The enzymatic A1 chain of cholera toxin retrotranslocates across the endoplasmic reticulum membrane into the cytosol, where it induces toxicity. Almost all other retrotranslocation substrates are modified by the attachment of polyubiquitin chains and moved into the cytosol by the ubiquitin-interacting p97 ATPase complex. The cholera toxin A1 chain, however, can induce toxicity in the absence of ubiquitination, and the motive force that drives retrotranslocation is not known. Here, we use adenovirus expressing dominant-negative mutants of p97 to test whether p97 is required for toxin action. We find that cholera toxin still functions with only a small decrease in potency in cells that cannot retrotranslocate other substrates at all. These results suggest that p97 does not provide the primary driving force for extracting the A1 chain from the endoplasmic reticulum, a finding that is consistent with a requirement for polyubiquitination in p97 function.

Cholera toxin (CT) enters host cells by co-opting two fundamental aspects of cell function that reverse membrane and protein transport in the secretory pathway. First, the toxin binds to a membrane lipid that carries it retrograde from plasma membrane to the endoplasmic reticulum (ER) (1). After arrival in the ER, a fragment of CT, the A1 chain, then enters the cytosol by hijacking the cellular machinery for degradation of terminally misfolded proteins (2, 3). Such proteins are recognized by ER luminal chaperones, transported to the cytosol, and degraded by the proteasome. This pathway is termed retrotranslocation, or ERAD for ER-associated protein degradation. Unlike misfolded proteins, the A1 chain escapes degradation by the proteasome (4) and induces toxicity by activating adenyl cyclase.

CT is a member of the AB5 family of toxins, where the A subunit couples non-covalently with a homopentameric B subcomplex. The cholera toxin A1 chain, however, can induce toxicity to the retrotranslocation of the A1 chain that requires the p97-Ufd1-Npl4 complex (7, 9), and the enzymatic A1 chain of cholera toxin retrotranslocates across the endoplasmic reticulum membrane into the cytosol by the ubiquitin-interacting p97 ATPase complex. The polyubiquitin chains are recognized by both p97 and its cofactor Ufd1, and this is required for p97 to pull the polyubiquitinated protein into the cytosol (6–8). The CT A1 chain, however, does not require polyubiquitination to induce toxicity (4), and how it crosses the ER to enter the cytosol remains a mystery.

In this paper, we test whether p97 provides the driving force for retrotranslocation of the CT A1 chain. We use adenovirus expressing WT and dominant-negative forms of p97 to infect two cell lines that are sensitive to CT. One cell line is established as a model for retrotranslocation of MHC Class I heavy chain that requires the p97-Ufd1-Npl4 complex (7, 9), and the other is an established model for CT entry into host cells (1). Our data show that the cytosolic ATPase p97 cannot provide the primary driving force for CT retrotranslocation across the ER, a finding that is consistent with the need for polyubiquitination in p97 function.

EXPERIMENTAL PROCEDURES

Materials—CT was from EMD Biosciences (San Diego, CA) or prepared in house (4). GM1 was from Matreya, Inc. (Pleasant Gap, PA), doxycycline was from Sigma, and antibodies were from Qiagen (RSG-His), Zymed Laboratories, Inc. (ubiquitin), Research Diagnostic (mouse monoclonal p97), and Molecular Probes (goat anti-mouse Alexa 568).

Cell Culture—T84, US11-, and U373-MG astrocytoma cells were cultured as described previously (1, 6). Adenovirus—Tetracycline-regulated adenoviral p97 constructs were created as described (10) with the modification that the tetracycline-regulated (tet-off) plasmid pAdtet (11) was used for virus construction. His-tagged WT, KA (ATP-binding mutant K524A) and QQ (ATP-hydrolysis mutant E305Q/E578Q) p97 (6) were subcloned into pAdtet by PCR amplification using the primers p97S (5’-CAACTAGCATGATGAGAAGATCGATCACC-3’), SpiI site underlined, start codon bolded) and
FIG. 1. Adenoviral expression of mutant p97 in US11 cells induces the unfolded protein response and completely inhibits retrotranslocation of the MHC Class I heavy chain. A, US11 cells infected or not with the indicated adenovirus and imaged by bright field phase contrast (upper panels) or immunofluorescence for the His-epitope (lower panels). B, immunoblots for p97 (upper panel), the His-epitope (middle panel), and ubiquitin (Ub, lower panel) of total cell lysates prepared from US11 cells from the same experiment infected with the indicated viruses and treated or not with doxycycline (Dox) as indicated. C, expression of KA and QQ mutant p97 in COS cells transfected with the reporter plasmid pCAX-F-XBP1∆DBD-venus induces the unfolded protein response as assessed by fluorescence microscopy for expression of green fluorescent protein (GFP, lower panel) and DAPI stain (upper panel). D, SDS-PAGE and phosphorimaging of immunoprecipitation (IP) for [35S]-labeled MHC Class I heavy chain (HC) from total cell lysates of US11 cells infected or not with the indicated adenovirus. Cells were pulse-labeled for 5 min with [35S]Cys/Met and chased with 100-fold excess cold Cys/Met for the time indicated. The graph shows the mean ± S.D. for 1–3 independent experiments. WB, Western blot.

