Shiga-like toxin I (Slt-I) is a ribosome-inactivating protein that undergoes retrograde transport to the endoplasmic reticulum to exert its cytotoxic effect on eukaryotic cells. Its catalytically active A1 domain subsequently migrates from the endoplasmic reticulum (ER) lumen to the cytoplasm. To study this final retrotranslocation event, a suicide assay was developed based on the cytoplasmic expression and ER-targeting of the cytotoxic Slt-I A1 fragment in *Saccharomyces cerevisiae*. Expression of the Slt-I A1 domain (residues 1–251) with and without an ER-targeting sequence was lethal to the host and demonstrated that this domain can efficiently migrate from the ER compartment to the cytosol. Deletion analyses revealed that residues 1–239 represent the minimal A1 segment displaying full enzymatic activity. This fragment, however, accumulates in the ER lumen when directed to this compartment. The addition of residues 240–251 restores the translocation property of the A1 chain in yeast. However, single mutations within this region do not significantly alter this function in the context of the 251-residue long A1 domain or affect the toxicity of the resulting Slt-I variants toward Vero cells in the context of the holotoxin. Since this mechanism of retrotranslocation is common to other protein toxins lacking a peptide motif similar in sequence to residues 240–251, the present results suggest that the ER export mechanism may involve the recognition of a more universal structural element, such as a misfolded or altered peptide domain localized at the C terminus of the A1 chain (residues 240–251) rather than a unique ER export signal sequence.

**A Role for the Protease-sensitive Loop Region of Shiga-like Toxin 1 in the Retrotranslocation of Its A1 Domain from the Endoplasmic Reticulum Lumen**

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Shiga-like toxin I (Slt-I) is a ribosome-inactivating protein that undergoes retrograde transport to the endoplasmic reticulum to exert its cytotoxic effect on eukaryotic cells. Its catalytically active A1 domain subsequently migrates from the endoplasmic reticulum (ER) lumen to the cytoplasm. To study this final retrotranslocation event, a suicide assay was developed based on the cytoplasmic expression and ER-targeting of the cytotoxic Slt-I A1 fragment in *Saccharomyces cerevisiae*. Expression of the Slt-I A1 domain (residues 1–251) with and without an ER-targeting sequence was lethal to the host and demonstrated that this domain can efficiently migrate from the ER compartment to the cytosol. Deletion analyses revealed that residues 1–239 represent the minimal A1 segment displaying full enzymatic activity. This fragment, however, accumulates in the ER lumen when directed to this compartment. The addition of residues 240–251 restores the translocation property of the A1 chain in yeast. However, single mutations within this region do not significantly alter this function in the context of the 251-residue long A1 domain or affect the toxicity of the resulting Slt-I variants toward Vero cells in the context of the holotoxin. Since this mechanism of retrotranslocation is common to other protein toxins lacking a peptide motif similar in sequence to residues 240–251, the present results suggest that the ER export mechanism may involve the recognition of a more universal structural element, such as a misfolded or altered peptide domain localized at the C terminus of the A1 chain (residues 240–251) rather than a unique ER export signal sequence.

**EXPERIMENTAL PROCEDURES**

Plasmids and Strains—pRSATT was created by amplifying two regions of the GAL1 promoter from pRS854 and cutting them into the multiple cloning site of pRS316. The first region was created with a sense primer (STATA2, GAG AGA GAA TTC tac gtt aat ctc aat gct tcg) complementary to the sequence upstream of the GAL4 repeats and an antisense primer (ATATA2, GAG AGA GGA TCC GAG AGA GCA TGC gtt aat aag cca aaa atc atc gtt ctc aat ctc) complementary to the sequence around the TATA element of the GAL1 promoter. This construct resulted in a product with an upstream EcoRI cloning site (underlined) and a downstream BamHI site (with a nested SphI site, both underlined), which was cloned into pRS316 between EcoRI and BamHI (creating an intermediate vector). The second region was created with a sylceramide or Gb3 (4–6). Receptor binding is followed by clathrin-mediated endocytosis and retrograde transport of the toxin through the Golgi apparatus en route to the ER lumen (3, 7). While migrating through the secretory pathway, a protease-sensitive loop (residues 242–261) located in the C-terminal region of the A chain is cleaved, dividing the A chain into a B pentamer-associated A2 domain (residues 252–293) and an enzymatic A1 domain (residues 1–251) (8). The A1 domain remains associated with the A2/B subunits complex by virtue of a disulfide bond between cysteines 242 and 261. This disulfide bond is ultimately released in the ER lumen, liberating the enzymatic A1 domain that is subsequently retrotranslocated to the cytosol. The N-glycosidase activity of the A1 fragment depurinates a single adenosine residue at position 4324 in the 28 S rRNA inhibiting protein synthesis and subsequently leading to cell death (9).

