Membrane Translocation of Diphtheria Toxin A-Fragment: Role of Carboxy-Terminal Region†

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ABSTRACT: The C-terminal end of diphtheria toxin A-fragment was altered and the consequences for toxicity and translocation of the A-fragment to the cytosol were studied. Mutations and deletions in the protease-sensitive, disulfide-bridged region linking the two functional parts of the toxin, the A- and B-fragments, reduced the toxicity of the protein as such, but when the mutant toxins were cleaved ("nicked") by trypsin before being added to cells, the toxicity was restored. Prevention of disulfide formation by removal of Cys186 resulted in complete loss of toxicity. To circumvent the nicking step, toxin was formed by reconstitution from separate A- and B-fragments where the A-fragments varied in the C-terminal sequences. The amino acids C-terminal to Cys186 were found not to be required for translocation. Furthermore, both charged and uncharged residues near the C-terminal end were compatible with translocation. The data indicate that the C-terminal amino acid sequence is not decisive for translocation of diphtheria toxin A-fragment to the cytosol.

An increasing number of proteins, most of them toxins, are found to be translocated from the cell exterior to the cytosol (Olsnes et al., 1988; Frankel & Pabo, 1988; Ward, 1987). Translocation of diphtheria toxin is the best developed model system to study this process. It is a crucial and limiting step in the action of the toxin that the enzymatically active A-fragment is translocated to the cytosol (Olsnes & Sandvig, 1988).

Diphtheria toxin is synthesized as a single polypeptide chain which is easily cleaved into an A- and a B-fragment by trypsin and other proteolytic enzymes (Drazin et al., 1971; Gill & Dinius, 1971). The B-fragment binds the toxin to cell surface receptors and facilitates translocation of the A-fragment to the cytosol. The A-fragment is an ADP-ribosylating enzyme that modifies and inactivates elongation factor 2 and thereby stops protein synthesis (Pappenheimer, 1977).

For translocation to occur, the toxin must be exposed to pH <5.3 (Moskaug et al., 1987, 1988). Normally, this occurs in acidic endosomes, but the translocation can be mimicked at the level of the plasma membrane when cells with surface-bound toxin are exposed to low-pH medium (Draper & Simon, 1980; Sandvig & Olsnes, 1980, 1981; Moskaug et al., 1988).

When proteins are translocated into the endoplasmic reticulum, the mitochondria, peroxisomes, and other organelles, they appear to be transported as extended polypeptides (Verner & Schatz, 1988; Hartl & Neupert, 1990; Simon & Blobel, 1991). The translocation is often dependent on a signal sequence in the one end of the polypeptide (von Heijne, 1983; Gierasch, 1989; Keegstra, 1989). In the case of diphtheria toxin, the N-terminal amino acid sequence of the A-fragment is not essential for translocation induced by low pH. Thus, we have earlier shown that the A-fragment can be modified by a number of different peptides linked to the N-terminal end without impeding translocation (Stenmark et al., 1991b). In the case of a diphtheria toxin–interleukin-2 fusion protein, however, the amino acid sequence near the N-terminal end was found to be important for toxicity (Chaudhary et al., 1991).

It is possible that toxin translocation is initiated by insertion of the B-fragment into the membrane (Moskaug et al., 1991). Since the C-terminal end of the A-fragment is disulfide-linked to the B-fragment, translocation of the A-fragment could start at the C-terminus. In the case of *Pseudomonas aeruginosa* exotoxin A, cholera toxin, and *Escherichia coli* heat-labile toxin, the enzymatically active parts have C-terminal sequences that may interact with the KDEL receptor (Chaudhary et al., 1990; Pelham, 1990; Brinkmann et al., 1991). Since removal of this sequence strongly reduced the toxicity, the possibility has been considered that interaction with the KDEL or a related receptor could facilitate membrane translocation of toxins (Seetharam et al., 1991). The A-fragment of diphtheria toxin does not contain a KDEL-like sequence, but it was conceivable that another amino acid sequence near the C-terminus could be involved in the translocation. In the present work we have carried out systematic modifications of the C-terminal end of diphtheria toxin A-fragment and studied the translocation capabilities of the modified proteins.

RESULTS

Tryspin Sensitivity of Toxin with Alterations in the Interfragment Region. In newly synthesized diphtheria toxin the regions corresponding to the A- and the B-fragments are linked by a 14 amino acid stretch flanked by a disulfide bond between Cys^{186} and Cys^{201} (Greenfield et al., 1983). In order for the toxin to be active, it must be proteolytically cleaved or "nicked" in this region (Sandvig & Olsnes, 1981). When the toxin is treated with trypsin, cleavage can occur next to any of three closely spaced arginine residues located in the disulfide-bridged region. Therefore a mixture of the three A-fragments is normally obtained. When this mixture is treated with carboxypeptidase B to remove terminal Arg residues, the translocation competence and the toxicity of the

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protein is increased 2–3-fold. Isoelectric focusing studies of the translocated material indicated that only A-fragment with one or no arginine residues in the C-terminal end is efficiently translocated (Moskaug et al., 1989).

In our first attempts to modify the C-terminal end of the A-fragment, we introduced a number of mutations and deletions into this region (Figures 1 and 2). In some cases we also removed Cys^{186} to prevent formation of the disulfide bond. Wild-type and mutant toxin genes were transcribed in vitro and the mRNA was translated as a single polypeptide chain in a rabbit reticulocyte lysate system (Olsnes et al., 1989).

We first tested the trypsin sensitivity of the products obtained. As shown in Figure 2a, in vitro-made, [^{35}S]-methionine-labeled toxin with the authentic amino acid sequence (except that Gly^1 was changed to Met), here denoted "wild type", was cleaved with low concentrations of trypsin into A- and B-fragments. Even 10-fold higher trypsin concentrations did not degrade the toxin further. The same trypsin sensitivity was obtained with ^{125}I-labeled natural toxin (McGill et al., 1989).