p97AS (5'-GTACATGTTAAGCCATACAGGTATCGT-3', SpeI site underlined, stop codon bolded), followed by digestion with Sphi and SpeI and ligation into pAdtet. All constructs were verified by sequencing the subcloned insert. Recombinant adenovirus was created by co-transfecting HEK293 Cre8 cells with the pAdtet-derived plasmid and pAd5 adenoviral DNA. After three rounds of harvesting virus and re-infecting Cre8 cells, virus stocks were produced by a large scale infection of HEK293 cells and virus purification using CsCl-gradients. Cells were co-infected (see below) with equal amounts of p97 and tet-transactivator TTA adenovirus. Optimal amounts of virus for uniform p97 expression while minimizing virus dose were determined empirically for each construct and batch of virus.

Viral Infections—U373-MG/US11 cells were trypsinized and infected in suspension before plating at original density ~16 h prior to experiments. 10 μM G418 was added during the infection where indicated. For infection of T84 monolayers, 0.33 cm² Transwell inserts (Corning) were washed twice in CaCl₂ and MgCl₂-free Hank's buffered salt solution, incubated for 2 h in CaCl₂ and MgCl₂-free Hank's buffered salt solution with 2.5 mM EDTA to open tight junctions and then transferred to medium supplemented with 1 mM CaCl₂. Adenovirus was added to the apical compartment and cells were incubated for 18 h prior to analysis. To suppress p97 expression, 1 ng/ml doxycycline was added. Aliquots of cells were taken at the indicated time points and were solubilized in 0.5% Nonidet P-40, 50 mM Tris-HCl, 150 mM sodium chloride, 5 mM magnesium chloride plus protease inhibitor cocktails. Cell extracts were subjected to immunoprecipitation with heavy chain antibodies, separated by SDS-PAGE, and analyzed by a phosphorimaging device.

Analysis of COS Cells for the Unfolded Protein Response—COS cells were seeded in 24-well plates and transfected with the plasmid pCAX-F-XBP1∆DBD-venus (14) using FuGENE 6 (Roche Applied Science) 15 h later. Cells were then infected with adenovirus expressing either wild type p97 or p97 defective in ATP hydrolysis 8 h after transfection. Cells were stained with DAPI 16 h after infection and were visualized by immunofluorescence microscope.

MHC Heavy Chain Degradation—US11-expressing U373-MG cells were infected as described above and assayed for MHC Class I heavy chain degradation exactly as described (6). Specifically, infected cells were trypsin-treated and incubated in Dulbecco's modified Eagle's medium lacking cysteine and methionine for 60 min at 37 °C. Cells were then labeled with 27.5 μCi of [35S]methionine/1.0 × 10⁶ cells for 5 min before an excessive amount of non-radiolabeled methionine and cysteine was added. Aliquots of cells were taken at the indicated time points and were solubilized in 0.5% Nonidet P-40, 50 mM Tris-HCl, 150 mM sodium chloride, 5 mM magnesium chloride plus protease inhibitor cocktails. Cell extracts were subjected to immunoprecipitation with heavy chain antibodies, separated by SDS-PAGE, and analyzed by a phosphorimaging device.

