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Membrane Translocation of Diphtheria Toxin Carrying Passenger Protein Domains

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For diphtheria toxin to be cytotoxic, the enzymatically active part (fragment A) must be translocated to the cytosol. We here demonstrate that additional proteins linked as N-terminal extensions can be translocated along with fragment A across the plasma membrane of toxin-sensitive cells. Thus, an extra fragment A of diphtheria toxin and some of apolipoprotein AI were translocated as passenger proteins along with mutant diphtheria toxin fragment A. Translocation was monitored by the cytotoxic effect of the additional fragment A as well as by the translocation of [^{35}S]methionine-labelled protein to a compartment protected from externally added pronase. Cytotoxicity experiments indicated that double A fragments can also be translocated across the membrane of intracellular vesicles. The results demonstrate that the translocation apparatus used for toxin translocation is not limited to a single A fragment but can accommodate additional proteins as well. The fact that proteins as large as 20 kDa can be brought into cells by way of diphtheria toxin under both in vitro and in vivo conditions opens up the possibility of using diphtheria toxin mutants for introducing molecules with biological activity into cells.

The enzymatic moiety of diphtheria toxin (fragment A) normally enters the cytosol of eukaryotic cells by translocation across the endosomal membrane. This process results in the inhibition of protein synthesis because of the inherent ability of fragment A to ADP-ribosylate elongation factor 2 (EF-2) (10). By exposing cells with receptor-bound toxin to a low pH, thus mimicking conditions inside endosomes, translocation directly across the plasma membrane can be induced (3, 13). In this way, the translocation is synchronized, and it is possible to manipulate in a controlled manner the conditions on each side of the surface membrane. By the use of a radiolabelled protein, one can trace the translocated protein, enabling studies of reduction and cleavage (8).

The C-terminal portion of diphtheria toxin, fragment B, undergoes a conformational change at a low pH and becomes stuck in the membrane, while fragment A is translocated across the membrane and into the cytosol (8). Fragment A undergoes partial unfolding at a low pH (2, 4, 12, 21), but it is unclear whether this unfolding is required for translocation across the membrane.

We previously demonstrated that at a low pH oligopeptides of up to 30 amino acids fused to the N terminus of fragment A can be translocated across the plasma membrane as passengers (17). In the present work, we studied whether whole protein domains can be translocated to the cytosol when present as N-terminal extensions. Furthermore, we investigated whether the translocation of passenger proteins can take place across the intracellular vesicle membrane as well as across the plasma membrane.

MATERIALS AND METHODS

Buffers. HEPES medium consisted of bicarbonate-free Eagle's minimal essential medium buffered with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and adjusted to various pHs. When the pH was adjusted to below 6.8, 10 mM Na gluconate and 10 mM MES [2-(*N*-morpholino)-ethanesulfonic acid] were added to increase buffering capacity. Phosphate-buffered saline (PBS) contained 140 mM NaCl and 10 mM Na_2HPO_4 (pH 7.2). Dialysis buffer consisted of PBS with 2 mM $CaCl_2$. Lysis buffer consisted of 0.1 M NaCl, 10 mM Na_2HPO_4 (pH 7.4), 1% Triton X-100 (Sigma), 1 mM EDTA, 1 μg of alpha-2-macroglobulin (Sigma) per ml, 1 mM iodoacetamide (Sigma), 1 mM *N*-ethylmaleimide (NEM) (Sigma), 10 μg of leupeptin (Sigma) per ml, 10 μg of pepstatin (Sigma) per ml, 10 μg of antipapain (Sigma) per ml, 10 μg of chymostatin (Sigma) per ml, 100 μM L-1-tosylamide-2-phenylethyl chloromethyl ketone (Sigma), and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma).

Bacterial strains. *Escherichia coli* JM105 and DH5α were used in the cloning procedures.

Cell culture. Vero cells were propagated under standard conditions and seeded into 12- or 24-well disposable Costar plates 2 days prior to the experiments. Cells were used at a density of 1×10^5 or 5×10^4 cells per well, respectively. [^{3}H]leucine was incorporated as described previously (15).

Plasmids and fusion proteins. Cloning was done as described by Maniatis et al. (6). Restriction enzymes, T4 DNA polymerase, S1 nuclease, T4 DNA ligase, and polynucleotide kinase were from New England BioLabs, Beverly, Mass. Oligonucleotides were from Medprobe, Oslo, Norway.

pBD-1 encodes protein A58, in which Glu-148 is substituted for by Ser (E148S mutation). This plasmid has been described before (7) as pBND-2. Diphtheria toxin E148S has been shown to be 800-fold less toxic than wild-type diphtheria toxin because of strongly reduced ADP-ribosylating activity (1a, 19). pBD-30 encodes diphtheria toxin fragment A. This plasmid has been described before (16). pBD-23 encodes fragment B starting at the sequence Met-Ala-Gly-Arg-193 (16). For construction of pBD-58, a 418-bp *Acc*I-*Msc*I fragment from pBD-1 was cloned into pBD-30 by fragment exchange. This plasmid encodes the truncated A fragment terminating at Val-191 and in which Glu-148 is

