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Accessibility
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Cholera toxin travels from the plasma membrane to the endoplasmic reticulum of host cells, where a portion of the toxin, the A1-chain, is unfolded and targeted to a protein-conducting channel for retrotranslocation to the cytosol. Unlike most retrotranslocation substrates, the A1-chain escapes degradation by the proteasome and refolds in the cytosol to induce disease. How this occurs remains poorly understood. Here, we show that an unstructured peptide appended to the N terminus of the A1-chain renders the toxin functionally inactive. Cleavage of the peptide extension prior to cell entry rescues toxin half-life and activity in vivo. We find that the addition of an N-terminal peptide allows retrotranslocation of the mutant toxin and that this occurs remains poorly understood. Here, we show that an unstructured peptide appended to the N terminus of the A1-chain renders the toxin functionally inactive. Cleavage of the peptide extension prior to cell entry rescues toxin half-life and function. The loss of toxicity is explained by rapid degradation by the proteasome after retrotranslocation to the cytosol. Degradation of the mutant toxin does not follow the N-end rule but depends on the two Lys residues at positions 4 and 17 of the native A1-chain, consistent with polyubiquitination at these sites. Thus, retrotranslocation and refolding of the wild-type A1-chain must proceed in a way that protects these Lys residues from attack by E3 ligases.

Like most toxins, cholera toxin (CT)3 must cross a cell membrane to enter the cytosol of host cells and induce disease. To do this, CT traffics retrograde from the plasma membrane to the endoplasmic reticulum (ER), where it co-opted the machinery for degradation of terminally misfolded proteins in the ER (1). Unlike the misfolded substrates, however, the toxin escapes rapid degradation by the proteasome and refolds to its native conformation so as to act enzymatically in the cytosol (2). This is evidenced most clearly by the lack of sensitivity to chemical inhibition of the proteasome (3). How the A1-chain avoids rapid degradation to induce toxicity, however, is not fully understood. The prevailing view is that the paucity of lysines explains the resistance to ubiquitination and proteasomal degradation (4). Another view is that cytosolic chaperones stabilize the fold of the A1-chain so as to prevent non-ubiquitin-dependent degradation by the 20S proteasome, but degradation of the A1-chain in vivo is slow, occurring after the induction of toxicity (5).

CT typifies the AB5 family of toxins and is the virulence factor responsible for the massive secretory diarrhea seen in Asiacal cholera. The A-subunit is comprised of an enzymatically active A1-chain linked non-covalently to the B-subunit via the A2-chain. The A1- and A2-chains are joined by a flexible loop containing a serine-protease cleavage site bridged by a disulfide bond. Both bonds must be broken before the A1-chain can enter the cytosol of host cells (2). The B subunit consists of five 11.5-kDa peptides assembled non-covalently into a stable homopentamer that binds to the ganglioside GM1 on the plasma membrane. The B-subunit-GM1 complex carries the A-subunit into the ER (6).

In the ER, protein disulfide isomerase (PDI) in its reduced state unfolds and dissociates the A1-chain from the B-subunit. When oxidized, PDI releases the A1-chain to subsequent steps in the retrotranslocation reaction (7). Presumably the A1-chain crosses the ER membrane in an unfolded conformation via transport through a protein-conducting channel, possibly involving the E3 ligase Hrd1, Derlin-1, and Sec61 (8–11). Unlike most other ER-associated degradation (ERAD) substrates, the A1-chain does not require ubiquitination (3) or interaction with the AAA-ATPase P97 for retrotranslocation (12), and the driving force for the retrotranslocation reaction remains unknown. It is possible that rapid and partial refolding of the A1-chain immediately upon transit across the ER membrane might actually drive retrotranslocation by creating the conditions for a molecular ratchet (3). Once in the cytosol, the A1-chain refolds to its native conformation and enzymatically activates adenyl cyclase. This causes the massive intestinal chloride and water secretion that is the hallmark of cholera.