Mutants where Val^{191} was changed to Asp (Figure 2b) or Arg^{190} to Ile (Figure 2c) exhibited the same trypsin sensitivity as wild-type toxin (Figure 2a). Two deletion mutants, Δ194–196 (Figure 2d) and Δ191–197 (Figure 2e), lacking three and seven amino acids, respectively, required slightly more trypsin for complete nicking. (The effect is most clearly visible in the appearance of the B-fragment since contaminating labeled material migrating in the same region as the A-fragment was obtained in some of the lysates.) However, even in the latter case (Figure 2e) where the length of the disulfide-bridged region was reduced from 14 to seven residues and the number of Arg residues from three to one, the toxin was cleaved by trypsin in an apparently correct manner. On the other hand, two constructs which lacked Cys^{186} (C186S and A185G Δ186–192) and which therefore were unable to form an interfragment disulfide, were not correctly nicked by trypsin (Figure 2f,g), suggesting incorrect folding of the protein.

Toxicity of Mutants with Altered Interfragment Region. Unnicked diphtheria toxin is fully active when it is given to cells in overnight experiments, apparently because it is nicked by proteases present in serum or at the cell surface. We therefore added unnicked (and unlabeled) toxins to cells and incubated them overnight at 37 °C in normal growth medium containing 10% fetal calf serum, and we then measured the ability of the cells to incorporate [^{3}H]leucine. We have earlier shown that wild-type toxin made in vitro is as toxic as natural toxin (Olsnes et al., 1989). As shown in Figure 3A, R190I was approximately as toxic as wild-type toxin, whereas V191D was approximately 10 times less toxic. The deletion mutants lacking three and seven amino acids (Δ194–196 and Δ191–197) were much less toxic.

To test if the reduced toxicity was due to inefficient "nicking" by proteases in the serum or associated with the cells, we treated the different constructs with 3 μg/mL trypsin as in Figure 2 before adding them to the cells. As shown in Figure 3B, the toxicity was largely restored under these conditions. This indicates that the alterations introduced into the C-terminal end of the A-fragment and in the N-terminal end of the B-fragment did not inhibit translocation once the toxin was cleaved. The two mutants lacking Cys^{186} (C186S and A185G Δ186–192), which were not correctly nicked by trypsin, were not toxic whether or not they were treated with trypsin before being added to cells.

When nicked R190I was treated with carboxypeptidase B to remove C-terminal Arg residues, its toxicity was increased 2–3-fold (data not shown). This is in accordance with our previous findings with natural toxin that removal of terminal Arg residues increases the translocation competence (Moskaug et al., 1989).

Reconstitution of Toxin with A-Fragments Containing C-Terminal Alterations. The nicking in the experiments above occurs in a poorly controlled manner, and data obtained with natural toxin has shown that it can occur next to any of the arginines in the interfragment region (Moskaug et al., 1989). In attempts to study in detail the effect of C-terminal alterations of the A-fragment on its translocation competence, we decided to circumvent the nicking step. For this purpose we formed full-length, active toxin by mixing and dialyzing A- and B-fragments that were synthesized separately in vitro. Under these conditions the fragments became associated and a disulfide bond was formed between them. As B-fragment we used the translocation product of a construct where the N-terminal end of the B-fragment was Met-Ala-Gly-Arg^{193}. The modified B-fragment thus obtained was found to bind specifically to Vero cells, insert into the membrane, and form cation-selective channels (Stenmark et al., 1991a). The different A-fragments were obtained by linearizing plasmids shown in Figure 1, followed by in vitro transcription and translation. The deduced structures next to the interfragment disulfide in the different constructs are indicated in Figure 4A.

We first tested the ability of the different A-fragments to associate with B-fragment. Reconstituted toxin was obtained in good yield as shown for constructs 1–5 in Figure 4B, lanes 1–5. Upon treatment with 2-mercaptoethanol, essentially all labeled material migrated corresponding to the free A- and B-fragments (lanes 6–10). It should be noted that in the unreduced samples most of the B-fragment was in all cases consumed, leaving an excess of A-fragment. This is important since excess B-fragment would interfere with the binding of the reconstituted toxin to the specific receptors. It should also be noted that an additional band (indicated with an asterisk in Figure 4B) was obtained in varying amounts, apparently representing disulfide complexes of the A-fragment with (unlabeled) globin chains that are present in very high concentration in the lysate (Stenmark et al., 1992). Similar results were obtained with constructs 6–14 (data not shown). When the A-fragment was deleted after Gly^{174} and the sequence Leu-Cys-Arg-Lys-Ser-Cys-Gln-Ala-Ile-Ser-Lys was added instead (obtained from pKD-53, not demonstrated), the yield of reconstituted toxin was strongly reduced, suggesting that the sequence preceding Cys^{186} is necessary for correct folding of the A-fragment.

Translocation of Toxin with A-Fragment Containing C-Terminal Alterations. As a preliminary test of the ability of the different constructs to translocate to the cytosol, we tested their ability to intoxicate cells. The dialyzed translation products were added to cells, and after allowing time for binding to occur, the cells were exposed to pH 4.8 to induce translocation across the plasma membrane. In this way we minimized the ability of proteases associated with the cells to remove extensions from the C-terminal end of the A-fragments. The cells were incubated overnight in normal medium containing 10 mM NH_4Cl to prevent subsequent entry of toxin by the natural route, and then we measured the ability of the cells to incorporate [^{3}H]leucine. In Figure 4C toxicity data are shown for constructs 1–5. In this assay all constructs were toxic, although constructs 1 and 3, each having two positive charges in the C-terminal end, were somewhat less toxic than construct 5, which has no positive charge in the

