA
S-S
PDI
SH
S-S
step 1: targeting
step 2: reduction and unfolding

Chapter 3

The E3 ubiquitin ligases Hrd1 and gp78 Bind to and Promote Cholera Toxin Retro-translocation

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 two necessary components for successful intoxication and co-opting of host cell pathways: a homopentameric B subunit (CTB) and a single A subunit (CTA) (Spangler, 1992). CTB binds the ganglioside receptor GM1 on the surface of host cells and the holotoxin is endocytosed and transported into the endoplasmic reticulum (ER) where CTB targets the holotoxin to the quality control pathway, ER-associated degradation (ERAD; Fujinaga et al., 2003; Bernardi et al., 2008). The ability of CTA to be recognized as a misfolded substrate of ERAD is essential for its entry into the cytosol. While CTB remains in the ER, CTA is reduced to generate the CTA1 peptide, unfolded and transferred into the cytosol where its catalytic activity leads to the inevitable opening of a chloride channel (Lencer and Tsai, 2003). The release of chloride ions and water from the cell ultimately results in diarrhea that characterizes this disease.

The ER events that allow for the correct processing of the toxin and subsequent translocation of its catalytic subunit into the cytosol are poorly understood. Elucidating how the toxin is able to utilize the ERAD pathway, yet avoid the proteosomal degradation that occurs for other ERAD substrates is essential to characterizing both toxin translocation and the basic ERAD mechanism. We previously found a role for ER lumenal oxido-reductase, protein disulfide isomerase (PDI) in the binding and unfolding of the reduced CTA1 subunit, a necessary step for the toxin to gain entry into the cytosol

(Tsai et al., 2001; Forster et al., 2006). We then discovered that Derlin-1, an ER transmembrane protein and component of the retro-translocon (Ye et al., 2004; Lilley and Ploegh, 2004) exists in a complex with PDI and facilitates the retro-translocation of CTA1 (Chapter 2; Bernardi, et al., 2008). Derlin-1's role in CTA1 retro-translocation is supported by an additional study (Dixit et al., 2008). These findings help link events occurring in the ER lumen directly to those at the ER membrane and provide a model whereby CTB targets the holotoxin to Derlin-1, allowing the Derlin-1 bound PDI to prepare the toxin for transport into the cytosol via an unfolding reaction. However, how the toxin reaches the cytosol after it associates with the PDI-Derlin-1 complex remains to be discovered.

Misfolded proteins targeted for retro-translocation are normally ubiquitinated at the ER membrane by the ubiquitination machinery associated with the retro-translocation channel (Tsai et al., 2002; Vembar and Brodsky, 2008; Hirsch et al., 2009). The ubiquitin modified substrate is then targeted to the proteasome for degradation. For CT to induce its toxicity in the cytosol, it must evade degradation by the proteasome. It is believed the ability of CTA1 to avoid degradation is due the low amount of lysine residues (the primary sites of ubiquitin modification) on the CTA1 chain (Hazes and Read, 1998). This hypothesis is supported by two observations. First, when the two lysine residues of CTA1 were mutated and its N-terminus (an additional site for ubiquitination) was chemically blocked, the toxin still retained activity similar to wild-type toxin (Rodighiero et al., 2002). Second, chemical inhibition of the proteasome also did not affect CTA1 toxicity (Rodighiero, et al., 2002). Thus, it remains a mystery whether the ubiquitination machinery associated with components of the retro-translocon is necessary for toxin retro-translocation. In addition to Derlin-1, the E3 ubiquitin ligases Hrd1 and gp78 are key components of the retro-translocon (Ye et al., 2005; Lilley and Ploegh, 2005; Schulze et al., 2005). As Derlin-1 has been implicated in the retro-translocation of CTA1 (Bernardi et al., 2008; Dixit et al., 2008), we sought to examine whether Hrd1 and gp78 play a role in this process.

Hrd1 and gp78 are responsible for the ubiquitination of a subset of misfolded substrates prior to their extraction from the ER membrane, including the commonly studied T cell receptor subunit TCRα and CD3δ (Fang et al., 2001; Kikkert et al., 2004; Chen et al., 2006). As orthologs 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 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, membrane and cytosol, and therefore are likely important factors that connect events across the ER membrane (Ye et al., 2005; Lilley and Ploegh, 2005; Hirsch et al., 2009).

In this study, we found that Hrd1 and gp78 are involved in the retro-translocation of CTA1. Expression of mutant versions of Hrd1, gp78 and an E2 conjugating enzyme dedicated to ERAD, Ube2g2, inhibits the retro-translocation of CTA1. Co-immunoprecipitation studies demonstrate that Hrd1 and gp78 interact with CT and PDI. Additionally, the binding studies describe a sequential transfer event of the toxin from Derlin-1 to Hrd1 prior to its exit from the ER. This study thus identifies Hrd1 and gp78 as novel components for cholera toxin retro-translocation and unveils a previously unknown role for ubiquitination in the retro-translocation of cholera toxin.

Results

Expression of Hrd1 Mutants Decrease Retro-translocation of Cholera Toxin.

We first expressed wild-type and mutant variants of Hrd1, each containing a C-terminal Myc tag, in 293T cells to examine whether they act as dominant negatives in CTA1 retro-translocation (Figure 1A). In this study, we chose to utilize the dominant negative C291A Hrd1, as a point mutation in this critical cysteine residue of the catalytic RING finger domain has been shown to inhibit the degradation of a variety of substrates (Kikkert et al., 2004; Yang et al., 2006; Okuda-Shimizu and Hendershot, 2007). Additionally, we generated two truncation variants of Hrd1 to assess their effect on CTA1 retro-translocation. TM1-6 Hrd1 Myc consists of only the six transmembrane domains of Hrd1, while cyt Hrd1 Myc contains only the cytosolic portion of wild-type

protein. We found that WT Hrd1 Myc, C291A Hrd1 Myc, TM1-6 Hrd1 Myc and cyt Hrd1 Myc were robustly expressed during transient transfections of 293T cells (Figure 3.1A top panel lanes 2-6). Importantly, over-expression of these Hrd1 proteins did not induce the expression of unfolded protein response (UPR) markers Bip and Derlin-1 (Oda et al., 2006), indicating that ER stress was not occurring in these cells. To ensure correct integration of TM1-6 Hrd1 Myc into the ER membrane, cells expressing this variant were subjected to alkali extraction. Similar to endogenous Derlin-1, TM1-6 Hrd1 Myc was found in the pellet fractions (Figure 3.1B, top and bottom panels, compare lane 1 to 2), thereby confirming its behavior as an integrated protein. Additionally, TM1-6 co-localized with the ER marker calnexin using fluorescent microscopy (data not shown), further validating its use in this study.

We sought to further characterize TM1-6 Hrd1 Myc and cyt Hrd1 Myc prior to examining their potential effect on CTA1 retro-translocation. It has previously been suggested that Hrd1 is able to dimerize (Schulze et al., 2005). Therefore, we asked whether these Hrd1 truncation mutants are able to interact with WT Hrd1. To this end, we generated an additional construct in which a FLAG tag was inserted onto the C-terminus of WT Hrd1 (WT Hrd1 FLAG). Cells were co-transfected with WT Hrd1 FLAG and either WT Hrd1 Myc, C291A Hrd1 Myc, TM1-6 Hrd1 Myc or cyt Hrd1 Myc, the lysates subjected to immunoprecipitation with a Myc antibody, and the samples analyzed by SDS-PAGE followed by immunoblotting with the indicated antibodies. We found that WT Hrd1 FLAG co-precipitated with Myc-tagged WT Hrd1, C291A Hrd1, TM1-6 Hrd1 and cyt Hrd1 (Figure 3.1C, top panel, lanes 1-4). This result was observed when the immunoprecipitation was also performed in reverse, as each Myc-tagged Hrd1 protein co-immunoprecipitated with WT Hrd1-FLAG (Figure 3.1D, top three panels, lanes 1-4). The binding of TM1-6 Hrd1 and cyt Hrd1 individually to WT Hrd1 suggests two distinct binding sites for Hrd1 dimerization, one located in a transmembrane region and the other in the cytosolic domain. Of note, the E3 ligase most similar to Hrd1, gp78 was recently found to contain two distinct oligomerization sites, one in its cytosolic domain and the other in a transmembrane segment (Li et al., 2009).

To demonstrate whether the expression of WT Hrd1, C291A Hrd1, TM1-6 Hrd1 or cyt Hrd1 Myc-tagged proteins are able to affect the retro-translocation of CT, we subjected cells to the ER-to-cytosol retro-translocation assay established previously in our laboratory (Forster et al., 2006; Bernardi et al., 2008; Chapter 2, Fig 2.1A). Briefly, cells were intoxicated with CT (10 nM) for 90 minutes, harvested and treated with a low concentration of digitonin to gently solubilize the plasma membrane, allowing for the release of only cytosolic content while leaving intracellular membranes intact. Cells were then subjected to fractionation by centrifugation to isolate cytosolic (supernatant) and membranous (pellet) fractions. As CTA1 is retro-translocated into the cytosol, we are able to determine if expression of a particular protein disturbs the transfer of the toxin into the cytosol by immunoblot analysis of CTA1 in the supernatant fraction obtained in this assay. Experimental controls to demonstrate proper separation of cellular components include the presence of the cytosolic chaperone Hsp90 in supernatant fractions and the ER lumenal protein, PDI, in pellet fractions (Figure 1E).

