p97 Is in a Complex with Cholera Toxin and Influences the Transport of Cholera Toxin and Related Toxins to the Cytoplasm*

Ramzy J. AbuJarour‡§, Seema Dalal¶, Phyllis I. Hanson¶, and Rockford K. Draper¶†

From the ‡Molecular and Cell Biology Department, The University of Texas at Dallas, Richardson, Texas 75083-0688 and the ¶Washington University School of Medicine, Department of Cell Biology and Physiology, St. Louis, Missouri 63110

Certain protein toxins, including cholera toxin, ricin, and *Pseudomonas aeruginosa* exotoxin A, are transported to the lumen of the endoplasmic reticulum where they retro-translocate across the endoplasmic reticulum membrane to enter the cytoplasm. The mechanism of retro-translocation is poorly understood but may involve the endoplasmic reticulum-associated degradation pathway. The AAA ATPase p97 (also called valosin-containing protein) participates in the retro-translocation of cellular endoplasmic reticulum-associated degradation substrates and is therefore a candidate to participate in the retro-translocation of protein toxins. To investigate whether p97 functions in toxin delivery to the cytoplasm, we measured the sensitivity to toxins of cells expressing either wild-type p97 or a dominant ATPase-defective p97 mutant under control of a tetracycline-inducible promoter. The rate at which cholera toxin and related toxins entered the cytoplasm was reduced in cells expressing the ATPase-defective p97, suggesting that the toxins might interact with p97. To detect interaction, the cholera toxin A chain was immunoprecipitated from cholera toxin-treated Vero cells, and co-immunoprecipitation of p97 was assessed by immunoblotting. The immunoprecipitates contained both cholera toxin A chain and p97, evidence that the two proteins are in a complex. Altogether, these results provide functional and structural evidence that p97 participates in the transport of cholera toxin to the cytoplasm.

Protein toxins of the AB type contain two components, an A subunit that has a deleterious enzymatic activity and one or more copies of a B subunit that binds to a cell surface receptor. The substrates for the enzymatic A subunit are within the cytoplasm; therefore, at least the A chain must be transported across a membrane barrier to access substrates. This transport process involves endocytosis and membrane traffic by the target cell. Some toxins, such as diphtheria toxin (DT) (1) and anthrax toxin (2), enter the cytoplasm by penetrating the endosomal membrane soon after endocytosis. However, other toxins, including cholera toxin (CT) (3), ricin (4), and *Pseudomonas aeruginosa* exotoxin A (ETA) (5), are endocytosed and traffic to the endoplasmic reticulum (ER) by retrograde transport before passing into the cytoplasm. CT contains five B subunits that bind the ganglioside receptor G_{M1}, whereas the A chain ADP-ribosylates a regulatory G protein and activates adenylate cyclase, which elevates intracellular CAMP (6). Ricin is a plant toxin that binds glycoconjugates containing galactose so that either glycoproteins or glycolipids displaying galactose can serve as receptors (7). The A chain of ricin arrests protein synthesis by inactivating 28 S ribosomal RNA (8, 9). ETA binds the α_{2} macroglobulin receptor/low density lipoprotein receptor-related protein and arrests protein synthesis by ADP-ribosylating elongation factor 2 in the cytoplasm, the same reaction catalyzed by the A chain of DT (10).

After receptor-mediated endocytosis, CT and related toxins enter retrograde trafficking pathways that transport them to the trans-Golgi network and then to the ER. The number of retrograde pathways available to toxins, and the molecular details of their operation, are not well understood (3, 11, 12). Once delivered to the lumen of the ER, there is indirect evidence that the catalytic chains of ricin (13), ETA (14), and CT (15, 16) retro-translocate from the lumen of the ER into the cytoplasm through the Sec61 pore. These studies suggest that the toxins may use the endoplasmic reticulum-associated degradation (ERAD) system to leave the ER (17–19). The ERAD system retro-translocates misfolded ER luminal and membrane proteins into the cytosol for degradation by the 26 S proteasome (20). If toxins use the ERAD pathway to leave the ER, they must somehow subvert the degradation capacity of the system to remain intact in the cytosol. Toxin A chains appear to avoid degradation by having evolved fewer sites for ubiquitination (19, 21) and by folding up quickly upon entering the cytoplasm, making them resistant to the proteasome (22).