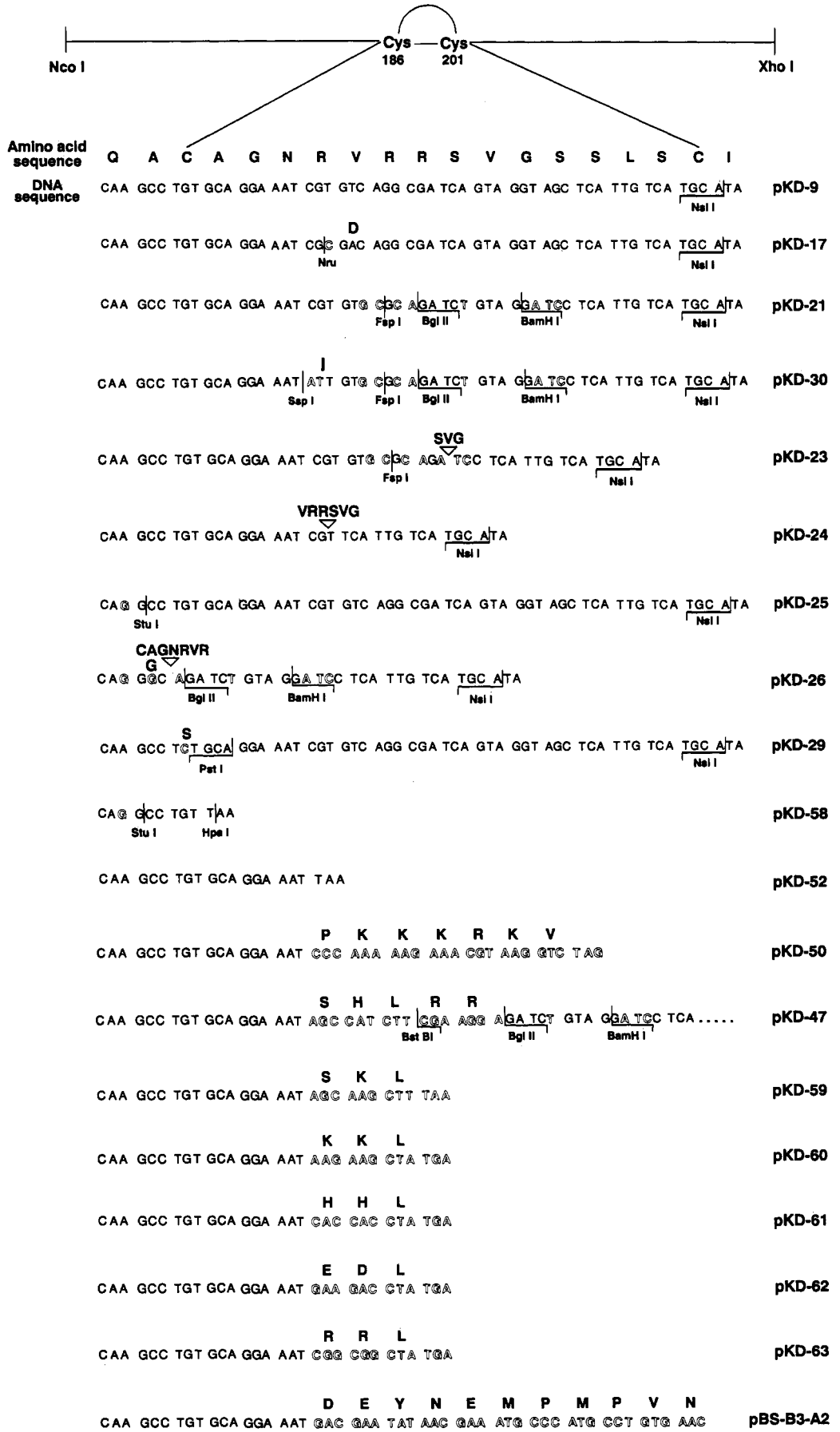

FIGURE 1: DNA sequence of the interfragment region of wild-type (pKD-9) and mutant genes and of the C-terminal end of A-fragments. Nucleotide changes are indicated with *open* letters; single amino acid changes are in boldface letters *above* and restriction sites are *below* the nucleotide sequence. Deletions are indicated with the deleted amino acids in boldface type above a triangle. The wild-type amino acid sequence is retained in pKD-21, but new restriction sites were introduced into the DNA sequence.

