

# Peptides Fused to the Amino-Terminal End of Diphtheria Toxin Are Translocated to the Cytosol

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**Abstract.** Diphtheria toxin belongs to a group of toxic proteins that enter the cytosol of animal cells. We have here investigated the effect of NH<sub>2</sub>-terminal extensions of diphtheria toxin on its ability to become translocated to the cytosol. DNA fragments encoding peptides of 12–30 amino acids were fused by recombinant DNA technology to the 5'-end of the gene for a mutant toxin. The resulting DNA constructs were transcribed and translated in vitro. The translation products were bound to cells and then exposed to low pH to induce translocation across the cell membrane. Under these conditions all of the oligopeptides tested, includ-

ing three viral peptides and the leader peptide of diphtheria toxin, were translocated to the cytosol along with the enzymatic part (A-fragment) of the toxin. Neither hydrophobic nor highly charged sequences blocked translocation. The results are compatible with a model in which the COOH-terminus of the A-fragment first crosses the membrane, whereas the NH<sub>2</sub>-terminal region follows behind. The possibility of using nontoxic variants of diphtheria toxin as vectors to introduce peptides into the cytosol to elicit MHC class I-restricted immune response and clonal expansion of the relevant CD8<sup>+</sup> cytotoxic T lymphocytes is discussed.

**N**EWLY synthesized proteins are translocated across a number of different membranes in cells (Blobel and Dobberstein, 1975; Wickner and Lodish, 1985; Schatz, 1987; Keegstra, 1989; Neupert et al., 1990). Whereas in most cases translocation occurs from the cytosol into organelles, a number of bacterial and plant toxins possess the ability to enter the cytosol from extracytosolic locations (Olsnes et al., 1988a). Among these toxins, the uptake mechanism has been most extensively studied in the case of diphtheria toxin (Olsnes et al., 1988b). Membrane translocation of diphtheria toxin can be regarded as a simple model system to study how a water-soluble protein becomes able to cross lipid bilayers.

Diphtheria toxin is synthesized as a single chain polypeptide, which is readily cleaved ("nicked") at a trypsin-sensitive site to yield two disulfide-linked fragments, A and B (Drazin et al., 1971). The B-fragment (37 kD) binds to cell surface receptors and facilitates membrane translocation of the A-fragment (21 kD) to the cytosol (Uchida et al., 1972) where it ADP-ribosylates elongation factor 2 (Honjo et al., 1968) and thereby inhibits protein synthesis and kills the cell (Yamaizumi et al., 1978). The insertion of the B-fragment into the cell membrane is understood to some extent (Moskaug et al., 1988; McGill et al., 1989; Moskaug et al., 1991), but little is known about the way the A-fragment traverses the membrane. In the present work we have investigated whether the translocation apparatus, consisting of the B-fragment and probably the toxin receptor (Stenmark et al., 1988), is specific for the A-fragment alone, or if additional sequences at the NH<sub>2</sub>-terminus of the A-fragment can be translocated.

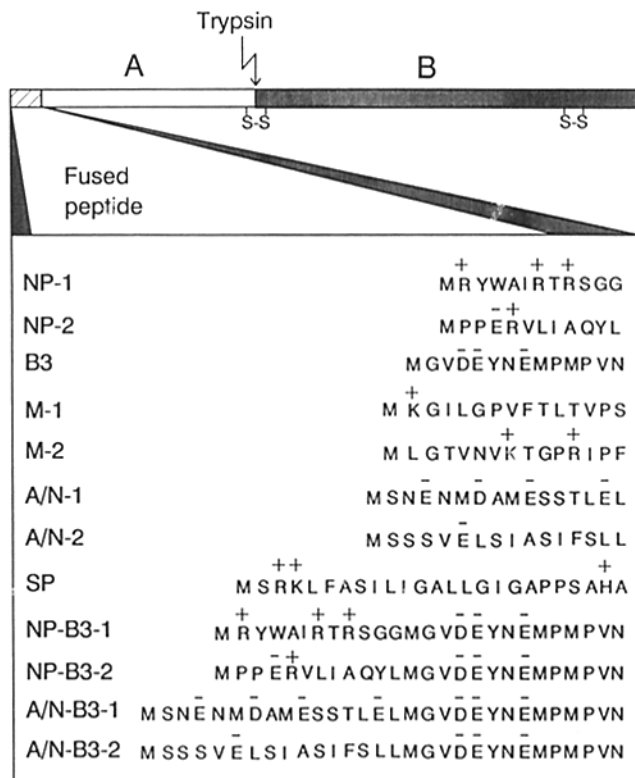
Translocation of the A-fragment to the cytosol normally occurs across the limiting membrane of endosomes (Marnell et al., 1984; Sandvig et al., 1984). At the low vesicular pH, the B-fragment exposes hydrophobic regions (Sandvig and Olsnes, 1981; Blewitt et al., 1985; Cabiaux et al., 1989), whereas the A-fragment undergoes conformational changes that may make it more translocation competent and thus bypass the need for molecular chaperones (Zhao and London, 1988; Dumont and Richards, 1988; Cabiaux et al., 1989). When cells with surface-bound toxin are exposed to acidic medium, translocation occurs from the cell surface (Draper and Simon, 1980; Sandvig and Olsnes, 1980, 1981). Because it enables us to distinguish between translocated and non-translocated material (Moskaug et al., 1987, 1988), we have here used this artificial system to study membrane translocation of A-fragment with and without NH<sub>2</sub>-terminal extensions.