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substituted for by Ser. For construction of pBD-59, a 45-bp linker

*Nco*I *Fsp*I *Bgl*II *Bam*HI
CATGGCAGGAAATCGTGTGCGCAGATCTGTAGGATCCTCATTGAG
CGTCCTTTAGCACACGCGTCTAGACATCCTAGGAGTAACTCGTAC

was inserted into pBD-30 that had been cut with *Nco*I. For construction of pBD-60, pKD-29 (see below) was restricted with *Pst*I, and the overhangs were removed with T4 DNA polymerase. The 800-bp fragment (*Pst*I-*Pst*I) was isolated, and a 10-mer *Nco*I linker (AGCCATGGCT) was ligated to the fragment. The linker-containing fragment was restricted with *Nco*I, and the 550-bp fragment was isolated and ligated into *Nco*I-restricted pBD-59. This plasmid encodes the A part of protein DD-4 (see Fig. 1). For construction of pBD-61, pBD-60 was restricted with *Nco*I, and the 550-bp fragment was ligated into *Nco*I-restricted pBD-58. This plasmid encodes the A part of protein DD-5 (see Fig. 1). For construction of pBD-67, the *Nco*I-*Cel*II fragment from pBD-59 was cloned into pBD-58. For construction of pBD-68, the *Nco*I fragment (550 bp) from pBD-60 was cloned into pBD-67, yielding the A part of protein DD-6 (see Fig. 1). For construction of pKD-29, pKD-9 (11) was modified by site-directed mutagenesis, changing Cys-186 to Leu and thereby introducing a *Pst*I site. pBLD-2 encodes B3-ApoAI$^{89-212}$-A fragment (tetradecapeptide B3-amino acids 89 to 212 of apolipoprotein AI-A fragment) (16). The B3 peptide has the amino acid sequence MGVDEYNEMPMPVN (16).

In vitro synthesis of proteins. Expression plasmids were linearized downstream of the insert with *Eco*RI. Plasmids were transcribed as described previously (7) with T3 RNA polymerase (GIBCO-BRL). Ethanol-precipitated transcripts were translated for 1 h at 31°C in micrococcal nuclease-treated reticulocyte lysates (Promega). Unlabelled amino acids (25 μM), excluding methionine, and L-[^{35}S]methionine (Amersham) at 0.25 mCi/200 μl (a 1 μM concentration in 200 μl) were added to the lysates. In some cases, 25 μM unlabelled methionine and no labelled methionine were added. After translation, the lysates were dialyzed against dialysis buffer to remove free methionine and the reducing agents present in the lysates (to allow disulfide bonds to form [16]).

When lysates for translocation experiments were made, fragment B was, in most cases, not radiolabelled. However, a small amount of radiolabelled fragment B was made in parallel. Fragment A was present in excess compared with fragment B during dialysis so that all of fragment B was consumed during the reassociation reaction. The association of fragments A and B was monitored by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) under nonreducing conditions.

Translocation assay. The translation products were added at a concentration of 1 nM to Vero cells growing as monolayers in 12-well microtiter plates and kept at 24°C for 20 min in the presence of 10 μM monensin (to inhibit the translocation of endocytosed protein). Also, 1 mM unlabelled methi-