Immunostaining/Microscopy—Cells on coverslips or Transwell filters were fixed in 4% paraformaldehyde, permeabilized with 0.2% saponin, blocked in 10% normal goat serum, and incubated in primary (1 μg/ml RGS-His, Qiagen) and secondary (5 mg/ml goat anti-mouse Alexa 568) antibodies in 10% normal goat serum. cAMP and Electrophysiology—Adenovirus-infected astrocytoma and US11 cells were studied for cAMP by enzyme-linked immunosorbent assay of cells cultured in 96-well plates following the directions of the manufacturer (Direct Biotrak EIA kit, Amersham Biosciences). To correct for the effect of mutant p97 on adenyl cyclase activity, cAMP responses were normalized to the forskolin response. Confluent T84 monolayers were studied for short circuit current (Isc) as described (1), and Isc responses were calibrated to baseline (B) and maximal levels.
Adenovirus Encoding Dominant-negative p97 Inhibits Retrotranslocation of MHC Class I Heavy Chain—We first constructed adenovirus containing cDNA for WT and two dominant-negative p97 variants that are defective in their ATPase activity (KA and QQ mutants) (6). All forms of p97 were His-tagged and placed under the control of a tetracycline-sensitive promoter. To test for expression of WT p97 and the KA and QQ mutants, we used U373-MG astrocytoma cells that stably express the cytomegalovirus protein US11 (US11 cells) (6, 9). US11 constitutively induces the retrotranslocation and degradation of MHC class I heavy chains in a reaction dependent on the p97-Ufd1-Npl4 complex (6). Conditions were established for adenoviral infection that resulted in uniform and highly efficient expression of WT and mutant p97 as assessed by immunofluorescence using antibodies for the His-epitope (Fig. 1A). US11 cells not infected with adenovirus did not stain. Immunoblots of whole cell lysates using antibodies for p97 and for the His-epitope show that the recombinant p97 proteins were expressed at equal levels and that their expression was fully inhibited by pretreatment with doxycycline (Fig. 1B, upper and middle panels). Based on the band densities of the immunoblot, we estimate the expression levels of virally encoded His-tagged p97 (WT or mutant forms) to be more than 3-fold higher than endogenous p97.

We next tested for a dominant-negative effect of the KA and QQ mutants. Expression of KA and QQ mutant p97 in US11 cells induces an increase in high molecular weight polyubiquitinated proteins as detected by immunoblot of total cell lysates using antibodies against ubiquitin (Fig. 1B, lower panel, compare lanes 5 and 7 with 1 and 2). This is reversed by treatment with doxycycline (compare lanes 5 and 7 with 6 and 8). Expression of WT p97 has no effect (Fig. 1B, lower panel, lanes 3 and 4). Thus, the KA and QQ mutant viruses result in the accumulation of polyubiquitinated proteins, in agreement with the known role of p97 in the ubiquitin-proteasome pathway (6, 12).

To test for inhibition of the retrotranslocation pathway, we examined the unfolded protein response, which is elicited when misfolded proteins cannot be exported from the ER (13). We used COS cells transiently transfected with the plasmid pCAX-FDBD-venus to express green fluorescent protein upon induction of unfolded protein response (14). Infection with WT p97 virus causes little expression of GFP as assessed by fluorescence microscopy (Fig. 1C). In contrast, cells infected with KA and QQ mutant virus display strong green fluorescent protein fluorescence and vacuolization of the ER. These results are consistent with previous studies (12, 15) and show that mutant p97 induces the unfolded protein response in US11 cells.

To test more directly for a dominant-negative effect on retrotranslocation, we examined the degradation rate of MHC Class I heavy chain. US11 cells were pulse-labeled for 5 min

![Image](image_url)
FIG. 3. Expression of mutant p97 in intestinal T84 cells has no detectable effect on CT function. A, expression of WT or mutant p97 by adenoviral infection of well differentiated polarized monolayers of T84 cells treated or not treated with doxycycline (Dox) as indicated. B, Immunoblots for p97 (upper panel), the polyhistidine-epitope (middle panel), and ubiquitin (Ub, lower panel) of total cell lysates prepared from T84 cells.
with [35S]methionine/cysteine and chased for 0, 5, or 10 min. The MHC Class I heavy chain was immunoprecipitated from total cell lysates and analyzed by SDS-PAGE and phosphoimaging. In US11 cells not infected with adenovirus or in cells overexpressing WT p97, the MHC Class I heavy chain is rapidly degraded with little heavy chain detected after 5 or 10 min of chase (Fig. 1D, compare lanes 1 with 2 and 3 and 4 with 5 and 6). In contrast, overexpression of the KA or QQ p97 mutants completely inhibits degradation of the heavy chain (lanes 7–12), and degradation is fully rescued by pretreatment with doxycycline (lanes 13–15). These results are quantified for up to 3 independent experiments in the associated graph. Thus, the KA and QQ p97 mutants inhibit retrotranslocation of MHC Class I heavy chain in US11 cells.

