Derlin-1 Facilitates the Retro-Translocation of Cholera Toxin

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Cholera toxin (CT) intoxicates cells by using its receptor-binding B subunit (CTB) to traffic from the plasma membrane to the endoplasmic reticulum (ER). In this compartment, the catalytic A1 subunit (CTA1) is unfolded by protein disulfide isomerase (PDI) and retro-translocated to the cytosol where it triggers a signaling cascade, leading to secretory diarrhea. How CT is targeted to the site of retro-translocation in the ER membrane to initiate translocation is unclear. Using a semipermeabilized-cell retro-translocation assay, we demonstrate that a dominant-negative Derlin-1-YFP fusion protein attenuates the ER-to-cytosol transport of CTA1. Derlin-1 interacts with CTB and the ER chaperone PDI as assessed by coimmunoprecipitation experiments. An in vitro membrane-binding assay showed that CTB stimulated the unfolded CTA1 chain to bind to the ER membrane. Moreover, intoxication of intact cells with CTB stabilized the degradation of a Derlin-1-dependent substrate, suggesting that CT uses the Derlin-1 pathway. These findings indicate that Derlin-1 facilitates the retro-translocation of CT. CTB may play a role in this process by targeting the holotoxin to Derlin-1, enabling the Derlin-1-bound PDI to unfold the A1 subunit and prepare it for transport.

INTRODUCTION

Cholera toxin (CT) produced by *Vibrio cholerae* is the virulence factor responsible for causing massive secretory diarrhea (Sears and Kaper, 1996). Structurally, the CT holotoxin consists of a receptor-binding homopentameric B subunit (CTB) that is noncovalently associated with a single catalytic A subunit (CTA). On secretion from *V. cholerae*, CTA is proteolytically nicked into the toxic A1 and the A2 domains (Spangler, 1992), which are linked by a disulfide bond and noncovalent interactions. To intoxicate cells, CTB binds to the ganglioside receptor GM1 and carries the CTA subunit from the plasma membrane to the lumen of the endoplasmic reticulum (ER; Fujinaga *et al.*, 2003). In this compartment, CTB remains attached to the luminal side of the ER membrane while CTA is reduced, generating the A1 peptide that is disassembled from the rest of the toxin. Subsequent translocation of the A1 peptide through a protein-conducting channel in the ER membrane enables the toxin to reach the cytosol, where it induces a signaling cascade that leads to the opening of a chloride channel (Lencer and Tsai, 2003). The ensuing secretion of chloride ions and water across the plasma membrane results in the massive diarrhea that typifies cholera.

The sequence of events within the ER lumen that prepares the toxin for transport into the cytosol is poorly defined. The ER oxido-reductase protein disulfide isomerase (PDI) was shown previously to bind, unfold, and disassemble the CTA1 subunit (Tsai *et al.*, 2001), a reaction essential for its

transport into the cytosol (Forster *et al.*, 2006). When the A1 peptide is released from PDI in vitro, the toxin refolds rapidly (Rodighiero *et al.*, 2002), a scenario that could prevent its efficient transport across the retro-translocation channel. Hence, it was postulated that the PDI-dependent unfolding reaction is coupled mechanistically to translocation across the channel (Tsai *et al.*, 2002). How these events are achieved is unknown. Furthermore, whether CTB plays a functional role in the ER during CT retro-translocation is also unclear.

Because CT is thought to masquerade as a misfolded protein to hijack the ER quality control system that normally targets misfolded ER proteins to the cytosol for proteasomal degradation (Hazes and Read, 1997), deciphering the mechanism of CT retro-translocation is likely to provide fundamental insight into the pathway by which misfolded proteins are targeted to the cytosol. Indeed, PDI has been implicated in the retro-translocation of misfolded proteins (Gillece *et al.*, 1999; Molinari *et al.*, 2002; Wahlman *et al.*, 2007).