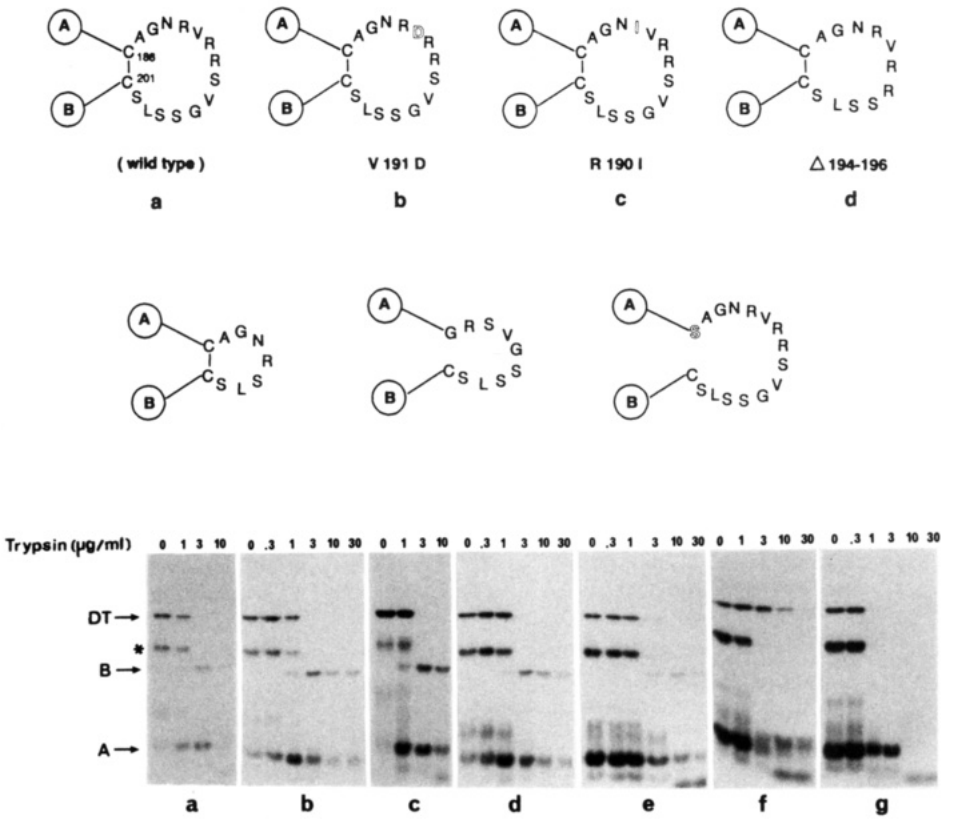

FIGURE 2: Trypsin sensitivity of diphtheria toxin with wild-type and altered amino acid sequence in the interfragment region. The *Sma*I–*Nsi*I fragment (A-fragment sequence) of the indicated plasmid was ligated to the *Nsi*I–*Apa*I fragment of pKD-1 (B-fragment sequence), cut with *Apa*I, and amplified by PCR as described in Experimental Procedures. The A-fragment sequence was derived from the following plasmids: from pKD-21 to obtain wild-type toxin (a); from pKD-17 to obtain V191D (b); from pKD-30 to obtain R190I (c); from pKD-23 to obtain Δ194–196 (d); from pKD-24 to obtain Δ191–197 (e); from pKD-26 to obtain A185G Δ186–192 (f); and from pKD-29 to obtain C186S (g). The amplified genes were transcribed and translated in vitro in the presence of [^{35}S]methionine. The labeled toxins were incubated with the indicated concentrations of TPCK-treated trypsin at 37 °C for 60 min. PMSF was then added to a final concentration of 1 mM and the samples were analyzed by SDS–PAGE, under *reducing* conditions. The material indicated with an asterisk appears to be due to downstream initiation at a preferred internal methionine.

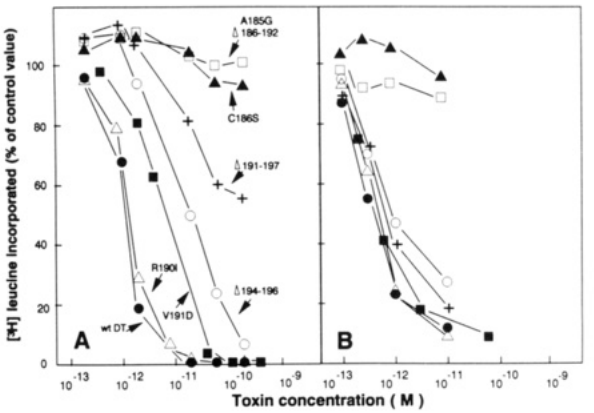

FIGURE 3: Ability of diphtheria toxin mutants to inhibit protein synthesis in Vero cells. Unlabeled wild-type or mutant diphtheria toxin formed in vitro was either not preincubated (A) or incubated with 3 μg/mL TPCK-treated trypsin for 60 min at 37 °C (B). Then increasing amounts were added to Vero cells in 24-well disposable trays, and after incubation overnight, the ability of the cells to incorporate [^{3}H]leucine was measured. ●, wild-type toxin; △, R190I; ■, V191D; ○, Δ194–196; +, Δ191–197; □, A185G Δ186–192; ▲, C186S.

C-terminus. Similar experiments with constructs 6–14 (data not shown) showed that constructs containing other positive residues replacing Arg, e.g., constructs 9, 10, 12, and 13, were also toxic, and this was also the case with constructs containing negative charges in the C-terminal end and with construct 6, where the A-fragment was terminated after Cys^{186}. In all cases the general impression was that positive charges in the C-terminal end moderately reduced the toxicity.

Since the C-terminal extensions might be removed by proteolysis before translocation took place, without affecting the enzymatic activity of the A-fragment, assessment of toxic effect is not a good measure of the amount of *extended* A-fragment translocated. Therefore, we analyzed the translocated material in a more direct way to see whether it represented extended A-fragment or if the extension had been removed. For this purpose we used toxin reconstituted from [^{35}S]methionine-labeled A-fragment and unlabeled B-fragment. The data in Figure 4D, upper panel, show that, after reduction of reconstituted toxin, the A-fragments migrated at somewhat different rates, as expected from the length of the extensions and deletions.

The reconstituted toxins were bound to cells and translocation was induced by exposure to low pH. Toxin remaining at the cell surface was removed with pronase and the protected material was analyzed. The data in Figure 4D, lower panel, show that the A-fragments of all constructs were translocated. In most cases the translocated A-fragments migrated at the same rates as before translocation (Figure 4D; compare upper and lower panels). To increase the sensitivity of the assay, we also compared the migration rate by running the untranslocated and translocated A-fragments next to each other in the same gel. In most cases we could not detect any difference in migration rate before and after translocation (not demonstrated). Only construct 8 migrated more rapidly after translocation, suggesting that only those A-fragments where