*p97 Mutants Have a Small Effect on CT Function*—We next tested for a role of p97 in the retrotranslocation of the CT A1 chain by assessing adenylyl cyclase activity in US11 cells after CT treatment. Initial studies showed that CT induced only a small cAMP response in US11 cells (Fig. 2A, column 2). This is likely explained by low levels of the toxin receptor GM1, because toxicity is strongly increased by the addition of exogenous GM1 (Fig. 2A, compare column 3 with columns 1 and 2). Under these conditions, toxicity is completely inhibited by treatment with brefeldin A, which blocks vesicular traffic from plasma membrane to ER, and no response is obtained using the CT mutant G33D that cannot bind GM1 (Fig. 2A, compare columns 4 and 5 with column 3), consistent with all previous studies on toxin action (2). All future studies of CT action on US11 cells were carried out with addition of GM1.

We next found that US11 cells exhibit the same cAMP response to CT as the parent astrocystoma cell line not expressing US11 (Fig. 2B). Thus expression of US11 does not have an effect on retrotranslocation of the CT A1 chain. Because our assay for retrotranslocation of the A1 chain depends on the activity of adenylyl cyclase, we next examined the effect of p97 on the cAMP response to the diterpene forskolin. Forskolin is cell-permeant and rapidly activates adenylyl cyclase by directly binding to the enzyme. Forskolin treatment caused a large increase in intracellular cAMP in US11 cells not virally infected or in cells overexpressing WT p97 (30–50-fold greater than baseline, Fig. 2C). Forskolin also induced the activation of adenylyl cyclase in US11 cells expressing the KA and QQ p97 mutants, but the maximal response was lower (only 10-fold greater than baseline). Pretreatment with doxycycline rescued the cAMP response to forskolin in these cells (Fig. 2C, compare column 5 with 6 and column 7 with 8). These results show that dominant-negative inhibition of p97 in US11 cells affects the endogenous function of adenylyl cyclase.

To control for this confounding variable, all subsequent experiments using cAMP to assay for retrotranslocation of the CT A1 chain were conducted with a one point calibration for adenylyl cyclase activity. In uninfected US11 cells or in cells expressing WT p97, CT (10 nM) induced a strong cAMP response (Fig. 2D, columns 1–4). Similarly, in cells expressing the dominant-negative KA and QQ mutant p97, CT also induced toxicity. These studies were performed side-by-side with our studies on MHC Class I heavy chain retrotranslocation (Fig. 1D) and demonstrated that the A1 chain reaches the cytosol in conditions where p97 function is greatly impaired. However, the cAMP response induced by CT in cells expressing mutant p97 was not always as strong as the maximal response induced by forskolin (Fig. 2D, columns 5–8). Also, the dose response to CT in cells expressing the KA mutant showed a slight reduction in potency (Fig. 2E). Thus, even though the A1 chain clearly retrotranslocates to the cytosol in cells expressing mutant p97, there appears to be a small effect on CT action. This small effect, however, could be indirect or nonspecific, as it contrasts starkly with the complete dependence on p97 function for retrotranslocation of MHC Class I heavy chain (compare Fig. 2, D and E with Fig. 1D).

**Studies on p97 in Intestinal T84 Cells**—We next examined the effect of p97 on CT entry into the human intestinal T84 cell line. In T84 cells, retrotranslocation of the CT A1 chain induces the cAMP-dependent secretion of Cl− ions. This can be measured in real time as an increase in Isc using standard methods of electrophysiology. Conditions were established for viral infection in well differentiated T84 cell monolayers so that the monolayers uniformly expressed equal amounts of WT p97 and the KA and QQ mutants as assessed by immunofluorescence microscopy (Fig. 3A, top panels) and by immunoblot of total cell lysates using antibodies for p97 and the His-epitope (Fig. 3B, lanes 1, 3, 5, and 7). Expression of the p97 transgene was fully inhibited by treatment of the monolayers with doxycycline (Fig. 3, A, lower panels, and B, lanes 4, 6, and 8). We estimate that the ratios of recombinant to endogenous p97 expression levels in T84 cell monolayers are >2:1 for infection with each of the adenovirus constructs.

To test for a dominant-negative effect of mutant p97 expression in T84 cells, we measured the content of polyubiquitinated proteins in total cell lysates as described for US11 cells. Unlike US11 cells, there is a background of polyubiquitinated proteins in T84 cells not infected with adenovirus or expressing the WT p97 (Fig. 3B). Even so, the expression of KA and QQ mutant p97 caused a clear increase in polyubiquitinated proteins, and this was completely reversed by treatment with doxycycline (Fig. 3B, lower panel compare lanes 5 with 6 and 7 with 8). Thus, in T84 cells, the KA and QQ p97 mutants inhibit p97 function in the proteasome pathway.