Ubiquitination is a modification of many proteins that leave the ER via the ERAD system, and several proteins implicated in this process are known in mammalian cells. One of them is p97 (also called valosin-containing protein), the mammalian homologue of the Cdc48 ATPase that is related to N-ethylmaleimide-sensitive factor protein (NSF) (23, 24). There is some uncertainty about whether p97 functions in homotypic fusion in the Golgi apparatus of mammalian cells as well as in the ERAD pathway, but there is no doubt that it is required for ERAD of many proteins (25, 26). During ERAD, p97 associates with other proteins, including Ufd1, Npl4, and Derlin-1 (27–30). An important question with respect to toxin retro-translocation is whether ubiquitination is essential for p97 to interact with substrates because toxins have evolved to avoid ubiquitination (and attendant proteasomal degradation). In fact, adding ubiquitination sites to ricin A chain enhances proteasomal degradation (21), and the CT A chain with all ubiquitination sites removed still reaches the cytoplasm (22). Recent work...
suggests, however, that ubiquitination is not essential for p97 to bind translocating substrates (28, 31). Thus, it is conceivable that p97 is involved with toxin translocation, even though the toxins are poor substrates for ubiquitination.

In this paper, we measured the effects of toxins in a cell line containing an inducible dominant-inhibitory mutant of p97 (25) and found that the rate at which toxins entered the cytoplasm was reduced, suggesting that p97 might interact with the toxins. To test for interaction, the CT A chain was immunoprecipitated from Vero cells, and both CT A and p97 were in the immunoprecipitates. These data provide functional and structural evidence that p97 and the CT A chain are together in a complex that participates in toxin delivery to the cytoplasm.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ricin, FITC-labeled ricin, bovine serum albumin, and rhodamine-labeled secondary antibodies were from Sigma. Alexa Fluor 647 secondary antibody was from Molecular Probes (Eugene, OR). CT was from EMD Biosciences (La Jolla, CA). ETA and diphtheria toxin were from List Biological Laboratories (Campbell, CA). Anti-GM130 was from Transduction Laboratories (Lexington, KY). Primary antibodies to Rab6 and Myc were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-p97 was from RDI Research Diagnostics (Flanders, NJ). Polyclonal anti-CT B chain was from Fitzgerald (Concord, MA). Antibodies to the CT A 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. Trans 35S label was from ICN Radiochemical (Irvine, CA).

**Cells and Cell Culture**—U2OS TRex cells stably expressing a tetracycline repressor (Invitrogen) and U2OS cells containing the NSF and p97 variants have been previously described (25). The cells were maintained with Dulbecco’s modified Eagle’s medium (Irvine Scientific, Santa Ana, CA) containing 10% tetracycline-free fetal bovine serum (HyClone, Logan, UT), 50 μg/ml hygromycin B, and 65 μg/ml zeocin (Invitrogen). The cells were exposed to 1 μg/ml tetracycline for 12 h to induce p97 and for 6 h to induce NSF. 80–90% of the cells expressed the exogenous, tagged proteins as determined by immunofluorescence microscopy. Vero cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured under standard conditions, as described previously (32).

**Protein Synthesis Assay**—Protein synthesis was measured by the incorporation of radioactivity from Trans 35S label into acid-insoluble protein essentially as described previously (33). Control and induced cells were pretreated with respective toxins at different concentrations for 3 h. Trans 35S label (1 μCi/ml) was added for the last hour, and the cells were washed and lysed, and the lysate was spotted within squares defined by gridlines drawn on filter paper. The paper was incubated with trichloroacetic acid containing 0.5 mg/ml methimethine 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 quantitation (Amersham Biosciences).

**Cholera Toxin Assay**—Control and induced cells were grown in 24-well culture plates and incubated for 3 h with different concentrations of cholera toxin. The cells were lysed, and intracellular CAMP levels were directly measured with an enzyme immunoassay system according to the instructions provided by the manufacturer (Amersham Biosciences).

**Kinetics of Intoxication**—The rate of intoxication by ricin, ETA, and DT was measured by assessing the incorporation of radioactivity from Tran 35S label into acid-insoluble protein as described previously (34) with modifications. The cells were seeded at 5 x 10^4 cells/well in 48-well culture dishes 1 day before an experiment. After induction with tetracycline, control and induced cells were incubated with Dulbecco’s modified Eagle’s medium (lacking methionine and cysteine) in the presence of 25 μg/ml ricin or ETA for corresponding time points as indicated by the figure legends, followed by a 10-min incubation with Tran 35S label (10 μCi/ml). The cells were washed and lysed, and radioactivity was measured as described in the previous section on protein synthesis assays. To determine the rate of intoxication by ETA, control and induced cells were incubated with 25 μg/ml ETA for indicated times, followed by cell lysis and measurement of intracellular CAMP levels as described in the preceding section.