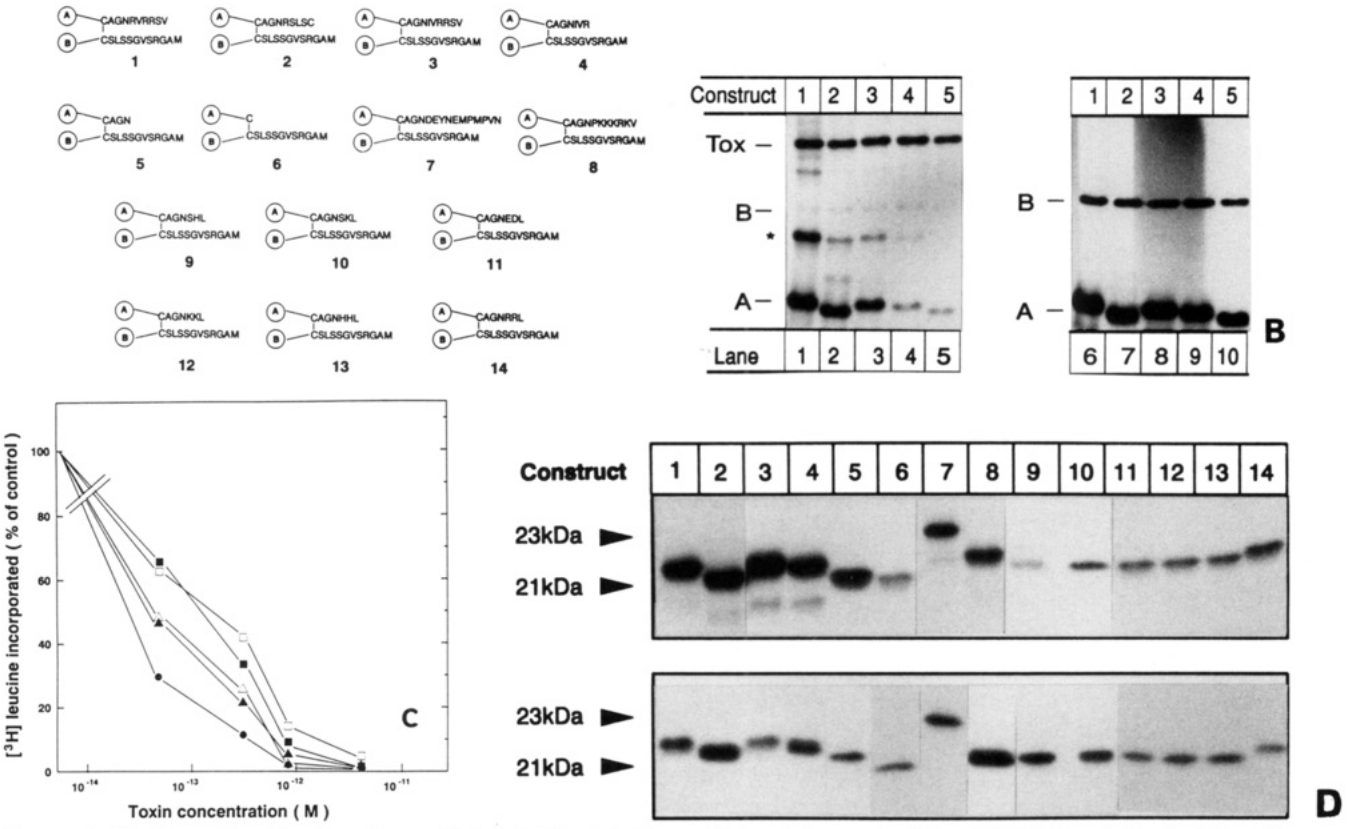

FIGURE 4: Binding and translocation of reconstituted diphtheria toxins containing A-fragments with modified C-terminal ends. Plasmids were linearized with enzymes as indicated below and transcribed and translated in vitro in the presence of [^{35}S]methionine to give toxin A-fragments varying in the C-terminal end. The translation products were mixed with unlabeled B-fragment formed in vitro with pBD-23 as a template, and the mixture was dialyzed overnight against PBS to remove reducing agents, allowing whole toxin to be formed. The interfragment region is shown schematically in (A). (B) Toxins were reconstituted from ^{35}S-labeled, in vitro-formed A- and B-fragments. Fragment A was obtained from the following plasmids: construct 1, pKD-21 linearized with *Bam*HI; construct 2, pKD-24 linearized with *Nsi*I; construct 3, pKD-30 linearized with *Bam*HI; construct 4, pKD-30 linearized with *Bgl*II; construct 5, pKD-52 linearized with *Xho*I. The lysates containing the fragments were mixed and dialyzed against Hepes medium and then analyzed by SDS–polyacrylamide gel electrophoresis in the absence (lanes 1–5) and presence (lanes 6–10) of 2-mercaptoethanol. (C) Constructs 1–5 were added in increasing amounts to Vero cells growing in 24-well microtiter plates in Hepes medium, pH 7.2, containing 10 mM monensin. After 15 min the medium was removed and the cells were incubated for 2 min at room temperature in Hepes medium adjusted to pH 4.8. Then the cells were transferred to leucine-free Hepes medium, pH 7.4, containing 10 μM monensin and incubated at 37 °C overnight. Finally the ability of the cells to incorporate [^{3}H]leucine during 30 min was measured. □, construct 1; ▲, construct 2; ■, construct 3; △, construct 4; ●, construct 5. (D, upper panel) SDS–PAGE of reconstituted toxins treated with 2-mercaptoethanol before electrophoresis. The numbering of the constructs is the same as in (A). (D, lower panel) Reconstituted toxin was added to Vero cells in Hepes medium containing 10 μM monensin. The cells were kept for 20 min at 24 °C and then exposed to Hepes medium, pH 4.8, for 2 min at 37 °C. The cells were subsequently treated with 4 mg/mL pronase E, then with 1 mM NEM, and finally washed and analyzed by *nonreducing* SDS–PAGE. Constructs 1–5 were made as described above. In the remaining constructs the A-fragments were obtained from the following plasmids: construct 6, pKD-58 linearized with *Nsi*I; construct 7, pBS-B3-A2 linearized with *Hin*dIII; construct 8, pKD-50 linearized with *Bgl*II; construct 9, pKD-47 linearized with *Bst*BI; construct 10, pKD-59 linearized with *Xho*I; construct 11, pKD-62 linearized with *Xho*I; construct 12, pKD-60 linearized with *Xho*I; construct 13, pKD-61 linearized with *Xho*I; construct 14, pKD-63 linearized with *Xho*I.