Here we demonstrate that overexpression of a dominant-negative Derlin-1, an ER membrane protein implicated as a component of the retro-translocation channel, decreased the ER-to-cytosol transport of the A1 peptide, suggesting that Derlin-1 facilitates CT retro-translocation. Coimmunoprecipitation studies showed that Derlin-1 associates with CTB and PDI. The dominant-negative Derlin-1 was found to interact with the holotoxin and impart conformational changes to endogenous Derlin-1. Thus the dominant-negative Derlin-1 likely exerts its inhibitory action by titrating CT from Derlin-1 and inducing a structural defect on Derlin-1. An in vitro membrane-binding assay showed that CTB stimulated the unfolded CTA1 chain to bind to the ER membrane. Moreover, CTB stabilized the degradation of CFTR, a Derlin-1-dependent retro-translocation substrate. These findings indicate that Derlin-1 mediates the retro-translocation of CT. CTB may play a role in this process by targeting the holo-

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Abbreviations used: CT, cholera toxin; PDI, protein disulfide isomerase; YFP, yellow fluorescent protein.

toxin to Derlin-1, allowing the Derlin-1-bound PDI to unfold the A1 subunit and prepare it for transport.

MATERIALS AND METHODS

Materials

Polyclonal antibodies against PDI and Hsp90 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), the polyclonal and monoclonal GFP antibodies and the monoclonal PDI antibody from Abcam (Cambridge, MA), the polyclonal antibody against α 1-antitrypsin and the mouse antibody against FLAG from Sigma (St. Louis, MO), the monoclonal CFTR (M3A7) antibody from Upstate Biotechnology (Lake Placid, NY), and the polyclonal calnexin antibody from StressGen (San Diego, CA). The CTA and CTB antibodies were provided by the W. Lencer (Harvard). The polyclonal and monoclonal ERp29 antibodies were generous gifts from S. Mkrtychian (Karolinska Institutet). 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 (National Institutes of Health). Purified CT and CTB were purchased from Calbiochem/EMD Biosciences (San Diego, CA). The pcDNA3.1 containing YFP construct and the mAb 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 α 1-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-yellow fluorescent protein (YFP) construct was generated by PCR amplification of the Derlin-1 coding sequence using a hemagglutinin (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 (La Jolla, 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 HBSS at 37°C for 45 or 90 min. For permeabilization, 2×10^6 cells were resuspended in 100 μ l of 0.02% digitonin in HCN buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM CaCl₂, and 10 mM *N*-ethyl maleimide [NEM], and protease inhibitors), incubated on ice for 10 min and centrifuged at $16,000 \times g$ for 10 min. The supernatant was removed and the pellet washed with PBS and resuspended in 100 μ l of the original buffer. Fractions were analyzed by nonreducing SDS-PAGE and immunoblot analysis.

cAMP Assay

CT-induced cAMP level was analyzed as previously described (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, Chatsworth, CA), and the cells were grown for an additional 48 h before 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, pH 7.5 (30 mM), MgCl₂ (4 mM), and NEM (10 mM) with either 1% Triton X-100 or 1% deoxyBigChap, and centrifuged at $16,000 \times g$ for 10 min, and the supernatant was used for immunoprecipitation experiments. Coimmunoprecipitation 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, monoclonal GFP, 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, Carlsbad, CA), washed,

and subjected to nonreducing 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 μ M), and reduced glutathione (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 nontransfected or overexpressing Derlin-1-YFP or Derlin-1-HA were incubated with 0.04% digitonin for 5 min at 4°C and centrifuged at 14,000 rpm for 10 min, and the pellet was resuspended in a buffer containing HEPES (50 mM, pH 7.4), KOAc (150 mM), sucrose (250 mM), and MgCl₂ (4 mM). The samples were incubated with 0.1 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 h before the experiment. Analysis of CFTR degradation in HEK293T cells followed a previously published protocol (Zhang *et al.*, 2002).