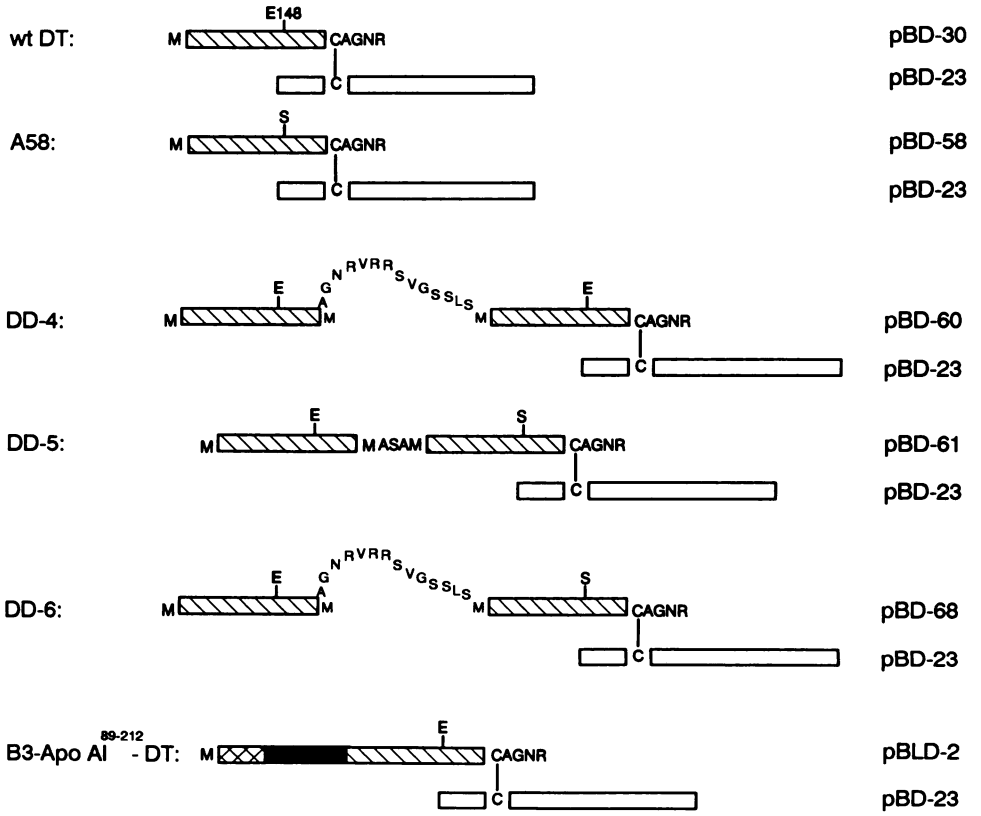

FIG. 1. Schematic representation of the different diphtheria toxin mutants and fusion proteins used. Symbols: ▩, B3 peptide; ▨, A fragment of diphtheria toxin (DT); ■, Apo AI$^{89-212}$; □, B fragment of diphtheria toxin. To the left are listed the protein designations used, and to the right are listed the plasmids from which the proteins were made by the procedures described in Materials and Methods. E (above the A fragment), Glu-148 (wild type); S, E148S mutation. wt DT, wild-type diphtheria toxin.

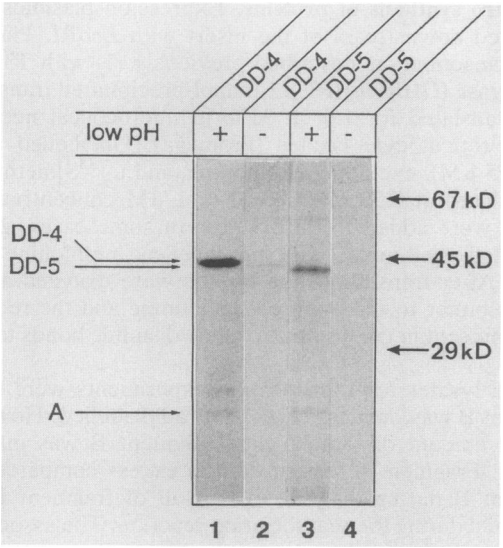

FIG. 2. Translocation of diphtheria toxin fusion proteins with two A fragments to a pronase E-inaccessible compartment. The A fragments of DD-4 and DD-5 were translated in the presence of [^{35}S]methionine, while the B fragment was translated in the absence of radiolabelled methionine, as described in Materials and Methods. The translation products were then mixed and dialyzed to allow the formation of disulfide bonds between the A and B fragments of DD-4 and DD-5. The resulting reconstituted proteins containing radiolabelled A fragments and unlabelled B fragment were bound to Vero cells in 12-well microtiter plates at 20°C for 20 min. The cells were exposed to HEPES-gluconate medium adjusted to pH 4.5 (lanes 1 and 3) or pH 7.0 (lanes 2 and 4) for 3 min at 37°C. The cells were then treated with 4 mg of pronase E per ml for 10 min with 10 μM monensin present in the medium. The detached cells were recovered by centrifugation and solubilized in lysis buffer (see Materials and Methods) at 4°C for 10 min. The samples were centrifuged, and the proteins in the supernatant fraction were precipitated as described in Materials and Methods. The precipitated proteins were analyzed by nonreducing SDS-PAGE (12% gel) and fluorography. The film was exposed for 14 days.