the C-terminal extension had been removed by proteolysis were translocated to the cytosol (see Discussion). Other A-fragments containing positively charged residues migrated after translocation at the same rate as the nontranslocated A-fragments (constructs 1–4, 9, 10, and 12–14). Constructs where the A-fragment was extended by oligopeptides containing negatively charged and hydrophobic amino acids but no positive charges (cnstructs 7 and 11) were also translocated and so was construct 6, where all amino acids C-terminal to Cys^{186} had been removed.

For technical reasons it is difficult to standardize the translocation system sufficiently to allow exact quantitative comparisons of the translocation *efficiency* of the different constructs. In particular, it should be noted that the efficiency of translocation cannot be deduced from the pictures in Figure 4D where the autoradiograms had been exposed for different periods of time. However, each experiment was carried out independently 3–10 times and the general impression was that the translocation efficiency was somewhat lower in constructs 1 and 3 than in the other constructs. In general, the translocation efficiency varied between 5% and 30% of the surface-bound toxin. Altogether, the data indicate that the amino acid sequence near the C-terminal end of the A-fragment is not decisive for translocation of in vitro translated and reconstituted toxin, although the presence of several positively charged residues appears to reduce the translocation efficiency.

DISCUSSION

The data presented here demonstrate that considerable alterations in the C-terminal amino acid sequence of the A-fragment can be carried out without blocking translocation. Some mutant toxins with alterations in the interfragment region were less toxic than wild-type toxin when added to cells in unnicked form, but when they were previously nicked with trypsin, they exhibited the same toxicity as wild-type toxin. Apparently, proteases present in the serum (Sandvig & Olsnes, 1981) or adsorbed to the cell surface (Blasi et al., 1986) were less able than trypsin to nick these constructs. All constructs

tested could be nicked by trypsin into A- and B-fragments provided the disulfide bridge was preserved. When Cys^{186} was removed to prevent formation of the disulfide, the toxin was not nicked correctly but was degraded at low concentrations of trypsin into material of low molecular weight.

Williams et al. (1990) found that a fusion protein consisting of the major part of diphtheria toxin and interleukin-2 was only toxic to cells when the Arg residue corresponding to position 193 in wild-type toxin was preserved. In their case the toxin was taken up by endocytosis and translocated across the limiting membrane of an intracellular compartment. It is therefore difficult to tell if modifications occurred before translocation.

In our translocation system with reconstituted toxin we are able to analyze the A-fragment before and after translocation. By comparing the migration rates in high-resolution SDS–PAGE we can monitor with reasonable accuracy changes in the length of the A-fragment. Our present data show that toxin reconstituted from A- and B-fragments was less sensitive than natural toxin (Moskaug et al., 1989) to positively charged residues at the C-terminal end of the A-fragment. The reason for this discrepancy is not clear. Only when five consecutive positive amino acids were added C-terminally to Cys^{186} (construct 8) was the full-length A-fragment not translocated. The translocated A-fragment was in this case reduced in size to approach that of the natural A-fragment and there was no trace of A-fragment migrating at the rate of the initial construct. Most likely the extension had been cleaved off by cell surface proteases before rather than after translocation. Thus, we have found that surface-bound toxin is degraded to some extent at the surface of Vero cells, whereas those extended A-fragments that were translocated appeared to be rather stable in the cytosol (our unpublished data).

We also found that A-fragment terminating with Cys^{186} was efficiently translocated to the cytosol. The amino acids C-terminal to this residue therefore appear not to play a decisive role in the translocation process.

We have recently shown that a number of different peptides can be added on to the *N-terminal* end of the A-fragment without interfering with translocation (Stenmark et al., 1991b). We here show that a number of small extensions can also be added to the C-terminal end, thus extending the possibilities of using the toxin A-fragment as a vector to bring peptide sequences into the cytosol.

Since it now appears that considerable alterations can be made in both ends of the A-fragment without impeding translocation, it is unlikely that the translocation is guided by a defined sequence in one end of the protein. Our attempts to remove internal regions of the A-fragment have so far led to proteins that did not reconstitute with the B-fragment and which are therefore presumably incorrectly folded. We are therefore not able to exclude the possibility that an internal amino acid sequence may guide the translocation, as is the case with ovalbumin translocation into the endoplasmic reticulum (Tabe et al., 1984). Another possibility is that the translocation is guided exclusively by the B-fragment, which inserts into the membrane in the process (Moskaug et al., 1991).

EXPERIMENTAL PROCEDURES

Materials. Pronase E (from *Streptomyces griseus*), monensin, phenylmethanesulfonyl fluoride (PMSF), L-1-tosylamide-2-phenylethyl chloromethyl ketone- (TPCK-) treated trypsin, trypsin inhibitor (from egg white), *N*-ethylmaleimide (NEM), and Hepes were purchased from Sigma, St. Louis, MO. [^{35}S]Methionine and [^{3}H]leucine were obtained from Amersham, Buckinghamshire, U.K. Diphtheria toxin (crude) was purchased from Connaught Laboratories, Willowdale, Canada, and purified as described (Sandvig & Olsnes, 1981). Micrococcal nuclease-treated rabbit reticulocyte lysate was purchased from Promega, Madison, WI. Restriction endonucleases, T4 DNA ligase, and T4 DNA polymerase were obtained from New England Biolabs, Beverly, MA. T3 RNA polymerase was obtained from Gibco-BRL, Eggenstein, FRG. Horse anti-diphtheria toxin serum was from the National Institute for Public Health, Oslo, Norway.