**Immunofluorescence Microscopy**—To study the uptake and intracellular trafficking of ricin, control and induced cells were incubated with FITC-labeled ricin (5 μg/ml) for 30 min on ice to bind the toxin to the cell surface. Toxin internalization was initiated by warming the cells to 37 °C for 5 min. The cells were then chilled on ice and washed twice with Dulbecco’s modified Eagle’s medium plus galactose (100 μM) and once with phosphate-buffered saline to remove ricin that was not internalized. The cells were then kept in Dulbecco’s modified Eagle’s medium for 3 h. Trans 35S label (1 μCi/ml) was added for the last hour, and the cells were washed and fixed with 4% paraformaldehyde for 10 min on ice (to prevent further transport of the toxin) and 10 min at room temperature. After permeabilization with 0.2% saponin for 10 min, the cells were stained with antibodies to Rab6 and Myc and viewed using a Leica TCS SP2 AOBS confocal microscope and a 63× lens (Leica Microsystems, Bannockburn, IL). Quantitative analysis of FITC-ricin accumulation in Golgi cisternae was performed using Leica software and by defining the region of interest as the pericentriolar region that is positive to Rab6. The ratio of FITC-ricin staining to Rab6 staining in the Rab6-positive region was then recorded.

**Transfection and Immunoprecipitation**—Vero cells were transiently transfected for 12 h with plasmids (25) encoding either wild-type p97 or p97(E578Q) using Lipofectamine 2000 (Invitrogen) following the protocol provided by the vendor. Transfection efficiency was between 60 and 70% as confirmed by immunofluorescence.

For immunoprecipitation, the cells were incubated with 10 μg/ml CT for 4 h at 37 °C, washed thoroughly with phosphate-buffered saline, and solubilized in IP buffer (0.5% Triton X-100, 30 mM HEPES, 100 mM NaCl, 5 mM MgCl2, 2 mM ATP, 1 mM dithiothreitol, EDTA-free protease inhibitors, pH 7.4). Anti-CT A chain antibody was incubated with the precleared cell extracts for 1 h at 4 °C, and protein A-Sepharose beads (Sigma) were added, and the mixture was incubated for additional 2 h at 4 °C. The beads were washed thoroughly and removed by centrifugation, and proteins in the supernatant and bead pellet were electroblotted in a polyacrylamide gel with sodium dodecyl sulfate, transferred to nitrocellulose, and immunoblotted with antibodies as described in the figure legends.

**RESULTS**

**Expressing the Dominant p97(E578Q) Mutant Inhibits the Cytotoxicity of CT, ricin, and ETA but Not That of DT**—U2OS-p97(E578Q) cells are stably transfected with an ATPase-defective dominant-interfering mutant of p97 under control of the tetracycline promoter (25). To measure the effect of expressing the dominant mutant on the cytotoxic activity of toxins, the cells were either exposed to tetracycline for 12 h to induce p97(E578Q) or were left untreated. Fig. 1 shows the response of induced and uninduced cells to different concentrations of CT, ricin, ETA, and DT after exposure to the toxins for 3 h. Table I presents a summary of the toxin concentrations required to elicit a 50% effect (IC50 values) corresponding to the dose-response curves of Fig. 1. The IC50 values for cells expressing p97(E578Q) were two to three times higher compared with uninduced cells for CT, ricin, and ETA, but not for DT. The resistance to CT and ricin upon p97(E578Q) induction was not the result of tetracycline alone or nonspecific effects of overexpressing p97 because U2OS cells transfected with wild-type p97 under control of the tetracycline promoter (25) showed no resistance to the toxins upon induction with tetracycline (Table II). CT, ricin, and ETA have in common that they enter the lumen of the ER by membrane traffic pathways and then must retro-translocate across the ER membrane to reach the cytoplasm where they encounter their substrates (3–5). In contrast, DT penetrates an endosomal membrane to reach the cytoplasm and does not require transport to the ER (1). Thus, expressing a dominant mutant of p97 inhibits toxins that pass through the ER en route to the cytosol but not a toxin that escapes to the cytosol before reaching the ER.