We found a similar amount of retro-translocated CTA1 in the supernatant in cells expressing WT Hrd1 Myc and yellow fluorescent protein (YFP; Figure 3.1E, top panel, compare lanes 1 and 2; quantified in Figure 3.1F), whereas expressing C291A Hrd1 Myc and TM1-6 Hrd1 Myc decreased the level of retro-translocated CTA1 (Figure 3.1E, compare lanes 3 and 4 to lanes 1 and 2; quantified in Figure 3.1F). Expression of cyt Hrd1 Myc did not affect the amount of CTA1 present in the supernatant (Figure 3.1E, top panel, compare lane 6 to lane 5). We conclude that expression of C291A Hrd1 Myc and TM1-6 Hrd1 Myc blocks 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 and TM1-6 Hrd1 Myc and found that when compared to the YFP control, cells expressing the mutant Hrd1 proteins exhibited less cAMP production. These findings are consistent with the results of the retro-translocation assay (Figure 3.1 E and F) and further implicate a role for Hrd1 in the retro-translocation of CTA1.

Hrd1 binds to CTB and CTA

To examine if Hrd1 is able to physically interact with the toxin, 293T and HeLa cells transiently transfected with YFP, WT Hrd1 Myc, C291A Hrd1 Myc, or TM1-6 Hrd1 Myc were incubated with CT (10nM) for 90 minutes, lysed with 1% Triton X-100 buffer and subjected to immunoprecipitation with a Myc antibody. The immunoprecipitation complexes were resolved by SDS-PAGE and probed for the presence of CTB. As expected YFP did not precipitate CTB, however, each Hrd1 protein expressed was found to interact with CTB (Figure 3.2A and 3.2B, top panels, compare lane 1 to lanes 2-4). Interestingly, in both cell types, TM1-6 Hrd1 precipitated a higher level of CTB compared to WT or C291A Hrd1 (Figure 3.2A and 3.2B, top panel, compare lane 4 to lanes 2 and 3). Thus, we conclude that CTB binds to Hrd1 as it does to Derlin-1 (Bernardi et al., 2008).

As we were unable to detect a Hrd1-CTA interaction under this lysis condition, we reasoned that CTA may interact with the retro-translocon components weakly and transiently for efficient transport of the toxin into the cytosol. Therefore, we incubated cells expressing the different Hrd1 proteins with a higher concentration of CT (100 nM) and lysed them with a gentler detergent (1% deoxyBigChap) in an effort to capture a potential Hrd1-CTA interaction. Cells expressing Derlin-1-YFP were used as a positive control as Derlin-1-YFP was previously shown to interact with CTA (Bernardi, et al., 2008; Chapter 2, Fig 2.3A). We found that Derlin-1-YFP and WT Hrd1 both co-precipitated CTA (Figure 3.2C, top panel, lanes 2 and 3). Interestingly, while TM1-6 Hrd1 immunoprecipitated a similar amount of CTA as WT Hrd1, C291A Hrd1 co-precipitated the toxin more efficiently (Figure 3.2C, top panel, compare lanes 4 and 6 to lane 5). This result suggests that C291A Hrd1 is able to inhibit CTA1 retro-translocation by trapping the substrate. Such an enhanced interaction with substrate due to the lack of a functional RING finger domain has been demonstrated with the Hrd1 dependent substrate, CD3δ (Kikkert et al., 2004).

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

We previously reported that CTB targets the holotoxin to Derlin-1 and that the dominant negative Derlin-1-YFP is able to bind and trap the toxin, thus inhibiting its retro-translocation (Bernardi et al., 2008; Chapter 2). In this study, we demonstrate that CTB also binds to Hrd1 (Figure 3.2A). Therefore, as Derlin-1 and Hrd1 exist in a complex with one another, we sought to elucidate the temporal order of CTA1 retro-translocation. We reasoned that if Derlin-1-YFP was able to block the interaction between CTB and Hrd1, then Derlin-1 is likely upstream of Hrd1 in this pathway. Cells expressing WT Hrd1 Myc and either YFP (control), Derlin-1-YFP, or Derlin-2-YFP (control) were intoxicated with CT (10 nM) for 90 minutes, lysed, the resulting lysates subjected to immunoprecipitation with a Myc antibody and the sample analyzed by SDS-PAGE and immunoblotted with the indicated antibody. While the expression of YFP or Derlin-2-YFP did not perturb the ability of Hrd1 to interact with CTB, expression of Derlin-1-YFP inhibited the Hrd1-CTB interaction (Figure 3.3A, top panel, compare lane 2 to lanes 1 and 3). Similarly, Derlin-1-YFP also blocked the TM1-6 Hrd1-CTB interaction (Figure 3B, top panel, compare lane 2 to lanes 1 and 3). These data suggest that Derlin-1 is upstream of Hrd1 in the CT retro-translocation pathway and provide mechanistic insight into the trafficking of the toxin and its interactions within the Derlin-1/Hrd1 complex.

Expression of a catalytic-inactive gp78 mutant decreases the retro-translocation of cholera toxin.

Similar to Hrd1, the E3 ubiquitin ligase gp78 is also an ortholog of the yeast Hrd1, possesses a cytosolic RING-H2 domain and demonstrates a similar membrane topology as Hrd1 (Fang et al., 2001; Chen et al., 2006), therefore making it an additional candidate for involvement in CT retro-translocation. While some substrates utilize both Hrd1 and gp78 (Fang et al., 2001; Kikkert et al., 2004; Chen et al., 2006), others including CFTR and HMG CoA reductase use only one (Song et al., 2005; Morito et al., 2008). In an effort to determine if gp78 could also serve a role in CT retro-translocation, WT gp78 Myc or a catalytically-inactive gp78 mutant in which cysteines 337 and 374 in the RING domain are mutated to serines (C337S:C374S gp78 Myc) were expressed in 293T cells (Figure 3.4A, top panel, lanes 2 and 3). Expression of C337S:C374S gp78 was

previously shown to inhibit the degradation of ERAD substrate CFTR deltaF508 (Morito et al., 2008). Similar to the expression of the Hrd1 proteins (Figure 3.1A), the expression of WT and C337S:C374S gp78 did not induce ER stress as indicated by the similar protein levels of Bip and Derlin-1 in cells expressing gp78 constructs compared to the YFP vector control (Figure 3.4A, second and third panels, compare lanes 2 and 3 to lane 1).

When cells expressing YFP, WT gp78 Myc or C337S:C374S gp78 Myc were incubated with CT (10 nM) for 90 minutes and subjected to the ER-cytosol retro-translocation assay described in Figure 3.1, we found less CTA1 in the supernatant fractions of cells expressing C337S:C374S gp78 Myc compared to YFP or WT gp78 Myc (Figure 3.4B, top panel, compare lane 3 to lanes 1 and 2; quantified in Figure 3.4C). Furthermore, compared to cells transfected with YFP, cells expressing C337S:C374S gp78 Myc also exhibited a decrease in cAMP production (Figure 3.4D). These data identify a role for gp78 in the retro-translocation of CT.

We next analyzed whether gp78 interacts with CT. Cells expressing WT gp78 Myc or C337S:C374S gp78 Myc were treated with CT (10 nM) for 90 minutes and lysed in 1% Triton X-100-containing buffer. Complexes were immunoprecipitated with Myc antibody and probed for CTB by immunoblot analysis. CTB interacted with WT gp78 Myc and to a lesser extent, C337S:C374S gp78 Myc (Figure 3.4E, top panel, compare lanes 1 and 2). When this same experiment was conducted with 100 nM CT and 1% deoxyBigChap buffer to detect a potential CTA interaction with gp78, we found that consistent with the CTB binding pattern, C337S:C374S gp78 Myc bound less strongly to CTA than WT gp78 Myc (Figure 3.4F, top panel, compare lanes 1 and 2). Thus, we conclude that while gp78 does interact with CT, the catalytic-inactive C337S:C374S gp78 Myc mutant can not bind the toxin efficiently. The inability of C337S:C374S gp78 Myc to facilitate the retro-translocation of CT could therefore be attributed to its inability to engage the toxin, contrary to the behavior of the catalytic-inactive C291A Hrd1 mutant which engages the toxin, but fails to release it (Figure 3.2C).

Expression of Ube2g2 Mutants Decrease CTA1 Retro-translocation

CT is presumed to be a non-ubiquitinated substrate, which is how it is thought to avoid degradation by the proteasome upon cytosolic entry (Rodighiero et al., 2002). However, the fact that the catalytic activity of both Hrd1 and gp78 are important for CT retro-translocation suggests a previously unpredicted role for ubiquitination in this essential step in CT trafficking. To further assess whether ubiquitination is involved in CTA1 retro-translocation, we examined whether defects in an additional component of the ubiquitination machinery could alter CT 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). Polyubiquitination reactions involving Ube2g2 and gp78 require the preassembly of ubiquitin chains on the catalytic cysteine of Ube2g2 and the subsequent transfer of this chain to the substrate via gp78 (Li et al., 2007). In order to build the ubiquitin chain, two Ube2g2 molecules are brought in close proximity to each other through the homodimerization of gp78, allowing for the E2 proteins to partake in a transfer reaction. Mutants of Ube2g2 unable to catalyze the transfer reaction fail to form polyubiquitin chains attached to Ube2g2. This reaction can be demonstrated in vitro through the use of purified recombinant Ube2g2 and the cytosolic domain of gp78 (gp78c; Li et al., 2007; Figure 3.5A). In the presence of WT Ube2g2 and gp78c, Ube2g2 is polyubiquitinated (Figure 3.5A, lane 3). However, when either mutant version of Ube2g2, Ube2g2 $_{H94K}$ or Ube2g2 $_{delta\ loop}$ were individually analyzed with gp78c, the formation of polyubiquitinated chains onto the catalytic cysteine of Ube2g2 was drastically hindered (Figure 3.5A, compare lanes 4 and 5 to lane 3). Similar results were obtained when the Ube2g2 mutants were incubated with a higher concentration of gp78c (Figure 3.5A, lanes 6-8). Thus, as Ube2g2 $_{H94K}$ and Ube2g2$_{delta\ loop}$ mutants are incapable of building a polyubiquitin chain onto Ube2g2, they are expected to be defective in the subsequent E3-dependent polyubiquitination of substrates.