RNA Isolation and mRNA Analysis by Semiquantitative RT-PCR

RNA extractions were carried out with the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. Cells, 1×10^6 , 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, Richmond, CA) according to the manufacturer's protocol. Two microliters of cDNA products were amplified using the Expand High Fidelity PCR system (Roche, Indianapolis, IN) in the MgCl₂-free buffer in the presence of specific primers for CFTR or GAPDH used at a concentration of 0.1 μ M each. MgCl₂ was added to the reaction at a concentration of 1 mM. Reactions were carried out in the Eppendorf Mastercycler Personal. A first cycle of 10 min at 95°C, 45 s at 65°C, and 1 min at 72°C was followed by 45 s at 95°C, 45 s at 65°C, and 1 min 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 GGC-AGGCAGTG-3'; GAPDH: 5'-ACCACAGTCCATGCCATCACTGCC-3' and 5'-TC CACCACCTGTGTCTGTAGCC-3'. CFTR yielded an amplification product of 765 base pairs and GAPDH of 453 base pairs, which were resolved by an agarose gel.

RESULTS

Derlin-1 Facilitates the Retro-Translocation of CT

We first established a retro-translocation assay in 293T cells that was developed previously in HeLa cells (Forster *et al.*, 2006), as 293T cells afford the high transfection efficiency that is required for this study. Cells were intoxicated with CT (A and B subunits), treated with a low concentration of digitonin to permeabilize the plasma membrane, and then fractionated by centrifugation. The supernatant contained cytosolic proteins as shown by the presence of the cytosolic Hsp90 protein in this fraction (Figure 1A, fourth panel, lanes 1 and 3). In contrast, the pellet but not the supernatant contained the ER-resident protein PDI (Figure 1A, third panel, compare lanes 2 and 4 to lanes 1 and 3), indicating that the ER was separated from the cytosol and remained intact. The plasma membrane and intracellular membranes are also expected to be in this pellet fraction.

When cells intoxicated with CT at 37°C were subjected to this protocol, a portion of the A1 peptide appeared in the supernatant fraction (Figure 1A, top panel, compare lanes 1 and 2), whereas the B subunit was absent in this fraction (Figure 1A, second panel, compare lanes 1 and 2) as expected. However, when cells were incubated with CT at 37°C in the presence of brefeldin A (BFA), a drug that blocks the arrival of CT to the ER (Fujinaga *et al.*, 2003), the A1 peptide did not appear in the supernatant (Figure 1A, top panel, compare lane 3 to lane 1). Thus, appearance of the A1

Figure 1. Derlin-1 facilitates the retro-translocation of CTA1. (A) 293T cells were incubated with CT (10 nM) for 90 min at 37°C with or without BFA. Cells were permeabilized and centrifuged, and the supernatant and pellet fractions were separated, subjected to non-reducing SDS-PAGE, and immunoblotted with the indicated antibodies. CTA, CTA1, and CTB are 28, 22, and 11 kDa, respectively. (B) 293T cells expressing YFP, Derlin-1-YFP, or Derlin-2-YFP were harvested and lysed, and the lysates were 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 or 90 min, the cells were permeabilized and centrifuged, and the supernatant fraction was analyzed as in A. The intensity of the CTA1 band generated from cells treated with CT for 90 min was quantified with ImageJ. Mean $\pm$ SD (error bars) of two to five independent experiments is 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).

peptide in the supernatant represents retro-translocated toxin. Toxin that did not undergo retro-translocation remained in the pellet.