Buffers. PBS, phosphate-buffered saline contained 10 mM sodium phosphate, pH 7.4, and 140 mM NaCl. Hepes medium was Eagle's minimum essential medium buffered with 20 mM Hepes to the pH indicated. When the pH was adjusted to values below pH 6.0, 10 mM sodium gluconate was added to increase the buffering capacity. Lysis buffer consisted of 0.1 M NaCl, 20 mM NaH_2PO_4, 10 mM EDTA, 1% Triton X-100, 10 mM NaF, 0.1 mM sodium vanadate, 1 mM PMSF, and 200 units/mL aprotinin, pH 7.4.

Cell Culture. Vero cells were propagated under standard conditions (Sandvig & Olsnes, 1981). Twenty-four hours prior to the experiments the cells were transferred to 24- or 12-well microtiter plates at densities ranging from 2×10^4 to 5×10^4 cells/mL.

Bacterial Strains. E. coli strain 71-81 was used as host during the site-directed mutagenesis step and *E. coli* DH5α during the cloning procedures.

Construction of Diphtheria Toxin Expression Plasmids. The numbering of amino acid residues refers to the diphtheria toxin sequence as published by Greenfield et al. (1983). The interfragment region of the different constructs is shown in Figure 1. The various constructs were prepared as follows:

pKD-9, derived from Bluescribe (Stratagene), carries behind a T3 promoter the coding region of a diphtheria toxin mutant lacking the C-terminal end of the B-fragment (Olsnes et al., 1989). pKD-17 was derived from pKD-9 by introducing a unique *Nru*I site and simultaneously changing Val^{191} to Asp by oligonucleotide-directed mutagenesis. pKD-21 was obtained by inserting a 35-bp linker into pKD-17 cut with *Nru*I and *Nsi*I. The linker introduced three new restriction sites (*Fsp*I, *Bgl*II, and *Bam*HI) within the coding region for the disulfide loop, without changing the amino acid sequence.

pBD-23 encodes B-37, which is essentially a full-length B-fragment (Stenmark et al., 1992).

pKD-23. pKD-21 was digested with *Bgl*II and *Bam*HI, followed by religation, after removal of the 9-bp fragment, which resulted in a loss of three amino acids within the disulfide-bridged region.

pKD-24. pKD-21 was digested with *Bam*HI, and the ends were made flush with S1 nuclease and then digested with *Xho*I. The 310-bp fragment thus obtained was cloned into pKD-17 cut with *Nru*I and *Xho*I. This resulted in a loss of seven amino acids within the disulfide-bridged region.

pKD-25. The DNA sequence of pKD-9 was changed at Gln^{184} from CAA to CAG by oligonucleotide-directed mutagenesis to incorporate a *Stu*I site without changing the amino acid sequence.

pKD-26. pKD-21 was digested with *Fsp*I and *Xho*I, and a 295-bp fragment was isolated and cloned into pKD-25 cut with *Stu*I and *Xho*I. This resulted in a loss of seven amino acids, including Cys^{186}, therefore preventing formation of the disulfide bridge.

pKD-29. Cys^{186} in pKD-9 was changed into Ser by oligonucleotide directed mutagenesis. A *Pst*I restriction site

was generated at Ala[187].

pKD-30. The codon for Arg[190] in pKD-21 (CGT) was changed to one for Ile (AAT) by oligonucleotide-directed mutagenesis to form pKD-30. An *Ssp*I site was formed in the process.

pKD-47. pKD-30 was cut partially with *Ssp*I and to completion with *Bgl*II. A linker

```
      BstBI
AGCCATCTTCGAAGGA
TCGGTAGAAGCTTCCTCTAG
```

coding for Ser-His-Leu-Arg-Arg was inserted.

pKD-50. pKD-30 was digested with *Ssp*I and ligated with a linker

```
                    BglII
              XbaI
CCCAAAAAGAAACGTAAGGTCTAGA
GGGTTTTTCTTTGCATTCCAGATCTCTAG
```

coding for the sequence Pro-Lys-Lys-Lys-Arg-Lys-Val. The ligated material was then digested with *Nco*I, and the *Nco*I–*Bgl*II fragment obtained was ligated into pKD-30 which had been cut with *Nco*I and *Bgl*II.

pKD-52. The 564-bp *Nco*I–*Ssp*I fragment from pKD-30 was cloned into pKD-30 that had been cut with *Nsi*I, had the overhangs removed by T4 polymerase, and then had been cut with *Nco*I. A stop codon (TAA) was thereby introduced after Asn[189].

pKD-53. pKD-25 was cut with *Stu*I and *Bal*I and religated. A 32-bp fragment was deleted in the process.

pKD-58. pKD-25 was cut with *Stu*I and *Nsi*I and a linker

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    HpaI
CCTGTTAACTGCA
GGACAATTG
```

coding for a stop codon (TAA) after Cys11[86] was cloned in. The *Stu*I site was reformed, whereas the *Nsi*I site was lost.

pKD-59. pKD-30 was cut partially with *Ssp*I and to completion with *Bgl*II. A linker

```
   HindIII
AGCAAGCTTTAAG
TCGTTCGAAATTCCTAG
```

coding for Ser-Lys-Leu was inserted.

pKD-60. pKD-30 was partially cut with *Ssp*I and to completion with *Bgl*II. A linker

```
      SacI
AAGAAGCTATGAGCTC
TTCTTCGATACTCGAGCTAG
```

coding for Lys-Lys-Leu was inserted.

pKD-61. pKD-30 was partially cut with *Ssp*I and to completion with *Bgl*II. A linker

