

Cholera Toxin Up-Regulates Endoplasmic Reticulum Proteins That Correlate with Sensitivity to the Toxin

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Cholera toxin (CT) contains one A chain and five B chains. The A chain is an enzyme that covalently modifies a trimeric G protein in the cytoplasm, resulting in the overproduction of cAMP. The B chain binds the glycosphingolipid G_{M1}, the cell surface receptor for CT, which initiates receptor-mediated endocytosis of the toxin. After endocytosis, CT enters the endoplasmic reticulum (ER) via retrograde vesicular traffic where the A chain retro-translocates through the ER membrane to reach the cytoplasm. The retro-translocation mechanism is poorly understood, but may involve proteins of the ER stress response, including the ER associated degradation (ERAD) pathway. We report here that treating cells with CT or CTB quickly up-regulates the levels of BiP, Derlin-1, and Derlin-2, known participants in the ER stress response and ERAD. CT did not induce calnexin, another known responder to ER stress, indicating that the CT-mediated induction of ER proteins is selective in this time frame. These data suggest that CT may promote retro-translocation of the A chain to the cytoplasm by rapidly up-regulating a set of ER proteins involved in the retro-translocation process. In support of this idea, a variety of conditions that induced BiP, Derlin-1, and Derlin-2 sensitized cells to CT and conditions that inhibited their induction de-sensitized cells to CT. Moreover, specifically suppressing Derlin-1 with siRNA protected cells from CT. In addition, Derlin-1 co-immunoprecipitated with CTA or CTB from CT-treated cells using anti-CTA or anti-CTB antibodies. Altogether, the results are consistent with the hypothesis that the B chain of CT up-regulates ER proteins that may assist in the

retro-translocation of the A chain across the ER membrane. *Exp Biol Med* 233:163–175, 2008

Key words: cholera toxin; Derlin-1; endoplasmic reticulum associated degradation; Shiga toxin; endoplasmic reticulum stress

Introduction

Cholera toxin (CT) is an AB₅ toxin that contains an A chain (CTA) noncovalently associated with 5 B chains (CTB) arranged in a shape resembling a torus. The A chain has two domains, A1 and A2, connected by a disulfide bond. The A1 domain contains a catalytic site that covalently transfers the ADP-ribosyl moiety of NAD⁺ to the trimeric G protein G_{sα}. This covalent modification constitutively activates G_{sα}, which in turn activates adenylyl cyclase, leading to massive overproduction of cAMP. To interact with G_{sα}, the CTA1 chain must be transported through a membrane and reach the cytoplasm. The transport process begins with binding of the toxin to G_{M1} receptors on the cell surface, mediated by the B chains, followed by uptake into endocytic vesicles.

After endocytosis, CT follows a trafficking pathway that delivers both the A and B chains to the lumen of the ER, although a clear understanding of the retrograde pathway used to get there is lacking. There are arguments that the toxin may be carried to the ER either in COPI-coated vesicles or COPI-independent vesicles, or both (1–3). Once within the ER, there is evidence that the CTA1 chain retro-translocates through the ER membrane, possibly exploiting elements of the endoplasmic reticulum associated degradation (ERAD) system (4–8). To thread through a proteinaceous pore in the ER membrane, it is believed that proteins must unfold and there are several lines of evidence that ER chaperones participate in CT unfolding. The export of the CTA1 chain from ER microsomal vesicles is reported to depend on the vital chaperone protein BiP (9). Separation of the CTA1 chain from the B chain complex and release of the unfolded A1 chain involves cleaving the disulfide bond

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bridging the CTA1 and CTA2 chains by protein disulfide isomerase (5, 10). Ero1, an ER oxidase, is also reported to participate in the release of unfolded reduced CTA1 so it can engage the retro-translocation apparatus (11). The mechanism by which ER proteins cross the membrane during ERAD is not well understood, but most models invoke the protein p97 (also called valosin-containing protein, VCP) and its partners in the process (12). There is evidence both for (13) and against (14) the involvement of p97 (VCP) in CT retro-translocation.

The levels of many ER proteins, including those that may be involved in the retro-translocation of CTA1 to the cytoplasm, are regulated in response to ER stress. Three robust and related responses to ER stress are known, reviewed recently by Schröder and Kaufman (15). One is the unfolded protein response (UPR) that senses the presence of unfolded proteins in the ER and, by a combination of signal transduction mechanisms, increases the production of chaperones and other ER proteins that assist proteins to fold. A second response is the endoplasmic reticulum associated degradation (ERAD) response that induces machinery to dispose of proteins that fail to fold. The molecular mechanisms of ERAD are poorly understood, but involve retro-translocation of misfolded proteins from the lumen of the ER to the cytoplasm where they are degraded. It is this system that is often invoked to explain how CTA1 passes through the ER membrane into the cytoplasm. A third response to the chronic presence of large amounts of unfolded proteins is apoptosis, thus eliminating cells whose functions are so compromised that they are a threat to the whole organism.

If the levels of ER proteins involved in the retro-translocation of CTA1 to the cytoplasm are up-regulated, then the sensitivity of cells to CT may be increased. This leads to the hypothesis that CT itself might up-regulate ER proteins that sensitize cells to the toxin. To explore this idea, we studied here whether treating cells with CT induced the ER proteins, BiP, Derlin-1, Derlin-2, and calnexin. We found that the levels of BiP, Derlin-1, and Derlin-2 quickly increased upon exposing cells to CT or CTB, but calnexin levels were unchanged. A variety of conditions that induced BiP, Derlin-1, and Derlin-2 sensitized cells to CT and conditions that inhibited their induction de-sensitized cells to CT. Suppressing Derlin-1 alone with siRNA also protected cells from CT and Derlin-1 co-immunoprecipitated with CTA or CTB from CT-treated cells using anti-CTA or anti-CTB antibodies. Altogether, the results are consistent with the hypothesis that the B chain of CT augments retro-translocation of the A1 chain through the ER membrane by inducing ER proteins that may participate in the transport process.

Materials and Methods

Materials. Bovine serum albumin, Saponin, cycloheximide (CH) and thapsigargin were from Sigma (St.

Louis, MO). Tunicamycin (Tm) was from Calbiochem (San Diego, CA). Ricin was from Vector Laboratories (Burlington, CA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). CT, CTA1, recombinant CTB, and ETA were from List Biological Laboratories (Campbell, CA). Pentameric CTB does not dissociate to monomers in SDS in the absence of reducing agent and greater than 95% of the recombinant CTB was pentamer as tested by electrophoretic migration in polyacrylamide gels with SDS (16). Shiga Toxin was from Toxin Technology Inc. (Sarasota, FL). Antibodies to the CTA1 chain were made by injecting the purified A chain into rabbits with Freund's adjuvant under standard conditions. An IgG fraction of high titer serum was prepared by ammonium sulfate precipitation. Rabbit anti-Derlin-1 raised against the C-terminal peptide (amino acid residues 239–251) was from MBL International (Japan); additional antibodies to Derlin-1 were made by injecting KLH-conjugated peptides corresponding to residues 2–15 and 197–213 (peptides and antibodies prepared by BioSynthesis Inc., Lewisville, TX). These two Derlin-1 antisera were mixed for use in immunoblots, except for the experiment in Figure 9. Rabbit anti-Derlin-1 antibody against peptides corresponding to residues 204–216 and 238–251 was provided by Drs. Y. Ye and T. A. Rapoport (Harvard University) and was used to stain Derlin-1 in immunoprecipitation experiments (Figure 9). Rabbit anti-Derlin-2 antibodies were from MBL International (Japan), mouse anti-BiP was from BD Biosciences (San Jose, CA), and mouse anti-Calnexin was from Abcam (Cambridge, MA). HRP conjugated Bovine anti-Rabbit and Bovine anti-Mouse secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit IgG TrueBlot™ reagent (18–8816) used for immunoprecipitations was from eBioscience (San Diego, CA). Anti-GM-130 was from BD Biosciences Pharmingen (San Jose, CA). Rabbit anti-Rab6 was from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit Alexaflor 488 and Goat anti-mouse Alexaflor 568 used for immunofluorescence studies were from Molecular Probes (Eugene, OR). The ECL Western Blotting system used for detecting protein bands and the cAMP Enzyme immunoassay Biotack™ (EIA) System used to detect intracellular cAMP were from GE Healthcare (Piscataway, NJ). siGENOME SMART pool reagent (a cocktail of four different siRNAs) for Derlin-1 was from Dharmacon RNA Technologies (Chicago, IL). Oligofectamine™ reagent used for siRNA transfection was from Invitrogen (Carlsbad, CA). Trans ³⁵S label was from ICN Radiochemical (Irvine, CA).

Cells and Cell Culture. Vero and A431 cells were obtained from the American Type Culture Collection (Manassas, VA). Vero cells were the primary model cell system used in this work as they are highly sensitive to CT and have been previously used for investigating the uptake and the trafficking of CT (17–19). Vero cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Irvine

Scientific, Santa Ana, CA), with 5% FBS. A431 cells were maintained in DMEM containing 10% FBS.