As the Ube2g2 mutants are defective in vitro, we reasoned that they may act as dominant negative proteins in cells by inhibiting the Hrd1/gp78 dependent polyubiquitination reactions required for ERAD. To test this hypothesis, FLAG-tagged WT Ube2g2,

Ube2g2 $_{H94K}$, Ube2g2$_{delta\ loop}$ or YFP were expressed in cells (Figure 3.5B, top panel, lanes 2-4). Bip and Derlin-1 protein levels were not up-regulated (Figure 3.5B, second and third panels, lanes 1-4), indicating that ER stress was not activated upon expression of these proteins. Cells were treated with CT (10 nM) for 90 minutes and subjected to the retro-translocation assay. We found that expression of Ube2g2 $_{H94K}$ or Ube2g2$_{delta\ loop}$ decreased the retro-translocation of CTA1 when compared to cells expressing WT Ube2g2 or YFP (Figure 3.5C). Thus, in addition to our studies using E3 mutants, we demonstrate that an E2 enzyme involved in ERAD also plays a role in mediating toxin retro-translocation. Collectively, these data reveal a novel and unexpected discovery that ubiquitination plays a key role in the retro-translocation of CTA1.

Hrd1 and gp78 Associate with PDI

As Hrd1 and gp78 are involved in the retro-translocation of CT, we sought to demonstrate their physical interaction with PDI, the ER lumenal chaperone that unfolds the CTA1 chain prior to its retro-translocation across the ER membrane (Tsai et al., 2001; Forster et al., 2006). To explore these potential interactions, 293T cells were co-transfected with PDI-FLAG and either WT Hrd1 Myc or WT gp78 Myc. Control cells were transfected with only PDI-FLAG to ensure that any PDI present in immunoprecipitation complexes was due to the presence and pull down of either Myc-tagged Hrd1 or gp78. Weak protein-protein interactions were preserved by the addition of the thiol cleavable crosslinker dithiobis (succinimidly propionate) (DSP) prior to cell lysis. Lysates were subjected to immunoprecipitation with Myc antibody to isolate the Hrd1 Myc or gp78 Myc complexes. Immunoprecipitated samples were subjected to reducing SDS-PAGE, following by immunoblotting with a FLAG antibody to probe for PDI. We found that PDI was present in both the Hrd1 Myc and gp78 Myc pull down (Figure 3.6A and B, top panels, lane 2), demonstrating that PDI exists in a complex with Hrd1 and gp78. As expected, PDI-FLAG was not immunoprecipitated in cells lacking Hrd1 Myc or gp78 Myc (Figure 3.6A and B, top panels, lane 1), thereby confirming the specificity of the interactions. This binding study, along with the previously isolated Derlin-1-PDI interaction (Bernardi et al., 2008), Derlin-1-Hrd1 and Derlin-1-gp78

interactions (Ye et al., 2005; Lilley and Ploegh, 2005) support the findings that each of these proteins is involved in the transport of CTA1 from the ER lumen into the cytosol. The formation of this protein complex likely provides efficient modification and subsequent retro-translocation of the toxin by connecting key events between the ER lumen and membrane.

Discussion

When CT reaches the ER, it hijacks the ERAD quality control pathway, is transported into the cytosol and somehow manages to avoid proteasomal degradation. How the toxin accomplishes each of these steps is not well understood. This study identifies novel ERAD components involved in CTA1 retro-translocation and provides mechanistic insight into the ER events responsible for its transport into the cytosol. We previously demonstrated that the ER membrane protein, Derlin-1, mediates the retro-translocation of CTA1 (Bernardi et al., 2008), which was also confirmed by a subsequent study (Dixit et al, 2008). As the E3 ubiquitin ligases Hrd1 and gp78 form complexes with Derlin-1 and are responsible for the degradation of a variety of substrates (Ye et al., 2005; Lilley and Ploegh, 2005; Hirsch et al., 2009), we sought to determine whether this ubiquitination machinery may also regulate toxin retro-translocation, despite the findings that the CTA1 chain is not ubiquitinated on its two lysine residues or its N-terminus upon its cytosolic entry (Rodighiero et al., 2002).

Using mutant versions of the Hrd1 protein, we show that Hrd1 is involved in the ER-to-cytosol transport of CTA1. Expression of the catalytic-inactive C291A Hrd1 and the truncated TM1-6 Hrd1 mutants inhibited the retro-translocation of the toxin, as assessed by two independent functional assays. ER stress was not up-regulated in cells expressing either of these mutants, thereby refuting the idea that CTA1 retro-translocation is attenuated due to the accumulation of misfolded substrates that inhibit CTA1 binding to the ERAD machinery. In fact, as C291A Hrd1 bound more strongly to CTA than WT Hrd1, it is apparent that expression of the Hrd1 mutants interferes with a specific step in CTA1 retro-translocation and is not generally disrupting ER homeostasis. Interestingly, while the catalytic activity of Hrd1 is not needed to initially bind the substrate, it is

necessary for the release and subsequent transport of the toxin to the cytosol. Similar to CTA, the Hrd1-dependent substrate CD3δ displayed a more stable interaction with C291A Hrd1 than to WT Hrd1 (Kikkert et al, 2004), suggesting that CTA1 and CD3δ may share a common mechanism in their association with Hrd1.

That the truncated TM1-6 Hrd1 decreased CTA1 retro-translocation suggests that communication between the transmembrane and cytosolic domains of Hrd1 is important during the translocation process. What might be the functions of the transmembrane and cytosolic domains? As we demonstrate in this study, CTB is able to bind TM1-6 Hrd1, suggesting that membrane and lumenal domains are important for interactions with substrate. Interestingly, recent findings indicate that the Hrd1 transmembrane domain transfers substrates from the ER into the cytosol (Omura et al., 2008) and senses the misfolded state of ERAD membrane substrates (Sato et al., 2009). While we observed more CTB binding to TM1-6 Hrd1 compared to WT Hrd1, it seems likely that in addition to its catalytic activity, the cytosolic domain is important in regulating the cycles of substrate binding and release from the E3 ubiquitin ligase. Expression of the Hrd1 cytosolic domain did not negatively affect CTA1 retro-translocation, perhaps because this domain neither binds to CT nor perturbs other ERAD components required for toxin translocation. However, the finding that the catalytic-inactive Hrd1 did effectively inhibit CTA1 retro-translocation suggests that ubiquitination of some protein or substrate, possibly even CTA1 (on non lysine residues, see below) is essential for CTA1 transport into the cytosol.

The number of E3 ligases implicated in ERAD continues to grow (Vembar and Brodsky, 2008; Hirsch et al., 2009). The E3 ubiquitin ligase gp78 is most similar to Hrd1 and shares a similar topology and RING-H2 domain essential for its function (Fang et al., 2001; Kikkert et al., 2004; Chen et al., 2006). We therefore tested whether gp78 plays a role in the transport of CTA1 into the cytosol. Through functional and interaction studies, we found that gp78 does mediate CTA1 retro-translocation, suggesting that gp78 and Hrd1 have functional similarities that allow them to accommodate the translocation and modification of some shared substrates. Other examples of functional conservation

between gp78 and Hrd1 include the degradation of the misfolded substrates TCRα and CD3δ (Fang et al., 2001; Kikkert et al., 2004). However, substrate specificity can exist with gp78 and Hrd1, as seen in studies examining the degradation of CFTR and HMG CoA reductase, which prefer a specific E3 ligase (Song et al., 2005; Chen et al., 2006; Morito et al., 2006). Thus, how a substrate is targeted to a particular E3 ligase for retro-translocation is currently difficult to explain.

We found that while the catalytic inactive Hrd1 bound more strongly to CT than WT Hrd1, the opposite result was obtained in the case of gp78, where the catalytic inactive mutant bound less to CT than WT gp78. Because expression of the mutant gp78 did not induce ER stress, we do not believe the decrease in binding of CT was due to an accumulation of misfolded proteins resulting in a lack of binding sites for CT on gp78. Instead, it is likely that the mutant gp78 endured structural changes that inhibited its ability to bind the toxin and/or other proteins that may recruit the toxin to the E3 ligase. Further studies are needed to clarify these possibilities.

How a particular substrate engages the various ERAD components in an ordered manner is not well understood. In this study, we found that CT is first recruited to Derlin-1 and then transferred to Hrd1. This finding is similar to a sequential transfer event that has been suggested in the Der1/Hrd1-dependent degradation of the yeast ERAD substrate CPY* (Gauss et al., 2006). Derlin-1, Hrd1 and gp78 interact with PDI. Thus, we believe that as the holotoxin is transferred from Derlin-1 to Hrd1, the Hrd1-bound PDI unfolds CTA1 to prepare it for transport into the cytosol. Undoubtedly, elucidating events downstream of Hrd1 that complete toxin retro-translocation is essential to understanding the entire pathway.