```
      EcoRI
CACCACCTATGAATTC
GTGGTGGATACTTAAGCTAG
```

coding for His-His-Leu was inserted.

pKD-62. pKD-30 was partially cut with *Ssp*I and to completion with *Bgl*II. A linker

```
      EcoRV
GAAGACCTATGATATC
CTTCTGGATACTATAGCTAG
```

coding for Glu-Asp-Leu was inserted.

pKD-63. pKD-30 was partially cut with *Ssp*I and to completion with *Bgl*II. A linker

```
      SacI
CGGCGGCTATGAGCTC
GCCGCCGATACTCGAGCTAG
```

coding for Arg-Arg-Leu was inserted.

pBS-B3-A1. pBSN-B3 (Stenmark et al., 1992) was cut with *Cla*I and *Xho*I, the overhangs were filled in with T4 polymerase, and the plasmid was religated.

pBS-B3-A2. The *Nco*I–*Ssp*I fragment from pKD-30 was ligated into pBS-B3-A1 that had been cut with *Nco*I and *Hin*cII.

Oligonucleotide-Directed Mutagenesis and DNA Sequence Determination. The mutagenesis was performed essentially according to the procedure described (Zoller & Smith, 1983), using a Boehringer Mannheim site-directed mutagenesis kit and synthetic oligonucleotides. Replicative-form bacteriophage DNA synthesized in vitro were transfected into *E. coli* 71-18mutS, and mutant bacteriophages were identified by restriction enzyme analysis of replicative-form DNA of transfectants. DNA sequence analysis by the dideoxy chain-termination method of Sanger et al. (1977) verified base changes and excluded additional mutations.

Formation of Enzymatically Active Toxin in Vitro. Since cloning of intact diphtheria toxin is considered hazardous, we used our previously developed method (Olsnes et al., 1989) of cloning the coding sequence for the A- and B-fragment separately and then we ligated the DNA pieces together. The ligated products were amplified by the polymerase chain reaction (PCR). The genes obtained were transcribed in vitro and the mRNA was translated in a rabbit reticulocyte lysate. The DNA containing the A-fragment was obtained by digesting the appropriate mutant plasmid (Figure 1) with *Sma*I and *Nsi*I and gel-isolating a ~800 base pair fragment. The second gene fragment containing the B-fragment was cut from pKD-1 (McGill et al., 1989), which was derived from Bluescribe KS and contains a diphtheria toxin gene with a triple mutation in the A-fragment changing Glu[148] to Ser (Barbieri & Collier, 1987; Wilson et al., 1990) and conferring a strong reduction in enzymatic activity whereas the B-fragment is intact. pKD-1 was digested with *Nsi*I and *Apa*I, and the 1120-bp fragment obtained was added in 5-fold excess to the first DNA fragment to ensure efficient ligation. After subsequent linearization with *Apa*I and *Sma*I, the gene was amplified by polymerase chain reaction (PCR) with two commercial sequencing primers (M13 reverse primer and KS primer, Stratagene).

DNA was transcribed with T3 RNA polymerase as described (McGill et al., 1989). The mRNA obtained was translated in micrococcal nuclease-treated rabbit reticulocyte lysate. To obtain toxin with high specific radioactivity for binding and translocation studies, the cell-free system contained 1 μM (0.25 mCi) [^{35}S]methionine and unlabeled amino acids (25 μM each, except methionine), whereas to obtain higher amounts of toxin for intoxication experiments, the radioactive methionine was replaced by 25 μM unlabeled methionine.

To estimate the amount of translation product in the latter cases, 5-μL aliquots were incubated in parallel in the presence of 5 μM [^{35}S]methionine in addition to the unlabeled methionine. The radioactive translation products were analyzed by SDS–PAGE and fluorography to localize the toxin band, which was then cut out from the gel and treated with Opti-fluor (Amersham) for 30 min and the radioactivity was

measured. Before use, all translation products were dialyzed extensively at 4 °C against Hepes medium, pH 7.4, to remove reducing agents and to allow disulfide bonds to form.

Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate (SDS-PAGE). Electrophoresis was carried out in 12% gels as described (Laemmli, 1970). Prior to drying, the gels were fixed for 30 min in 4% acetic acid/27% methanol and then treated for 30 min with 1 M sodium salicylate, pH 5.8, containing 2% glycerol. Dried gels were exposed to Kodak XAR-5 film, at −80 °C, in the absence of intensifying screens.

Toxin Binding. Vero cells on 12-well microtiter plates were incubated in Hepes medium, pH 7.3, containing 10 μM monensin for 20 min at 24 °C with radiolabeled toxin or mutants in the absence and presence of 10 μg/mL unlabeled diphtheria toxin. The cells were then incubated with lysis buffer for 15 min on ice, transferred to an Eppendorf tube, and centrifuged, and proteins in the supernatant were precipitated with trichloroacetic acid, extracted with diethyl ether, and analyzed by SDS–PAGE.

Translocation Assay. Vero cells in 12-well plates were incubated for 20 min at 24 °C with radiolabeled toxin or mutants in Hepes medium, pH 7.3, in the presence of 1 mM unlabeled methionine and 10 μM monensin. The cells were then washed twice with Hepes medium, pH 7.4, and then exposed to Hepes medium, pH 4.8, for 2 min at 37 °C. Subsequently, the cells were treated with pronase (4 mg/mL) in Hepes medium containing cycloheximide and monensin for 5 min at 37 °C. During the protease treatment, the cells detached from the plastic and were transferred to an Eppendorf tube, pelleted by centrifugation, and washed with Hepes medium containing 1 mM PMSF and 1 mM NEM. The cells were then dissolved in lysis buffer and the undissolved cell fragments and nuclei were removed by centrifugation. The proteins present in the supernatant were precipitated with 20% trichloroacetic acid, extracted with diethyl ether, and analyzed by SDS–PAGE under nonreducing conditions.

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