To assess the role of Derlin-1 and Derlin-2, two ER membrane proteins shown to mediate the transport of misfolded proteins from the ER into the cytosol (Ye *et al.*, 2004; Lilley and Ploegh, 2004; Oda *et al.*, 2006), in CT retro-translocation, we tested whether overexpression of the previously characterized dominant-negative Derlin constructs (i.e., Derlin-1-GFP and Derlin-2-GFP, 13) in cells affects the appearance of the A1 chain in the supernatant. (Throughout this study, YFP was used instead of GFP). Transient transfection experiments showed that YFP, Derlin-1-YFP, and Derlin-2-YFP were expressed robustly in 293T cells (Figure 1B, lanes 1–3). When cells were intoxicated with CT for 45 min, the level of A1 chain that appeared in the supernatant in cells expressing Derlin-1-YFP was less compared with cells expressing YFP or Derlin-2-YFP (Figure 1C, top panel, compare lane 2 to lanes 1 and 3, quantified in the bottom graph). A similar trend was seen when cells were intoxicated with CT for 90 min (Figure 1C, top panel, compare lane 5 to lanes 4 and 6, quantified in the bottom graph); coexpression of Derlin-1-YFP and Derlin-2-YFP also decreased the A1 peptide in the supernatant (Figure 1C, quantified in the bottom graph), an effect attributed to the expression of Derlin-1-YFP. On reaching the cytosol, the catalytic A1 subunit ADP ribosylates the Gas protein, activating adenylate cyclase that then generates cAMP. We therefore measured the CT-induced cAMP response and found that the cAMP level was reduced in Derlin-1-YFP transfected cells, when compared with the

YFP-transfected cells (Figure 1D). These findings are consistent with data from the retro-translocation assay and suggest that Derlin-1 facilitates the ER-cytosol transport of the A1 chain. It is also possible that overexpression of Derlin-1-YFP may perturb other factors in the ER so as to affect the mechanics of retro-translocation of the A1 chain indirectly.

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

To test if Derlin-1 interacts physically with the toxin, 293T cells were incubated with or without CT (30 nM), lysed in 1% Triton X-100, and the Derlin-1 and Derlin-2 proteins were immunoprecipitated and probed for coimmunoprecipitation with the toxin subunits. We found that CTB coimmunoprecipitated with Derlin-1, but not Derlin-2, ERp29 (an ER luminal protein), or a nonspecific IgG (Figure 2A, fifth panel, compare lane 3 to lanes 4–6). As expected, CTB was not detected in the Derlin-1 immunoprecipitate derived from a nonintoxicated cell (Figure 2A, fifth panel, lane 1). Neither the CTA nor CTA1 subunits were detectable in this assay (Figure 2A, fourth panel). Cells intoxicated with a lower CT concentration (10 nM) showed a similar result: CTB coimmunoprecipitated with Derlin-1 but not Derlin-2 or a nonspecific IgG (Figure 2B, third panel, compare lane 5 to lanes 6 and 8); a very low level of CTB was precipitated using an antibody against the ER membrane channel Sec61 (Figure 2B, third panel, lane 7). CTB also coimmunoprecipitated with Derlin-1 in cells intoxicated with CTB alone (Figure 2C, lane 1). We conclude that the B subunit of CT interacts with Derlin-1, a finding consistent with the role of

Figure 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, and the cells were 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 (10 nM) or CTB (10 nM). (D) CT (200 nM) and PDI (2 μ g/ml) were incubated with or without proteoliposomes or with PK-RM, and the samples were 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 were analyzed in SDS-PAGE followed by immunoblotting with an antibody against CTA.

Derlin-1 but not Derlin-2 in CT retro-translocation (Figure 1). Whether CTB binds directly to Derlin-1 is unknown. Because CTB remains bound to ganglioside GM1 in the ER membrane (Fujinaga *et al.*, 2003), it is possible that the association between CTB and Derlin-1 in Triton-cell extracts is mediated by the GM1 molecule.