## Materials and Methods

### Materials

Rabbit reticulocyte lysate (micrococcal nuclease-treated) and RNasin ribonuclease inhibitor were from Promega Biotec (Madison, WI). Pronase E, *N*-ethylmaleimide, PMSF, NaF, monensin, MES (2[*N*-morpholino] ethanesulfonic acid), trypsin (*N*-tosyl-L-phenylalanine-treated), and Triton-X-100 were from Sigma Chemical Co. (St. Louis, MO). Na-vanadate was from Ventron (Karlsruhe, Germany). Aprotinin was obtained from Bayer (Leverkusen, Germany). Restriction enzymes, T4 DNA polymerase and SP6 RNA polymerase were from New England Biolabs (Beverly, MA). T3 RNA polymerase was obtained from Gibco-BRL (Eggenstein, Germany). Diphtheria toxin was purified from a crude preparation obtained from Connaught Laboratories (Willowdale, Ontario, Canada) as described (Sandvig and

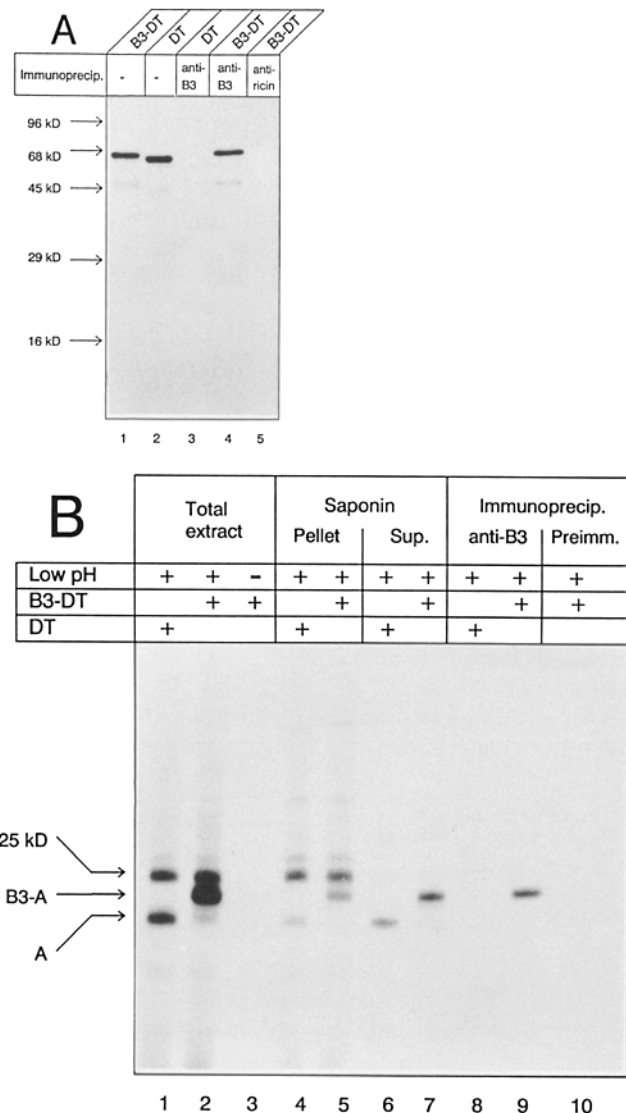




**Figure 1.** Schematic presentation of DT with NH<sub>2</sub>-terminal extensions. The diphtheria toxin mutant DT (535 residues) is identical to wild-type diphtheria toxin, except that Gly<sub>1</sub> was replaced by methionine and Glu<sub>148</sub> by serine (numbering of amino acids refers to the diphtheria toxin sequence as published by Greenfield et al. (1983)). The A-fragment is represented by an open box, and the B-fragment by a filled box. The site of interfragment trypsin cleavage is indicated. The position of the peptide fusions is indicated by a hatched box. Amino acid sequences of the different fused peptides are shown in the single-letter code. Basic amino acids (Arg, Lys, and His) are marked with + and acidic residues (Glu and Asp) with -. B3 is a peptide corresponding to the COOH-terminal end of a band 3-like protein (Demuth et al., 1986) with the additions of initiator methionine and a terminal asparagine for cloning purposes. M-1 is an immunogenic peptide from the matrix protein of influenza A virus, strain A/NT/60/68 (Gotch et al., 1987). A/N-1 and NP-1 are immunogenic peptides from the nucleoprotein of the same virus (Townsend et al., 1985). M-2, A/N-2, and NP-2 are encoded by reverse linkers for A/N-1, NP-1, and M-1, respectively. NP-B3-1, NP-B3-2, A/N-B3-1, and A/N-B3-2 are fusions of NP-1, NP-2, A/N-1, and A/N-2, respectively, with B3. SP, signal peptide of diphtheria toxin.

medium. Nontranslocated material was subsequently removed by pronase digestion. The cells were finally assayed for the presence of pronase-protected radiolabeled material. To inhibit translocation from acidic vesicles, monensin was present during all incubations, except during the brief low pH pulse.

As found earlier (Moskaug et al., 1988), full-length DT gave rise to two protease-protected polypeptides of 25 and 21 kD (Fig. 2 B, lane 1). The 25-kD polypeptide represents the COOH-terminal ~230 residues of the B-fragment (Moskaug et al., 1991), whereas the 21-kD band represents the A-fragment (Moskaug et al., 1988). Since electrophoresis was carried out under *nonreducing* conditions, cell-



**Figure 2.** DT with NH<sub>2</sub>-terminally fused B3 oligopeptide. (A) In vitro translation and immunoprecipitation. Aliquots (1  $\mu$ l) of in vitro translated, [<sup>35</sup>S]methionine-labeled DT (lanes 2 and 3) and B3-DT (lanes 1, 4, and 5) were analyzed by SDS-PAGE, either directly or after immunoprecipitation with anti-B3 or anti-ricin, as indicated. Fluorography was for 16 h. The arrows indicate the gel migration of size markers. (B) Translocation to the cytosol. DT and B3-DT (~1 nM) were added to Vero cells, translocation was induced by low pH and the cells were treated with pronase as described in Materials and Methods. In some cases (lanes 1-3 and 8-10), the cells were then dissolved with lysis buffer, nuclei were removed by centrifugation, and the protein in the supernatant fraction was precipitated with 5% TCA (total extract) or immunoprecipitated with anti-B3 or preimmune serum. In other cases (lanes 4-7) the cells were treated with 50  $\mu$ g/ml saponin in PBS containing 1 mM *N*-ethylmaleimide and 1 mM PMSF to release translocated A-fragment, and then the proteins both in the pellet and in the supernatant fractions were precipitated with TCA. In all cases the precipitated material was analyzed by SDS-PAGE under nonreducing conditions. Fluorography was for 6 d.

mediated reduction of the interfragment disulfide bridge must have taken place. Conceivably, the reduction occurs upon exposure of the disulfide to the reducing cytosol (Moskaug et al., 1987). The interfragment disulfide bridge arises

from the NH<sub>2</sub>-terminal part of the B-fragment, but we did not observe any protease protection of this part of the molecules. This is probably because only a very small part of the NH<sub>2</sub>-terminal region of the B-fragment is membrane inserted, and it may therefore be difficult to detect (Moskaug et al., 1991).

Pronase protection experiments with DT containing the B3 extension (B3-DT) show that two major fragments (25 and 23 kD) were protected in addition to small amounts of 21 kD fragment (Fig. 2 B, lane 2). The latter material probably represents A-fragments where the translation was initiated downstream of the first ATG (the ATG at the start of the A-fragment is in an optimal initiation context), in accordance with the scanning ribosome model (Kozak, 1989). When the exposure to low pH was omitted, no fragments were protected (lane 3). The 23-kD fragment, which corresponds in size to the A-fragment with attached peptide, was precipitated by anti-B3 (lane 9), but not with preimmune serum (lane 10). Protected A-fragment without the oligopeptide was not precipitated with anti-B3 (lane 8). The apparently larger amount of protected B3-A-fragment (lane 2) than A-fragment alone (lane 1) is due to more radioactivity incorporated, as the former contains 8 methionines, whereas the latter contains 5.