The observations that expression of two catalytic inactive E3 ligases and a catalytic inactive E2 conjugating enzyme dedicated to ERAD decreased the retro-translocation of CTA1 led us to conclude that an intact ubiquitination system is required for the ER-to-cytosol transport of CTA1. This finding is unexpected because previous reports suggested that CT is a non-ubiquitinated ERAD substrate (Rodighiero et al., 2002; Kothe

et al., 2005). These conclusions are based on experiments in which mutation of its two lysines and chemical blockage of its N-terminus does not affect the toxin's activity. However, as recent studies have identified alternate sites of ubiquitination, including serine, threonine and cysteine residues (Cadwell and Coscoy, 2005; Wang et al., 2007), it remains possible that CTA1 (which contains numerous serine and threonine residues and one cysteine residue) may actually be ubiquitinated (Figure 3.7, A). Notably, while the degradation of TCRα is dependent on an intact ubiquitination system (Yu and Kopito, 1999), it does not require ubiquitin modification of its lysine residues for degradation (Yu et al., 1997). As CTA1 is thought to spontaneously refold upon cytosolic entry (Rodighiero et al., 2002), it seems that addition of ubiquitin molecules (albeit small in size) onto the A1 chain may hinder this refolding event that would be necessary for successful toxicity to occur. A possible solution to this problem would be the utilization of de-ubiquitinating enzymes (DUBs), which have been shown to remove ubiquitin moieties from substrates, thus saving them from proteasomal degradation (Rumpf and Jentsch, 2006). In the case of CT, ubiquitination of CTA1 may be necessary for cytosolic entry, but the activity of DUBs could save it from degradation and allow it to refold as needed (Figure 3.7, B). As we have not observed any high molecular weight CTA1 species that would correspond to ubiquitinated CTA1, it is possible that either DUBs do act to quickly remove this modification from CTA1, or that CTA1 is indeed not ubiquitinated on any residue.

If CTA1 is not ubiquitinated, two additional possibilities exist for the role of the ubiquitination machinery in CTA1 retro-translocation. First, it has been hypothesized that in the US11-mediated retro-translocation of MHC class I molecules where a functional ubiquitination system is necessary (Shamu et al., 1999; Kikkert et al., 2001), substrate transport into the cytosol is not due to ubiquitination of the substrate itself (Hassink et al., 2006), but rather to adaptor proteins that recruit components to the retro-translocation complex. A similar mechanism could operate in CTA1 retro-translocation (Figure 3.7, C). As auto-ubiquitination of Hrd1/gp78 has been demonstrated (Fang et al., 2001; Kaneko et al., 2002; Nadav et al., 2003; Kikkert et al., 2004), it remains formally possible that this self modification recruits adaptor proteins that mediate the cytosolic

entry of CTA1. Conversely, the second possibility is that CTA1 "shuttles" on an ubiquitinated misfolded substrate to reach the cytosol (Figure 3.7, D). Certainly elucidating the critical role of the ubiquitination machinery in CT retro-translocation is essential to understanding CT pathogenesis and ERAD.

Experimental Methods

Materials

Polyclonal antibodies against PDI and Hsp90 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), the polyclonal GFP and CTB antibodies from Abcam (Cambridge, MA), the monoclonal Bip antibody from BD Biosciences (San Jose, CA), 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 crosslinker 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 (San Diego, CA). The Hrd1 Myc constructs (WT, C291A, TM1-6 and cyt) were from E. Wiertz (Leiden University Medical Center), 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), and the PDI-FLAG construct was described previously (Chapter 2, Bernardi, et al. 2008).

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.

Retro-translocation Assay

As described previously in Chapter 2 (Bernardi, et al. 2008).

cAMP Assay

As described previously in Chapter 2 (Bernardi, et al. 2008).

Cell Transfection

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

Immunoprecipitation

293T or HeLa cells were incubated with or without CT (10 nM or 100 nM) for 90 minutes. 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 minutes on ice. Cells were centrifuged at 16,000 g for 15 minutes and the supernatant was used for immunoprecipitation experiments. Co-immunoprecipitation 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 crosslinker DSP (2 mM) for 30 minutes 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, Carlsbad, CA), 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 were resuspended in 150 µl $NaCO_3$ (0.1 M, pH 11.6). Cells remained on ice for 30 minutes. 50 µl of each sample were subjected to centrifugation in an ultracentrifuge using the TLA100 Rotor (Beckman) at 100,000 g for 30 minutes at 4°C. Supernatant and pellet fractions were harvested and subjected to reducing SDS-PAGE and immunoblot analysis.

Chemical Crosslinking

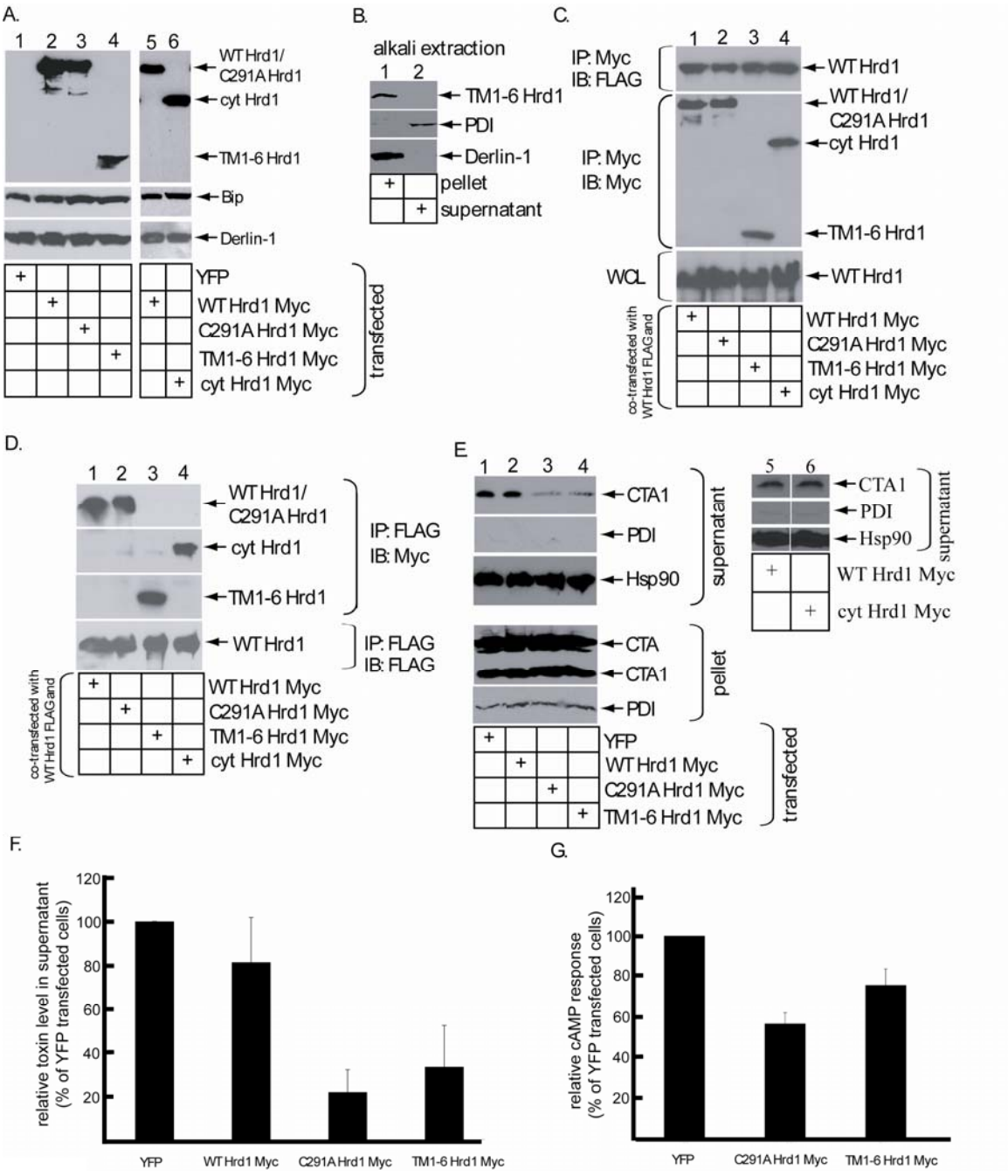
DSP was dissolved in DMSO (10 mg/ml). 800 µl of the DSP was further diluted into 9.2 ml of PBS. 293T cells from a confluent 10 cm dish were harvested and resuspended in

1.4 ml of the DSP in PBS and incubated at room temperature for 30 minutes. Cells were pelleted and the DSP removed. After washing with PBS, cells were lysed in a buffer containing 1% Triton X-100 and subjected to immunoprecipitation described above.

In Vitro Ubiquitination Assay

Purified recombinant WT Ube2g2 (400 nM), Ube2g2 delta loop (400 nM), Ube2g2 H94K (400 nM), E1 (60 nM), and ubiquitin (10 μM) 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 non-reducing SDS-PAGE and immunoblotted with an Ube2g2 antibody. Adapted from Li et al., 2007.

Figure 3.1. Expression of Hrd1 Mutants Decreases the 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. Membrane association of TM1-6 Hrd1 Myc. Cells expressing TM1-6 Hrd1 Myc were subjected to alkali extraction, pelleted, and the supernatant and pellet fractions separated. Samples were immunoblotted with a Myc antibody, a PDI antibody (soluble marker), and a Derlin-1 antibody (membrane marker). C and D. 293T cells expressing WT Hrd1 FLAG and either WT Hrd1 Myc, C291A Hrd1 Myc, TM1-6 Hrd1 Myc or 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. E. Cells expressing YFP, WT Hrd1 Myc, C291A Hrd1 Myc, TM1-6 Hrd1 Myc or cyt Hrd1 Myc were treated with 10 nM CT and subjected to the retro-translocation assay. Supernatant and pellet fractions were analyzed by non-reducing SDS-PAGE, followed by immunoblotting with indicated antibodies. CTA is 28 kD and CTA1 is 22 kD. F. The intensity of the CTA1 band generated in E was quantified with ImageJ. Mean ± SD (error bars) of 5 independent experiments is shown. G. 293T cells expressing YFP, C291A Hrd1 Myc or TM1-6 Hrd1 Myc were incubated with CT for 90 minutes and the cAMP level was measured with a cAMP Biotrak Enzyme Immunoassay System (GE Healthcare). Data were normalized against the forskolin-induced cAMP level, as demonstrated previously (Forster, et al. 2006).