In an approach to model retrotranslocation of the A1-chain in vitro, we prepared two mutant toxins with essentially the same N- and C-terminal peptide extensions of the A1-chain that would allow for development of a trans-membrane transport assay by protease protection. When tested in vivo, however, we found the toxin with the N-terminally extended A1-chain to be nearly non-functional. Here, we describe how the peptide extension renders the mutant toxin inactive. We find that the addition of an N-terminal peptide allows retrotranslocation but acts as a degron by rendering the two amines...
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present in the native A1-chain (Lys-4 and Lys-17) substrates for ubiquitination. Degradation of the mutant A1-chain is rapid, causing the loss of toxicity, and it is blocked by proteasome inhibitors or by mutation of the Lys residues to Arg. Thus, Lys-4 and Lys-17 of the native A1-chain are capable of being ubiquitinated, implying that retrotranslocation of the wild-type (WT) toxin must proceed in a way that protects these residues from attack by E3 ligases. This allows for escape from degradation by the proteasome and is required for induction of a physiologic response.

EXPERIMENTAL PROCEDURES

Materials—Carboxybenzyl-Leu-Leu-Leu-vinyl sulfone (ZL3VS) was a gift from Dr. H. Ploegh (Whitehead Institute for Biomedical Research). Antibodies were purchased against hemagglutinin (HA) (Roche Applied Science) and BiP (Santa Cruz Biotechnology; Santa Cruz, CA). Antibodies against CT were described previously (13). AcTev protease was from Invitrogen. CT used in the A2-chain experiment was from Calbiochem. Figures were made using BioDraw (Cambridgesoft; Cambridge, MA).

Plasmids—Sequences encoding WT CT, HA-CT, and CT-HA were cloned into pLMP3 and transferred into the arabino-nucleic pARC plasmid. The non-cleavable mutant, R192G, was previously described (1). The G33D mutant was a gift from Dr. R. Holmes. The E112D, K4R, and K17R mutations were introduced into WT CT and HA-CT within pLMP3 as described previously (3) and transferred into the pARC.

Protein Carbamylation and Electrophysiology—performed as described previously (3, 14). Where indicated, intestinal T84 cells were pretreated with 5 μM brefeldin-A (Sigma-Aldrich), 10 μM ZL3VS in dimethyl sulfoxide (DMSO), 10 μM lactacystin (Sigma-Aldrich), or vehicle alone.

Recombinant Toxins and GM1 Beads—Toxins were expressed in Escherichia coli BL21 cells. Cells were grown at 30 °C in 25 μg/ml chloramphenicol to an A600 of 0.6–0.8. Expression was induced with 0.5% arabinose for 3 h. Bacteria were harvested and resuspended in equilibration buffer (50 mM Na2HPO4, 300 mM NaCl, pH 7). Polymyxin B (Sigma-Aldrich), or vehicle alone.

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RESULTS

Structure and Function of the Toxin Mutants—Two mutant toxins were prepared containing the HA epitope linked to the N or C terminus of the A1-chain by a tobacco etch virus protease cleavage site (TEV) andFLAGanked at both ends by Gly and Ser residues (Fig. 1, A and B, named HA-CT and CT-HA, respectively, and see supplemental Fig. 1). Toxin function was examined using intestinal T84 cells grown in monolayer culture, which respond to CT by producing a CAMP-dependent Cl− secretory response. This is measured in real time as a transepithelial short-circuit current (Isc).

The secretory response induced by the HA-CT mutant toxin was strongly attenuated when compared with WT CT (Fig. 1C,
and not the B-subunit or intact A-subunit. We confirmed that the A1-chain is the only retrotranslocation substrate in separate experiments using WT CT by testing for the ability of PDI to dissociate the A1- and A2-chains from the B-subunit in vitro. The results show that only the A1-chain is released by PDI and that the A2-chain remains with the B-subunit (supplemental Fig. 2).