**Expressing p97(E578Q) Increases the Time Required for CT and Related Toxins to Reach the Cytosol**—The kinetics of intoxication by protein toxins begins with a lag period during which no effects are seen, followed by a first order inactivation of protein synthesis (35). The lag period is dose-dependent until a minimum lag is reached that cannot be decreased by increasing the toxin concentration, corresponding to saturation of surface receptors. The minimum lag period represents the time required by cells to transport and process toxins prior to release into the cytoplasm. To see whether the resistance to CT, ricin, and ETA...
induced by p97(E578Q) correlated with changes in intoxication kinetics, induced and uninduced cells were exposed to high doses of toxin (to ensure saturation of surface receptors), and the responses were measured as a function of time. Two consequences on the kinetics of intoxication were observed (Fig. 2). One was an increase of 25–50% in the minimum lag periods for the toxins (summarized in Table III). This increase suggests that a cellular event occurring during transport of the toxins to the cytoplasm takes longer when the dominant p97 mutant is expressed. Second, the generation of a response was delayed after the minimum lag period was over. Moreover, the response did not appear to be first order, probably because of the heterogeneous level of p97(E578Q) expression in the total cell population. Expressing wild-type p97 in U2OS cells had no effects on the kinetics of intoxication by CT, ricin, or ETA (data not shown). Overall, it is evident that interfering with p97 function slows the rate at which ricin and related toxins enter the cytoplasm.

The Accumulation of FITC-labeled Ricin within Golgi Membranes Is Not Impaired upon Expressing p97(E578Q)—The dominant interfering mutant of p97 could in principal cause toxin resistance by affecting any one or more of numerous events in the cytotoxic process, including the binding of the toxin to cell surface receptors, endocytosis, transport to the Golgi, transport of the toxin from the Golgi to the ER, and escape from the ER to the cytoplasm. The Golgi compartment is an intermediate point in these transport pathways, and the steady state accumulation of a toxin in the Golgi region should reflect changes in upstream or downstream trafficking pathways. For example, a reduction in toxin surface receptors, a decrease in the rate of endocytosis, or impaired delivery of endocytosed toxin to the trans-Golgi network, should reduce the accumulation of a toxin in the Golgi complex. Moreover, if there is less export of a toxin from the Golgi to the ER, the toxin should overaccumulate within Golgi membranes. Therefore, we measured the accumulation of FITC-labeled ricin in the Golgi region of cells by quantitative confocal fluorescence microscopy. To validate the approach, we first studied the accumulation of FITC-ricin in cells under conditions that should inhibit the delivery of endocytosed material to the Golgi, namely in cells expressing a dominant ATPase-defective mutant of NSF.

U2OS-NSF(E329Q) cells contain a dominant mutant of NSF under control of the tetracycline promoter (25). Because dissociation of soluble NSF attachment protein receptor complexes mediated by NSF is essential for many membrane fusion events, disrupting NSF function should impair the pathways of membrane traffic that depend on membrane fusion, and it has been shown that inducing the NSF(E329Q) mutant in U2OS cells disorganizes the Golgi apparatus and impairs intra Golgi transport (25). The cytotoxicity of toxins that require transport into the ER should also be inhibited by expressing NSF(E329Q), and the IC_{50} values for ricin, ETA, and CT were increased when the NSF mutant was induced (Fig. 3). Further, the rate at which ricin, ETA, and CT intoxicated cells was reduced in kinetic assays when the mutant NSF was ex-
pressed (data not shown). Cells expressing the dominant NSF mutant were only slightly resistant to DT (Fig. 3 D), suggesting that events necessary for DT action, including receptor-mediated endocytosis and acidification of early endosomes, are not severely disrupted by the dominant NSF mutant.

To determine whether the resistance to ricin caused by expressing NSF(E329Q) correlated with reduced uptake of FITC-labeled ricin into the Golgi apparatus, induced and uninduced cells were pulsed with FITC-labeled ricin for 5 min and chased for 0, 10, or 30 min prior to staining with antibodies to Rab6 (a trans-Golgi network marker) and to Myc (to identify Myc-tagged NSF(E329Q)). Images of cells at the 10-min chase time are shown in Fig. 4. In uninduced control cells, there was no staining for Myc, indicating a very low level of NSF(E329Q)-Myc expression without induction, and FITC-ricin co-localized with Rab6-positive Golgi cisternae (Fig. 4, top panels). When NSF(E329Q) was induced, strong staining for NSF(E329Q)-Myc was observed, and there was little detectable FITC-ricin in Rab6-positive structures (Fig. 4, bottom panels). To quantify the absence of FITC-ricin in Rab6-positive structures, the ratio of FITC to tetramethylrhodamine isothiocyanate in Rab6-positive regions was compared in uninduced and induced cells at different chase times. FITC-ricin co-localized with Rab6 first increased and then declined in uninduced cells, whereas very little FITC-ricin was within Rab6-positive structures at any time for induced cells (see Fig. 6A).