Cholera Toxin Assays. Cells were seeded in 48-well culture plates at a density of 5×10^4 /well and incubated overnight. After treatment with various reagents as described in the figure legends, the cells were challenged with the given concentrations of CT for the indicated times. Next, the cells were lysed and intracellular cAMP levels were directly measured with an enzyme immunoassay system according to the instructions provided by the manufacturer (GE Healthcare).

Protein Synthesis Inhibition Assays. Protein synthesis was measured by the incorporation of radioactivity from Trans ^{35}S label into acid-insoluble protein essentially as described previously (20). Cells were treated with the indicated toxins as described in figure legends, Trans ^{35}S label (6 $\mu\text{Ci}/\text{ml}$) was added for 10 min, the cells were washed, lysed, and the lysate was spotted within squares defined by gridlines drawn on filter paper. The filter paper was incubated in 5% trichloroacetic acid containing 0.5 mg/ml methionine for 30 min at room temperature, washed twice for 5 min in 100% ethanol, and dried. Radioactivity within each square of the grid was measured with a PhosphorImager using volume quantization (Amersham Biosciences).

Preparation of Microsomes. Cells were seeded in 100 mm culture plates at a density of 3×10^7 cells/plate and exposed the next day to various treatments as described in figure legends. To prepare microsomes, the cells were washed three times with cold PBS and once with homogenization buffer (0.25 M Sucrose, 10 mM TEA, 1 mM EDTA, and pH 7.4); 500 μl of homogenization buffer with 0.5 mM phenylmethylsulfonyl fluoride was added to each culture plate; the cells were scraped from the dishes, counted and homogenized with a metallic dounce homogenizer. A post nuclear supernatant was prepared by centrifuging the cells at 3000 g for 20 min. The post nuclear supernatant was centrifuged at 15,000 g for 30 min and the resulting supernatant was centrifuged at 100,000 g for 1 h to obtain the microsomal fraction. The pellet was washed and resuspended in homogenization buffer and centrifuged again for 45 min at 100,000 g. The microsome preparation was assessed by the recovery of the ER protein calnexin, estimated by immunoblotting. From the post nuclear supernatant to the microsomal fraction, calnexin recovery was virtually 100% while the protein concentration was reduced by ten-fold. Microsomes were lysed in lysis buffer (0.5% Triton X-100, 30 mM HEPES, 100 mM NaCl, 5 mM MgCl_2 , EDTA-free protease inhibitors, pH 7.4) for 30 min at 4°C. The lysed microsomal suspension was vortexed gently and then was centrifuged again at 100,000 g for 45 min to remove any un-lysed microsomes. The resulting supernatant was used in immunoblot analyses or immunoprecipitations.

Immunoblotting. Proteins were reduced with DTT and equal amounts of microsomal protein were loaded onto

a 12% SDS-PAGE gel. After electrophoresis, proteins were transferred to PDVF membrane and blocked overnight in PBS containing 5% nonfat milk and 0.5% Tween 20 (PBS-T). The next day, the appropriate lanes of the membrane were cut and blotted with various antibodies for 1.5 h and washed with PBS-T for 15, 5, 5, and 5 min. The membranes were then incubated for 1 h in the HRP-conjugated secondary antibody and again washed as after the primary antibody. The membrane was developed with the ECL Western Blotting system using the manufacturer's directions. Band intensities were quantified by phosphor-imaging. The dependence of the Derlin-1 signal on the amount of Vero cell microsomal protein that was applied to gels in controls was tested numerous times throughout the course of these experiments and was in the linear range between 0.05 and $\sim 4 \mu\text{g}$ of applied protein.

Immunoprecipitation. Cells were incubated with CT (1 $\mu\text{g}/\text{ml}$) for 3 h at 37°C in serum-free media, microsomes were prepared as described above, and solubilized in lysis buffer. Anti-CTA1, anti-CTB, anti-calnexin, or control antibody was preincubated with the microsomal lysate for 1 h at 4°C, anti-rabbit Ig IP beads (eBiosciences) were added and the mixture was incubated for an additional 2 h at 4°C. The beads were washed thoroughly and removed by centrifugation. Proteins in the supernatant and bead pellet were reduced with DTT, electrophoresed by SDS-PAGE, transferred to PVDF membrane, and treated with primary antibodies as previously described for immunoblotting. Since the molecular weight of the immunoglobulin light chain is similar to that of Derlin-1, the light chain would interfere with the Derlin-1 bands in the immunoprecipitation samples after SDS-PAGE. Therefore, TrueBlotTM HRP conjugated-goat-anti-rabbit antibody (eBiosciences) that detects only the intact and not the reduced form of antibody was used as the secondary, thus eliminating interference caused by the immunoprecipitating light and heavy chains. As a control, CTA1 was added for 30 min at 4°C to microsomes from cells not treated with CT, followed by microsome lysis, to check for possible interaction of CTA1 with target proteins after release from microsomes.

Immunofluorescence Microscopy. Vero cells (plated the previous day at 30,000 cells on 12 mm glass coverslips in 4-well dishes) were incubated with CT or CTB at 5 $\mu\text{g}/\text{ml}$ in 5% BSA DMEM-FBS for 60 min at 37°C. The cells were fixed with 4% w/v paraformaldehyde for 10 min and permeabilized with 0.1% w/v saponin in PBS for 10 min on a shaker. Cells were treated with 1% w/v BSA for 10 min on a shaker to block possible non-specific antibody reactions. Primary antibody treatment was with rabbit anti-Derlin-1 (MBL anti-Derlin-1) at 1:100 v/v in 1% w/v BSA in PBS and monoclonal anti-GM130 at 1:400 v/v in 1% w/v BSA in PBS for 10 h at 4°C. This was followed by washing with PBS and 10 min blocking on the shaker with 1% w/v BSA. Secondary antibody treatment was goat anti-rabbit Alexaflor 488 at 1:100 v/v in 1% w/v BSA and goat anti-

mouse Alexaflor 568 at 1:400 v/v in 1% w/v BSA for 10 h at 4°C. The stained cells were washed with water, were treated with 0.1 µg/mL DAPI for 10 min, and the cover slips were mounted with Fluoromount G.

Images were taken under a 60× oil immersion lens (NA 1.45) on a Nikon Eclipse TE2000-U wide field microscope with a Photometrics Cascade 512B digital camera. Derlin-1 and GM-130 images were obtained with a 5 ms exposure. For Derlin-1, regions of interest were defined in Metamorph software by drawing an outline of each cell with an indentation that excluded the nucleus and the average fluorescence intensity (in arbitrary units) of the region of interest was obtained. GM130 regions of interest were defined as the visible Golgi for each cell measured. Derlin-1 and GM130 regions were taken from the same cell. The background from each field was measured in an area where there were no cells and subtracted from the fluorescence intensities of the cells in that field. The background-corrected average fluorescence intensities of the cells in each experimental condition were averaged to yield the final values. The data sets for untreated control cells and cells treated with CT were from 3 separate experiments, each with five fields studied where 5 cells per field were analyzed for a total of 75 cells per condition. For cells treated with CTB, the data set was from 4 experiments, each with 5 fields studied, 3 cells per field for a total of 60 cells.

Derlin-1 Suppression. Vero cells were used in this study because they are very sensitive to CT. The genome for the African green monkey (from which Vero cells are derived) was not available; therefore, we searched the PubMed data bases of the human, bovine, chimp and mouse genomes to look for Derlin-1 cDNA sequences that were conserved in all four genomes. Since Derlin-1 is a relatively conserved protein among these species, we used the Smart Pool siRNA cocktail from Dharmacon designed for human Derlin-1, reasoning that the probability of the region being conserved in the African green monkey would be very high. Two siRNAs among the four in the original cocktail that gave the maximum suppression were mixed and used for further experiments. The sequences of these two siRNAs are 3'-GAACAGAGACAUGAUUGUAUU and 3'-GAUAUG-CAGUUGCUGAUGAUU. Vero cells at a density of 5×10^4 cells/ml were transfected with a cocktail of 15 nM of each of the two siRNAs with Oligofectamine™ Reagent using the manufacturers protocol (Invitrogen). As an additional control, an irrelevant siRNA against Rab6a/c (5'-CAGAAAGAGGAAGUGAUGU) was used as a non-specific siRNA sequence to check for nonspecific suppression of Derlin-1 or other proteins in our system. After 72 h the cells were harvested and tested for Derlin-1 suppression by immunoblotting using Derlin-2, calnexin, and Rab6a as control proteins. During and after exposure to siRNA, the cells displayed a normal morphology, suggesting that the procedure did not damage the cells. For cAMP assays, the cells were transfected in 48-well plates and cAMP levels were determined as described earlier. Control cells in cAMP

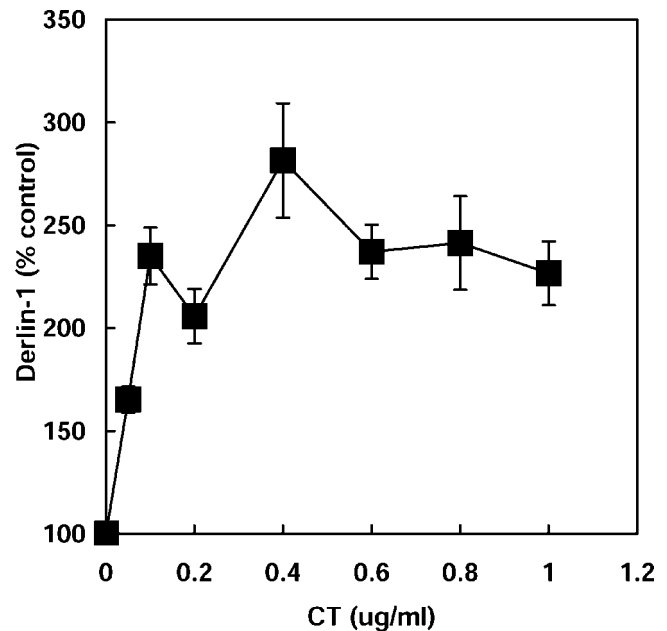


Figure 1. Derlin-1 is up-regulated as a function of CT dose. Vero Cells were incubated with the indicated concentration of CT for 45 minutes at 37°C. Microsomes were prepared and immunoblot analysis for Derlin-1 was as described in Materials and Methods. Error bars show the standard error of the mean for four experiments. Error bars are absent where the symbols were larger than the bars.