The mechanism by which the catalytic domain of Slt-I and other ERTs is exported from the ER lumen to the cytosol remains poorly defined. Proteolysis of the Slt-I A chain to an A1 fragment is a required event for toxicity to occur in mammalian cells (10). Proteolysis exposes a hydrophobic peptide present in the A chain of Slt-I and other ERTs, an event that may facilitate its interaction with ER-resident proteins or the ER membrane (11–13). However, studying the role of this region in retrotranslocation in isolation of other processes of retrograde traffic and processing remains challenging in the context of cell-based assays.

Yeast represents an attractive model for analyzing how the enzymatic A1 domain of Slt-I is able to escape from the lumen in the ER and kill the host cell. We report the development of a suicide assay in yeast where the fully active enzymatic A1 fragment of Slt-I is rapidly targeted to the ER before it can accumulate in the cytosol. The ability of the Slt-I A1 fragment to subsequently retrotranslocate to the cytosol from the ER is lethal to the host and was the basis of a screen to define a region of the toxin involved in this relocation process. This yeast assay was used to identify a C-terminal region of the Slt-I A1 fragment involved in retrotranslocation.
### TABLE I

| Product                                      | Description                                      |
|----------------------------------------------|--------------------------------------------------|
| AC SacI 251 TAG TGA                          | Primers used in this study to construct expression vectors |
| AC SacI 245 TAG TGA                          |                                                    |
| AC SacI L240R 240                            |                                                    |
| AC SacI 242 TAG TGA                          |                                                    |
| AC SacI 238 TAG TGA                          |                                                    |
| D1 and D6 (1–251 and 1–239 fragments, respectively) and cloned into combination with the antisense primers (D1 through D13). The primers were generated by PCR using the sense primer P2 in combination with | |
Expression of the Slt-I A1 Fragment in Yeast—Two yeast expression vectors were constructed to study the retrotranslocation of the A1 fragment of Shiga-like toxin 1 (Fig. 1A). The first vector, termed pRSATT, was constructed to express Slt-I A chain variants directly into the cytosol under the control of an attenuated GalI promoter, while pRSATT-ER was designed to express Slt-I A chain constructs harboring the N-terminal signal sequence (SS) able to direct their localization to the ER lumen. B, schematic diagram of the yeast assay. Slt-I A variants are shown as rectangles with the cryptic hydrophobic domain hydrophobic domain depicted as a gray area and the HAT signal sequence required for ER-targeting as a black rectangle. Cloned toxin elements expressed in the cytosol lead to cell death. Targeting of Slt-I A variants to the ER lumen will also lead to cell death unless a mutation (M) in a putative retrotranslocative domain prevents its retrotranslocation.

FIG. 1. A, yeast vector constructs used in this study. The pRSATT vector was used for the cytosolic expression of Slt-I A chain variants from an attenuated GalI promoter, while pRSATT-ER was designed to express Slt-I A chain constructs harboring the N-terminal signal sequence (SS) able to direct their localization to the ER lumen. B, schematic diagram of the yeast assay. Slt-I A variants are shown as rectangles with the cryptic hydrophobic domain hydrophobic domain depicted as a gray area and the HAT signal sequence required for ER-targeting as a black rectangle. Cloned toxin elements expressed in the cytosol lead to cell death. Targeting of Slt-I A variants to the ER lumen will also lead to cell death unless a mutation (M) in a putative retrotranslocative domain prevents its retrotranslocation.