Considering that the impairment of ricin transport to the Golgi complex was readily detected in cells expressing the dominant NSF mutant, we repeated the experiments in cells expressing the dominant p97 mutant. Images of the 10-min chase time point are shown in Fig. 5. In control cells in which p97(E578Q) was not induced, FITC-labeled ricin co-localized after 10 min in typical Golgi structures with Rab6, and no Myc staining was observed (Fig. 5, top panels). When p97(E578Q) was induced with tetracycline, Myc staining was intense, verifying induction, and there was no detectable change in the ability of FITC-labeled ricin to co-localize with Rab6 (Fig. 5, bottom panels). The ratio of ricin to Rab6 as a function of chase time was identical with induced and uninduced cells (Fig. 6B). Thus, in contrast to results with NSF, interfering with p97 function caused no detectable change in the passage of a pulse of ricin through the Golgi apparatus.

The CT A Chain Is in a Complex with p97 in Vivo—The cytotoxicity assays and the kinetics experiments provided indirect evidence for the involvement of p97 in the transport of ER-targeted toxins to the cytoplasm. To assess the possibility that p97 and CT might be in a complex, co-immunoprecipitation experiments were done. The cell line of choice in these experiments was Vero cells because they are very sensitive to CT and accumulate the CT A chain in their ER (36). Vero cells transiently expressing either wild-type p97 or p97(E578Q) were incubated with CT at 37 °C for 4 h, washed, and lysed, and the lysates were incubated with either antibody to CT A or a control antibody (anti-GM130). Protein A-Sepharose beads were added, the samples were centrifuged, and proteins in the supernatant and the bead pellet were analyzed by immunoblots to detect the CT A chain and Myc-labeled p97 (Fig. 7). Regardless of whether the immunoprecipitating antibody was anti-CT A or a control antibody, CT A was present in the

| Toxin | Experiment | Minimum lag (p97(E578Q) off) | Minimum lag (p97(E578Q) on) |
|-------|------------|-----------------------------|-----------------------------|
| CT    | 1          | 12                          | 16                          |
|       | 2          | 15                          | 19                          |
| Ricin | 1          | 28                          | 35                          |
|       | 2          | 10                          | 14                          |
| ETA   | 1          | 6                           | 11                          |
|       | 2          | 12                          | 18                          |

Minimized lag periods for CT, ricin, and ETA in cells expressing p97(E578Q) from two independent experiments

The results in Experiment 1 for the indicated toxins are also shown in Fig. 2.
supernatants after the beads were removed, as expected; however, CT A was only detected on the beads when the anti-CT A antibody was used, evidence that the immunoprecipitation was specific (Fig. 7, top two gels). With untransfected cells, p97-Myc could not be detected, either in the supernatant or in the immunoprecipitate, as expected, because the antibody is specific for exogenous Myc-tagged proteins (Fig. 7, lane 1, bottom two gels). In transfected cells, p97-Myc was in all of the supernatants but was not immunoprecipitated with the control antibody (Fig. 7, lanes 4 and 5, bottom two gels). The anti-CT A antibody, however, did co-immunoprecipitate both wild-type p97-Myc and p97(E578Q)-Myc (Fig. 7, lanes 2 and 3, bottom gel). These data are evidence that the CT A chain and p97 are in the same complex, independent of the ATP hydrolytic activity of p97.