Unexpectedly, we also found that the KA and QQ mutants increased the Isc in resting T84 cell monolayers, and this was completely reversed by treatment with doxycycline (Fig. 3C). We do not yet understand how p97 may act on the regulation of ion transport in T84 cells. Nonetheless, these results show further evidence for inhibition of p97 function in T84 cells by the KA and QQ mutant viruses.

To test whether retrotranslocation of the CT A1 chain in T84 cells depends on p97, we examined the time course of CT-induced Cl− secretion (Fig. 3D). All studies on T84 cells were controlled by a two-point calibration (see “Experimental Procedures”). The raw data and normalized time courses for CT-induced Cl− secretion for three doses of toxin are shown (Fig. 3D, top and lower panels). Monolayers not infected with virus or those infected WT or mutant p97 virus exhibit the same time course and maximal Cl− secretory response to CT. Identical results are obtained at all doses of toxin examined (Fig. 3, D and E). Thus, inhibition of p97 function in T84 cells has no monolayers infected or not with the indicated viruses. Some monolayers were treated with doxycycline as indicated. C, resting short circuit current (Isc) in T84 cell monolayers infected or not with the indicated viruses and treated with doxycycline as indicated (mean ± S.D., n = 3). D, time course of CT-induced Cl− secretion (Isc) in control (no virus) or WT and mutant p97 adenovirus-infected T84 monolayers. CT was applied apically at 0 min. 10 μM forskolin was added after 150 min to calibrate the assay for the maximal possible secretory response. The top panels show the absolute values from the experiment (mean ± S.D., n = 3). The bottom panels show normalized values as described under “Experimental Procedures.” E, dose response for the CT-induced Isc response in control (no virus) or WT and mutant p97 adenovirus-infected T84 monolayers (mean ± S.D., n = 3, labels same as previous panel). WB, Western blot.
detectable effect on CT action or retrotranslocation of the CT A1 chain.

**DISCUSSION**

In this paper, we showed that the CT A1 chain still reaches the cytosol in US11 cells that cannot retrotranslocate the MHC Class I heavy chain at all. We also found that the intestinal T84 cell line responds normally to CT under conditions where p97 function is greatly impaired. These results suggest that the cytosolic AAA-ATPase p97 cannot provide the primary driving force for retrotranslocation of the CT A1 chain.

Because the A1 chain does not require polyubiquitination to reach the cytosol (4), it is possible that our results define a general rule for retrotranslocation via the p97-Ufd1-Npl4 pathway. So far, in yeast and mammalian cells, all retrotranslocation substrates that depend on the p97 pathway are also modified by polyubiquitin (16, 17), and the modification is required for transport (18). There is one other example, the yeast pre-pro cyclase. Nevertheless, the authors interpret their results might even be caused indirectly by inhibition of adenylate cyclase.

A recent study published during the writing of this manuscript examined the role of p97 on the toxicity of a number of different toxins, including CT (20). In agreement with our results, the authors report only small effects of a dominant-negative p97 mutant on CT toxicity, which our experiments suggest might even be caused indirectly by inhibition of adenylate cyclase. Nevertheless, the authors interpret their results differently, i.e. that p97 is required for the retrotranslocation of CT. However, we feel that this conclusion is not warranted, because we found complete inhibition of another retrotranslocation substrate under the same condition where CT retrotranslocation is only marginally affected. The conclusions by Abujarour et al. (20) are also based on the results of co-immunoprecipitation experiments that show a physical association between p97 and the A1 chain in the lysates of toxin-exposed cells. However, the co-immunoprecipitation studies are not conclusive. The complex formed between p97 and the CT A1 chain might be nonspecific because very high concentrations of toxin were used or be representative of an interaction unrelated to retrotranslocation, perhaps caused by p97 function as a general chaperone for abnormal proteins in the cytosol.

So how does the A1 chain cross the ER membrane? One possibility is that the A1 chain may ratchet or pull itself out of the ER because it can rapidly and spontaneously refold as it emerges into the cytosol (4). Another possibility is that the ATPases in the 19 S proteasome may pull the A1 chain from the ER as has been proposed for the pre-pro α-factor in yeast (19). Much remains to be learned, and the next most informative steps may be to model the retrotranslocation reaction of the toxins in vitro.

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