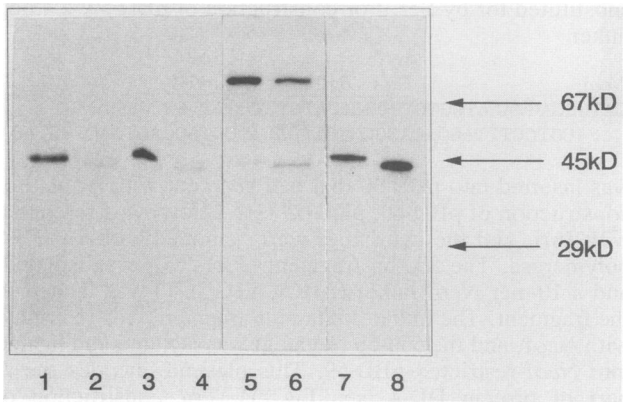

FIG. 3. Stability of translocated fusion proteins. The duplicated A fragments of the constructs were translated in the presence of [^{35}S]methionine, while the B fragment was translated in the presence of unlabelled methionine. The fragments were reconstituted by mixing and dialysis. (Lanes 1 to 4) The resulting fusion proteins were bound to Vero cells in a 12-well plate for 15 min at room temperature. The cells were washed five times with ice-cold HEPES medium and then exposed to HEPES-gluconate medium adjusted to pH 4.5 for 3 min at 37°C. The cells were then treated with pronase as described in Materials and Methods. In some cases, the cells were lysed and treated as described in the legend to Fig. 2, while in other cases, the cells were incubated for an additional 6 h in HCO_3^--containing medium with 10% fetal calf serum before being lysed. The precipitated proteins were subjected to nonreducing SDS-PAGE (12% gel) and fluorography. Lanes: 1, DD-4, cells lysed immediately after translocation; 2, DD-5, cells lysed immediately after translocation; 3, DD-4, cells lysed 6 h after translocation; 4, DD-5, cells lysed 6 h after translocation. The film was exposed for 14 days. (Lanes 5 to 8) One-microliter quantities of lysates containing DD-4 (lanes 5 and 7) or DD-5 (lanes 6 and 8) were run as size markers under nonreducing conditions (lanes 5 and 6) and under reducing conditions (lanes 7 and 8). The film was exposed for 12 h.

onine was added to the translation mixture to inhibit the translation of radiolabelled proteins due to traces of radiolabelled methionine. The cells were washed five times with ice-cold HEPES medium and then incubated at 37°C for 5 min with HEPES-gluconate medium adjusted to pH 4.5. The cells were then treated with 4 mg of pronase E (Sigma) per ml in HEPES medium (pH 7.4) containing 10 μM monensin for 10 min at 37°C. The cells that became detached from the plastic by the treatment were recovered by centrifugation and washed once with HEPES medium containing 1 mM NEM and 1 mM PMSF. The cells were then treated with lysis buffer at 4°C for 10 min. The samples were centrifuged at 12,000 rpm in an Eppendorf centrifuge (model 5415 C) for 3 min. To the supernatant fraction was added 10% trichloroacetic acid, and the proteins were precipitated on ice for 10 min. The samples were centrifuged in an Eppendorf centrifuge (model 5415 C) for 5 min at 12,000 rpm. The pellets were extracted with diethyl ether overnight. Finally, the pellets were boiled in nonreducing sample buffer and analyzed by SDS-PAGE as described earlier (7).

RESULTS

Translocation of diphtheria toxin fusion proteins with two A fragments. To study whether an extra protein domain can be translocated along with diphtheria toxin to the cytosol, we constructed a fusion protein consisting of a toxin with strongly reduced cytotoxicity (because of a triple base mutation [1a]) as a vector and wild-type fragment A as a passenger. This construct should allow us to use the enzymatic activity of the passenger fragment A to monitor translocation to the cytosol.

To be translocation competent, diphtheria toxin must be proteolytically cleaved in the arginine-rich region between fragments A and B (14). This cleavage can be circumvented when the A fragment is synthesized separately from the B fragment and the toxin is subsequently reconstituted by dialysis. Such reconstitution is an advantage when additional protease-sensitive sequences exist in fusion proteins. Reconstitution also provides the advantage of labelling only one of the fragments. Such labelling facilitates the interpretation of experiments when a radiolabelled protein is being traced.

When Vero cells with surface bound, nicked diphtheria toxin are exposed to medium with a low pH, the toxin is translocated across the surface membrane (3, 13). When a radiolabelled toxin is used and the cells are subsequently treated with pronase E to remove nontranslocated material, pronase-protected fragments can be visualized by SDS-PAGE and then autoradiography or fluorography (8). In this way, full-length fragment A was earlier demonstrated to be translocated to the cytosol, while the C-terminal 25-kDa portion of fragment B was found to be inserted into the membrane (8, 9).

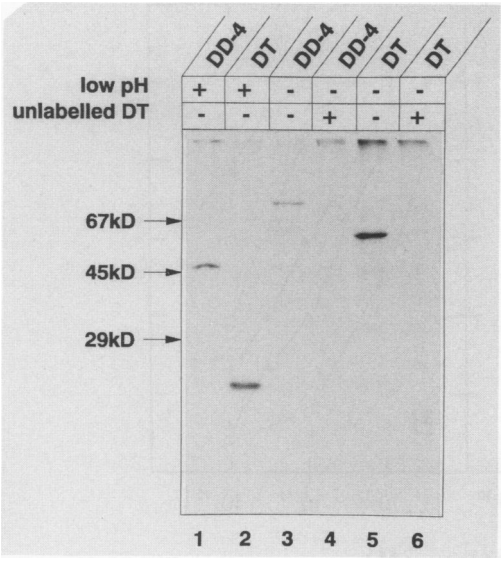

FIG. 4. Efficiency of translocation of DD-4 and diphtheria toxin (DT). Fragment A and the duplicated fragment A were translated in the presence of [^{35}S]methionine, while fragment B was translated in the presence of unlabelled methionine. The two A fragments were reconstituted with the same low concentration of fragment B by mixing and dialysis. (Lanes 1 and 2) The reconstituted proteins were bound to Vero cells in a 12-well plate for 15 min at room temperature. The cells were washed five times with ice-cold HEPES medium and then exposed to HEPES-gluconate medium adjusted to pH 4.5 for 3 min at 37°C. The cells were then treated with pronase as described in the legend to Fig. 2. (Lanes 3 to 6) The reconstituted proteins were bound to Vero cells in a 12-well plate for 15 min at room temperature in the presence (lanes 4 and 6) or in the absence (lanes 3 and 5) of excess amounts of unlabelled diphtheria toxin. The cells were lysed, and the precipitated proteins were subjected to nonreducing SDS-PAGE (12% gel) and fluorography. The film was exposed for 6 days. Twice as many cells were used in lanes 1 and 2 as in lanes 3 to 6.