The immunoprecipitates studied in Fig. 7 were from cells over-expressing transfected forms of p97 displaying the Myc epitope, and it is possible that the high concentration of p97-Myc could have induced an unnatural complex between p97 and the CT A chain. Therefore, the immunoprecipitation experiments were repeated with untransfected cells, and endogenous p97 was detected in immunoblots with anti-p97 antibody rather than anti-Myc antibody. Untransfected Vero cells were either left untreated or treated with CT and lysed, and the lysates incu-
bated with anti-CT A chain antibody followed by the addition of protein A-Sepharose beads and preparation of supernatants and pellets by centrifugation. The samples were immunoblotted with either anti-CT A antibody or anti-p97 antibody. CT A was not detected in either the supernatant or pellet from cells left untreated with CT but was present in both the supernatant and immunoprecipitated pellet in CT-treated cells (Fig. 8, lanes 1 and 2, top two gels). p97 was present in the supernatant, but not the pellet, of cells left untreated with CT (Fig. 8, lane 1, bottom two gels), demonstrating that the p97 antibody reacted with p97 as expected; however, p97 was present not only in the supernatant of lysates immunoprecipitated with anti-CT A, but also in the pellet (Fig. 8, lane 2, bottom two gels). This is evidence that endogenous wild-type p97 is in a complex with CT A.

Several additional controls were done to demonstrate the specificity of the immunoprecipitation reaction that detected both CT A and endogenous p97 in a complex. With a control immunoprecipitating antibody in place of anti-CT A (anti-GM130), neither CT A nor p97 was immunoprecipitated (Fig. 8, lane 3), indicating that anti-CT A was essential for the immunoprecipitation reaction. Further, if lysates were prepared from cells not treated with CT, and the CT A chain was added post lysis, followed by immunoprecipitation with anti-CT A chain, no p97 was found in pellets, indicating that CT A chain was not reacting with p97 after the cells had been lysed (data not shown). In addition, when lysates prepared from cells treated with CT were immunoprecipitated with anti-CT B chain, CT A chain was detected in both supernatants and pellets (Fig. 8, lane 4, top two gels), as expected, because most of the CT A chain detected in the cell consists of heterodimers of the A and B chains covalently attached by disulfide bonds. Most importantly, however, is that the anti-CT B chain did not co-immunoprecipitate p97 (Fig. 8, lane 4, bottom two gels). This indicates that p97 is not reacting with intact CT or with the B chain alone.

**DISCUSSION**

Several protein toxins that cross the ER membrane barrier physically associate with the Sec61 translocon (13–15). The unfolding of the CT A chain, which is probably required for retro-translocation, is also dependent on protein disulfide isomerase and the ER oxidase Ero1 (37). However, except for Sec61, protein disulfide isomerase, and Ero1, other proteins that mediate the dislocation of toxins across the ER barrier remain uncertain. The AAA ATPase p97 has been implicated in the retro-translocation of ERAD substrates (25, 27, 38), and we studied here whether p97 participates in the retro-translocation of ER-targeted protein toxins. The most important findings in this study are that an ATPase-defective mutant of p97 slows the transport of several toxins to the cytoplasm and that the CT A chain is in a complex with p97.

**FIG. 7. CT A is in a complex with the transfected p97 variants.** Vero cells were either mock-transfected or transfected for 12 h with Myc-tagged variants of either wild-type (wt) p97 or the p97(E578Q) mutant. The cells were incubated with 10 µg/ml CT for 4 h at 37 °C, washed, and lysed. Precleared lysates were then immunoprecipitated (IP) with antibodies to the A subunit of CT or anti-GM130 (a control antibody). Protein A beads were added, and the extract was centrifuged to prepare a supernatant and a pellet, with the latter containing immunoprecipitated proteins. Immunoprecipitates and 5% of the supernatant were electrophoresed with sodium dodecyl sulfate, transferred to nitrocellulose, and immunoblotted with either anti-CT A or anti-Myc.

**FIG. 8. CT A is in a complex with endogenous p97.** Nontransfected Vero cells were either incubated without (lane 1) or with (lanes 2–4) 10 µg/ml CT for 4 h at 37 °C, washed, and lysed. Precleared lysates were immunoprecipitated with anti-CT A chain (lanes 1 and 2), anti-GM130 (control antibody; lane 3), or antibody to the B chain of CT (lane 4). Electrophoresed immunoprecipitates and 5% of the supernatant were immunoblotted with either anti-CT A or with antibody to p97.