When cells with translocated diphtheria toxin are treated with a low concentration of saponin, allowing cytoplasmic

marker enzymes to leak out of the cells without dissolving the membranes, the translocated A-fragment is released into the medium whereas the B-fragment-derived 25-kD polypeptide remains associated with the membrane fraction (Moskaug et al., 1988). This indicates that the A-fragment is translocated to the cytosol, whereas the 25-kD polypeptide is inserted into the membrane. Also most of the B3-A-fragment was released with saponin (Fig. 2 B, lane 7) in the same way as normal A-fragment (lane 6), whereas the 25-kD fragment was associated with the membranes (lanes 4 and 5). Therefore, it appears that most of the translocated A-fragment with B3 peptide is, like natural A-fragment, free in the cytosol.

### Binding and Trypsin Susceptibility of DT with Different NH<sub>2</sub>-terminal Extensions

To investigate whether diphtheria toxin can be used for peptide translocation in general, or whether only certain types of peptides can be translocated, we fused to the A-fragment a number of peptides with different charge, length, and hydrophobicity (Fig. 1). The translation products, analyzed by SDS-PAGE and fluorography, are shown in Fig. 3 A. Occasionally, contamination with unidentified proteins of lower molecular weight was observed (lane 4). These proteins probably represent molecules translated from prematurely