A.
1 2 3 4 5 6
WT Hrd1/ C291A Hrd1
cyt Hrd1
TM1-6 Hrd1
Bip
Derlin-1
YFP
WT Hrd1 Myc
C291A Hrd1 Myc
TM1-6 Hrd1 Myc
cyt Hrd1 Myc
transfected
B.
alkali extraction
1 2
TM1-6 Hrd1
PDI
Derlin-1
pellet
supernatant
C.
IP: Myc
IB: FLAG
IP: Myc
IB: Myc
WCL
WT Hrd1
WT Hrd1/ C291A Hrd1
cyt Hrd1
TM1-6 Hrd1
co-transfected with WT Hrd1 FLAG and
WT Hrd1 Myc
C291A Hrd1 Myc
TM1-6 Hrd1 Myc
cyt Hrd1 Myc
D.
IP: FLAG
IB: Myc
IP: FLAG
IB: FLAG
E.
CTA1
PDI
Hsp90
supernatant
CTA
pellet
F.
relative toxin level in supernatant (% of YFP transfected cells)
G.
relative cAMP response (% of YFP transfected cells)
YFP
WT Hrd1 Myc
C291A Hrd1 Myc
TM1-6 Hrd1 Myc

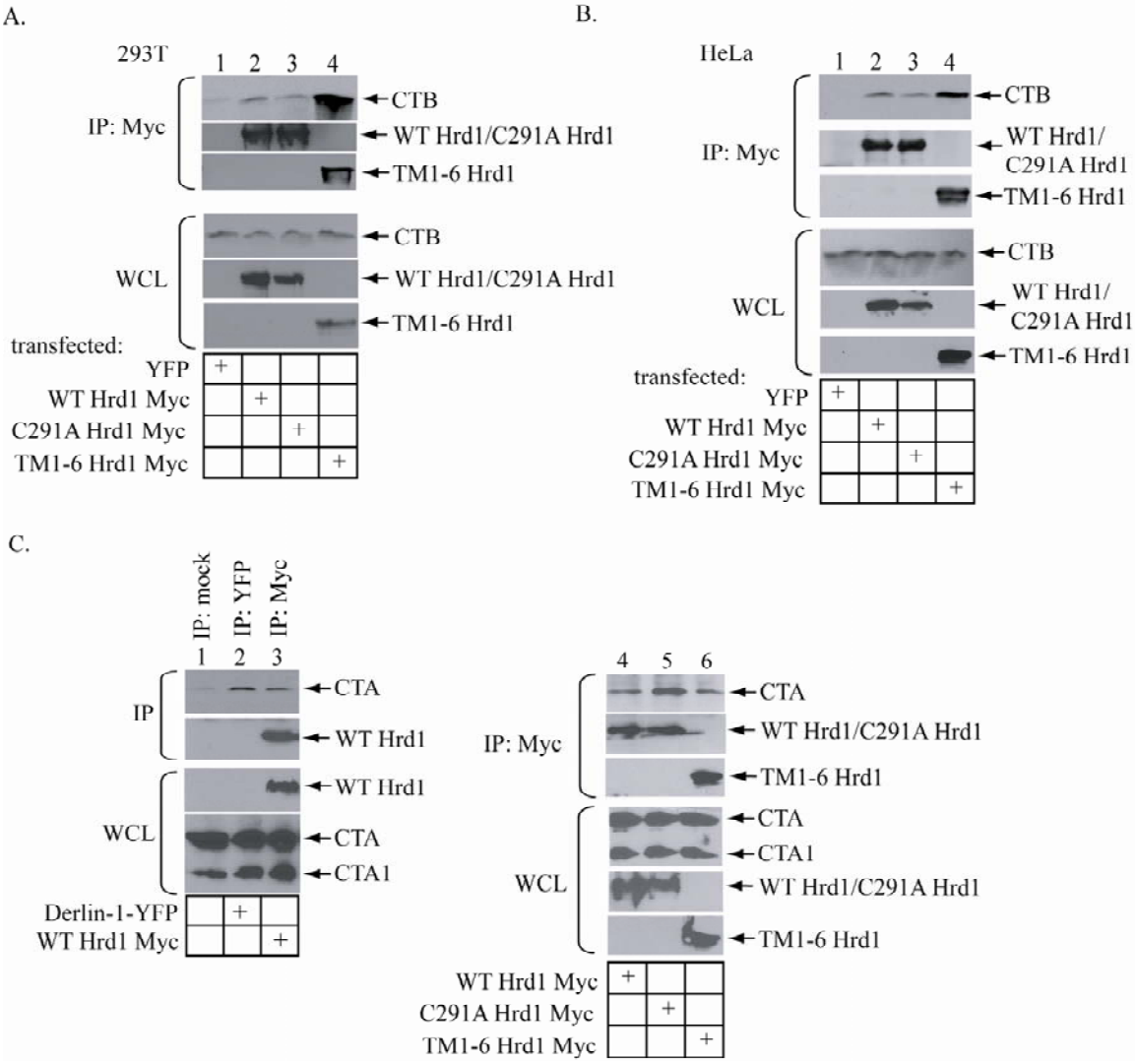


Figure 3.2. Hrd1 Binds to CTB and CTA. A. 293T cells expressing YFP, WT Hrd1 Myc, C291A Hrd1 Myc or TM1-6 Hrd1 Myc were treated with 10 nM CT for 90 minutes. Cells were harvested and lysed in 1% Triton X-100 buffer. Lysates were subjected to immunoprecipitation with a monoclonal Myc antibody. The precipitated complexes were subjected to non-reducing 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.

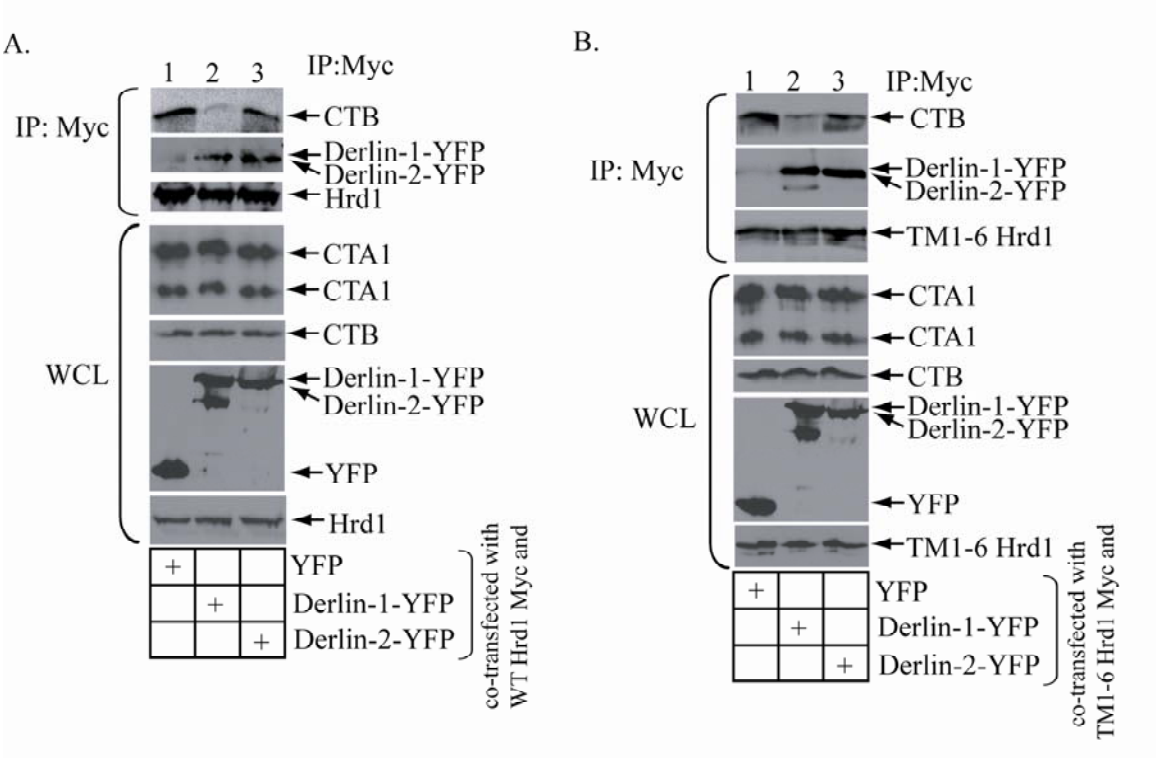


Figure 3.3. Expression of Derlin-1-YFP Blocks the Interaction Between WT Hrd1/TM1-6 Hrd1 and CTB. A. 293T cells expressing WT Hrd1 Myc and either YFP, Derlin-1-YFP or Derlin-2-YFP were treated with 10 nM CT for 90 minutes, harvested, and lysed in a 1% Triton X-100 buffer. Lysates were subjected to immunoprecipitation with monoclonal Myc antibody. Precipitated complexes were analyzed by non-reducing SDS-PAGE and immunoblotted with the indicated antibodies. B. As in A, except TM1-6 Hrd1 Myc were expressed.