In the present work, we used in vitro-synthesized, [^{35}S]methionine-labelled fusion proteins for translocation studies. For constructs DD-4 and DD-5 (Fig. 1), only the A fragments were synthesized in the presence of [^{35}S]methionine, and the proteins were then mixed with unlabelled B fragments and dialyzed against buffer without reducing agents to allow the formation of disulfide bonds. After exposure to a low pH and pronase treatment, protected fragments appeared for both constructs DD-4 and DD-5 (Fig. 2, lanes 1 and 3). When the low-pH exposure was omitted (Fig. 2, lanes 2 and 4), no protected material was observed. When the labelled proteins were added to cells in the presence of excess amounts of unlabelled diphtheria toxin to inhibit binding to the diphtheria toxin receptor, pronase-protected fragments were not observed (data not shown).

For construct DD-4 (Fig. 2, lane 1), the full-length labelled protein (duplicated A fragment) was protected, as were three lower-molecular-weight bands of weak intensity. These bands either can represent degradation products, the smallest of which has the same mobility as authentic fragment A, or may be the result of the translocation of incomplete proteins with lower molecular weights due to downstream initiation of translation.

Construct DD-5 has two A fragments, the first of which has a C-terminal deletion of five amino acids. The link between the two A fragments does not contain arginines (Fig. 1). DD-5 was translocated mainly as a full-length protein at a low pH. Also, a faint band migrating at the same size as fragment A was seen (Fig. 2, lane 3). The results indicate that a duplicated A fragment was translocated to the cytosol. DD-5 was clearly less efficiently translocated to the cytosol than was DD-4 (Fig. 2). This result can be explained by a measurable reduction in specific receptor binding (data not shown).

Once inside the cytoplasm, the translocated portions of DD-4 and DD-5 were stable for at least 6 h (Fig. 3). When the cells were incubated in serum-containing medium after pronase treatment, no breakdown of the full-length translocated protein into lower-molecular-weight bands was observed. The intensity of the translocated portion of DD-4 was the same immediately after translocation as after additional incubation for 6 h (Fig. 3, compare lanes 1 and 3). The same was true for DD-5 (Fig. 3, compare lanes 2 and 4). These results are in accordance with the earlier finding that fragment A has a long half-life inside cells (20).

Efficiency of translocation of fusion proteins. The results presented in Fig. 2 suggested that diphtheria toxin might be used for practical purposes to carry protein domains into the cytosol. It was therefore of interest to determine how efficiently fusion proteins were translocated in comparison with authentic diphtheria toxin. For this purpose, wild-type fragment A and the double A fragments encoded by pBD-60 were dialyzed with the same batch of unlabelled fragment B. It is important to avoid the presence of free fragment B in the dialyzed toxin preparation, because fragment B binds to diphtheria toxin receptors with a higher affinity than full-length diphtheria toxin (7) and may block the binding and translocation of reconstituted toxin. Fragment A was therefore present in excess to consume all of fragment B (data not shown). The translocation of reconstituted fusion protein and diphtheria toxin is shown in Fig. 4, lanes 1 and 2, respectively. Figure 4, lanes 3 and 4, shows the total amount of fusion protein bound to cells in the absence and presence of excess unlabelled diphtheria toxin, respectively, while Fig. 4, lanes 5 and 6, shows the corresponding binding for diphtheria toxin. When the translocated material was compared with the amount specifically bound, it appeared that the translocation was efficient and that the translocation of the fusion protein was as efficient as the translocation of diphtheria toxin fragment A.

Cytotoxicity of fusion proteins with two A fragments. The translocation experiments described above strongly indicated that fragment A with N-terminal extensions can be translocated to the cytosol under in vitro conditions. We then went on to study whether the extended fragment A also can be translocated under in vivo conditions. In this case, the process of translocation was studied by monitoring the arrest of cellular protein synthesis when the fusion proteins were added to cells and allowed to be endocytosed. In all constructs used for this purpose, wild-type, enzymatically active fragment A containing Glu-148 at the active site was the N-terminal fragment A, while as the C-terminal fragment A was used, in most cases, the E148S mutant, which has strongly reduced enzymatic activity (1a, 19).

The fusion proteins were added to Vero cells as dilutions of nonradiolabelled translation products. Wild-type toxin was made by reconstitution of the A and B fragments, as were DD-4, DD-5, and DD-6. In parallel, a small amount of radiolabelled protein was made, so that the amounts of the various full-length proteins could be determined.