We next asked whether the CTB-Derlin-1 interaction established in cells could be recapitulated *in vitro*. Purified CT was incubated in the presence or absence of proteoliposomes. These proteoliposomes contain essentially all ER membrane proteins with their orientations randomized, enabling CTB to interact with the luminal domain of Derlin-1. The samples were solubilized, the Derlin-1 and Derlin-2 proteins immunoprecipitated, and the precipitated samples were probed for the presence of CTB. We found that CTB coimmunoprecipitated with Derlin-1, whereas a very low level coprecipitated with Derlin-2 in this *in vitro* reaction (Figure 2D, compare lane 3 to 4). No significant level of CTA was detected in the Derlin-1-immunoprecipitated sample (not shown). In contrast to proteoliposomes, microsomes stripped of ribosomes (i.e., PK-RM) in which the orientations of the membrane proteins were not randomized, poorly supported the CTB-Derlin-1 interaction (Figure 2D, compare lane 6 to lane 5), suggesting that the CTB-Derlin-1 association in proteoliposomes was not due to artifactual binding. Moreover, using proteoliposomes, we found that less CTB was immunoprecipitated with a Sec61 antibody when compared with the Derlin-1 antibody (Figure 2E, compare lane 2 to lane 1), consistent with the results from 293T cells (Figure 2B). Collectively, these findings further establish the ability of CTB to interact specifically with Derlin-1.

We next tested whether the CTB-Derlin-1 interaction has a functional significance. Using a membrane-pelleting assay, we showed previously that the PDI-unfolded CTA1 chain binds to an ER membrane protein (Tsai and Rapoport, 2003); the identity of the ER membrane protein is unknown, but it could be the actual retro-translocation channel of the A1 chain. To test whether CTB stimulates the ability of the A1

chain to bind to the ER membrane protein, we incubated the ER proteoliposomes with either the isolated CTA chain or CT in the presence of PDI and compared the level of the A1 chain that bound to the ER membrane (i.e., in the pellet fraction). We found a higher level of CTA1 in the pellet when CT (but not CTA) was used as the substrate (Figure 2F, compare lane 4 to 2). Because the PDI-unfolded A1 chain is released from CTB (Tsai *et al.*, 2001), the increased binding of the A1 peptide to the ER membrane is not simply due to its association with CTB (which binds to Derlin-1 in the ER membrane). Instead, the released A1 peptide is likely captured by an ER membrane protein. This result suggests that CTB functions to efficiently transfer the A1 chain to an ER membrane protein, perhaps through its ability to bind to Derlin-1, thereby targeting the holotoxin to the retro-translocation machinery.

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

How might Derlin-1-YFP decrease CT retro-translocation? One possibility is that Derlin-1-YFP interacts with the CT holotoxin, thereby competing with endogenous Derlin-1 for access to the substrate. To test this possibility, 293T cells expressing YFP, Derlin-1-YFP, or Derlin-2-YFP were intoxicated with CT, and the lysate was subjected to immunoprecipitation using a GFP antibody. We found that CTB coimmunoprecipitated with Derlin-1-YFP, but not with YFP or Derlin-2-YFP (Figure 3A, third panel, compare lane 2 to lanes 1 and 3), consistent with the observation that Derlin-1 but not Derlin-2 interacts with CTB (Figure 2). In contrast to endogenous Derlin-1 (Figure 2), CTA coimmunoprecipitated with Derlin-1-YFP (Figure 3A, middle panel, lane 2). Moreover, in cells intoxicated with CTB alone, CTB also coimmunoprecipitated with Derlin-1-YFP (Figure 3B, lane 2). These data indicate that Derlin-1-YFP binds to the holotoxin, likely through CTB; CTA coimmunoprecipitated with Derlin-1-YFP presumably because it remained attached to CTB. Thus,

Figure 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 were lysed in a buffer containing 1% Triton X-100, and the lysate was 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 overexpressing Derlin-1-YFP or Derlin-1-HA were incubated with 0.1 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.

the inhibitory action of Derlin-1-YFP on CT retro-translocation could be mediated by titration of CT away from endogenous Derlin-1.