RESULTS

Expression of the Slt-I A1 Fragment in Yeast—Two yeast expression vectors were constructed to study the retrotranslocation of the A1 fragment of Shiga-like toxin 1 (Fig. 1A). The first vector, termed pRSATT, was constructed to express Slt-I A chain variants directly into the cytosol under the control of an attenuated GalI promoter (16). The second vector, pRSATT-ER, was designed such that Slt-I A1 variants would be expressed with an N-terminal ER-targeting sequence (17) to ensure the concomitant synthesis and translocation of the nascent A1 chains into the ER lumen. Folded A1 mutant chains unable to retrotranslocate from the ER (or another compartment of the secretory pathway) back to the cytosol would result in a survival phenotype. An expression of toxicity levels observed for cloned Slt-I A1 variants expressed from pRSATT and pRSATT-ER was the basis of a method to delimit region(s) of the A1 chain involved in retrotranslocation (Fig. 1B).

Identification of a Retrotranslocative C-terminal Peptide in the Slt-I A Chain—We hypothesized that distinct peptide domains mediated cytotoxicity and retrotranslocation and that a minimum cytotoxic fragment of the Slt-I A1 fragment would not retain its ability to escape from the ER lumen. A series of cassettes coding for the wild-type Slt-I A1 fragment (residues 1–251) and C-terminally truncated variants were created and cloned into pRSATT and pRSATT-ER. The expression of the 1–251 Slt-I A1 fragment from pRSATT was lethal to the host (Fig. 2A; fragment 1–251). Slt-I A1 fragments truncated at their C terminus also remained lethal to the host until residue 239 was deleted (Fig. 2A; fragments 1–238 and 1–239). The viability of yeast cells transformed with the pRSATT vector coding for residues 1–238 was indistinguishable from that observed in yeast transformed with the empty vector (pRSATT only) and in yeast expressing a catalytically inactive E167A/R170A double mutant termed DETOX (Fig. 2A). Wild-type Slt-I holotoxin and its detoxified variant as well as Slt-I A1 fragments coding for either residues 1–238, 1–239, or the entire A chain were then separately expressed in bacteria, purified, and subsequently tested to confirm their ability to inactivate ribosomes in vitro (N-glycosidase activity) in a rabbit reticulocyte lysate (TNT) assay (Fig. 2C). As expected, the enzymatic activities of the A chain and of the 1–239 fragment were comparable with that of the wild-type Slt-I toxin, while the 1–238 fragment...
and the DETOX form of the toxin had no detectable catalytic activity (Fig. 2C). Genes coding for Slt-I A1, 1–251 and 1–239 were then cloned into pRSATT-ER to determine whether the deleted C-terminal region (residues 240–251) was essential for routing the A1 fragment from the ER lumen to the cytosol. Both Slt-I A1, 1–251 and 1–239 were equally toxic when expressed into the cytosol of yeast cells (Fig. 2B), while only the 1–251 construct remained toxic to cells when expressed with an N-terminal signal sequence (Fig. 2B, fragment ER 1–251). A survival phenotype was observed from the ER-routed A1, 1–239 variant implying that the truncated protein was unable to relocate back to the cytosol from the ER lumen after removal of the signal sequence. Finally, the fragment expressing residues 1–239 fused to the signal-cleaved A1 chain ( Fig. 2B, fragment DETOX holotoxin, and of 1–238 fragment in a rabbit reticulocyte lysate assay (TNT assay). A loss of luciferase activity (relative light units) correlates with the blockage of protein synthesis.