Figure 3.4. 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, lysed, and the lysates subjected to SDS-PAGE and analyzed with the indicated antibodies. B-D. As in Figure 1, except gp78 constructs were used. E. As in Figure 2, except gp78 constructs were used. F. 293T cells expressing WT gp78 Myc or C337S:C374S gp78 were treated with 100 nM CT for 90 minutes, harvested and lysed in a 1% deoxyBigChap buffer. Lysates were subjected to immunoprecipitation with a monoclonal Myc antibody. Immunoprecipitation complexes were subjected to non-reducing SDS-PAGE and analyzed for the presence of CTA. *, unidentified protein.

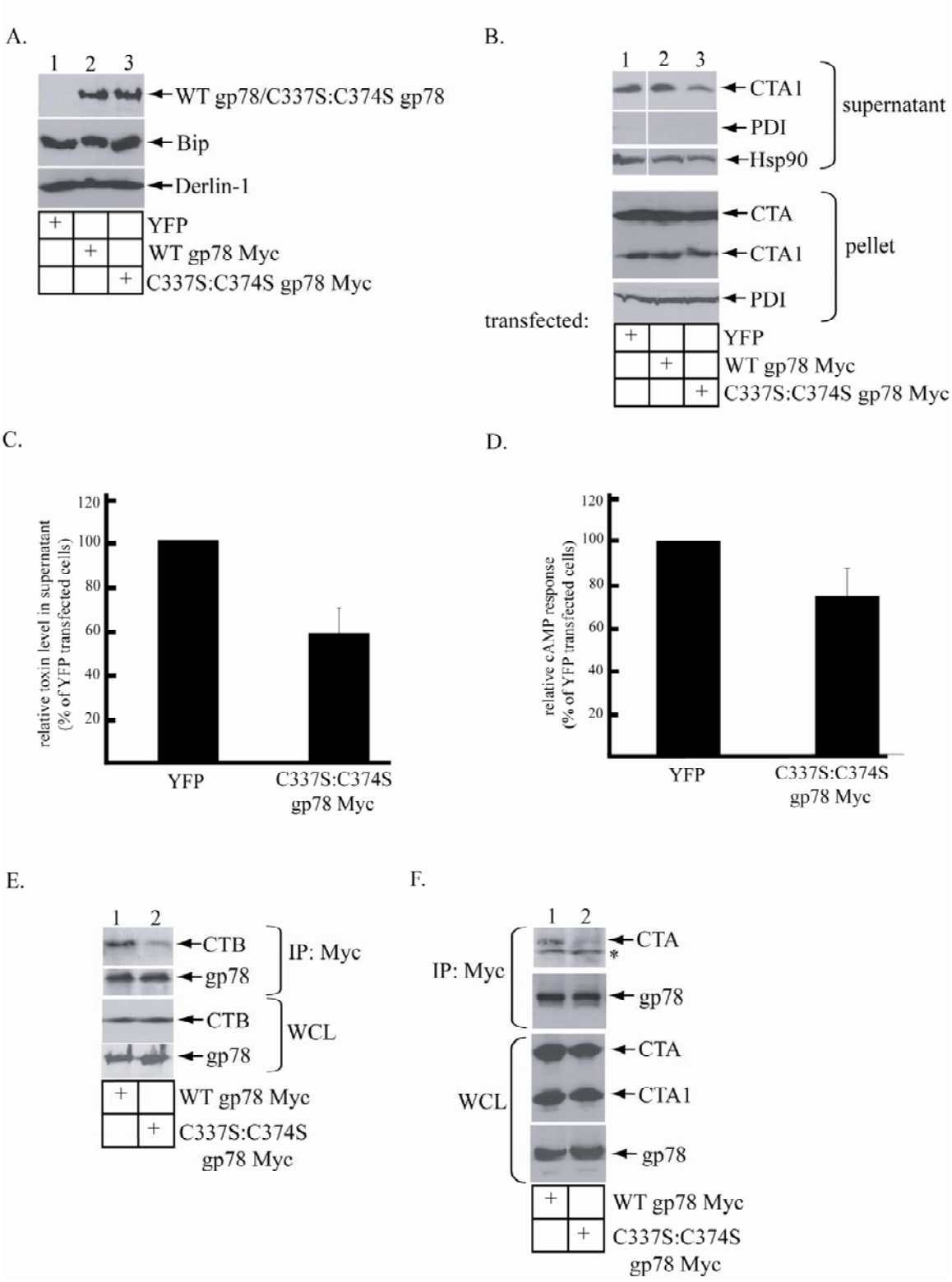
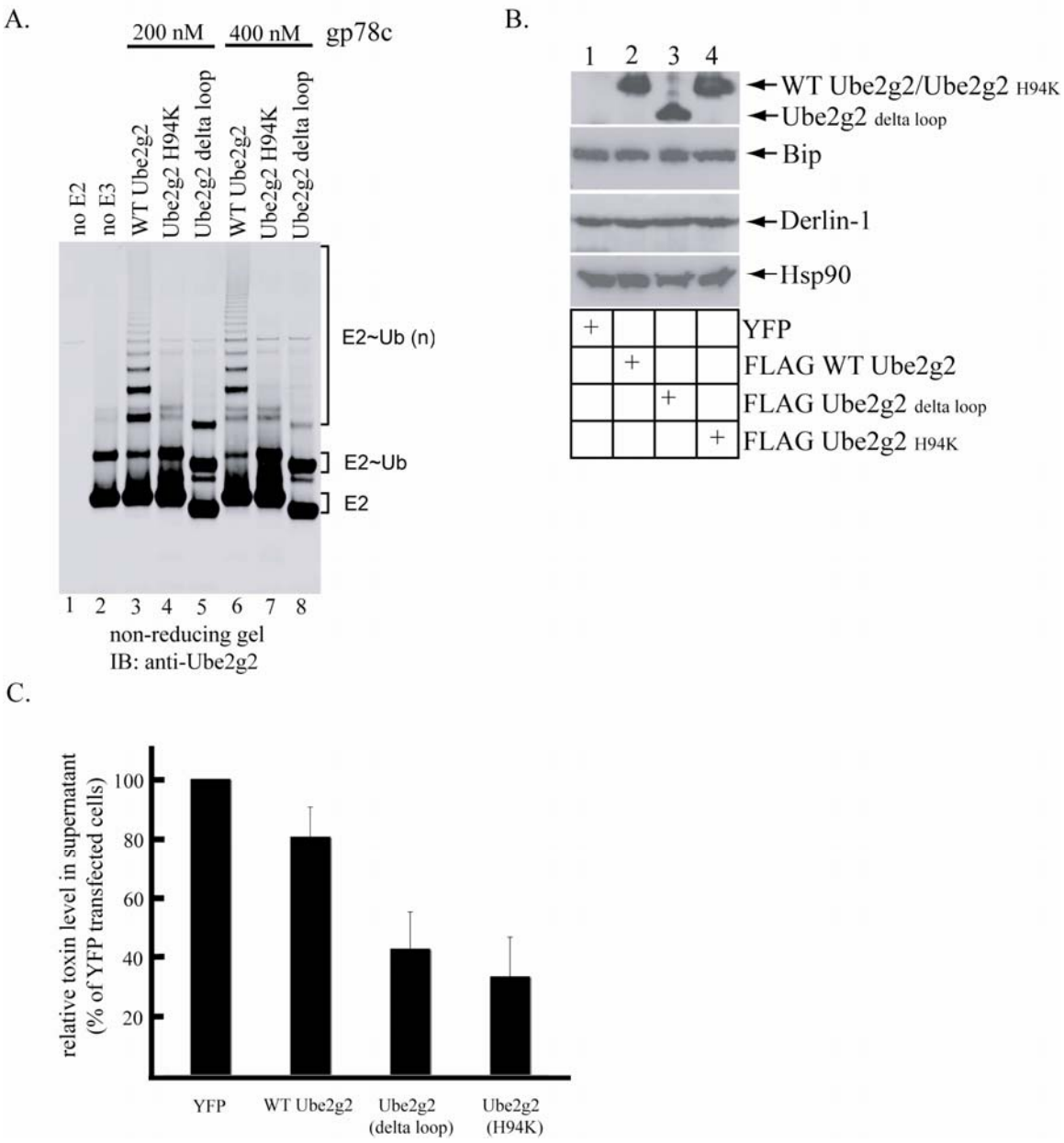

A.
1 2 3
WT gp78/C337S:C374S gp78
Bip
Derlin-1
YFP
WT gp78 Myc
C337S:C374S gp78 Myc
B.
1 2 3
CTA1
PDI
Hsp90
supernatant
CTA
CTA1
PDI
pellet
transfected:
YFP
WT gp78 Myc
C337S:C374S gp78 Myc
C.
relative toxin level in supernatant (% of YFP transfected cells)
120
100
80
60
40
20
YFP
C337S:C374S gp78 Myc
D.
relative cAMP response (% of YFP transfected cells)
120
100
80
60
40
20
YFP
C337S:C374S gp78 Myc
E.
1 2
CTB
gp78
IP: Myc
CTB
gp78
WCL
WT gp78 Myc
C337S:C374S gp78 Myc
F.
1 2
IP: Myc
CTA
*
gp78
WCL
CTA
CTA1
gp78
WT gp78 Myc
C337S:C374S gp78 Myc

Figure 3.5. **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 non-reducing 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 Ube2g2 FLAG, $Ube2g2_{delta\ loop}$ FLAG, or $Ube2g2_{H94K}$ FLAG were harvested, lysed, and the lysates subjected to immunoblot analysis with the indicated antibodies. C. 293T cells expressing YFP, WT Ube2g2 FLAG, $Ube2g2_{delta\ loop}$ FLAG, or $Ube2g2_{H94K}$ FLAG were treated with 10 nM CT for 90 minutes and subjected to the retro-translocation assay described in Figure 1. The intensity of the CTA1 band in the supernatant fraction was quantified with ImageJ. Mean ± SD (error bars) of 3 independent experiments is shown.