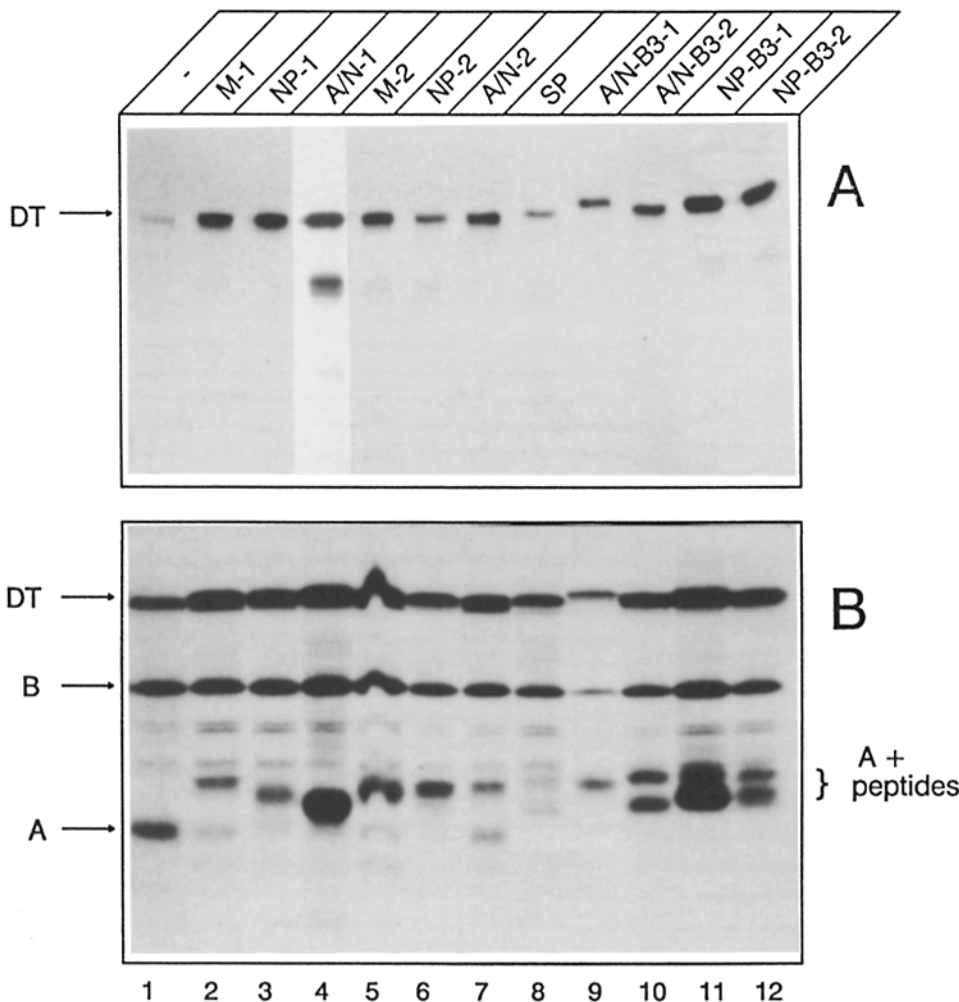


Figure 3. Binding and nicking of DT with various NH<sub>2</sub>-terminal extensions. (A) SDS-PAGE of translated proteins. 1  $\mu$ l <sup>125</sup>I-labeled diphtheria toxin (lane 1) or in vitro translated, <sup>35</sup>S-labeled mutant proteins containing the NH<sub>2</sub>-terminal extensions indicated (lanes 2-12) were analyzed by SDS-PAGE under reducing conditions. Fluorography was for 12 h. (B) Binding to cells and trypsin sensitivity. Vero cells were incubated with 100  $\mu$ l of HEPES medium containing <sup>125</sup>I-labeled natural diphtheria toxin (lane 1) or <sup>35</sup>S-labeled in vitro translated DT with NH<sub>2</sub>-terminal extensions (lanes 2-12) and binding was allowed to occur for 20 min at 24°C. The cells were then washed 4 times, and incubated with 0.4  $\mu$ g/ml trypsin for 5 min at 24°C. The cells were subsequently washed three times with cold PBS and dissolved in 200  $\mu$ l lysis buffer; nuclei were removed by centrifugation. Proteins were precipitated with TCA and analyzed by SDS-PAGE under reducing conditions, followed by fluorography. The exposure time was 3 d.

terminated transcripts. In contrast to the full-length proteins, the low molecular weight material does not bind to cells (data not shown).

Unnicked diphtheria toxin is translocation incompetent (Sandvig and Olsnes, 1981; Moskaug et al., 1988). However, several of the fused peptides contain arginine and lysine residues and might be degraded by the trypsin treatment necessary to nick the toxin. To test this, we bound the different fusion proteins to Vero cells and added a low concentration of trypsin. The nicked, cell-bound proteins were then analyzed by SDS-PAGE under reducing conditions.