FIG. 2. A, delimited the minimum cytotoxic domain of Slt-I A1. Yeast cells were transformed either with the empty pRSATT vector, the vector expressing a catalytically inactive mutant of Slt-I A1 (DETOX), full-length Slt-I A1 (1–251), or C-terminal truncated forms of Slt-I A1 (1–239 and 1–238). 10-Fold serial dilutions of each yeast culture were spotted on SD-Ura plates supplemented with 2% galactose. B, the deletion of residues 240–251 from the C terminus of Slt-I A1 abolishes retrotranslocation. The toxicities of Slt-I A1, 1–251 constructs expressed in the cytosol (1–251) or directed to the ER lumen (ER 1–251) of yeast cells were comparable with that of cytoscopically expressed Slt-I A1, 1–239 (1–239). ER-targeted Slt-I A1, 1–239 (ER 1–239) was not cytotoxic. C, N-glycosidase activity of wild-type holotoxin ( C), of full-length 1–239 A chain ( C), of 1–239 fragment, of DETOX holotoxin ( C), and of 1–238 fragment in a rabbit reticulocyte lysate assay (TNT assay). A loss of luciferase activity (relative light units) correlates with the blockage of protein synthesis.

and the DETOX form of the toxin had no detectable catalytic activity (Fig. 2C). Genes coding for Slt-I A1, 1–251 and 1–239 were then cloned into pRSATT-ER to determine whether the deleted C-terminal region (residues 240–251) was essential for routing the A1 fragment from the ER lumen to the cytosol. Both Slt-I A1, 1–251 and 1–239 were equally toxic when expressed into the cytosol of yeast cells (Fig. 2B), while only the 1–251 construct remained toxic to cells when expressed with an N-terminal signal sequence (Fig. 2B, fragment ER 1–251). A survival phenotype was observed from the ER-routed A1, 1–239 variant implying that the truncated protein was unable to relocate back to the cytosol from the ER lumen or any other compartments along the secretory pathway or was simply sequestered by cytoplasmic compartments. More specifically, myc-tagged versions of the 1–251 and 1–239 fragments were cloned into a high copy number vector (pRS416) and expressed under the control of the wild-type Gal1 promoter, with and without the ER signal sequence. W303a yeast cells were then transformed with these vectors and grown in galactose to induce the expression of the toxin variants. The yeast cells were fractionated into cytosolic and ER fractions, and the myc-tagged protein was immunoprecipitated from each fraction. Immunoprecipitated material was then separated by SDS-PAGE and Western blots were performed using anti-Slt-I A chain polyclonal antibodies. As expected, 1–251 and 1–239 toxin fragments were recovered from the cytosol but not from ER fractions of yeast cells expressing the 1–251 and 1–239 toxin fragments lacking the ER signal sequence (29-kDa band; Fig. 3B). Similarly, toxicity was only recovered from the cytosol of yeast cells expressing the 1–251 fragment fused to the ER signal sequence (33-kDa band; Fig. 3B). In this case, a larger molecular mass band (33 kDa; 1–251 fragment with ER signal sequence) is observed in the cytosol as expected from the cytoplasmic overexpression of this construct as well as a dominant cleaved A1 chain band (29 kDa) corresponding to the mass of fragment 1–251 lacking the ER signal sequence. These results suggest that the A1 chain has been directed into the ER and then rerouted to the cytosol after removal of the signal sequence. Finally, the fragment expressing residues 1–239 fused to the signal-cleaved ER signal sequence was predominantly found in the ER fraction of yeast cells (29-kDa band) suggesting that it accumulated there after translocation and signal cleavage. Predictably, some of the overexpressed ER signal-containing version of the 1–239 fragment (33 kDa band) was also observed in its unprocessed form the cytosolic fraction.