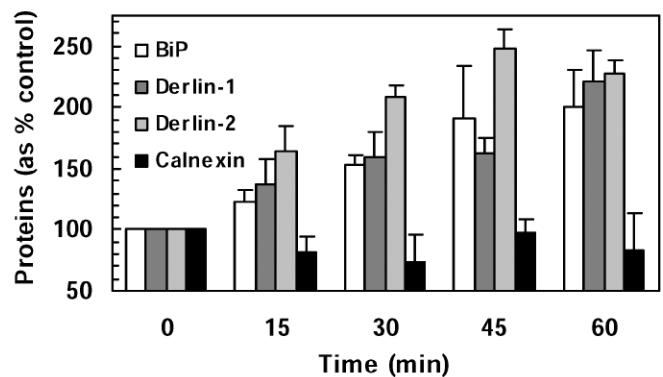
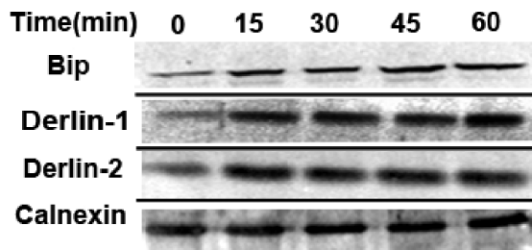
assays were mock treated with Oligofectamine™ but without siRNA, or were treated with siRNA that had a scrambled sequence unrelated to Derlin-1. Neither siRNA nor control treatments non-specifically reduced protein levels, judged by band intensities of control proteins after SDS-PAGE.

Results

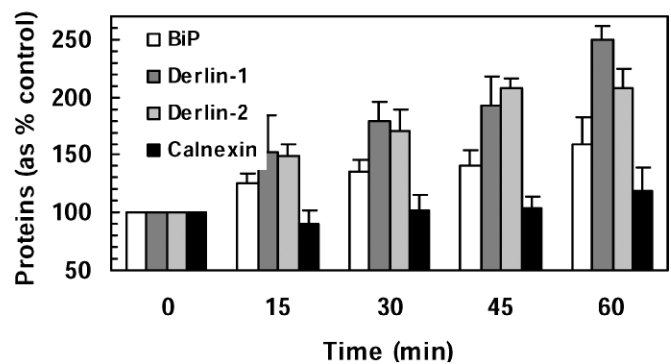
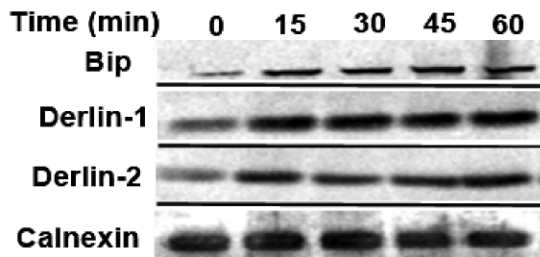
CT Rapidly Induces BiP, Derlin-1, and Derlin-2. Derlin-1 is an integral membrane protein of the ER that is up-regulated by ER stress and is required for ERAD of certain substrates (21, 22). To explore the possibility that CT induced Derlin-1, Vero cells were exposed to different concentrations of CT for 45 min, and the Derlin-1 levels were measured by immunoblotting. Derlin-1 increased as a function of CT concentration and by ~0.5 µg/ml of CT the increase in Derlin-1 leveled off at just over twice the amount found in untreated cells (Fig. 1). These data suggest that CT up-regulates Derlin-1 in a dose dependent manner.

The rate at which CT up-regulated Derlin-1 was assessed by treating cells for different times with the toxin, followed by immunoblot analysis. We also compared the effect of CT on the levels of the ER proteins Derlin-2, BiP, and calnexin. BiP was chosen because it is integral to the ER stress response (15, 23) and is up-regulated early when a UPR is induced (24). Treating cells with a combination of CT and phorbol myristate acetate induces BiP within 4 h (25), but to our knowledge, an effect of CT alone at earlier time points has not been reported. Derlin-2 is related to Derlin-1

A



B



C

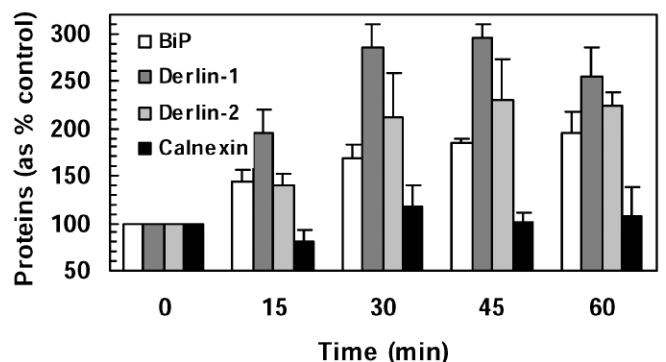
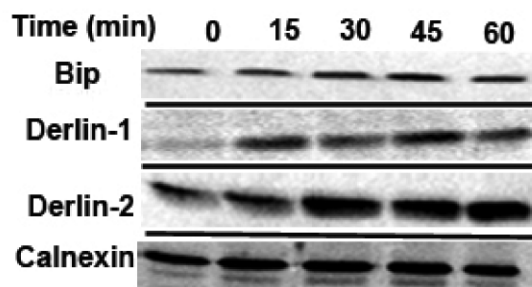
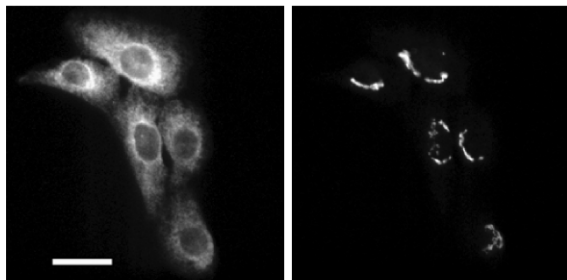


Figure 2. CT, Tm, and CTB rapidly up-regulate BiP, Derlin-1 and Derlin-2. Vero cells were treated with 1 µg/ml (11 nM) of CT (A), 10 µg/ml of Tm (B), or 0.6 µg/ml (10 nM) of recombinant CTB (C) for the indicated times. Microsomes were prepared, lysed, and subjected to SDS-PAGE. After transfer to a PVDF membrane, proteins were identified by immunoblotting with anti-BiP, anti-Derlin-1, anti-Derlin-2 or anti-calnexin antibodies and the levels quantified as described in Materials and Methods. Each experiment was done three times and one representative composite image for each treatment is shown on the left. Horizontal lines separate images that were either from separate gels, or from separate areas of the same gel. The mean band intensities (error bars show the standard deviation from the mean) from three independent experiments for each protein are shown in the bar graphs on the right for each time point. The statistical significance of the increase in the proteins with reference to the zero time point was assessed by carrying out analysis of variance using the F test. The probability that increases in BiP, Derlin-1, and Derlin-2 over time was due to chance is less than 0.005. The difference in calnexin levels over time was not statistically significant.

and is also involved in ERAD (21, 22, 26), although it may function differently than Derlin-1 (26). Calnexin, an ER membrane protein, was chosen because it participates in the calnexin/calreticulin chaperone cycle by binding to glucose residues on glycoproteins (27), and there is no reason to

predict a relationship between CT and calnexin because CT is not normally glycosylated (28). Immunoblotting revealed that BiP, Derlin-1, and Derlin-2 levels increased within 30 min of CT addition and were elevated between about 1.5-fold and 2.5-fold within 60 min, whereas calnexin was not