As shown in Fig. 3 B, the trypsin concentration was sufficiently high to partially nick cell-bound natural toxin (lane 1). The faint bands between A- and the B-fragment presumably represent partially degraded B-fragment. Like natural toxin, all the different fusion proteins were partially nicked (lanes 2-12). Whereas the B-fragment obtained after nicking had as expected the same molecular weight in all cases, the apparent molecular weight of the obtained A-fragments varied depending on the nature of the attached peptides.

It should be noted that the electrophoretic migration rate of the A-fragment constructs was not only influenced by the size of the attached peptide, but also by its charge. Most remarkably, A-fragment fused with the highly acidic 16-mer A/N-1 peptide (lane 4) showed a significantly higher migration rate than when fused with the 14-mer basic peptide M-1 (lane 2). When the amount of bound material (B) is compared with 1  $\mu$ l of the toxin solution added to the cells (A), it appears that DT fused with A/N-B3-1 (lane 9), which contains as much as seven acidic residues, binds less well than the other constructs. This was confirmed by other experiments (data not shown).

In several cases, the attached peptide was partially degraded by the trypsin treatment. Degradation was most pronounced in the case of NP-B3-1 (lane 11), which is a fusion between a basic peptide, NP-1, and the B3 peptide. Apparently, the arginines in NP-1 were highly trypsin susceptible when placed in front of B3-DT (lane 11), but not when placed in front of DT alone (lane 3). Similar results were obtained

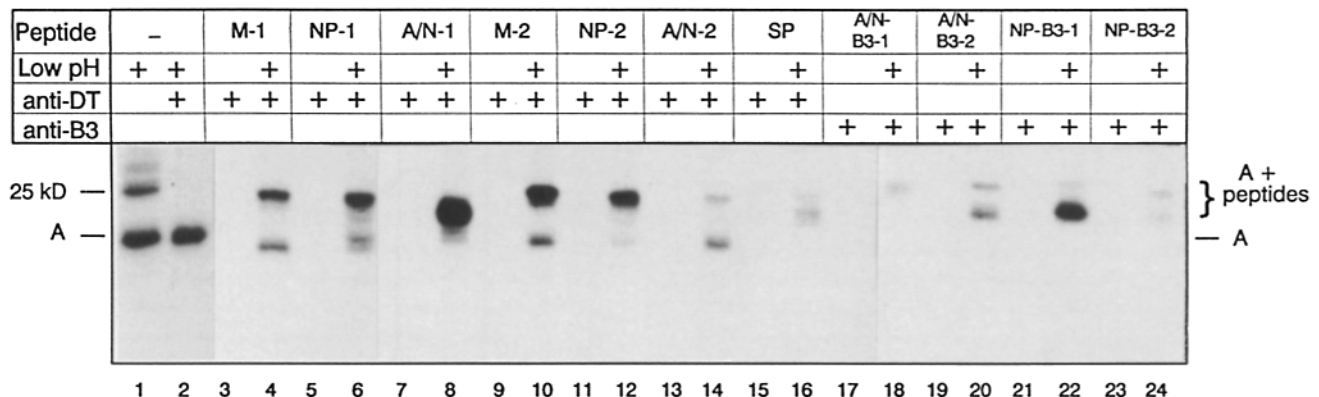
when the constructs were treated with trypsin without being bound to cells (data not shown). The observed degradation must therefore be caused by the trypsin treatment, and not by cellbound proteases.