Last, a genetic approach was devised to confirm that the survival phenotype of yeast expressing the ER-targeted 1–239 fragment was due to ER import and not due to a lack of enzymatic activity. Our strategy was based on the use of a cold-sensitive yeast strain, which expresses a mutant form of Sec61p that conditionally limits ER import. The strain RSY1295 is non-viable at 17 °C but propagates in the temperature range from 23 to 37 °C where the disruption in ER import is not so severe as to prevent cell growth (19, 20). Transforming RSY1295 with the vector expressing ER-targeted 1–239 (ER 1–239; Fig. 3C) and growing this particular strain at a temperature causing a partial blockage of ER import should lead to a reduction in yeast viability due to the accumulation of newly synthesized toxic 1–239 fragments directly into the cytosol. At the permissive temperature for ER import (30 °C), RSY1295 displayed the expected sensitivity to cytosolically expressed DETOX, 1–251 and 1–239, and ER-targeted 1–251 and 1–239 (Fig. 3C; DETOX, ER 1–251, 1–239, ER 1–251, and ER 1–239) as was seen for the isogenic wild-type RSY1293 strain transformed with the same vectors. However, at 37 °C, RSY1295 was 2 orders of magnitude more sensitive to the ER-targeted 1–239 than RSY1293 (Fig. 3C, ER 1–239). The same effect was observed, albeit to a lesser extent, at 24 °C (Fig. 3C) indicating that the toxic A1 fragment 1–239 with the N-terminal ER-targeting sequence was produced but poorly shuttled to the ER lumen, thereby resulting in ribosome inactivation. Similar results were obtained for all non-retrotranslocative A1 mutants expressed in the cold-sensitive RSY1295 yeast strain (data not shown).

Taken together, these results suggest that A1 chains fused to the ER signal sequence are produced as enzymatically active molecules and targeted to the ER lumen. Furthermore, the data indicate that the 1–251 fragment of the Slt-I A chain efficiently retrotranslocates from the ER, while the 1–239 fragment accumulates there.
Progressively longer versions of the Slt-I A1 fragment were cloned into pRSATT-ER and expressed in yeast to determine the minimal peptide segment within the region 240–251 necessary to facilitate the retrotranslocation of the A1 chain from the ER lumen to the cytosol. The addition of Leu240 to the C terminus of 1–239 had a dramatic effect on retrotranslocation, restoring toxicity to nearly that of the wild type A1 domain (Fig. 4A, ER 1–240 and ER 1–251). The additions of Asn241 and Cys242 appear to partly mask the retrotranslocative potential of Leu240, while the addition of the three consecutive histidines from residues 243 to 245 restored retrotranslocation to the same level observed for 1–251 (Fig. 4A, ER 1–241, ER 1–242, and ER 1–245). The toxicity of all lengths tested was indistinguishable from wild type when expressed from pRSATT (data not shown).

Substitution of Leucine for Aspartic Acid at Position 240 in the 1–240 and 1–251 Slt-I A1 Fragment Results in a Reduction in Retrotranslocative Potential—The addition of Leu240 to the 1–239 fragment of the Slt-I A chain restored most of its retrotranslocative potential. To determine whether this effect was due to the presence of a specific amino acid at position 240 or simply a chain length effect, leucine 240 was replaced in the 1–240 Slt-I A1 fragment with alanine, asparagine, arginine, and aspartic acid and tested for cytotoxicity when expressed and routed to the ER using the pRSATT-ER vector. The toxicity of these ER-targeted variants was reduced (Fig. 4B) in relation to the ER-targeted 1–240 and 1–251 fragments (Fig. 4B). Full-length Slt-I A1 mutants (1–251) were then constructed to determine whether mutations at position 240 were blocking retrotranslocation specifically because of their location at the C terminus of the peptide. Leucine at position 240 was replaced either with alanine, asparagine, arginine, or aspartic acid in the context of the full-length Slt-I A1 (1–251), and the constructs were tested for toxicity when expressed from pRSATT-ER. The retrotranslocation defects conferred by mutations at position 240 were blocking retrotranslocation specifically because of their location at the C terminus of the peptide. Leucine at position 240 was replaced either with alanine, asparagine, arginine, or aspartic acid in the context of the full-length Slt-I A1 (1–251), and the constructs were tested for toxicity when expressed from pRSATT-ER.