Molecular controls further verify the retrotranslocation assay. The A1-chain of the mutant toxin containing Gly for Arg at position 192 (R192G), which prevents cleavage of the A-subunit and thus release of the A1-chain from the B-subunit (18, 19), is not seen in the cytosolic fraction (Fig. 2A, lane 6). The A1-chain of another mutant holotoxin G33D, which is deficient in binding GM1 and cannot traffic into the ER of host cells (20), is also absent from the cytosol (data not shown). Finally, a toxin mutated within its enzymatic active site, E112D, but otherwise capable of ER transport is seen in the cytosol as predicted (Fig. 2A, lane 5). In all cases, the B-subunit monomer is found only in the membrane fraction (except for the G33D mutant, which cannot bind GM1), and the ER lumenal chaperone BiP is found only in the membrane fraction, indicating that lumenal ER proteins are not released (Fig. 2B). Thus, the assay is robust and specific for measuring retrotranslocation of the A1-chain (Fig. 2A, compare middle and right panels).

When tested in this assay, the A1-chain of the C-terminally extended mutant toxin CT-HA was found in the cytosol of host cells, but the A1-chain of the N-terminally extended HA-CT was not (Fig. 2C, middle and top panels, compare lane 7 with lane 4). Cleavage of the peptide extensions by treatment with TEV protease rescued the retrotranslocation reaction for the HA-CT mutant (Fig. 2C, middle panel, compare lanes 4 and 5), consistent with rescue of toxicon function by TEV protease (Fig. 1C). Immunoblots using antibodies against the HA epitope are negative in these rescue experiments because the tag has been cleaved. Recombinant WT CT was used as a positive control (Fig. 2C, middle panel, lane 2). In all cases, the B-subunit monomers were absent from the cytosolic fractions, providing an internal control showing specificity for retrotranslocation of the A1-chain. Also, treatment of host cells with brefeldin A completely inhibited the retrotranslocation reaction as expected for blockade of toxin entry into the ER (Fig. 2C, top and middle panels, lanes 3, 6, 8, and 10). Thus, the A1-chain of the N-terminally extended mutant HA-CT was either blocked in the retrotranslocation reaction or rapidly degraded after arrival in the cytosol.
Inhibition of the Proteasome Rescues Function of the HA-CT Mutant—To test for rapid degradation of the A1-chain in the cytosol, we pretreated host cells with the proteasome inhibitor lactacystin or vehicle control alone and assayed for retrotranslocation. Lactacystin blocked proteasome function in Vero cells, as assayed by SDS-PAGE of total cell lysates and immunoblot for polyubiquitinated proteins (data not shown). As predicted by our previous studies (3), lactacystin had no effect on the appearance of the WT A1-chain in the cytosol after retrotranslocation (Fig. 3A, lower panel, compare lanes 3 and 4). For the N-terminally extended HA-CT mutant, however, the inhibitor rescued the appearance of the A1-chain in the cytosol, where no A1-chain was present in the absence of lactacystin (Fig. 3A, compare lanes 5 and 6). The same results were obtained using the proteasome inhibitor ZL3VS (data not shown). Thus, unlike the WT A1-chain, the N-terminal mutant A1-chain is a substrate for proteasomal degradation after arrival in the cytosol.

These results were confirmed for the N-terminally extended HA-CT mutant using functional studies for toxin-induced Cl⁻/H⁺ secretion (Isc). Treatment of T84 cell monolayers with lactacystin had minimal or no effect on the viability of monolayers was demonstrated by applying the cAMP agonist, forskolin. C, summarized data from two independent experiments, each done in triplicate, of maximal Isc for untreated or lactacystin (Lac) pretreated T84 cells is shown. Error bars in C and D indicate S.D.
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As before, the A1-chain of WT CT appears in the cytosol following incubation with host cells, and lactacystin has no measurable effect (Fig. 4A, middle panel, compare lanes 6 and 7). The A1-chain of the HA-CT mutant does not appear in the cytosol unless the cells are pretreated with lactacystin (Fig. 4A, top and middle panels, compare lanes 10 and 11). Substitution of the two Lys residues in the HA-CT mutant (HA-CT K4RK17R), however, rescues the presence of the A1-chain in the cytosolic fractions, independent of lactacystin treatment (Fig. 4A, middle and upper panels, lanes 12 and 13). The same results were obtained after carbamylation of the HA-CT K4RK17R mutant to block the N-terminal α-amino from ubiquitination (HA-CT K4RK17R*, Fig. 4A, middle and upper panels, lanes 14 and 15). In control studies, Arg substitutions and blockade of the N-terminal α-amino in the WT toxin (WT K4RK17R*) had no effect (Fig. 4A, middle panel, lanes 8 and 9), consistent with our previous experiments (3). Thus, the addition of a small peptide to the N terminus somehow renders Lys-4 and Lys-17 of the native toxin substrates for ubiquitination.