In some cases, contaminating A-fragments, probably translated due to initiation downstream of the first ATG, were observed. For instance, A/N-2-A-fragment was contaminated with natural-length A-fragment (lane 7), which could be explained by translational initiation at Met<sub>1</sub> of the natural A-fragment in this construct. (Neither the A/N-2 peptide nor the NH<sub>2</sub>-terminus of the A-fragment contain trypsin-susceptible arginines or lysines.) Similarly, A/N-B3-2-A-fragment was contaminated with B3-A-fragment, also probably due to downstream initiation. Despite the problems with degradation and downstream initiation products, we found that in all cases sufficient amounts of the correctly extended A-fragments were formed to carry out translocation experiments.

#### Membrane Translocation of A-fragment with Attached Peptides of Different Charge

The translocation competence of A-fragment containing the various extensions was assayed by pronase protection experiments. To distinguish between translocated, extended A-fragment and the 25-kD B-fragment-derived polypeptide, we immunoprecipitated pronase-protected material with an antidiphtheria toxin serum that recognizes the A-fragment, but not the 25-kD polypeptide. That the A-fragment, but not the 25-kD polypeptide, is precipitated by this antiserum is evident from Fig. 4, lanes 1 and 2, which show total protected material and immunoprecipitated material, respectively.

The results in Fig. 2 B indicated that the B3 peptide, which contains three acidic residues, was efficiently translocated. We also examined the translocation competence of A-fragment containing two other, unrelated acidic peptides, A/N-1 (Fig. 4, lanes 7 and 8) and A/N-2 (lanes 13 and 14). These 16-mer peptides contain, respectively, 4 and 1 acidic residues. In both cases, fragments corresponding in size to A-fragment with extensions were protected against pronase and immunoprecipitated after low pH exposure (lanes 8 and 14), but not when low pH was omitted (lanes 7 and 13).



**Figure 4.** Membrane translocation of DT containing NH<sub>2</sub>-terminal peptides with different properties. <sup>125</sup>I-labeled natural diphtheria toxin (lanes 1 and 2) and <sup>35</sup>S-labeled, in vitro translated DT with the indicated NH<sub>2</sub>-terminal extensions (lanes 3-24) were bound to cells and, where indicated, membrane translocation was induced by low pH. After pronase treatment, the cells were lysed in lysis buffer and nuclei were removed by centrifugation. Proteins in the supernatant fraction were either precipitated with TCA (lane 1) or immunoprecipitated with antiserum that recognizes the A-fragment but not the 25-kD fragment (lanes 2-16) or with anti-B3 serum (lanes 17-24). The precipitated proteins were analyzed by SDS-PAGE under nonreducing conditions, followed by fluorography. The exposure time was 10 d.

In several systems, stretches of positively charged amino acids have been found to interfere with membrane translocation (von Heijne, 1986; Li et al., 1988; Nilsson and von Heijne, 1990; Boyd and Beckwith, 1990). We therefore examined the ability of three basic peptides, M-1 (Fig. 4, lanes 3 and 4), NP-1 (lanes 5 and 6) and M-2 (lanes 9 and 10), containing respectively one, three, and two basic residues, to be translocated together with the A-fragment. As indicated by the results in lanes 4, 6, and 10, all three peptides were translocated together with the A-fragment, suggesting that the net charge of the attached peptide does not to a significant extent influence the membrane translocation of the A-fragment. Consistent with this, also NP-2, a peptide containing one basic and one acidic residue was efficiently translocated at low pH (lanes 11 and 12).

### Effect of Peptide Length on Diphtheria Toxin-aided Translocation

The results in Fig. 4, lanes 3–14 indicated that peptides of 12–16 residues were efficiently translocated when attached to the NH<sub>2</sub>-terminus of diphtheria toxin A-fragment. To study whether longer peptides (26–30 residues) can be translocated, we tested the translocation competence of B3-A-fragment fused with NP-1, NP-2, A/N-1 and A/N-2, yielding A-fragment with the extensions NP-B3-1, NP-B3-2, A/N-B3-1 and A/N-B3-2, respectively (see Fig. 1). Pronase-protected polypeptides were immunoprecipitated with anti-B3 serum.