A



B

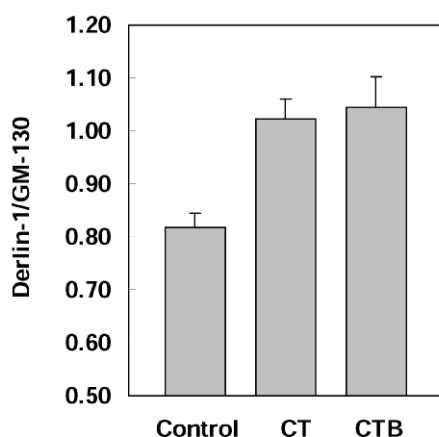
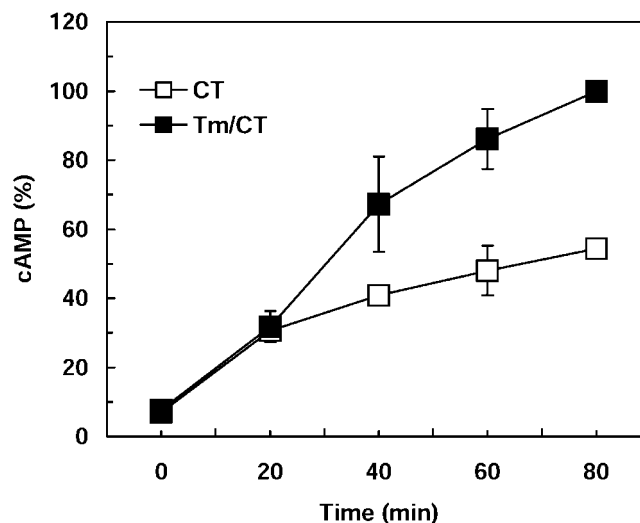


Figure 3. The effect of CT and CTB on Derlin-1 levels measured by immunofluorescence microscopy. Vero cells were either untreated or exposed to CT or CTB (5 μ g/ml) for 60 min at 37°C, fixed, and stained for dual color immunofluorescence with antibodies to Derlin-1 and GM-130 as described in Materials and Methods. (A) Image example of staining in control cells for Derlin-1 (left) and GM-130 (right). The scale bar is 30 microns. (B) The ratio of the Derlin-1 signal to the internal GM-130 control for each cell was analyzed as described in Materials and Methods. Bars show the mean ratios plus the standard error of the mean. Student's unpaired t test returned a value of 4.45 for comparing the control with CT-treated samples and a value of 4.29 for comparing the control with CTB-treated samples. The probability that the differences in these mean values are due to chance is less than 0.0001.

induced by 60 min (Fig. 2A). Note that calnexin also serves as an internal control for the amount of microsomes loaded on the gels because it was approximately constant over the time course of the experiments.

Tunicamycin (Tm) is known to induce many ER proteins, and we compared the effects of Tm with CT on the up-regulation of BiP, Derlin-1, Derlin-2, and calnexin over the same time course as used with CT in Vero cells. The effects of Tm were very similar to those of CT: the levels of BiP, Derlin-1, and Derlin-2 increased whereas calnexin did not (Fig. 2B). Thus, the rapid up-regulation of several key ER proteins by CT is similar to that of Tm, a known ER stressor.

A



B

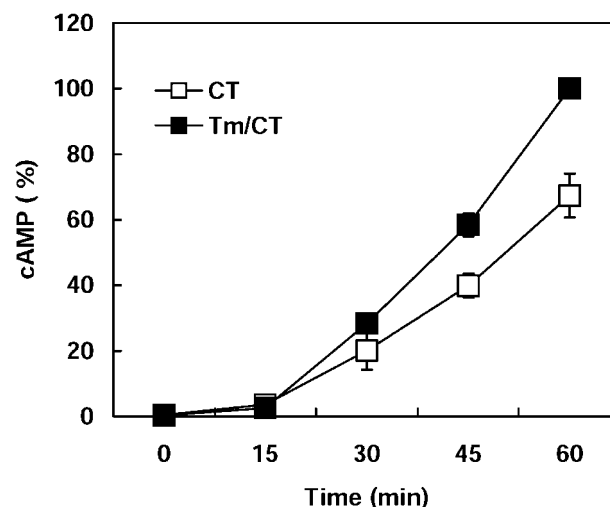


Figure 4. Tm sensitizes cells to CT. Vero cells (A) or A431 cells (B) were either untreated or exposed to 10 μ g/ml of Tm for 45 min, followed by addition of 2 μ g/ml (22 nM) of CT for the indicated times. Cells were lysed and the intracellular cAMP levels were measured. The maximum response in each experiment was taken as 100%. Error bars represent standard deviations of three independent experiments. Error bars are absent where the symbols were larger than the error bars. The statistical significance of the data at each time point was assessed by analysis of variance using the F test. With Vero cells (A), the probability that the differences between the two cAMP average values at 40, 60, and 80 min were due to chance is less than 0.001. With A431 cells (B), the probability that the differences between the two cAMP average values at 30, 45, and 60 min were due to chance is 0.007 or less.

CTB Alone Rapidly Induces BiP, Derlin-1, and Derlin-2. The ability of CT to selectively induce ER proteins could be associated with the CTA chain, the CTB chain, or both. To see if CTB was sufficient to evoke the

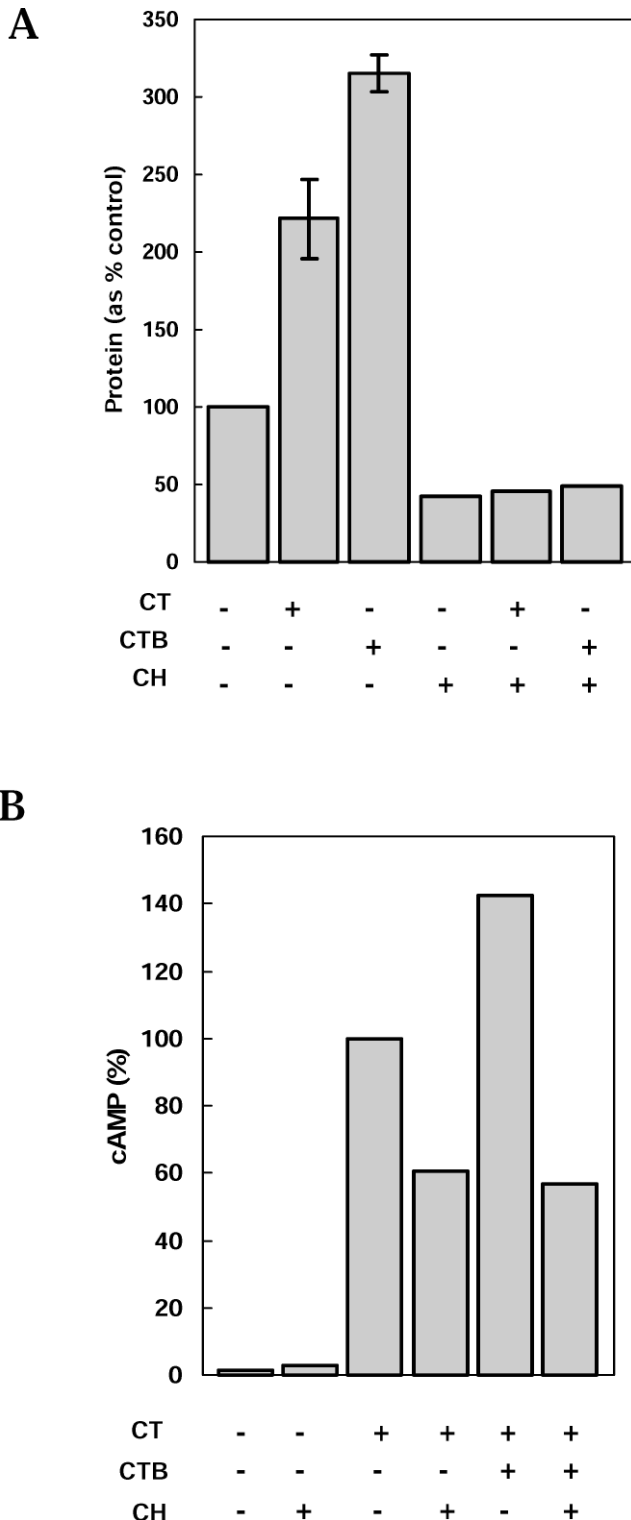


Figure 5. Cycloheximide protects cells against CT or CTB induced CT sensitization. (A) Vero cells were either untreated or exposed to 10 μ g/ml of cycloheximide for 40 min followed by addition of either 2 μ g/ml (22 nM) of CT or 2 μ g/ml (33 nM) of CTB for 60 min as indicated. Microsomes were prepared and analyzed by immunoblotting with Derlin-1 antibodies. Derlin-1 levels in control cells with no treatment were set at 100%. Error bars represent standard deviations of three independent experiments. (B) Vero Cells were either untreated or exposed to 10 μ g/ml of CH for 40 min as indicated and challenged with either 2 μ g/ml CT or a mixture of 2 μ g/ml each of

response, cells were exposed to recombinant CTB in the absence of the A chain and the levels of BiP, Derlin-1, Derlin-2, and calnexin in the ER were measured. The results were similar to those observed with intact CT: the amounts of BiP, Derlin-1, and Derlin-2 increased, whereas calnexin did not change (Fig. 2C), indicating that the up-regulation of these proteins does not require the CTA chain, and is therefore not a consequence of increased levels of cAMP in the cells catalyzed by the A chain.

To determine whether the increase in BiP and Derlin-1 levels in response to CTB occurred in a cell line other than Vero cells, A431 cells (a human kidney cell line) were exposed to CTB for an hour followed by assessing Derlin-1 and BiP in microsomes by immunoblotting. In triplicate measurements, Derlin-1 and BiP increased by $3.7 \pm .4$ times and $2.1 \pm .4$ times, respectively, compared to untreated controls. Thus, the induction of Derlin-1 and BiP by CTB is not a response restricted to Vero cells.