A.
200 nM
400 nM
gp78c
no E2
no E3
WT Ube2g2
Ube2g2 H94K
Ube2g2 delta loop
WT Ube2g2
Ube2g2 H94K
Ube2g2 delta loop
E2~Ub (n)
E2~Ub
E2
1 2 3 4 5 6 7 8
non-reducing gel
IB: anti-Ube2g2
B.
1 2 3 4
WT Ube2g2/Ube2g2 H94K
Ube2g2 delta loop
Bip
Derlin-1
Hsp90
YFP
FLAG WT Ube2g2
FLAG Ube2g2 delta loop
FLAG Ube2g2 H94K
C.
relative toxin level in supernatant
(% of YFP transfected cells)
100
80
60
40
20
YFP
WT Ube2g2
Ube2g2
(delta loop)
Ube2g2
(H94K)

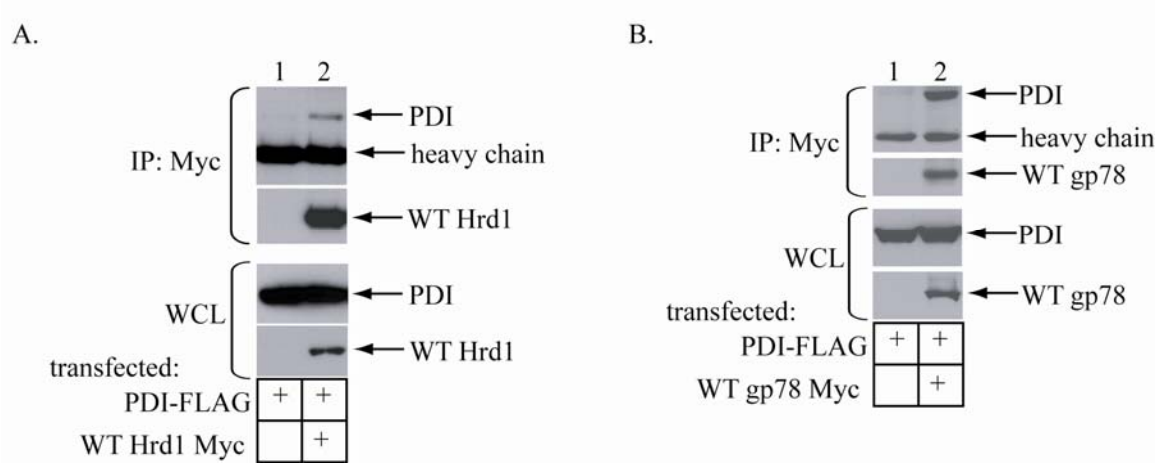
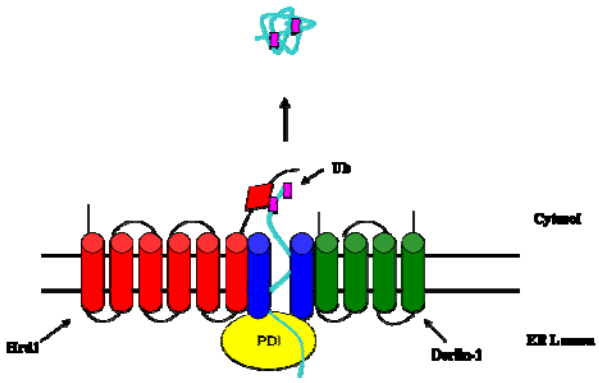


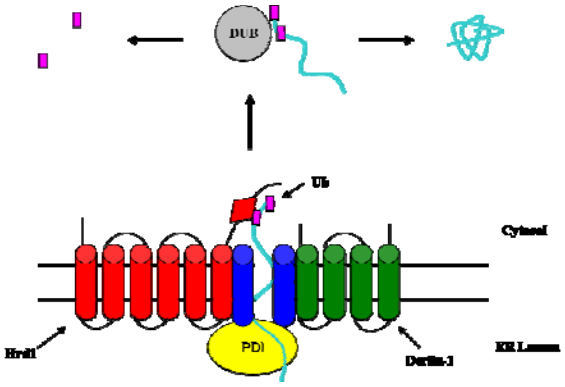
Figure 3.6. Hrd1 and gp78 Bind to PDI. A. 293T cells were transfected with PDI FLAG (lane 1) or co-transfected with PDI-FLAG and WT Hrd1 Myc (lane 2). Cells were harvested, treated with DSP for 30 minutes, then lysed in a 1% Triton X-100 buffer. Lysates were subjected to immunoprecipitation with a monoclonal 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.

Figure 3.7. Potential Roles of the Ubiquitination System in CT Retro-translocation. A. CTA1 is ubiquitinated on serine, threonine and/or cysteine residues via the Hrd1/gp78 RING Finger domain as it exits the ER. CTA1 spontaneously refolds upon cytosolic entry despite the presence of ubiquitin moieties. B. CTA1 is ubiquitinated on serine, threonine and/or cysteine residues via the Hrd1/gp78 RING Finger domain as it exits the ER. However, these ubiquitin moieties are quickly removed by a de-ubiquitinating enzyme (DUB), allowing CTA1 to avoid proteasomal degradation and refold. C. CTA1 is not ubiquitinated on any residue. Instead, the ubiquitination of an ERAD component (potentially auto-ubiquitination of Hrd1/gp78) recruits other factors that help mediate CTA1 retro-translocation. D. CTA1 is not ubiquitinated. Instead, it shuttles through the ER membrane with a misfolded ERAD substrate that is itself ubiquitinated. Upon reaching the cytosol, CTA1 refolds to induce toxicity while the ubiquitinated substrate is targeted to the proteasome for degradation.

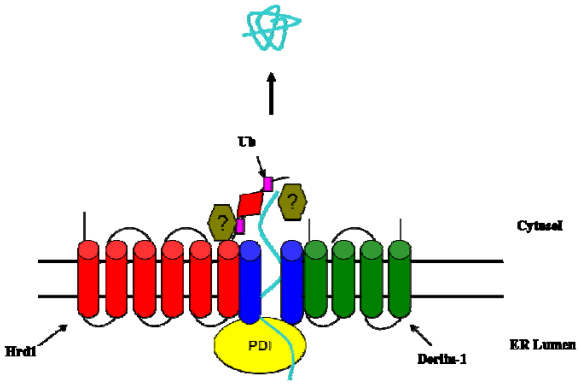
A.



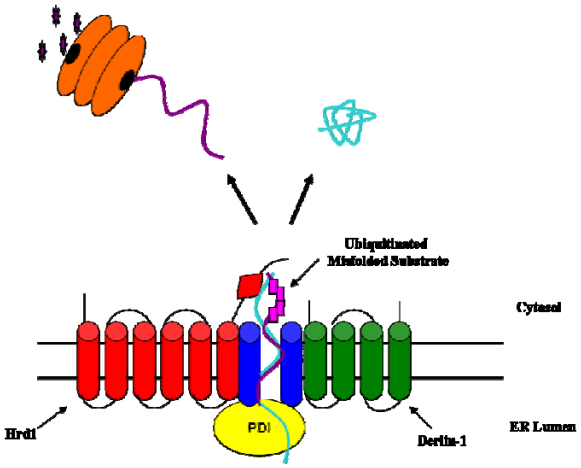
B.



C.


Ub
?
?
Cytosol
Hrd1
PDI
Derlin-1
ER Lumen

D.


Ubiquitinated
Misfolded Substrate
Cytosol
Hrd1
PDI
Derlin-1
ER Lumen

Chapter 4

Conclusion

The targeting of ERAD substrates to distinct complexes in the ER membrane for retro-translocation is proving to be a complicated process to decipher, as many ERAD chaperones and membrane proteins remain to be identified or functionally analyzed. The work presented in this thesis focuses on the mechanism by which CT co-opts the ERAD pathway, specifically revealing key events and interactions at the ER membrane. We demonstrate that CT is targeted to the retro-translocation complex containing Derlin-1 and Hrd1/gp78 via its CTB subunit. As PDI was found to interact with this complex, we predict a model whereby strategic targeting of the CTA1 subunit to these proteins mediates its efficient reduction, unfolding and subsequent ubiquitin-dependent retro-translocation. These findings provide important details in CT transport and ERAD that may emulate the retro-translocation of ER-derived substrates.

The ability of CTB to target the holotoxin to the ERAD machinery is a novel finding that provides significant mechanistic insight into the retro-translocation of CTA1 (Figure 2.2, 2.4). This newly identified role of CTB is similar to that of the human cytomegalovirus US11 membrane protein, which aids in viral infection by targeting MHC class I heavy chains to a retro-translocation complex consisting of Derlin-1 (Ye et al., 2004; Lilley and Ploegh, 2004). Notably, the importance of localizing a substrate to the correct retro-translocation machinery is not specific to pathogenic virulence factors, as this mechanism is also utilized in ERAD of endogenous substrates (Vembar and Brodsky, 2008). For example, the ERAD membrane chaperone BAP31 is essential for presenting CFTRdeltaF508 to Derlin-1 for degradation (Wang et al., 2008). Depletion of BAP31 greatly decreases the amount of CFTRdeltaF508 associated with Derlin-1 and subsequently inhibits the degradation of the substrate (Wang et al., 2008). How these

chaperones interact with the membrane retro-translocation machinery remains to be clarified. Interestingly, our finding that excess CTB inhibits the degradation of CFTR (Figure 2.4) suggests that the two targeting factors, CTB and BAP31, may bind Derlin-1 in a similar manner. Mapping the domains responsible for these interactions could provide essential insight into ERAD and may also help to dissect whether these interactions are direct or facilitated by other factors. Of note, as CTB remains associated with GM1 ganglioside in the ER (Fujinaga et al., 2003), it seems feasible the ganglioside may also mediate interactions with ERAD machinery. Future studies are required to examine this possibility.