Although not mutually exclusive, another possibility to explain Derlin-1-YFP's inhibitory effect is that it altered the conformation of endogenous Derlin-1, thereby affecting Derlin-1's function. To test this possibility, we assessed the structure of endogenous Derlin-1 in the presence of Derlin-1-YFP by using limited proteolysis. We found that Derlin-1 in the 293T cell lysate was sensitive to proteinase K digestion at either the low (0.1 mg/ml) or high (1 mg/ml) concentrations (Figure 3C, top panel, compare lanes 2 and 3 to lane 1). In contrast, in the presence of Derlin-1-YFP, endogenous Derlin-1 was partially resistant to proteinase K digestion at the low but not high concentration (Figure 3C, top panel, compare lane 5 to lanes 6 and 4). Protease resistance of Derlin-1 was not due to protein overexpression, because overexpression of Derlin-1 that is tagged with HA at its C-terminus (Derlin-1-HA) did not confer protease resistance of endogenous Derlin-1 at the low concentration (Figure 3C, top panel, compare lane 8 to lane 7), consistent with the observation that Derlin-1-HA does not act as a dominant-negative factor in the retro-translocation of CT (not shown). Importantly, Derlin-1-YFP did not protect endogenous Derlin-2 against protease digestion (Figure 3D, compare lane 5 to lane 4). These results suggest that Derlin-1-YFP imparted a structural change on Derlin-1 but not Derlin-2, consistent with previous findings that Derlin-1-GFP binds preferentially to Derlin-1 and not Derlin-2 (Lilley and Ploegh, 2005). Hence, in addition to Derlin-1-YFP's ability to bind to the holotoxin, Derlin-1-YFP's dominant-negative behavior on toxin retro-translocation may also be attributed to its ability

to impart conformational changes on endogenous Derlin-1 to affect Derlin-1's function.

Intoxication by CTB Stabilizes a Derlin-1-dependent Substrate

Our data suggest that CTB targets the toxin to Derlin-1 for retro-translocation of the A1-chain. If so, it is possible that excess CTB might compete with other substrates that use Derlin-1 for retro-translocation to the cytosol. To test this prediction, we examined the degradation of cystic fibrosis transmembrane conductance regulator (CFTR) as Derlin-1 was shown previously to facilitate the retro-translocation and proteasomal degradation of CFTR (Sun *et al.*, 2006; Younger *et al.*, 2006). The effect of CTB on CFTR degradation was examined using metabolic-labeling, pulse-chase experiments. Cells were treated with CTB (100 nM) for 3 h, labeled with [³⁵S]methionine for 30 min and chased for the indicated time; then the cell lysate was subjected to immunoprecipitation with a CFTR antibody, and the sample analyzed by SDS-PAGE followed by autoradiography. We found that CTB stabilized the degradation of the immature B band and increased the level of the mature C band of CFTR (Figure 4A, compare lanes 5 and 6 to lanes 2 and 3; see quantification below); the increased level of the C band is likely due to more B band being available for maturation. We note that CTB treatment increased the total level of labeled CFTR (Band B and Band C) slightly (~10%) at time = 0 when compared with no CTB addition. This effect may reflect CTB's ability to stabilize CFTR within the 30 min labeling time.

Semiquantitative RT-PCR analysis further demonstrated that CTB did not induce the level of CFTR mRNA (Figure

Figure 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, and harvested at the indicated chase times, and the resulting cell lysate was 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, SD. Band B, the immature core-glycosylated form of CFTR; Band C, the mature complex-glycosylated form. (B) RNA isolated from cells incubated with or without CTB (100 nM) was subjected to semiquantitative 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 was subjected to SDS-PAGE and immunoblotted with an antibody against NHK.

4B, compare lane 2 to lane 1), indicating that the CTB-induced stabilization of CFTR is unlikely because of an effect at the transcriptional level. CTB treatment also did not affect the expression of the ER-resident proteins PDI, ERp72, or Derlin-1 (Figure 4C, compare lane 2 to lane 1). Moreover, CTB appears to act specifically on the Derlin-1 pathway because intoxication of cells with CTB had no detectable effect on the steady-state level of the Derlin-2-dependent substrate, the null Hong Kong (NHK) mutant of α 1-proteinase inhibitor (Oda *et al.*, 2006; Figure 4D, compare lane 2 to lane 1). Together, these results are consistent with an effect

of CTB on the retro-translocation of CFTR in the Derlin-1 pathway, further supporting the idea that after arrival to the ER, CTB and Derlin-1 associate *in vivo*.