The results in Fig. 4, lanes 17–24 indicate that all 4 peptides were translocated upon exposure to low pH. In the cases of NP-B3-2 (lane 20), A/N-B3-1 (lane 22), and A/N-B3-2 (lane 24), two pronase-protected polypeptides were immunoprecipitated with anti-B3 serum after low pH exposure. The upper bands correspond to A-fragment with full-length (26 or 30 residues) peptide extension, whereas the lower bands conceivably represent degraded material or B3-A-fragment, formed by downstream initiation. When we compare the relative intensities of the upper and lower band with the A-fragments obtained after nicking (compare Fig. 4, lanes 20, 22, and 24 with Fig. 3 B, lanes 10, 11, and 12, respectively), it appears that the larger and the smaller A-fragments were translocated with approximately the same efficiency. This result is promising, since it suggests that even longer peptides could be translocated to the cytosol together with the A-fragment. However, our efforts to demonstrate this have so far been hampered by the trypsin susceptibility and reduced receptor affinity of longer constructs (data not shown).

### Membrane Translocation of A-fragment Containing the Diphtheria Toxin Signal Peptide

To test if a hydrophobic peptide could be translocated with the A-fragment, we chose the natural signal peptide (sp) of diphtheria toxin, which consists of 25 residues (Fig. 1). Like other signal peptides (von Heijne, 1988), it contains a highly hydrophobic core region. Fig. 4, lane 15, shows that no part of the protein was pronase protected and immunoprecipitated when low pH incubation of the cells was omitted. However, when the cells were exposed to low pH before the pronase treatment, a polypeptide corresponding in size to A-fragment with attached signal peptide was pronase-protected and immunoprecipitated (lane 16, top band). Also a degradation product (bottom band) was translocated (com-

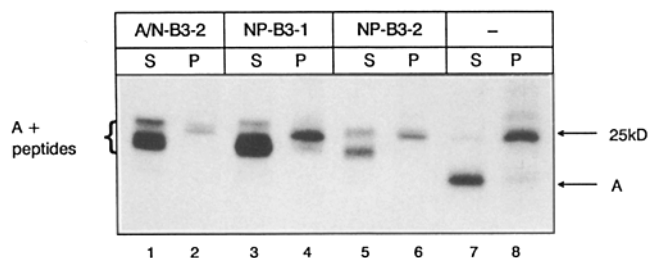


Figure 5. Saponin fractionation of cells with translocated A-fragment with and without NH<sub>2</sub>-terminal extensions. <sup>35</sup>S-labeled mutant toxins containing the extensions indicated (lanes 1–6) and <sup>125</sup>I-labeled natural diphtheria toxin (lanes 7 and 8) were bound to Vero cells and translocation was induced by low pH. The cells were then treated with pronase as described, and saponin was added to the pelleted cells, as described in the legend to Fig. 2. After saponin treatment, proteins in the supernatant fraction (S) and in the pellet (P) were analyzed by SDS-PAGE under nonreducing conditions, as described in legend to Fig. 2. Fluorography was for 7 d.

pare with Fig. 3 B, lane 8). The results indicate that even the hydrophobic signal peptide of diphtheria toxin can be translocated across the Vero cell plasma membrane.

### Saponin Fractionation of Translocated Extended A-fragments

The possibility existed that some of the peptide extensions were stuck in the membrane upon translocation. To study if A-fragment containing various NH<sub>2</sub>-terminal extensions were membrane-associated, we used the saponin fractionation assay described in Fig. 2 B. The results obtained with some of the constructs are shown in Fig. 5. They indicate that A-fragments extended with A/N-B3-2, NP-B3-1, and NP-B3-2 were all released into the saponin supernatant (lanes 1, 3, and 5), in the same way as natural A-fragment (lane 7). Similar results were obtained with the other constructs tested, including the hydrophobic signal peptide (data not shown). As expected, the membrane-associated 25-kD B-fragment-derived polypeptide, was recovered from the pellet fraction in all cases (lanes 2, 4, 6, and 8). The low amount of 25-kD polypeptide in lane 2 compared with the amount of protected extended A-fragment in lane 1 is probably due to inefficient pronase digestion. The data indicate that even extensions of 30 residues are completely translocated into the cytosol, without being arrested in the membrane.