FIG. 3. A, RT-PCR and amplification of cDNA from total RNA. mRNA could be detected in total RNA preparations from yeast cells expressing ER-routed 1–239, L240D1–240, and L240D1–251 as well as DETOX but not in yeast cells transformed with an empty vector (pRSATT). The PCR product in all cases is represented by the ~720-bp band. B, yeast cells overexpressing a catalytically inactive (E167A/R170A), myc-tagged 1–251 and 1–239 Slt-I A1 fragment and ER-targeted 1–251 Slt-I A1 fragment accumulate protein in the cytosol (uncleaved ~33-kDa fragment) and in the ER (cleaved ~29-kDa fragment). C, the production and routing of ER-targeted 1–239 Slt-I A1 fragment were confirmed using a temperature-sensitive Sec61p mutant yeast strain (defect in ER import). The expression of ER-targeted Slt-I A1 fragment 1–239 (ER 1–239) in the RSY1295 yeast strain is ~100-fold more toxic at 24 and 37 °C (cytoplasmic accumulation) in comparison with its expression in the isogenic wild-type strain 1293. Slt-I A1 1–251 constructs expressed in the cytosol (1–251) and ER-targeted (ER 1–251), as well as cytosolically expressed Slt-I A1 1–239 (1–239) and inactive variant (DETOX), displayed comparable toxicities in the RSY1295 strain at 24, 30, and 37 °C temperatures. 10-Fold serial dilutions of each yeast culture were spotted on SD-Ura-Leu plates supplemented with 2% galactose.
retained full enzymatic activity (data not shown).

Finally, the impact of a single L240D mutation in retrotranslocation was further addressed in an assay involving the entire AB5 Slt-I holotoxin. Wild-type and L240D Slt-I holotoxins were expressed, purified, and their cytotoxicity toward VERO cells monitored to verify if the L240D mutant was less toxic toward mammalian cells. Interestingly, both the wild-type and L240D holotoxins exhibited identical toxicity profiles toward VERO cells (Fig. 5) (see “Discussion”).

Fragment 1–239 and Full-length Slt-I A1 Domain (1–251) Adopt Similar Overall Structures in Solution—The ER lumen hosts resident proteins and mechanisms that probe the folding and structural integrity of nascent proteins (21, 22). Misfolded proteins are subsequently routed (retrotranslocated) to the cytosol for degradation (19, 23). One of these pathways is typically exploited by antigen-presenting cells to degrade foreign viral proteins and to present a complex spectrum of peptide antigens to a limited set of major histocompatibility complex class I molecules in the ER lumen (24, 25). In summary, ER-resident mechanisms can recognize a broad range of altered or foreign proteins that may be perceived as misfolded elements (22). Such mechanisms are inherently less restrictive than the traditional concept of distinct receptors recognizing unique peptide signals. The Slt-I A1 domain (1–251) as well as other ER-routed toxins may thus utilize such routing pathways, although none of these toxins share in common a domain homologous in sequence to residues 240–251. If the absence of these residues blocks the ER export of fragment Slt-I A1 (1–239) to the cytosol, one could conclude that the first 239 amino acids of Slt-I A1 are not adopting a misfolded geometry in relation to the native Slt-I A1 (1–251). Both segments Slt-I A1 1–239 and 1–251 display comparable enzymatic activity (Fig. 2C) and thus must not differ significantly in terms of their overall structure. To confirm that these fragments do adopt comparable structures in solution, we expressed, purified, and recorded the circular dichroism spectrum of Slt-I A1 1–238 (inactive A1 chain; Fig. 2), 1–239, and 1–240 from 195 to 260 nm. All spectral profiles essentially overlap (Fig. 5B), suggesting that their overall secondary structures are similar. More precisely, neither the loss of catalytic activity nor the loss of retrotranslocation can be associated with a major conformational change. The addition of residues 240–251 may thus restore the ER...
export event potentially through the presentation of a local misfolded C-terminal patch. This form of recognition would not depend on the preservation of a unique peptide motif and would explain the minimal impact of single mutations within residues 240–251 of the 1–251 peptide domain.