A58 (containing the E148S mutation) was >100-fold less toxic than the wild-type toxin (Fig. 5A), in accordance with

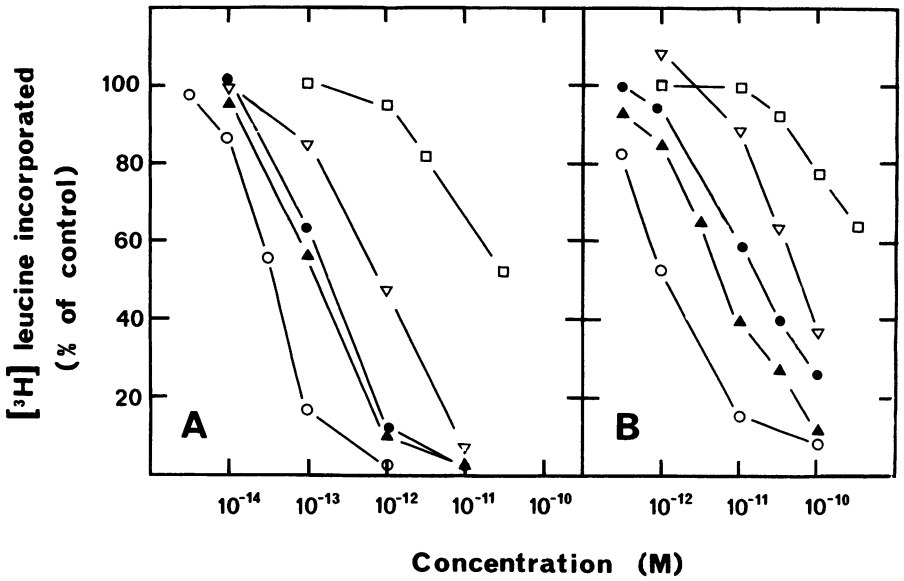

FIG. 5. Cytotoxicity of diphtheria toxin, the E148S mutant of diphtheria toxin, and fusion proteins with extra A fragments. (A) Dialyzed translation products containing the indicated constructs were added to Vero cells in 24-well microtiter plates, and the cells were incubated at 37°C overnight. (B) Dialyzed translation products containing the indicated constructs were added to Vero cells in 24-well microtiter plates, and the cells were incubated at 4°C for 2 h. The cells were washed twice with ice-cold HEPES medium and then incubated with HEPES-gluconate medium (pH 4.5) for 5 min. Subsequently, ordinary growth medium with 10% fetal calf serum, 10 μM monensin, and 10 μl of anti-diphtheria toxin antiserum was added, and the cells were incubated overnight at 37°C. In both panels A and B, residual protein synthesis was measured by assaying the incorporation of [^{3}H]leucine (Amersham) over 15 min. Symbols: ○, wild-type diphtheria toxin; □, A58; ▲, DD4; ▽, DD5; ●, DD6.

the results of Barbieri and Collier (1a). DD-6 was approximately 10-fold less toxic than the wild-type toxin (Fig. 5A). DD-4 was slightly more toxic than DD-5 and DD-6, the reason probably being that both A fragments are wild type. The reason that the fusion proteins were less toxic than normal diphtheria toxin could be that the enzymatic activity of wild-type fragment A is decreased in the context of a fusion protein. The fact that all the fusion proteins were more toxic than A58 demonstrates that the N-terminal fragment A was indeed translocated to the cytosol. In DD-5 and DD-6, there was a contribution to cytotoxicity from the C-terminal fragment A because of some residual enzymatic activity of the E148S mutant. However, since all these constructs have much higher cytotoxicity than the E148S mutant, A58, obviously cytotoxicity is largely due to the N-terminal fragment A.

In the experiments presented in Fig. 5A, the toxic proteins were added to the cells and incubation was carried out overnight. Under these conditions, the translocation of diphtheria toxin occurs across the membrane of intracellular vesicles and not across the surface membrane. To test toxicity under conditions more like those used for Fig. 2, we first bound the fusion proteins to cells and then pulsed them through the surface membrane at a low pH. Under these conditions, the various constructs were more toxic than A58 and approximately 10 times less toxic than wild-type diphtheria toxin (Fig. 5B). The cytotoxicity of the various constructs relative to that of the wild-type toxin was essentially the same under both types of experimental conditions. This result suggests that the mechanisms of translocation of the fusion proteins are the same in vivo and in vitro.