The up-regulation of Derlin-1 by CT and CTB with Vero cells was also observed using immunofluorescence microscopy. Cells were exposed to either CT or to CTB for 60 min, fixed, and stained with both anti-Derlin-1 and anti-GM-130. GM-130 is a Golgi-associated protein and serves as an internal fluorescent control. Control cells were untreated with toxin. The ratio of the Derlin-1 signal to the GM-130 signal in the regions of interest was determined, as described in Materials and Methods. Relative to the internal control GM-130, Derlin-1 was increased by both CT and CTB compared to untreated cells (Fig. 3). The average increase in the Derlin-1/GM-130 ratio in CT or CTB treated cells was significant by Student's t-test (see legend to Fig. 3). Thus, the ability of CT and CTB to elevate Derlin-1 was observed with both isolated microsomes and cells. It is not presently clear why the fold increase in Derlin-1 as assessed in immunoblots was more than the increase seen with immunofluorescence. The difference may result from difficulties in the antibody accessing all the Derlin-1 in fixed cells using the immunofluorescence approach.

BiP, Derlin-1 and Derlin-2 Levels Correlate with Sensitivity to CT and Shiga Toxin. Since BiP, Derlin-1, and Derlin-2 could be involved in the process by which CTA1 escapes the ER, up-regulating these proteins could conceivably augment CTA1 transport to the cytoplasm and enhance the sensitivity of cells to CT. Since Tm rapidly induced all three proteins in Vero cells, we compared cAMP production in CT-treated cells in the presence and absence of Tm and found that the rate of cAMP production was increased within 40 minutes of Tm treatment (Fig. 4A). A431 cells were also sensitized to CT by Tm, indicating that the effect was not restricted to Vero cells (Fig. 4B).

CT and CTB for 60 min. The cells were lysed and the intracellular cAMP levels were measured. The cAMP level for CT was taken as 100%. Results are the average of duplicate samples.

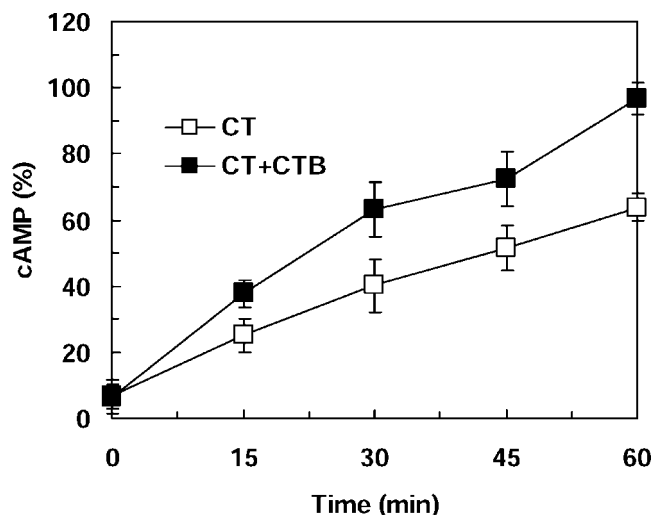


Figure 6. CTB sensitizes cells to CT. Vero cells were treated with either 29 nM of CT or a mixture of 29 nM of CT and 23 nM of CTB pentamer for the indicated times. The cells were lysed and the intracellular cAMP levels were measured. The maximum response in each experiment was taken as 100%. Error bars represent the standard deviation of the average of three independent experiments. The statistical significance of the data at each of the time points was determined by the analysis of variance technique. The probability that the differences between the two cAMP average values at 30, 45, and 60 min was due to chance is less than 0.003 as revealed by the F test.

If up-regulating BiP, Derlin-1, and Derlin-2 sensitizes cells to CT, then blocking mRNA translation of these proteins with cycloheximide (CH) should block the contribution they make to enhancing CT action, thus partially protecting cells from CT. As a control to test this, we first confirmed that treating cells with CH blocked the induction of Derlin-1 by both CT and CTB: Derlin-1 levels went up in cells treated with CT or CTB, but went down when CH was present (Fig. 5A). When cells were pretreated with CH, the production of cAMP in response to CT was reduced (Fig. 5B), as predicted. We also challenged Vero cells with equimolar amounts of CTB and CT to compare the effect of the CTB on cAMP production by CT. The presence of CTB increased the rate of cAMP production (Fig. 6), despite the fact that CTB should have reduced cAMP production by competing with CT for G_{M1} binding to the surface. Moreover, the presence of CH prevented the enhanced activity of CT in CTB treated cells (Fig. 5B). While several potential mechanisms could underlie this effect of CTB, it could be explained by elevated induction of ER proteins by CTB, leading to more efficient delivery to the cytoplasm of the A chain carried by CT that was co-internalized with CTB.

If the ER proteins upregulated by CT enhances CT activity, it may also enhance the activity of other toxins that transit through the ER en route to the cytoplasm, such as Shiga toxin, *Pseudomonas aeruginosa* Exotoxin A (ETA), and ricin. Treating cells with CTB increased the sensitivity of Vero cells to Shiga toxin (Fig. 7A), but not to ETA (Fig.

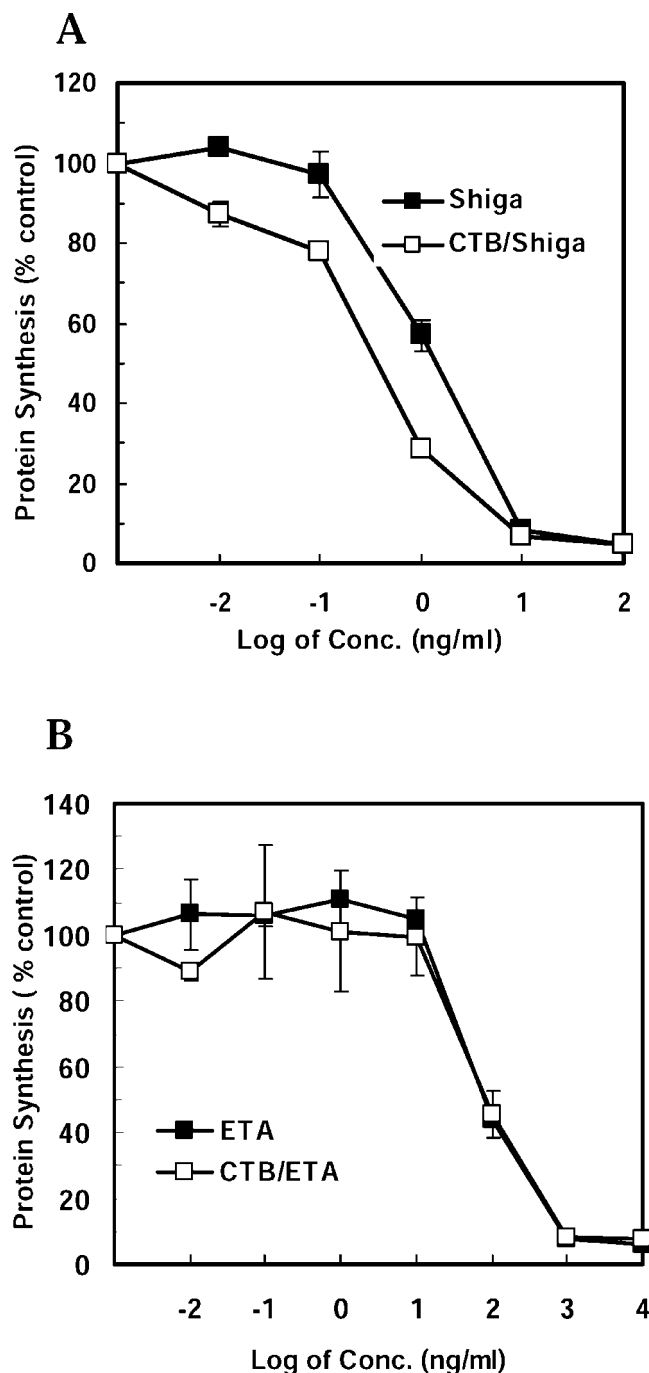


Figure 7. CTB sensitizes cells to Shiga Toxin but not ETA. Vero Cells were pre-treated with 2 μ g/ml (33 nM) of recombinant CTB chain for 30 min, then treated with either increasing concentrations of Shiga toxin (A) or ETA (B) for 1.5 h. Trans 35 S-label was added for 10 min and the incorporation of radioactivity into acid-insoluble material was determined. Error bars show the standard deviation from the mean for triplicate experiments. Where error bars are not visible, they were smaller than the symbols. The F test was used to assess the statistical significance of the data at each concentration by the analysis of variance technique. In graph A, the probability that the differences between the two % protein synthesis average values at 10^{-2} , 10^{-1} , and 1 ng/ml were due to chance is less than 0.001. In graph B, the differences between the two % protein synthesis average values were not significant at any concentration.