We did not observe any evidence for ubiquitinated A1-chain in any of our immunoblots, and our attempt to demonstrate ubiquitination of the A1-chain using Vero cells expressing Myc-ubiquitin were not successful. These results typify most studies on ubiquitinated proteins degraded by the proteasome, even in the presence of proteasome inhibitors. Thus, we sought to confirm the role of Lys-4 and Lys-17 using the electrophysiology assay for toxin function in T84 cells. As predicted by our biochemical studies, we found that lactacystin had no further effect on the toxicity of the HA-CT K4RK17R mutant when carbamyalted at the N-terminal α-amino (Fig. 4B and D). The increase induced by the HA-CT K4RK17R mutant that was not carbamyalted at the N terminus, however, was enhanced in cells pretreated with lactacystin (Fig. 4C and D). Thus, mutation of Lys-4 and Lys-17 does not fully rescue the HA-CT mutant from attack by the proteasome, implying that the α-amino of the N-terminal residue in the HA-CT K4RK17R mutant can also act as a site for ubiquitination.

HA-CT Refolds Poorly after Release from PDI in Vitro—How does the N-terminal peptide act as a degron? Certain E3 ligases recognize substrates that are unfolded or have unfolded domains (21). Because the peptide appended to the A1-chain is likely unstructured (as evidenced by protease degradation in vitro, data not shown), it is possible that the peptide extension itself might target the refolded A1-chain for ubiquitination after retrotranslocation. Alternatively, the peptide extension might block the normal refolding of the A1-chain as it emerges into the cytosol.

time course of $I_{sc}$ induced by WT CT, consistent with our previous studies (3) (Fig. 3B, compare filled and open squares). In contrast, lactacystin partially rescued the $I_{sc}$ induced by HA-CT (Fig. 3B, compare filled and open triangles). The same result was found for ZL3VS (data not shown). Treatment of these mono-layers with forskolin at the end of the time course (arrows) induced a further increase in $I_{sc}$, showing that the cells were physiologically fully intact. The effect of lactacystin on maximal $I_{sc}$ for six independent studies is summarized in Fig. 3C. These results show that the mutant A1-chain of HA-CT undergoes retrotranslocation but is rapidly degraded by the proteasome after arrival in the cytosol. Rescue of toxin function by the proteasome inhibitors, however, was not complete, so the N-terminal extension of the refolded A1-chain for ubiquitination after retrotranslocation implies that the peptide extension might block the N-terminal α-amino of the HA-CT K4RK17R mutant to block the proteasome.

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Degradation of Cholera Toxin A1-chain

A)

\[
\begin{array}{c}
\text{HSV-Tev} \\
\text{GSSGGQPELAPEDPEDLEENLYFGGSS} \\
\text{Linker} \\
\text{HSV Tag} \\
\text{Tev Site} \\
\text{Link} \\
\text{A2} \\
\text{R192} \\
\text{A1} \\
\end{array}
\]

B)

\[
\begin{array}{c}
\text{HSV-CT} \\
\text{+ AcTev} \\
\text{Mean Isc} \\
\text{Time (minutes)} \\
\text{0} \\
\text{40} \\
\text{80} \\
\text{120} \\
\text{160} \\
\text{200} \\
\end{array}
\]