Saponin fractionation of cells with translocated fusion protein DD-4. The cytotoxicity experiments indicated that the N-terminal passenger domains were indeed translocated to the cytosol. Since the toxicity was reduced almost 10-fold compared with that of the wild-type toxin, the possibility existed that most of fusion protein DD-4 could be stuck in the membrane upon translocation. To test whether the full-length fusion protein was localized to the cytoplasm upon translocation, we subjected cells with translocated DD-4 to saponin fractionation after treatment with pronase. Translocated authentic fragment A was observed only in the supernatant fraction (Fig. 6, lanes 1 and 2), while the 25-kDa fragment derived from fragment B as well as undigested fragment B were observed only in the pellet fraction (Fig. 6, lanes 5 and 6), indicating localization in the membrane. Approximately equal amounts of fusion protein DD-4 were present in the supernatant and pellet fractions (Fig. 6, lanes 3 and 4), indicating that 50% of the full-length fusion protein was completely translocated to the cytosol, whereas the rest appeared to be stuck in the membrane. To exclude the possibility that some of the membrane-embedded fusion protein could cause cytotoxicity due to membrane effects and not due to ADP-ribosylation of EF-2, we added DD-4 to Vero cells that were EF-2 mutants. In these cells, which cannot be killed by ADP-ribosylation of EF-2, there was no inhibition of protein synthesis (data not shown).

The fact that a portion of fusion protein DD-4 appeared to be stuck in the membrane upon translocation does not necessarily mean that the delivery system has approached an upper size limit for protein translocation. We recently demonstrated the translocation of a fusion protein consisting of DD-4 and a 16-kDa N-terminal extension (unpublished data). This fusion protein was more readily released from the cytosol after saponin treatment than was DD-4 alone.

Translocation of some of apolipoprotein AI as a passenger of diphtheria toxin. To investigate whether diphtheria toxin can also translocate proteins different from diphtheria toxin

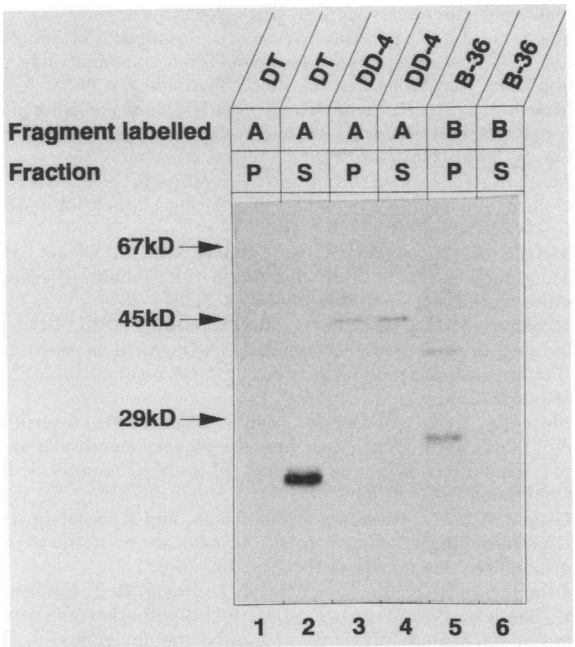

FIG. 6. Saponin fractionation of cells with translocated natural diphtheria toxin (DT) or fusion protein DD-4. Reconstituted toxins consisting of ^{35}S-labelled A fragments and unlabelled B fragments (lanes 1 to 4) were bound to Vero cells, and translocation was induced by exposure to HEPES-gluconate medium adjusted to pH 4.5. As a control, ^{35}S-labelled fragment B (B-36) was bound to cells, and insertion was induced at pH 4.5 (lanes 5 and 6). The cells were then treated with pronase as described in Materials and Methods and with 50 μg of saponin per ml in HEPES medium containing 1 mM PMSF and 1 mM NEM to release protein translocated to the cytosol. The proteins in both the supernatant (S) and the pellet (P) fractions were precipitated with trichloroacetic acid. In all cases, the precipitated proteins were analyzed by SDS-PAGE (12% gel) under nonreducing conditions. Fluorography was done for 10 days.