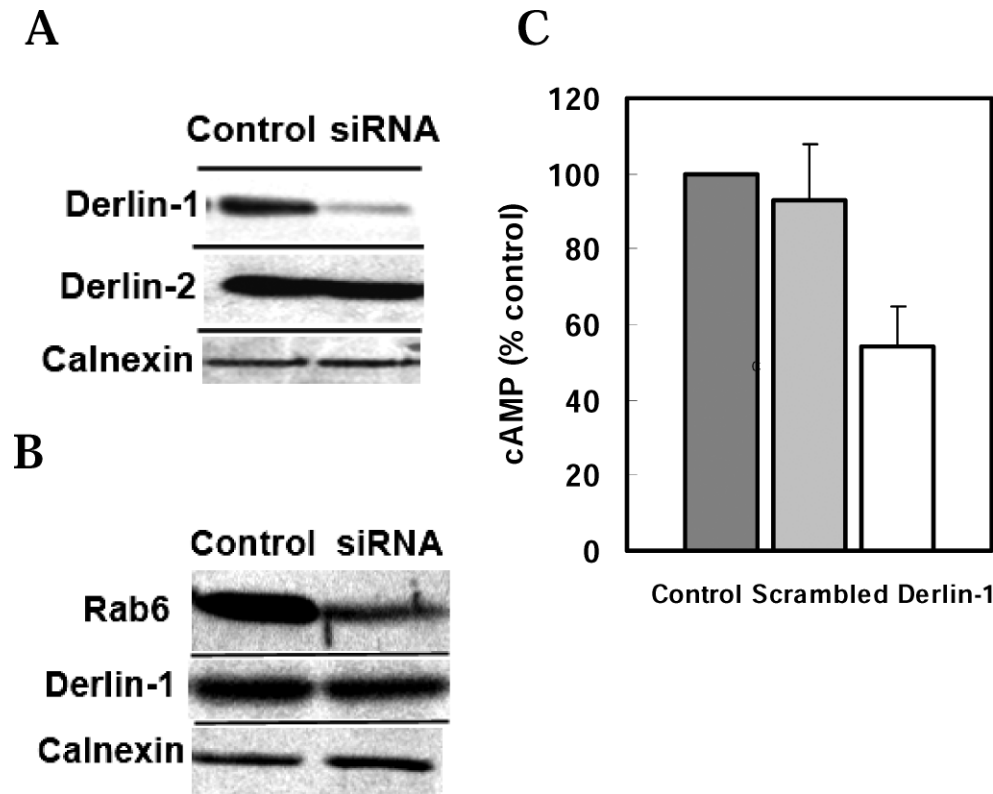


Figure 8. Derlin-1 suppression protects cells against CT. (A) Vero cells were transfected with Derlin-1 siRNA using Oligofectamine™ reagent. After 72 h, cells were lysed and analyzed by immunoblotting with Derlin-1 antibodies to check for suppression. Derlin-2 and Calnexin were used as controls. (B) As an additional control, cells were transfected with siRNA to Rab6a/c and the levels of Rab6a, Derlin-1 and calnexin were assessed. Rab6a declined, but Derlin-1 and calnexin were unchanged, indicating that a non-specific siRNA did not affect Derlin-1 levels. (C) Vero cells were transfected with Derlin-1 siRNA as above or with a scrambled siRNA unrelated to Derlin-1 or with no siRNA. After 72 h, 2 µg/ml (22 nM) of CT was added to cells for 60 min. Cells were lysed and cAMP levels were determined. Error bars show the standard deviation from the mean for triplicate experiments.

7B). Ricin was also not significantly affected by pre-treating the cells with CTB (data not shown). Shiga toxin is an AB₅ toxin, like CT, whereas ETA and ricin are AB toxins. Thus, the ability of CTB to enhance the effect of toxins may be restricted to toxins of the AB₅ family.

siRNA Suppression of Derlin-1 Inhibits CT and Derlin-1 Co-immunoprecipitates with Both CTA1 and CTB. In the preceding section, several conditions that induce ER stress-related proteins were found to sensitize cells to CT whereas conditions that prevent induction de-

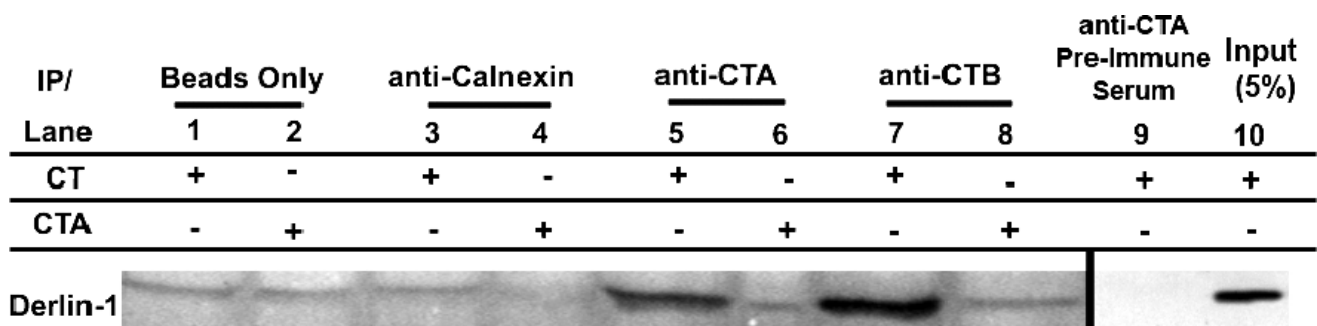


Figure 9. CTA1 and CTB co-immunoprecipitate with Derlin-1. Vero Cells were treated without or with 1 µg/ml (11 nM) of CT for 3 h at 37°C and microsomes were prepared. For lanes 2, 4, 6 and 8 microsomes from non-toxin treated cells were lysed in the presence of CTA and were incubated for 30 min at 4°C. Immunoprecipitations were performed with beads only (lanes 1 and 2), anti-calnexin (lanes 3 and 4), anti-CTA1 (lanes 5 and 6), anti-CTB (lanes 7 and 8) and anti-CTA1 pre-immune serum (lane 9). 5% of the input sample was run in lane 10. All samples were electrophoresed in a 12.5% reducing SDS-PAGE gel and immunoblotted with Derlin-1 antibodies and TrueBlot™ HRP conjugated goat-anti-rabbit secondary antibody.

sensitized cells. To see whether this correlation could be extended to specifically reducing Derlin-1 levels in cells, we suppressed Derlin-1 with siRNA and measured the effect on CT. Vero cells were treated with Derlin-1 siRNA for 72 h and immunoblotting revealed that Derlin-1 was suppressed approximately 80% (Fig. 8A). Derlin-2 and calnexin, controls in this experiment, were not suppressed, evidence that the observed suppression of Derlin-1 was specific. Rab6 suppression did not affect the levels of Derlin-1 or calnexin (Fig. 8B) confirming again the specificity of the Derlin-1 siRNA suppression. As shown in Fig. 8C, Derlin-1 suppression inhibited the production of cAMP in CT-treated cells by almost 50% after a one hour exposure to the toxin. A partial inhibition of CT action is consistent with the fact that there is still residual Derlin-1 in the ER after siRNA treatment that may contribute to CT processing or retro-translocation. Thus, the sensitivity of cells to CT correlates with Derlin-1 levels, even when Derlin-1 is specifically suppressed.

Given that CT induced Derlin-1 and that suppression of Derlin-1 protected cells from CT, we determined whether Derlin-1 co-immunoprecipitated with CTA1 or CTB from CT-treated cells using anti-CTA1 or anti-CTB antibodies. Microsomes were prepared from cells that had been incubated with or without CT and lysed. Prior to lysis, CTA1 was added to the microsomes from cells not treated with CT as a control to ensure that any possible interaction between CTA1 and Derlin-1 did not occur after the proteins were released by lysis of microsomes. Derlin-1 was not recovered in immunoprecipitations when only anti-rabbit IgG beads were used, or when the precipitating antibody was anti-calnexin (Fig. 9 lanes 1–4). Derlin-1 was co-precipitated with anti-CTA1 from CT-treated cells (Fig. 9 lane 5); however, Derlin-1 was not recovered when CTA1 was added to microsomes from untreated cells prior to lysis (Fig. 9 lane 6), demonstrating that CTA1 did not interact with Derlin-1 after release from microsomes. Derlin-1 was also recovered from cells treated with CT when the precipitating antibody was anti-CTB, but not in cells that received no CT but did have CTA1 present before microsome lysis (Fig. 9 lanes 7 and 8). Derlin-1 was not immunoprecipitated with pre-immune serum (Fig. 9 lane 9), providing additional evidence for the specificity of the immunoprecipitation system. Figure 9, lane 10, shows the Derlin-1 band from a sample containing 5% of the total input used in the immunoprecipitations. Thus, the amount of Derlin-1 immunoprecipitated by anti-CTA1 or anti-CTB represents only a few percent of the total microsomal Derlin-1. The interaction of CTA1 and CTB with Derlin-1 may foretell a role for Derlin-1 in either the induction of Derlin-1 by CT, or the process by which CTA1 escapes the ER, or both.