Interaction of PDI with the Derlin Family Proteins

We showed previously that PDI unfolds the A1 peptide (Tsai *et al.*, 2001), a reaction that prepares the toxin for retro-translocation (Forster *et al.*, 2006). However, it remains unknown whether PDI unfolds the A1 subunit initially and then brings the toxin to the retro-translocation site or alternatively, that CT is first targeted to the retro-translocation site where CTA is reduced and then unfolded by PDI. Our current data suggest that CT is targeted to Derlin-1 by CTB, perhaps in complex with the ganglioside GM1. In this environment, CTA may be reduced, and the resulting A1 peptide unfolded by PDI. This model implicates that PDI would interact with Derlin-1. Indeed, we found that, under a gentle solubilization condition, a portion of PDI coimmunoprecipitated with Derlin-1 and Derlin-2 (Figure 5A, top panel, lanes 1 and 2). Under this condition, hetero-dimerization of Derlin-1 and Derlin-2 was observed, as Derlin-2 coimmunoprecipitated with Derlin-1, and vice-versa (Figure 5A, second and third panels, lanes 1 and 2), as observed previously (Lilley and Ploegh, 2005). The interaction of PDI with Derlin-1 and Derlin-2 was also found in HeLa cells (Figure 5B, lanes 1 and 2), indicating that the Derlin-PDI complex can be found in different cell types. Furthermore, Derlin-1-YFP also binds to PDI (Figure 5C, lane 2). We next asked whether Derlin-1 is a substrate of PDI. Mutation of the isoleucine residue at position 272 to tryptophan (I272W) in human PDI results in a substrate-binding mutant of PDI (Pirneskoski *et al.*, 2004). We transfected mouse FLAG-tagged PDI with the corresponding mutation (I272W) and wild-type PDI and found that the mutant and wild-type mouse PDI bound with equal efficiency to Derlin-1 (Figure 5D, compare lane 2-1), indicating that Derlin-1 is unlikely a substrate of PDI but instead a stable binding partner. We conclude that PDI associates with the Derlin proteins, suggesting that *in vivo* PDI unfolds and dissociates the A1 chain from the CTB subunit after the holotoxin is targeted to Derlin-1.

DISCUSSION

A central goal in elucidating the retro-translocation process is to clarify the mechanism by which a specific misfolded substrate engages the retro-translocation machinery on the ER membrane. Although recent data have begun to unravel the complex retro-translocation machineries on the ER membrane (Carvalho *et al.*, 2006; Denic *et al.*, 2006; Gauss *et al.*, 2006), it remains unclear how a particular retro-translocation substrate is targeted to these sites.

This study was initiated to examine the role of the Derlin proteins, components of the retro-translocation machinery, in mediating the ER-cytosol transport of CT. We found that expression of the dominant-negative Derlin-1 attenuated the ER-to-cytosol transport of CTA1, likely because the dominant-negative Derlin-1 competed with endogenous Derlin-1 for CTB, and/or because dominant-negative Derlin-1 imparted a structural change on Derlin-1 to affect its function. The coimmunoprecipitation studies uncovered two novel interactions. First, Derlin-1 was found to associate with CTB. Although CTB was shown previously to play a crucial role in carrying the CTA subunit from the plasma membrane to the ER (Fujinaga *et al.*, 2003), its role in the ER during the retro-translocation process is unknown. The ability of CTB to associate with a component of the retro-translocation machinery suggests that it may play an instructive role in

Figure 5. Association of PDI with Derlins. (A) 293T cells were lysed in a buffer containing 1% deoxyBigChap, and the lysates were 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.

the ER during CT retro-translocation. Specifically, the *in vitro* membrane binding study implicated a role of CTB in delivering the unfolded A1 chain to an ER membrane protein. We envision a scenario wherein, upon reaching the ER, CTB targets the holotoxin to Derlin-1, enabling the A1 chain to undergo its subsequent unfolding and translocation reactions. Because CTB is bound to GM1 in the ER membrane, GM1 may mediate the association of CTB with Derlin-1. This process of CTB targeting the A1 chain to Derlin-1 is reminiscent of the mechanism by which the US11 membrane protein (encoded by the human cytomegalovirus) targets the MHC class I heavy chains to Derlin-1 for retro-translocation (Ye *et al.*, 2004; Lilley and Ploegh, 2004).