### Discussion

In this work, evidence is presented that diphtheria toxin A-fragment can be extended at its NH<sub>2</sub> terminus, without loss of translocation competence. The NH<sub>2</sub> terminus is probably not involved in initiating the translocation process. This would be different from proteins destined for mitochondria, chloroplasts, and the ER, where NH<sub>2</sub>-terminal leader peptides are crucial for membrane translocation (von Heijne, 1988).

The possibility that the protected extended A-fragments contained material from the B-fragment rather than the NH<sub>2</sub>-terminally fused peptides is unlikely for several reasons: (a) The electrophoretic migration rate of the protected fragments

was the same as that of the extended A-fragments obtained after chemical reduction. (b) In several cases the protected fragments were immunoprecipitated with antibodies against the N-terminal extensions. (c) In those cases where it has been tested we found that part of the bound toxin with NH<sub>2</sub>-terminal extensions was reduced by the cells upon exposure to low pH (data not shown), as earlier found with natural toxin (Moskaug et al., 1987). It should also be noted that the cells were treated with *N*-ethylmaleimide before lysis or saponin treatment to exclude the possibility of reduction taking place after opening of the cells. We therefore feel that peptides fused to the NH<sub>2</sub>-terminal end of the A-fragment are indeed translocated to the cytosol.

Since a number of peptides differing in size, charge, and hydrophobicity were found to translocate along with the toxin A-fragment, the translocation apparatus confined by the B-fragment/receptor is not restricted to the A-fragment as such, but accepts considerable NH<sub>2</sub>-terminal variation. So far we have only demonstrated translocation of comparatively small extra sequences (10–30 residues), but work is in progress to study if longer peptides and whole proteins can be translocated along with the A-fragment.

Chaudhary et al. (1990) found that additional peptide material could be added close to the COOH-terminal end of *Pseudomonas* exotoxin A without reducing its toxic effect. Possibly the additional peptide follows the enzymatically active domain III into the cytosol. However, since the additional peptide material was introduced outside (behind) the ADP-ribosylating region of the toxin, it cannot be excluded from the presented data that it was trimmed off before membrane translocation.

The finding that mutant diphtheria toxin of low toxicity can deliver foreign oligopeptides into the cytosol makes it an interesting tool in experimental biology, in cases where rapid and synchronized introduction of specific peptide sequences into cells is required. The intracellular concentration of translocated material obtained is low (the maximal intracellular concentration of A-fragment obtained in Vero cells is ~2 nM), but it could still be sufficient for certain purposes, such as tracing the intracellular fate of radiolabeled sequences, or introducing regulatory proteins.

At present we are interested in exploring the possibility of using the toxin-induced peptide translocation in vaccine development. Antigen presentation by major histocompatibility antigens (MHC) of class I requires that the antigen to be presented is found in the cytosol or in the ER (Townsend et al., 1985; Moore et al., 1988; Townsend and Bodmer, 1989; Yewdell and Bennink, 1990). Externally added polypeptides therefore do normally not elicit a class I response. On the other hand, if the antigen is artificially introduced into the cytosol, presentation by MHC class I may occur (Moore et al., 1988; Yewdell et al., 1988). Convenient and nondamaging methods to introduce foreign peptides, such as viral antigens, into the cytosol could therefore be useful for vaccine purposes to expand the relevant population of CD8<sup>+</sup> MHC class I-restricted cytotoxic T-lymphocytes.

Most human tissues possess receptors for diphtheria toxin, and it is likely that toxin with fused oligopeptides would be endocytosed by most cells, with subsequent translocation to the cytosol. If the oligopeptide used were a viral peptide, it might be presented at the surface, with resulting clonal expansion of the relevant CD8<sup>+</sup> cytotoxic T lymphocytes. In fact,

three of the peptides tested in this study, NP-1, M-1, and A/N-1 (Fig. 1), are (except for the NH<sub>2</sub>-terminal Met residue) influenza virus peptides known to be presented by defined MHC class I allotypes and to elicit cytotoxic T-cell response (Townsend et al., 1985; Gotch et al., 1987). Work is now in progress to study if the peptides are also presented by class I molecules when they are introduced into cells by aid of diphtheria toxin.

The diphtheria toxin gene used here encodes a protein with a slight residual toxicity (1/800 of wild-type) (Barbieri and Collier, 1987), but we are now trying to prepare completely nontoxic mutants which are translocation competent and which could safely be used for vaccination. The possibility should also be considered that enzymatically inactive mutants of other protein toxins that enter the cytosol, such as abrin, ricin, Shigella toxin, and cholera toxin (Olsnes and Sandvig, 1985) could be used as vectors for peptides.

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