C)

\[
\begin{array}{c}
\text{HSV-CT} + \text{lactacystin} \\
\text{Mean Isc} \\
\text{Time (minutes)} \\
\text{0} \\
\text{5} \\
\text{10} \\
\text{15} \\
\text{20} \\
\text{25} \\
\text{30} \\
\end{array}
\]

FIGURE 5. Development and functional analysis of HSV-CT. A, a schematic of the N-terminal peptide extensions; HA-CT and HSV-CT, is shown. Arg-192 (R192) is located at the cleavage site separating the A1- and A2-chains. A disulfide bond, in addition to other non-covalent interactions, links together the two chains. B, in a time course of CT-induced Cl− secretion (Isc), T84 cells were incubated with HSV-CT (filled circle) or HSV-CT pretreated with AcTev protease to remove the tag (HSV-CT, open circle). C, this was performed as in panel B, but cells were treated with HSV-CT (filled circle) or lactacystin-pre-treated HSV-CT (open circle). Error bars in B and C indicate S.D.

DISCUSSION

Most ERAD substrates in mammalian cells and yeast are retrotranslocated to the cytosol and targeted to the proteasome by polyubiquitination (22). Ubiquitination, in fact, may contribute fundamentally to the retrotranslocation reaction by allowing for stable interaction between the ERAD substrate and the cytosolic AAA-ATPase P97 (22, 23). P97 provides the driving force for these reactions by pulling the ERAD substrate into the cytosol from the ER membrane (23). The toxins, however, are an exception. They do not require P97 and are presumed to escape ubiquitination and proteasomal degradation long enough to act enzymatically in the cytosol and induce disease. This is partially explained by the paucity of lysine residues in the enzymatic chains of the toxins (4). Here, we find that other factors must also be involved.

CT contains two Lys residues in the N-terminal domain of the A1-chain that are conserved in the closely related E. coli heat labile toxin type I (24). Both are dispensable for toxin function (3). In this study, we show that a short peptide extension to the N terminus of the A1-chain of CT inhibits toxin action by...
our results. Rather, it is the presence of an additional peptide chain, located at the N terminus, which induces ubiquitination. The primary structure of the peptide extension is dispensable as another peptide of different amino acid sequence elicits the same effect. Both peptide extensions tested are predicted to be loosely structured (27, 28), so this feature might be an essential factor. Placement of the degron at the N terminus in close proximity to the two Lys residues (29) also appears to be required as the analogous peptide appended to the C terminus has little effect on toxicity. Furthermore, recent studies show that ubiquitination may not be sufficient to ensure degradation by the proteasome and that an unstructured domain is needed to initiate unfolding and physical entry into the proteasome channel (16, 29). The unstructured peptide extension of HA-CT might also act in this way.

**What Protects Lys-4 and Lys-17 of the Native Toxin from Ubiquitination after Retrotranslocation to the Cytosol?**—Because retrotranslocation of the A1-chain does not require P97 in intact cells (12), we previously speculated that rapid and partial refolding of the A1-chain immediately upon transit across the ER membrane might drive the retrotranslocation reaction by creating the conditions for a molecular ratchet (3). These ideas led us to test whether the N-terminal peptide might act as a degron by interfering with refolding of the A1-chain as it emerges from the ER into the cytosol, thus rendering the amines of the A1-chain substrates for attack by E3 ligases. Our in vitro results are consistent with this hypothesis. The peptide extension of the HA-CT mutant inhibits spontaneous refolding of the A1-chain after release from PDI.

Circular dichroism and fluorescence spectroscopy studies by Pande et al. (5), however, show that the A1-chain on its own is thermally unstable in vitro and may not refold spontaneously after retrotranslocation in vivo. This study also shows that binding of ADP-ribosylating factor 6 (ARF6), an enzymatic cofactor of CT, stabilizes the fold of the A1-chain, and the authors raise the important idea that cytosolic chaperones might be required for refolding of the A1-chain in the cytosol. Chaperone binding might then, directly or indirectly, protect the A1-chain from ubiquitination and rapid degradation immediately upon exit from the ER. Extension of the A1-chain at the N terminus could block this reaction, especially if the N terminus leads the peptide through the retrotranslocation channel.