Our observation that only one member of the Derlin family facilitates CTA1 retro-translocation is consistent with previous reports suggesting strict substrate specificity for these proteins (Ye et al., 2004; Lilley and Ploegh, 2004; Oda et al., 2006; Lilley et al., 2006; Sun et al., 2006; Younger et al., 2006; Schelhass et al., 2007; Okuda-Shimizu and Hendershot, 2007; Bernardi et al., 2008; Dixit et al., 2008; Gao et al., 2008; Schwieger et al., 2008; Wang et al., 2008; Rutledge et al, 2009; Jiang et al., 2009). Why does CT choose Derlin-1 for retro-translocation as opposed to Derlin-2? It is possible that this specificity may lie within the Derlin proteins themselves, or perhaps is achieved through the binding of different accessory proteins to each Derlin protein. Currently, studies have found interacting partners of Derlin-1 and Derlin-2 to be quite similar (Ye et al., 2005; Lilley and Ploegh, 2005; Mueller et al., 2008), with the exclusive EDEM-Derlin-2 interaction being the exception (Oda et al., 2006). Therefore, it is difficult to address any role of known binding partners in defining the specificity, unless future studies provide evidence that these proteins bind each Derlin in a different capacity that would affect function. Importantly, it is likely that the factors attributing specificity have yet to be identified. As it is expected that the retro-translocation complexes are dynamic, observing potentially transient interactions that may provide specificity could prove experimentally challenging. The substrate selectivity of Derlin-1 and Derlin-2 makes it difficult to understand the nature of their interaction with each other (Lilley and Ploegh, 2005). If this hetero-oligomerization were essential for function, one may expect to see more overlap in the degradation of substrates. However, it is important to note that while

many recent studies have implicated the Derlins in retro-translocation of a subset of proteins, many more substrates have yet to be tested and perhaps it is these future studies that will provide more clues to how these proteins function in their complexes.

The finding that Derlin-1 facilitates CTA1 retro-translocation does not support or refute the suggested role of Sec61 in this process (Schmitz et al., 2000). It remains possible that after the toxin interacts with Derlin-1, it is transferred to Sec61 for transport into the cytosol. Accordingly, it has been postulated that Derlin-1 may act as an accessory protein to switch the Sec61 channel from forward translocation to retro-translocation (Kalies et al., 2005). Clearly, the potential relationship between Derlin-1 and Sec61 in CTA1 retro-translocation requires clarification.

Following its association with Derlin-1, CT surprisingly utilizes the catalytic activity of E3 ubiquitin ligases to further its transport through ERAD (Chapter 3). The finding that Hrd1 and gp78 are both involved in CTA1 retro-translocation suggests substrate redundancy, which has been previously noted with these two ligases in the degradation of TCRα and CD3δ and is probably a testament to their similarities in structure and catalytic activity (Fang et al., 2001; Kikkert et al., 2004; Chen et al., 2006). However, our findings may also suggest that Hrd1 and gp78 function in the same pathway for a subset of proteins. While the concerted effort of gp78 and Hrd1 in the degradation of a particular substrate has not directly been demonstrated, gp78 has been shown to facilitate the degradation of CFTRdeltaF508 along with the E3 ubiquitin ligase RMA1 (Morito et al., 2008), suggesting a Hrd1/gp78 shared pathway scenario may occur.

Interestingly, gp78, but not Hrd1, facilitates ubiquitination of CFTRdeltaF508 (Morito et al., 2008). Recent studies have demonstrated distinct differences between Hrd1 and gp78 that may account for their substrate specificity (Song et al., 2005; Morito et al., 2008). In particular, the cytosolic CUE domain of gp78 is responsible for the ability of the ligase to act as an E4 (multiubiquitination chain assembly factor) in CFTRdeltaF508 ubiquitination after the E3 activity of RMA1 (Morito et al., 2008). This is in sharp contrast to Hrd1, which does not possess a CUE domain and E4 activity (Morito et al,

2008). Therefore, while Hrd1 and gp78 interact with Derlin-1 and with each other (Ye et al, 2005; Lilley and Ploegh, 2005), they may also exist in separate complexes to regulate the ubiquitination of different substrates that are perhaps chosen based on domain specific activities and interactions. Certainly, in addition to cytosolic domain analysis, the contributions of the transmembrane domains of Hrd1 and gp78 in defining substrate binding and recognition (Chapter 3; Sato et al., 2009), as well as the role of Hrd1/gp78 dimerization in mediating this process (Chapter 3; Li et al., 2009), will need further evaluation. It should also be noted that in addition to the catalytic activity of Hrd1 and gp78, their ability to form homo-oligomers and hetero-oligomers (Fang et al., 2001; Ye et al., 2005; Li et al., 2009; Chapter 3) has contributed to the notion that they may contribute to the formation of the retro-translocon. Future domain studies will be essential to determining the specific roles of these large, multi-functional E3 ligases and the complexes they form.

In addition to identifying a role for the E3 ubiquitin ligases Hrd1 and gp78 in CTA1 transport, we found that dominant negative versions of an E2 dedicated to ERAD, Ube2g2, inhibited the retro-translocation of CTA1. These observations strongly suggest that an intact ubiquitination system is necessary for CTA1 retro-translocation. As CTA1 avoids ubiquitination of its two lysine residues and N-terminus (Rodighiero et al., 2002), it previously seemed unlikely that the ubiquitination system would serve any role in CTA1 retro-translocation. However, our findings challenge the field to re-evaluate the ubiquitination state of CTA1 (possibly modified on serine, threonine or cysteine residues) and to further examine the possibility that ubiquitination of other proteins could stimulate the retro-translocation of a non-ubiquitinated species, which is similar to the current conclusions being drawn for the US11 substrate MHC class I heavy chain (Shamu et al., 1999; Kikkert et al., 2001; Hassink et al., 2006). Perhaps in the latter scenario, auto-ubiquitination of Hrd1/gp78 is important for recruiting accessory proteins to the retro-translocation machinery for CTA1 transport or the E3 ligases may themselves be used as the substrates that "shuttle" CTA1 through the membrane (Fang et al., 2001; Kaneko et al., 2002; Nadav et al., 2003; Kikkert et al., 2004). Whether auto-ubiquitination of Hrd1/gp78 leads to its own degradation similar to the fate of other RING finger E3

ligases or whether this modification instead targets the protein for different functions, is not known (Joazeiro and Weissman, 2000; Shen et al., 2007). If indeed the ubiquitination of an additional protein is required for CTA1 retro-translocation, identification of this protein is obviously imperative. As the toxin must refold upon cytosolic entry to induce toxicity, the addition of ubiquitin moieties onto the CTA1 chain itself would seem unfavorable for this refolding event (Rodighiero et al., 2002). However, it is possible that prior to refolding, ubiquitin moieties on CTA1 chains are removed by de-ubiquitinating enzymes (DUBs; Love et al., 2007). Substrates targeted for proteasomal degradation are de-ubiquitinated prior to their proteasomal degradation (Love et al., 2007; Vembar and Brodsky, 2008). Additionally, DUBs are able to rescue proteins from degradation (Rumpf and Jentsch, 2006), a role that may be essential in CTA1 activity. The large number of DUBs in the cell makes it difficult to analyze this model (Love et al., 2007). However, Ataxin-3 is a DUB that interacts with the Derlin-1 complex through the substrate extraction protein p97 (Ye et al., 2001; Wang et al., 2006) and therefore may be a good candidate for this role. Although p97 does not appear to play a major role extracting the CTA1 chain from the ER membrane (Abujarour et al., 2005; Kothe et al., 2005), analyzing the role of Ataxin-3 in CTA1 retro-translocation may nevertheless provide essential information as to how the toxin is extracted from the ER membrane and evades degradation.

Collectively, the findings presented in this thesis provide a great deal of insight into the retro-translocation of CTA1 through the identification of a membrane complex important for its cytosolic entry. Future studies should focus on whether other Derlin-1 interacting components such as Sel1L and Herp also contribute to CTA1 transport. Sel1L is an ER transmembrane chaperone implicated in the US11 mediated degradation of MHC Class I heavy chains (Mueller et al., 2006). In addition to existing in a 1:1 complex with Hrd1, Sel1L also interacts with Derlin-1 and PDI (Lilley and Ploegh, 2005; Mueller et al., 2008), further implicating it as a potential facilitator of CTA1 retro-translocation. Herp is an ERAD chaperone that associates with Derlin-1 via Hrd1 and is implicated in the degradation of CD3δ and Bip substrates (Schulze et al., 2005; Okuda-Shimizu and Hendershot, 2007). As Sel1L also interacts with Bip (Mueller et al., 2008), which has

been implicated in CTA1 retro-translocation (Winkeler et al., 2003), it seems possible that a rather large complex is responsible for facilitating the transport of CTA1 into the cytosol. The complete characterization of this complex, although challenging, will be greatly beneficial to understanding aspects of CTA1 retro-translocation and the fundamental mechanism of ERAD.

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