DISCUSSION

ER-routed toxins, such as ricin, cholera toxin, and Shiga toxin, are endocytosed by eukaryotic cells and migrate in a retrograde fashion eventually reaching the ER lumen. Their catalytic domain must subsequently escape from this penultimate compartment and reach their cytosolic targets. The A1 chain of Slt-I represents one such catalytic domain and was dissected in the present study to identify a C-terminal region serving a role in its retrotranslocation to the cytosol. More precisely, the Slt-I A1 chain contains residues 1–251 of Shiga-like toxin 1 A subunit. It harbors a hydrophobic segment within its C-terminal region (residues 224–242; Fig. 6A), a region postulated to be involved in its ER export mechanism (12). Such hydrophobic domains have also been identified in the A chain of Shiga and Shiga-like toxins and in other ribosome-inactivating proteins such as abrin (12) and ricin (26) (Fig. 6B).

To define a role for this region of the A1 domain in retrotranslocation, S. cerevisiae cells were transformed with a suicide vector that either expressed the 251-residue-long cytotoxic A1 domain in the cytosol of yeast cells or was directly routed to the ER lumen. This experimental approach essentially bypassed other routing events commonly associated with the intracellular trafficking of ER-routed toxins and directly addressed parameters associated with this final translocation event. As expected, the expression of either construct led to the eventual routing or deposition of this domain in the cytosol of yeast cells, resulting in the inactivation of ribosomes and in cell death (Fig. 2). The C terminus of the A1 chain was subsequently truncated to delimit both the minimal catalytic domain and the retrotranslocative domain of the 251-residue-long A1 domain of Slt-I. Our study revealed that the first 239 amino acids of the A1 chain represented the minimal region displaying full catalytic activity when expressed in the cytosol of yeast cells. In contrast, the same fragment harboring an N-terminal ER-targeting sequence lacked the ability to migrate from the ER compartment to the cytoplasm and was consequently not toxic to yeast cells. (Figs. 2 and 3).