Inducing p97(E578Q), followed by exposing the cells to toxins, increased the concentration of CT, ricin, and ETA required for a 50% response and also slowed the rate at which the toxins appeared in the cell cytoplasm. Inducing wild-type p97 had no effect on the toxins, suggesting that ATPase activity of p97 influences the process by which toxins enter the cytoplasm. The fact that the ATPase-defective mutant of p97 did not completely protect cells from toxins like CT is probably because the high concentration of endogenous p97 in the cells supports partial activity in the presence of the dominant ATPase mutant. Expressing p97(E578Q) had no effect on the action of
p97 Is in a Complex with Cholera Toxin

15871

diphtheria toxin, which reaches the cytoplasm without need for translocation across the ER membrane. Thus, the effects of p97(E578Q) were specific for toxins that pass through the ER en route to the cytoplasm.

Inducing p97(E578Q) in U2OS cells results in the accumulation of the mutant p97 on the ER and morphological changes in ER structure (25). One explanation for the effects of p97(E578Q) on ER-targeted toxins is that the mutant p97 interferes with the secretory function of the ER, which could indirectly affect toxins. For example, the placement of toxin receptors or other proteins in the plasma membrane or endosomal compartments could be altered to secondarily reduce the rate at which toxins are delivered to the Golgi compartment. To determine whether the delivery of ricin to the Golgi apparatus was affected when p97(E578Q) was expressed, the accumulation of FITC-labeled ricin in the Golgi region was compared between cells induced or not induced for the ATPase-defective dominant p97 mutant. As a control in these experiments, the effect of inducing an ATPase-defective dominant mutant of NSF on the accumulation of ricin in the Golgi complex was assessed because impairing NSF function should inhibit delivery of ricin to the Golgi. There was a clear decline in the amount of ricin entering the Golgi complex when the ATPase-defective form of NSF was induced, but not when the ATPase-defective form of p97 was induced. These data suggest that the effect of p97(E578Q) on the kinetics of ricin intoxication is not the result of inhibiting steps that deliver the toxin to the Golgi apparatus.

If p97 participates in toxin retro-translocation, then it is likely that the two proteins interact or at least are components of a larger complex. Complex formation was confirmed by the observation that an antibody to the A chain of CT immunoprecipitated both the CT A chain and p97 in Vero cells transfected with a plasmid encoding Myc-labeled p97. Co-immunoprecipitation occurred in cells expressing both the wild-type p97 and the ATPase-defective p97(E578Q) mutant, indicating that ATP bound to the D2 domain of p97 was not necessary for complex formation. Moreover, the complex was detected in untransfected cells upon immunoblotting with an antibody to endogenous p97, indicating that complex formation was not an artifact of p97-Myc overexpression. Interestingly, immunoprecipitating with anti CT B chain did not co-immunoprecipitate p97, although it did co-immunoprecipitate the CT A chain, suggesting that the complex containing p97 and CT A does not contain the CT B chain. Because the CT B chain enters the ER with the A chain (39), this is further evidence of the specificity by which the anti-CT A antibody immunoprecipitates the complex containing CT A and p97 and suggests that only A chain free of the B chain interacts with p97 located on the cytoplasmic side of the ER membrane.

During ERAD, p97 is believed to pull substrates through a translocation pore coincident with ATP hydrolysis (40), and it may have a similar function with toxin A chains. For example, p97 may bind to the CT A chain as it emerges from a pore on the cytoplasmic side of the ER membrane and promote dislocation. Overexpressing the wild-type p97 has no effect on the toxin, because the endogenous level of p97 is not limiting and is sufficient for full dislocation activity. The inhibition of cytotoxicity caused by overexpressing p97(E578Q) could be due to a failure of the ATPase-defective form of p97 to dissociate from the toxin/pore complex, blocking further dislocation. In light of this model, it is interesting that complex formation with CT A chain and p97 did not depend on the ATPase activity of p97. This suggests that complex formation and translocation may be distinguished by the requirement for ATP hydrolysis.

Acknowledgment—We thank Carole Mikoryak for reading the manuscript.