We also note that inhibition of the proteasome does not fully rescue retrotranslocation of the A1-chain nor its toxicity. These results might be explained by the inability of lactacystin or ZL3VS to fully inhibit proteasome function, but they are also consistent with an inhibitory effect of the N-terminal peptide on the retrotranslocation reaction itself. Unfolding and dissociation of the mutant A1-chains by PDI appear to be intact, at least as assessed in vitro. Perhaps the N terminus of the A1-chain helps target the protein to the retrotranslocation channel or to another ER chaperone essential for the reaction, and this is blocked in our N-terminal peptide mutant. However, the primary sequence of the extreme N- or C-terminal domains of the A1-chain cannot be required because both mutant A1-chains (HA-CT and CT-HA) clearly enter the cytosol from the ER when degradation by the proteasome is inhibited.

**Degradation of Cholera Toxin A1-chain**

![Figure 6. HA-CT refolds poorly after release from PDI in vitro.](http://www.jbc.org/)

*Figure 6. HA-CT refolds poorly after release from PDI in vitro.* Briefly, we incubated all toxins with GM1-coupled beads and PDI in reducing conditions. PDI binds, unfolds, and releases the A1-chain into the supernatant from the B-subunit-GM1 magnetic beads. The supernatants were left untreated or were treated with GSSG to induce release of the A1-chain from PDI, and refolding was measured as resistance to trypsin degradation. All samples were analyzed by SDS-PAGE under non-reducing conditions, except the 4-ng control, which was reduced to separate the A1-chain. Our results show that rapid degradation by the proteasome is inhibited against attack by E3 ligases, either during or very quickly after the A1-chain enters the cytosol. Our results show that rapid degradation by the proteasome is inhibited.

*How Does the N Terminus of the Mutant A1-chain Act as a Degron?*—The N-terminal residue of the HA mutant A1-chain is glycine, which is predicted to stabilize the peptide against degradation according to the N-end rule (25, 26). Also, cleavage of the peptide by TEV protease leaves the activated A1-chain with the same residue (Gly) at the N terminus, and this renders the mutant toxin fully active. So the N-end rule cannot explain rendering these Lys substrates for ubiquitination, thus causing rapid degradation by the proteasome. In nature, however, retrotranslocation of the WT A1-chain does not result in rapid degradation. Thus, these same residues must somehow be protected against attack by E3 ligases, either during or very quickly after the A1-chain enters the cytosol. Our results show that this is required for induction of a physiologic response. Such escape from ubiquitination for WT CT is now of particular interest because recent studies implicate the E3 ligases Hrd1 and GP78 from ubiquitination for WT CT is now of particular interest after the A1-chain enters the cytosol. Our results show that this is blocked in our N-terminal peptide mutant. However, the primary sequence of the extreme N- or C-terminal domains of the A1-chain cannot be required because both mutant A1-chains (HA-CT and CT-HA) clearly enter the cytosol from the ER when degradation by the proteasome is inhibited.
Degradation of Cholera Toxin A1-chain

Overall, our results show that the primary effect of the appended peptide is to target the A1-chain for ubiquitination and degradation by the proteasome. Both the N-terminal ω-amine and the two Lys residues near the N terminus provide reactive sites. Ubiquitination, however, does not occur for the WT toxin (3). Thus, we propose that the structure of the N-terminal domain in the native toxin allows for protection of the A1-chain against attack by E3 ligases. This could occur by allowing for rapid refolding of the peptide as it emerges from the ER, perhaps by association with cytosolic factors. Whatever the mechanism, such protection against ubiquitination explains how the A1-chain escapes rapid degradation by the proteasome after retrotranslocation, and this is essential for toxin function in the pathogenesis of disease.

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