Discussion

The work in this paper had two main objectives. The first was to determine whether any ER proteins were up-

regulated by CT. The second was to assess the possibility that proteins up-regulated by CT might contribute to the retro-translocation of the CTA1 chain across the ER membrane, thus enhancing CT activity. Regarding the first objective, multiple lines of evidence suggest that CT rapidly up-regulates several ER proteins. CT increased Derlin-1 levels in Vero cells as a function of toxin concentration by a maximum of about two-fold in 45 minutes. The dose of CT that induced Derlin-1 to half-maximum levels was about 0.1 $\mu\text{g/ml}$, similar to published data on the CT dose that increases the production of cAMP by 50% in Vero cells (29). As a function of CT exposure time, BiP, Derlin-1, and Derlin-2 reached levels approximately 1.5-fold to 2.5-fold higher than in uninduced cells within 60 min of toxin addition. A fourth ER protein, calnexin, did not increase in this time frame, suggesting that elevated levels of the other three proteins were not a generalized response. BiP, Derlin-1, and Derlin-2 increased in Vero cells treated with recombinant CTB at a rate that was similar to that seen with the holotoxin, indicating that the enzymatic activity of the CTA1 chain, and hence elevated levels of cytosolic cAMP, were not necessary to induce the proteins. The rate and extent of up-regulation of BiP, Derlin-1, and Derlin-2 by CT and CTB was comparable to that seen with Tm. The increase in BiP and Derlin-1 levels in response to CT, CTB, or Tm was also observed in A431 cells, demonstrating that the effect was not specific to the Vero cell line. Moreover, up-regulation of Derlin-1 was verified by an independent method, immunofluorescence microscopy. These data argue that the CTB chain, either alone or in the intact toxin, quickly up-regulates at least 3 ER proteins, BiP, Derlin-1 and Derlin-2, that could be involved in delivery of the CTA1 chain to the cytosol. Interestingly, preliminary experiments to follow the levels of Derlin-1 and Derlin-2 exposed to CTB for times longer than an hour suggest that they begin to decline by 3 hours (G. Dixit and R. Draper, unpublished results).

The transduction mechanism used by CT, CTB, and Tm to quickly up-regulate BiP, Derlin-1, and Derlin-2 is not clear. The induction of ER proteins in response to unfolded proteins and ER stress by transcriptional mechanisms involving the inositol requiring kinase 1 (IRE1) and activating transcription factor 6 (ATF6) are well known (15, 23). However, the rapid up-regulation we observed here is probably too fast to be explained by a transcriptional mechanism, suggesting that translational control may underlie the induction. For example, the induction of BiP within 10–20 minutes after thapsigargin addition to cells was attributed to translational control (24). The mechanism of translational up-regulation of ER proteins is not completely understood and there may be more than one pathway operating. Gulow *et al.* (2002) suggested that rapid up-regulation of BiP was due to increased ribosome transit that enhanced BiP translation efficiency. A second well-studied mechanism is the activation of PERK kinase by ER stress, which rapidly phosphorylates eIF2 α , inhibiting cap-

dependent initiation of protein synthesis. However, many proteins, including BiP, contain internal ribosome entry sites that permit and sometimes even increase the rate of translation using the alternative initiation sites (30–32). A third conceivable mechanism for rapid translational up-regulation is the recently discovered mRNA degradation activity of IRE1 upon stress induction, which opens the possibility that some mRNAs may be less sensitive to degradation than others, leading to preferential synthesis (33). It is also interesting to note that CTB increases free calcium by both intracellular store release and external calcium uptake (34) and that G_{M1} in the ER up-regulates BiP and calcium release from the ER, causing ER stress (35). Thus, another model for how CT and CTB up-regulate ER proteins is that they upset ER Ca^{2+} homeostasis by relocating G_{M1} from the plasma membrane to the ER. A better understanding of how CT and CTB rapidly induce BiP, Derlin-1 and Derlin-2 awaits further study.

The second objective of this work was to assess whether proteins up-regulated by CT might contribute to the translocation of the CTA1 chain across the ER membrane. In one approach to this, we first determined what effect Tm had on the sensitivity of cells to CT. Tm increased the rate of cAMP production in both Vero and A431 cells treated with CT by approximately 1 to 2% per minute. Because Tm causes an early up-regulation of several proteins similar to that seen with CT and CTB, it is reasonable to suggest that this sensitization is related to one or more ER proteins that are up-regulated. Supporting this idea is the observation that thapsigargin, another well-studied ER-stressor, is also known to rapidly sensitize A431 cells to CT (36). Thapsigargin depletes ER Ca^{2+} stores by inhibiting the Ca^{2+} -ATPase responsible for Ca^{2+} accumulation in the ER (37), a mechanism distinct from that of Tm. Thus, two drugs that induce ER stress proteins in different ways have a similar effect on sensitizing cells to CT, consistent with the idea that the common denominator underlying their effects on CT is the up-regulation of ER proteins. More direct evidence that a specific ER protein augments CT action comes from studies with Derlin-1. Derlin-1 levels almost doubled within 60 minutes of CT or CTB addition to cells. Inhibiting protein synthesis blocked the induction of Derlin-1 by CT and CTB, and this block correlated with reduced CT activity, measured by cAMP production. Inhibition of CT action by protein synthesis inhibitors has been noted before (38). Suppression of Derlin-1 levels by siRNA also inhibited the production of cAMP by CT. Thus, under several conditions of exposure to CT, when Derlin-1 levels increased, the rate of cAMP production went up, and when Derlin-1 levels decreased, the rate of cAMP production went down. These observations are consistent with the hypothesis that induction of Derlin-1 by CT facilitates CTA1 transport to the cytoplasm.

There is no evidence for a direct role of Derlin-1 in CTA1 retro-translocation through the ER membrane; however, Derlin-1 co-immunoprecipitated with CTA1 and

CTB, whereas another ER protein, calnexin, did not. This argues that Derlin-1 and CT either bind one another, or are components of a larger complex, even if they may not directly interact. It is also interesting to note that p97 co-immunoprecipitates with CTA (13) and that Derlin-1 is a component of a larger complex that includes not only p97, but also VIMP and other proteins involved in retro-translocating substrates through the ER membrane (21, 22, 39, 40).

Shiga toxin is an AB₅ toxin that binds the glycolipid globotriaosyl ceramide and traffics to the ER (41, 42). The Shiga toxin B subunit also binds BiP (43), suggesting that up-regulating BiP could influence the sensitivity of cells to Shiga toxin. Indeed, we found that simultaneously treating cells with CTB and Shiga toxin did sensitize cells to the toxin. Interestingly, CTB did not enhance the cytotoxicity of ETA or ricin, which are AB toxins containing a single B and A chain. It is possible that AB₅ and AB toxins have different requirements with respect to ER proteins used in toxin delivery to the cytoplasm. This notion is supported by the recent observation that suppressing Derlin-1 and Derlin-2 did not impair the cytotoxic activity of ricin (44).

The data presented here suggest that CTB has a function beyond that of a passive carrier in delivering CTA1 to the ER. Similarly, it is clear that CTB has a variety of immunological and cell signaling activities that are not related to the delivery of CTA1 to cells (2, 45). For example, CTB activates B cells (46), inhibits proliferation of CD8⁺ T cells (47), and inhibits the activation of CD4⁺ T cells by an NF- κ B-dependent pathway (48). The mechanisms underlying these activities are complex, and the observation here that CTB induces certain ER proteins may contribute to the effects of CTB on cells of the immune system. It is also interesting to note that *Vibrio cholerae* can secrete pentameric CTB along with intact CT (49) and that assembled CTB₅ can be secreted in the absence of the A chain (50). Thus, it is possible that the up-regulation of ER proteins induced in various tissues by free CTB in cholera patients could influence the course of the disease.

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1. Chen A, Hu T, Mikoryak C, Draper RK. Retrograde transport of protein toxins under conditions of COPI dysfunction. *Biochim Biophys Acta* 1589:124–139, 2002.
2. De Haan L, Hirst TR. Cholera toxin: A paradigm for multi-functional engagement of cellular mechanisms (Review). *Mol Membr Biol* 21:77–92, 2004.
3. Spooner RA, Smith DC, Easton AJ, Roberts LM, Lord JM. Retrograde transport pathways utilised by viruses and protein toxins. *Virology* 3:26, 2006.
4. Schmitz A, Herrgen H, Winkeler A, Herzog V. Cholera toxin is