REFERENCES

1. Eidels, L., and Draper, R. K. (1988) in Handbook of Natural Toxins (Hard- edge, M. C., and Tu, A. T., eds) pp. 217–247, Marcel Dekker, Inc., New York
2. Chandy, G. J., Moyeri, M., Liu, S., and Leppla, S. H. (2002) Trends Microbiol. 10, 58–62
3. Lencer, W. I., and Tsai, B. (2003) Trends Biochem. Sci. 28, 639–645
4. Sandvig, K., and Van Deurs, B. (2002) Annu. Rev. Cell Dev. Biol. 18, 1–24
5. Jackson, M. E., Simpson, J. C., Girod, A., Pepperkok, R., Roberts, L. M., and Lord, J. M. (2002) J. Cell Sci. 115, 467–475
6. Spangler, B. D. (1992) Microbiol. Rev. 56, 622–647
7. Sandvig, K., and Van Deurs, B. (2000) EMBO J. 19, 5943–5950
8. Kin, Y., Mitsui, K., Motizuki, M., and Tsuuri, K. (1997) J. Biol. Chem. 262, 5902–5912
9. Endo, Y., and Tsuuri, K. (1987) J. Biol. Chem. 262, 8128–8130
10. Kounnas, M. Z., Morris, R. E., Thompson, M. K., FitzGerald, D. J., and Saulnier, C. B. (1992) J. Biol. Chem. 267, 12420–12425
11. Sannerud, R., Saraste, J., and Goud, B. (2003) Curro. Opin. Cell Biol. 15, 438–445
12. Chen, A., Hu, T., Mikoryak, C., and Draper, R. K. (2002) Biochim. Biophys. Acta 1589, 124–139
13. Wesche, J., Rapak, A., and Olnes, S. (1999) J. Biol. Chem. 274, 34443–34449
14. Kooppman, J. O., Albring, J., Huter, E., Bulbuc, N., Spee, P., Neefjes, J., Hammerling, G. J., and Momburg, F. (2000) Immunity 13, 117–127
15. Schmitz, A., Herrgen, H., Winkelker, A., and Herzog, V. (2000) J. Cell Biol. 149, 1203–1212
16. Tsai, B., Rodighiero, C., Lencer, W. I., and Rapoport, T. A. (2001) Cell 104, 937–948
17. Lord, J. M., and Roberts, L. M. (1998) J. Cell Biol. 140, 733–736
18. Lord, J. M., Deeks, E., Marsden, C. J., Moore, K., Pateman, C., Smith, D. C., Spooner, R. A., Watson, P., and Roberts, L. M. (2003) Biochem. Soc. Trans. 31, 1260–1262
19. Hazes, B., and Read, R. J. (1997) Biochemistry 36, 11051–11054
20. Jarosh, E., Lenk, U., and Sommer, T. (2000) Int. Rev. Cytol. 225, 39–81
21. Deeks, E. D., Cook, J. P., Day, P. J., Smith, D. C., Roberts, L. M., and Lord, J. M. (2002) Biochemistry 41, 3405–3413
22. Rodighiero, C., Tsai, B., Rapoport, T. A., and Lencer, W. I. (2002) EMBO Rep. 3, 1222–1227
23. Jarosh, E., Geiss-Friedlander, R., Meusser, B., Walter, J., and Sommer, T. (2002) Traffic 3, 530–536
24. Jarosh, E., Taxix, C., Volkwein, C., Bordallo, J., Finley, D., Wolf, D. H., and Sommer, T. (2002) Nat. Cell Biol. 4, 134–139
25. Dalal, S., Rosser, M. F., Cyr, D. M., and Hanson, P. I. (2004) J. Biol. Chem. 279, 3980–3985
26. Chen, A., Abualour, J., and Draper, R. K. (2003) J. Cell Sci. 116, 3503–3510
27. Hu, T., Kao, C.-Y., Hudson, R. T., Chen, A., and Draper, R. K. (1999) Mol. Biol. Cell 10, 921–933
28. Bau, M.-Y., and Draper, R. K. (1993) J. Biol. Chem. 268, 19393–19394
29. Neville, D. M., and Hudson, T. H. (1986) Annu. Rev. Biochem. 55, 195–224
30. Majol, I. V., Bastianex, P. I. H., and Soling, H. (1996) J. Biol. Chem. 271, 777–789
31. Tsai, B., and Rapoport, T. A. (2002) J. Cell Biol. 159, 207–216
32. Bays, N. W., Wilhovsky, S. K., Goradia, A., Hodgkiss-Harlow, K., and Hampton, R. Y. (2001) Mol. Biol. Cell 12, 4114–4128
33. Fujinaga, Y., Wolf, A. A., Rodighiero, C., Wheeler, H., Tsai, B., Allen, L., Jobling, M. G., Rapoport, T., Holmes, R. K., and Lencer, W. I. (2003) Mol. Biol. Cell 14, 4783–4793
34. Tsai, B., Ye, Y., and Rapoport, T. A. (2002) Nat. Rev. Mol. Cell. Biol. 3, 246–255