We found that endogenous Derlin-1 did not bind to the CTA or CTA1 chains, perhaps because the catalytic subunit is translocated rapidly such that no stable association with Derlin-1 can be detected. However, the dominant-negative Derlin-1-YFP protein interacted with both CTB and CTA, but not CTA1. This finding suggests that Derlin-1-YFP, in part, prevents the proper processing of CTA, thereby enabling it to remain stably bound to Derlin-1-YFP.

We also found that PDI associates with Derlin-1 and Derlin-2. That only a small amount of PDI associates with these ER membrane proteins is not surprising as PDI is a soluble luminal protein. PDI was shown previously to bind and unfold the A1 chain (Tsai *et al.*, 2001), priming the toxin for retro-translocation (Forster *et al.*, 2006). As the toxin refolds rapidly upon release from PDI (Rodighiero *et al.*, 2002), a scenario that may impede its transport across the retro-translocation channel, it was postulated that the PDI-depend-

ent unfolding reaction would occur adjacent to the retro-translocation channel such that release of the toxin is coupled to its translocation (Tsai *et al.*, 2002). The finding that PDI associates with Derlin-1 would support this model (Figure 5): upon delivery of the holotoxin by CTB to Derlin-1 (step 1), CTA is reduced (by an unidentified reductase) and the A1 peptide unfolded precisely at the site of retro-translocation by the Derlin-1-bound PDI (step 2), thereby coupling the unfolding and translocation events. The ability of Derlin-1-YFP to behave as a dominant negative factor in decreasing toxin retro-translocation is unlikely due to the absence of PDI bound to Derlin-1-YFP. It is possible that Derlin-1-YFP failed to interact with other unidentified ER components mediating toxin retro-translocation or, as our finding indicated, affected the function of endogenous Derlin-1. Not all toxins that interact with PDI in the ER rely on Derlin-1 for retro-translocation, because retro-translocation of ricin, a plant toxin that interacts with PDI (Spooner *et al.*, 2004), was not affected by dominant-negative Derlin-1 (Slominska-Wojewodzka *et al.*, 2006).

Although Derlin-1 is likely a component of the CT retro-translocation channel, the Sec61 channel has also been shown previously to mediate the transport of CT (Schmitz *et al.*, 2000). Our studies do not exclude a role of Sec61 in the transport process. Hence, it is formally possible that, after targeting to Derlin-1 via CTB, the unfolded CTA1 chain may be transferred to Sec61. Future studies are required to clarify this possibility.

The mechanistic description of the early stages of CT retro-translocation described here may be applicable to the

transport of misfolded ER substrates. Similar to CT, misfolded substrates would first target to the family of Derlin proteins, allowing the Derlin-associated chaperones such as PDI, Bip, and Yos9 (Carvalho *et al.*, 2006; Denic *et al.*, 2006; Gauss *et al.*, 2006) to prepare the substrates for translocation. Juxtaposing the ER factors that prime the misfolded substrates for retro-translocation to the channel itself enables an efficient means of transport. Of note, retro-translocation of the nonglycosylated prepro- α -factor requires PDI (Gillece *et al.*, 1999; Wahlman *et al.*, 2007) and Derlin-1 (Wahlman *et al.*, 2007). Similarly, the ER-to-cytosol transport of the murine polyomavirus also relies on PDI (Gilbert *et al.*, 2006) and Derlin-2 (Lilley *et al.*, 2006). Whether other ERAD substrates that rely on PDI for retro-translocation, such as the human β -secretase variant BACE457 (Molinari *et al.*, 2002), also use the Derlin proteins remains to be established.

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