The lengthening of the chain (240–251) within the context of the ER-targeted A1 chain subsequently demonstrated that the addition of either leucine 240 alone or the entire segment 240–251 achieved a similar effect in terms of restoring the ability of the A1 chain to exit the ER lumen and kill yeast cells.
A series of single mutations at position 240 revealed that the impact of changes at this site was not sufficient to prevent the retrotranslocation event in the presence of the remaining C-terminal peptide (241–251) within the framework of the complete A1 chain (residues 1–251) (Fig. 4). This finding was further confirmed when the cytotoxic activity of the wild-type AB$_2$ toxin and a variant harboring a L240D mutation displayed identical toxicity profiles toward Vero cells (Fig. 5A). These results suggest that the nature of the translocation mechanism is not based on the recognition of a specific peptide sequence but rather on a potentially less stringent peptide element, possibly the exposure of a misfolded or hydrophobic sequence. This hypothesis is further supported by the fact that the sequence alignment of ER-routed toxins did not reveal the presence of a peptide motif similar to residues 240–251 of Shiga-like toxin 1 (Fig. 6B). An examination of the crystal structure of the Shiga holotoxin indicates that residues 236–240 of its A chain (part of its hydrophobic domain, underlined in Fig. 6) are normally buried in the intact holotoxin (27). An analysis of the surface exposure of residues 236–240 in the A1 fragment further suggests that only leucine 240 would be partially exposed to an aqueous environment upon removing the A$_x$ and B subunit domains, such as in our yeast system (as calculated using the software program NACCESS). The 1–239 A$_1$ fragment (with no exposed hydrophobic residues in the C-terminal region) may be perceived as stable or folded in the secretory pathway and thus not a substrate for ER chaperones. The addition of hydrophobic leucine 240 may locally destabilize the A$_1$ fragment making it a substrate for the retrotranslocative machinery of the cell. The substitution of leucine 240 with a polar residue such as aspartic acid (L240D) may favor the solvation of this region of the A$_1$ fragment, a conformational event that is masked in the context of the 1–251 A$_1$ fragment in yeast (Fig. 4). The fact that the L240D mutation had no effect in the context of the Shl-I holotoxin is not surprising, since this residue is not exposed in the presence of the A$_x$ fragment and B subunits. The overlapping circular dichroism profiles (195–260 nm) observed for A$_1$ fragments 1–238, 1–239, and 1–251 indicate that all three fragments share a similar if not identical overall fold. The solvent exposure of residues 240–251 in A$_1$ fragment 1–251 would thus be expected to only result in a local structural difference beyond residues 1–238 (Fig. 5B). This finding and the fact that both A$_1$ fragments 1–239 and 1–251 are equally active (Fig. 2C) would suggest that the peptide element (conformationally altered or misfolded peptide element) that triggers the local exposure of a hydrophobic patch in the A$_1$ fragment is localized to the region 240–251. Further support for this concept comes from two additional sources. In the context of the complete Shiga-like toxin 1, the A chain is proteolytically cleaved by furin during cellular trafficking. The cleavage site (Am$_{251}$ Met$_{252}$ (S)) is situated in the A chain protease-sensitive loop (residues 242–261), a region constrained by a single disulfide bond linking Cys$^{242}$ to Cys$^{261}$. Experiments aimed at altering or removing this furin cleavage site or at cleaving the A chain with other proteases (trypsin, calpain) have demonstrated that the processing of the A chain to an A$_1$ fragment dramatically increases the toxicity profile of the toxin but does not alter the catalytic activity of A chain in a cell free system (28, 29). Since the A$_1$ fragment is only released from the holotoxin A$_x$ and B subunits in the ER lumen as the disulfide bond between Cys$^{242}$ and Cys$^{261}$ is finally reduced, the proteolysis results suggest that it is at the level of the retrotranslocation step that ER-resident proteins may preferentially recognize the cleaved A$_1$ domain. Second, studies with cholera toxin have recently demonstrated that the cleavage of its protease-sensitive loop is required for the interaction of its A chain fragment with the ER-resident protein, protein disulfide isomerase (30, 31). Protein disulfide isomerase is a chaperone, which recognizes a broad spectrum of misfolded proteins and prevents their aggregation by catalyzing the rearrangement of their disulfide linkages (32). Native proteins do not represent good protein disulfide isomerase substrates in comparison with unfolded proteins suggesting that the mechanism of substrate recognition is based on misfolded peptide elements (33, 34).

It has been suggested that a large number of ERTs reach the cytosol by exploiting the ER-associated degradation system (ERAD) (35). A short misfolded peptide element exposed, in the context of their respective ERT, through processing could thus serve as a universal remodeling mechanism leading to the recognition of A$_1$-like fragments by chaperones and ER-resident proteins linked to the ERAD system. One can envision a retrotranslocation model where the cleavage of the protease-sensitive loop within the A chain of Slt-I (Fig. 6) results in the exposure of a short peptide element located at the C terminus of the A$_1$ fragment. This peptide signature encompassing residues 240–251 of the A$_1$ fragment triggers a local remodeling event leading to an interaction with ER-resident proteins involved in the recognition and retrotranslocation of misfolded proteins (ERAD pathway).

One cannot rule out the possibility that the C terminus of the A$_1$ fragment forms part of a ligand region recognized by an ER receptor distinct from chaperones. Such an interaction could act to retain the A$_1$ fragment in the ER for a sufficient period of time for retrotranslocation to occur. The disruption of this interaction may allow the A$_1$ chain to further migrate to other compartments of the secretory pathway making it unavailable for the ER retrotranslocation event. One may recall that the holotoxin Shl-I normally reaches the ER through the interaction of its B subunits with the glycolipid Gb3 (3). The A$_1$ chain is subsequently released from the B pentamer in the ER lumen. The ER-targeting feature is not associated with the free A$_1$ chain itself. We did observe a small amount of the signal-cleaved 1–239 fragment in the cytosol of yeast cells overexpressing the ER-translocated 1–239 fragment (Fig. 3B). This observation is consistent with the idea that the 1–239 fragment can retrotranslocate to the ER but may not be retained long enough in that compartment for an efficient retrotranslocation event to take place.

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