- exported from microsomes by the Sec61p complex. *J Cell Biol* 148: 1203–1212, 2000.
5. Tsai B, Rodighiero C, Lencer WI, Rapoport TA. Protein disulfide isomerase acts as a redox-dependent chaperone to unfold cholera toxin. *Cell* 104:937–948, 2001.
 6. Lord JM, Roberts LM. Retrograde transport: going against the flow. *Curr Biol* 8:R56–58, 1998.
 7. Lord JM, Deeks E, Marsden CJ, Moore K, Pateman C, Smith DC, Spooner RA, Watson P, Roberts LM. Retrograde transport of toxins across the endoplasmic reticulum membrane. *Biochem Soc Trans* 31: 1260–1262, 2003.
 8. Hazes B, Read RJ. Accumulating evidence suggests that several AB-toxins subvert the endoplasmic reticulum-associated protein degradation pathway to enter target cells. *Biochem* 36:11051–11054, 1997.
 9. Winkler A, Godderz D, Herzog V, Schmitz A. BiP-dependent export of cholera toxin from endoplasmic reticulum-derived microsomes. *FEBS Lett* 554:439–442, 2003.
 10. Orlandi PA. Protein-disulfide isomerase-mediated reduction of the A subunit of cholera toxin in a human intestinal cell line. *J Biol Chem* 272:4591–4599, 1997.
 11. Tsai B, Rapoport TA. Unfolded cholera toxin is transferred to the ER membrane and released from protein disulfide isomerase upon oxidation by Ero1. *J Cell Biol* 159:207–216, 2002.
 12. Jarosch E, Lenk U, Sommer T. Endoplasmic reticulum-associated protein degradation. *Int Rev Cytol* 223:39–81, 2003.
 13. AbuJarour R, Dalal S, Hansen M, Draper RK. p97 is in a complex with cholera toxin and influences the transport of cholera toxin and related toxins to the cytoplasm. *J Biol Chem* 280:15865–15871, 2005.
 14. Kothe M, Ye Y, Wagner JS, DeLuca H, Kern E, Rapoport TA, Lencer WI. Role of p97 AAA-ATPase in the retro-translocation of the cholera toxin A1 chain, a non-ubiquitinated substrate. *J Biol Chem* 280:28127–28132, 2005.
 15. Schröder M, Kaufman RJ. The mammalian unfolded protein response. *Annu Rev Biochem* 74:739–789, 2005.
 16. Hardy SJ, Holmgren J, Johansson S, Sanchez J, Hirst TR. Coordinated assembly of multisubunit proteins: Oligomerization of bacterial enterotoxins in vivo and in vitro. *Proc Natl Acad Sci U S A* 85:7109–7113, 1988.
 17. Majoul IV, Bastiaens PIH, Söling H-D. Transport of an external Lys-Asp-Glu-Leu (KDEL) protein from the plasma membrane to the endoplasmic reticulum: studies with cholera toxin in Vero cells. *J Cell Biol* 133:777–789, 1996.
 18. Majoul I, Sohn K, Wieland FT, Pepperkok R, Pizza M, Hillemann J, Söling HD. KDEL receptor (Erd2p)-mediated retrograde transport of the cholera toxin A subunit from the Golgi involves COPI, p23, and the COOH terminus of Erd2p. *J Cell Biol* 143:601–612, 1998.
 19. Bastiaens PI, Majoul IV, Verveer PJ, Söling HD, Jovin TM. Imaging the intracellular trafficking and state of the AB5 quaternary structure of cholera toxin. *EMBO J* 15:4246–4253, 1996.
 20. Hu T, Kao C-Y, Hudson RT, Chen A, Draper RK. Inhibition of secretion by 1,3-cyclohexanebis(methylamine), a dibasic compound that interferes with coatamer function. *Mol Biol Cell* 10:921–933, 1999.
 21. Lilley BN, Ploegh HL. A membrane protein required for dislocation of misfolded proteins from the ER. *Nature* 429:834–840, 2004.
 22. Ye Y, Shibata Y, Yun C, Ron D, Rapoport TA. A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature* 429:841–847, 2004.
 23. Bernales S, Papa FR, Walter P. Intracellular signaling by the unfolded protein response. *Annu Rev Cell Dev Biol* 22:487–508, 2006.
 24. Gulow K, Bienert D, Haas IG. BiP is feed-back regulated by control of protein translation efficiency. *J Cell Sci* 115:2443–2452, 2002.
 25. Wong WL, Brostrom MA, Kuznetsov G, Gmitter-Yellen D, Brostrom CO. Inhibition of protein synthesis and early protein processing by thapsigargin in cultured cells. *Biochem J* 289 (Pt 1):71–79, 1993.
 26. Oda Y, Okada T, Yoshida H, Kaufman RJ, Nagata K, Mori K. Derlin-2 and Derlin-3 are regulated by the mammalian unfolded protein response and are required for ER-associated degradation. *J Cell Biol* 172:383–393, 2006.
 27. Ellgaard L, Helenius A. Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell Biol* 4:181–191, 2003.
 28. Fujinaga Y, Wolf AA, Rodighiero C, Wheeler H, Tsai B, Allen L, Jobling MG, Rapoport T, Holmes RK, Lencer WI. Gangliosides that associate with lipid rafts mediate transport of cholera and related toxins from the plasma membrane to endoplasmic reticulum. *Mol Biol Cell* 14:4783–4793, 2003.
 29. De Wolf MJ, Dams E, Dierick WS. Interaction of a cholera toxin derivative containing a reduced number of receptor binding sites with intact cells in culture. *Biochim Biophys Acta* 1223:296–305, 1994.
 30. Fernandez J, Yaman I, Sarnow P, Snider MD, Hatzoglou M. Regulation of internal ribosomal entry site-mediated translation by phosphorylation of the translation initiation factor eIF2alpha. *J Biol Chem* 277:19198–19205, 2002.
 31. Sarnow P. Translation of glucose-regulated protein 78/immunoglobulin heavy-chain binding protein mRNA is increased in poliovirus-infected cells at a time when cap-dependent translation of cellular mRNAs is inhibited. *Proc Natl Acad Sci U S A* 86:5795–5799, 1989.
 32. Macejak DG, Sarnow P. Internal initiation of translation mediated by the 5' leader of a cellular mRNA. *Nature* 353:90–94, 1991.
 33. Hollen J, Weissman JS. Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. *Science* 313:104–107, 2006.
 34. Gouy H, Deterre P, Debre P, Bismuth G. Cell calcium signaling via GM1 cell surface gangliosides in the human Jurkat T cell line. *J Immunol* 152:3271–3281, 1994.
 35. Tessitore A, del PMM, Sano R, Ma Y, Mann L, Ingrassia A, Laywell ED, Steindler DA, Hendershot LM, d'Azzo A. GM1-ganglioside-mediated activation of the unfolded protein response causes neuronal death in a neurodegenerative gangliosidosis. *Mol Cell* 15:753–766, 2004.
 36. Sandvig K, Garred O, van Deurs B. Thapsigargin-induced transport of cholera toxin to the endoplasmic reticulum. *Proc Natl Acad Sci U S A* 93:12339–12343, 1996.
 37. Ikari A, Sakai H, Takeguchi N. ATP, thapsigargin and cAMP increase Ca²⁺ in rat hepatocytes by activating three different Ca²⁺ influx pathways. *Jpn J Physiol* 47:235–239, 1997.
 38. Hagmann J, Fishman PH. Inhibitors of protein synthesis block action of cholera toxin. *Biochem Biophys Res Commun* 98:677–684, 1981.
 39. Ye Y, Shibata Y, Kikkert M, van Voorden S, Wiertz E, Rapoport TA. Inaugural Article: Recruitment of the p97 ATPase and ubiquitin ligases to the site of retrotranslocation at the endoplasmic reticulum membrane. *Proc Natl Acad Sci U S A* 102:14132–14138, 2005.
 40. Lilley BN, Ploegh HL. Multiprotein complexes that link dislocation, ubiquitination, and extraction of misfolded proteins from the endoplasmic reticulum membrane. *Proc Natl Acad Sci U S A* 102: 14296–14301, 2005.
 41. Johannes L, Tenza D, Antony C, Goud B. Retrograde transport of KDEL-bearing B-fragment of Shiga toxin. *J Biol Chem* 272:19554–19561, 1997.
 42. Sandvig K, Garred O, Prydz K, Kozlov JV, Hansen SH, van Deurs B. Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. *Nature* 358:510–512, 1992.
 43. Falguieres T, Johannes L. Shiga toxin B-subunit binds to the chaperone BiP and the nucleolar protein B23. *Biol Cell* 98:125–134, 2006.
 44. Slominska-Wojewodzka M, Gregers TF, Walchli S, Sandvig K. EDEM is involved in retrotranslocation of ricin from the endoplasmic reticulum to the cytosol. *Mol Biol Cell* 17:1664–1675, 2006.

45. Plant A, Williams NA. Modulation of the immune response by the cholera-like enterotoxins. *Curr Top Med Chem* 4:509–519, 2004.
46. Francis ML, Ryan J, Jobling MG, Holmes RK, Moss J, Mond JJ. Cyclic AMP-independent effects of cholera toxin on B cell activation. II. Binding of ganglioside GM1 induces B cell activation. *J Immunol* 148:1999–2005, 1992.
47. Elson CO, Holland SP, Dertzbaugh MT, Cuff CF, Anderson AO. Morphologic and functional alterations of mucosal T cells by cholera toxin and its B subunit. *J Immunol* 154:1032–1040, 1995.
48. Rouquette-Jazdanian AK, Foussat A, Lamy L, Pelassy C, Lagadec P, Breitmayer JP, Aussel C. Cholera toxin B-subunit prevents activation and proliferation of human CD4+ T cells by activation of a neutral sphingomyelinase in lipid rafts. *J Immunol* 175:5637–5648, 2005.
49. Finkelstein RA, LoSpalluto JJ. Pathogenesis of experimental cholera. Preparation and isolation of cholera toxin and cholera toxinogen. *J Exp Med* 130:185–202, 1969.
50. Hirst TR, Sanchez J, Kaper JB, Hardy SJ, Holmgren J. Mechanism of toxin secretion by *Vibrio cholerae* investigated in strains harboring plasmids that encode heat-labile enterotoxins of *Escherichia coli*. *Proc Natl Acad Sci U S A* 81:7752–7756, 1984.