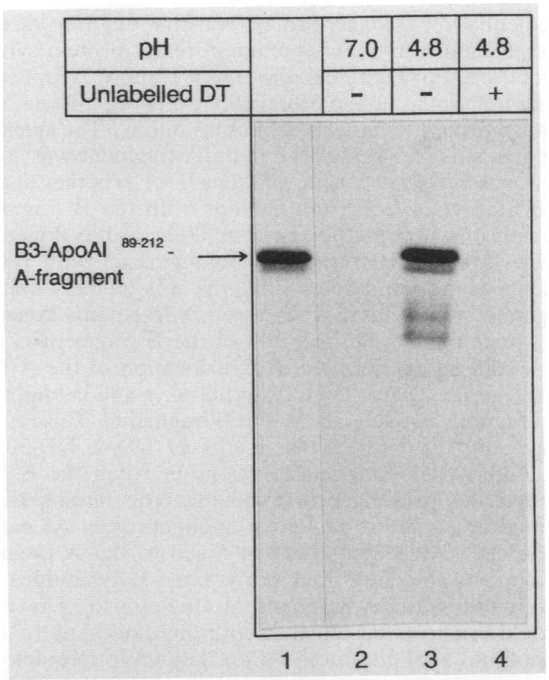

FIG. 7. Translocation of diphtheria toxin with an N-terminal extension of peptide B3 and amino acids 89 to 212 of apolipoprotein AI to a pronase E-inaccessible site. B3-ApoAI$^{89-212}$-A fragment was translated in the presence of [^{35}S]methionine, while fragment B was translated in the presence of unlabelled methionine. Both were reconstituted during dialysis (16) and bound to Vero cells in a 12-well plate in the absence (lanes 2 and 3) and presence (lane 4) of 20 μg of unlabelled diphtheria toxin (DT) per ml for 2 h at 4°C. The cells were washed with ice-cold HEPES medium and then exposed to HEPES-gluconate medium adjusted to pH 7.0 (lane 2) or pH 4.8 (lanes 3 and 4) for 3 min at 37°C. The cells were then treated with pronase as described in the legend to Fig. 2, and the precipitated proteins were finally subjected to nonreducing SDS-PAGE (12% gel) and fluorography. The film was exposed for 14 days. For comparison, 0.02 μl of a lysate containing B3-ApoAI$^{89-112}$-A fragment that had been dialyzed with fragment B was run as a size marker under reducing conditions (lane 1).

domains, we used a construct consisting of tetradecapeptide B3 (against which we had an antiserum) and amino acids 89 to 212 of apolipoprotein AI fused to fragment A of diphtheria toxin (Fig. 1). On the basis of the sequence of amino acids 89 to 212 of apolipoprotein AI, one can assume that the protein consists of several amphipathic alpha helices. This construct fused to fragment A was earlier demonstrated to be efficiently reconstituted with diphtheria toxin fragment B (16). The reconstituted protein bound to cells, although the binding to specific receptors was lower than that of diphtheria toxin (data not shown).

The A and B fragments of the fusion protein were made separately, with [^{35}S]methionine present only in the translation mixture for the A fragment. When Vero cells with bound reconstituted fusion protein were exposed to a low pH and then treated with pronase, a pronase-protected fragment corresponding to the full-length extended A fragment was seen (Fig. 7, compare lanes 1 and 3). This protected fragment could also be immunoprecipitated with antibodies to the B3 peptide (data not shown), as demonstrated earlier for the protected A fragment extended with the B3 peptide (17). In the absence of low-pH exposure, no pronase-protected fragment was observed (Fig. 7, lane 2). Also, when binding was carried out in the presence of excess amounts of unlabelled diphtheria toxin, no protected fragment was observed (Fig. 7, lane 4), indicating that the protected material represents protein translocated via an interaction with the diphtheria toxin receptor and not material adhering to the membrane in a nonspecific manner.

DISCUSSION

In the present work, we demonstrated that N-terminal extensions of diphtheria toxin with 135 to 202 amino acids can be translocated along with fragment A into the cytosol of eukaryotic cells. Translocation can take place across the plasma membrane as well as across the membrane of intracellular vesicles. The fact that proteins as large as 20 kDa can be translocated along with the toxin is important, because it may offer the opportunity to introduce biologically active molecules into cells.

When translocated to the cytosol, the fusion proteins that we used had a long half-life. In future work, it will be important to find ways to obtain cleavage in the cytosol of the delivered protein from the delivering protein. It should be noted that at least in the model system with double A fragments, the efficiency of translocation appeared to be the same for fusion proteins as for authentic diphtheria toxin.

The reason that we chose to use an extra diphtheria toxin

A fragment as a passenger protein was that the translocation of this fragment can readily be monitored as protein synthesis inhibition. For technical reasons, it is more complicated to make functional fusion proteins between diphtheria toxin and other protein synthesis-inhibiting toxins. The question hence arises as to whether the extra A fragment was translocated passively as a true passenger or whether it was translocated via specific interactions with the B fragment. We regard the latter possibility as unlikely, as it is difficult to envisage how the B fragment would interact with two A fragments at the same time. Similarly, it is unlikely that the B fragment would interact with two A fragments consecutively, since membrane insertion of the B fragment is irreversible and tightly coupled to translocation of the A fragment (8). Recent data (1) indicate that Cys-186 is important for interactions between the A and B fragments. This residue is deleted in the extra A fragments of DD-4, DD-5, and DD-6, highly disfavoring interactions between the B fragment and the passenger A fragment. The finding that a presumed amphipathic region of apolipoprotein AI can be translocated to the cytosol when fused to the A fragment also supports the view that fairly large polypeptides can passively follow the A fragment into the cytosol. However, we would like to point out that both the diphtheria toxin A fragment and apolipoprotein AI are known to interact with lipids. It may well be that only certain kinds of proteins, e.g., lipid-reactive proteins or proteins that assume loose folding at a low pH, can actually be translocated together with the A fragment. At present, we are investigating this issue, but so far we have encountered the problem that most fusion proteins that we have made are unable to bind specifically to diphtheria toxin receptors (unpublished results). Since receptor binding has been demonstrated to be a prerequisite for translocation to the cytosol (5, 18), these fusion proteins are of no use without further engineering. Knowledge of the three-dimensional structure of diphtheria toxin will presumably increase the chance of constructing fusion proteins with conserved receptor-binding ability.

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