Replacement of Negative by Positive Charges in the Presumed Membrane-inserted Part of Diphtheria Toxin B Fragment

EFFECT ON MEMBRANE TRANSLOCATION AND ON FORMATION OF CATION CHANNELS*

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Diphtheria toxin B fragment is capable of forming cation-selective channels in the plasma membrane. Such channels may be involved in the translocation of the toxin A fragment to the cytosol. Seven negatively charged amino acids in the B fragment were replaced one by one by lysines, followed by studies of cytotoxicity and channel-forming ability of the different mutants. The mutant D392K showed a strong reduction in binding to cell surface receptors. Of the six mutants that showed wild-type binding affinity, the two mutants D295K and D318K were very inefficient in forming channels. These two mutants had the lowest ability to mediate A fragment translocation. The mutant E362K was able both to induce cation channel formation and to mediate A fragment translocation at a higher pH value than the wild-type B fragment. The results support the notion that formation of cation channels is of importance for the translocation of the A fragment across the plasma membrane, and they indicate that the pH requirement for translocation of the A fragment to the cytosol is partly determined by the B fragment.

Several bacterial and plant toxins are capable of entering the cytosol of animal cells. The entry mechanism is known in most detail for diphtheria toxin. This toxin, which is the main pathogenicity factor in diphtheria, is secreted by Corynebacterium diphtheriae as a single polypeptide of 58 kDa (1). The protein can readily be nicked by trypsin into two fragments, A and B (2). Fragment B is responsible for the binding of the toxin to cell surface receptors and for facilitating A fragment translocation to the cytosol (3). The A fragment has an enzymatic activity which enables it to inactivate elongation polymerase  was obtained from GIBCO-BRL, Eggenstein, Germany. [3H]leucine, [22NaCl, [35S]methionine were obtained from Amer sham, United Kingdom. Crude diphtheria toxin from Con naught Laboratories, Willowdale, Canada, was purified as described (17). Micrococcal nuclease-treated rabbit reticulocyte lystate and RNAsin ribonuclease inhibitor were from Promega, Madison, WI. T7 RNA polymerase was obtained from Gibco-BRL, Eggenstein, Germany. T7 DNA ligase and restriction nucleases were from New England Biolabs, Beverly, MA.

EXPERIMENTAL PROCEDURES

Materials—PrOnase E (from Streptomyces griseus), L-tosyl-aminiodo-2-phenylethylchloromethyl ketone (TPCK), trypsin (TPCK-treated), dithiothreitol, phenylmethylsulfonyl fluoride (PMSF), oas bain, N-ethylmaleimide (NEM), aprotinin, para-chloromercuribenzoic acid, iodoacetamide, soybean trypsin inhibitor, a2-macroglobulin, and pepstatin A were from Sigma. Amiloride was a gift from Merck, Sharp, and Dohme, Drammen, Norway. Chymostatin and leupeptin were from Fluka BioChemika, Buchs, Switzerland. [1H]uracil, [22NaCl, [35S][12C]DMO, and [22S]methionine were obtained from Amer sham, United Kingdom. Crude diphtheria toxin from Con naught Laboratories, Willowdale, Canada, was purified as described (17). Micrococcal nuclease-treated rabbit reticulocyte lystate and RNAsin ribonuclease inhibitor were from Promega, Madison, WI. T7 RNA polymerase was obtained from Gibco-BRL, Eggenstein, Germany. T7 DNA ligase and restriction nucleases were from New England Biolabs, Beverly, MA.

1 The abbreviations used are: TPCK, L-tosyl-aminiodo-2-phenylethylchloromethyl ketone; DMO, dimethylxalolazine-2,4-dione; HEPES, N-2-hydroxyethylpipеразине-N'-2-этансульфонная кислота; MES, 2-(N-morpholino)этансульфокислота; NEM, N-этилмалеимид; PBS, phosphate-buffered saline; phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; bp, base pair(s).

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Bacterial Strains—Escherichia coli strain TG-1 was used as a host for M13 mp18 during mutagenesis, and E. coli strain DH5α was used in the cloning procedures.

Buffers—HEPES medium consisted of bicarbonate- and serum-free Eagle’s minimal essential medium buffered with 20 mM HEPES to pH 7.4. Lysis buffer consisted of 0.1 mM NaCl, 20 mM NaH₂PO₄, 10 mM EDTA, 1% Triton X-100, 10 mM NaF, 0.1 mM Na- vanadate, 1 mM PMSF, 1 mM NEM, 200 units/ml aprotinin, 1 μg/ml chymostatin, 1 μg/ml soybean trypsin inhibitor, 1 mM iodoacetamide, 1 mM para-chloromercuribenzoic acid, 1 μg/ml ω-macroglubulin, 1 μg/ml TPCF, pH 7.4. MES-glucuronate buffer: 140 mM NaCl, 20 mM MES, 5 mM sodium glutonate. Phosphate-buffered saline (PBS) consisted of 140 mM NaCl and 10 mM NaH₂PO₄, pH 7.4. Dialysis buffer consisted of 140 mM NaCl, 20 mM HEPES, and 2 mM CaCl₂ adjusted to pH 7.0 with NaOH.

Construction of Plasmids—Site-directed mutagenesis was performed using the Amersham mutagenesis kit and oligonucleotide primers from MedProbe, Oslo, Norway. The plasmid pGD-1 (18) which encodes the diphtheria toxin mutant DT-Ser-148 that has been mutated to introduce the five new unique restriction sites. The resulting plasmid was then used to introduce the six indicated mutations.

The four in vivo transcription and translation (3 pg of A fragment/100 μl of translation mixture), and A and B fragments were allowed to cotranslate in a 20-μl reaction mixture with T7 RNA polymerase as described (18). The mRNA was precipitated with ethanol and dissolved in 10 μl of H₂O containing 10 mM dithiothreitol and 0.1 unit/μl RNasin. The translation was performed in micrococcal nuclease-treated rabbit reticulocyte lysate using 5 μl of the dissolved mRNA/100 μl of lysate. The radioactive proteins were made with lysates containing 1 μM [³⁵S]methionine and 25 μM of each of the 19 other amino acids. Labeled methionine was replaced by 25 μM unlabeled methionine when making non-radioactive proteins. The amount of protein in the non-labeled lysates was estimated as earlier described (21) by translating in vitro a 10-μl aliquot of the lysate in the presence of [³⁵S]methionine. The translation mixture was dialyzed overnight against dialysis buffer to remove free methionine and reducing agent.

In Vitro Reconstitution of Active Toxin—When A and B fragments are mixed together under reducing conditions, and the reducing agent subsequently is removed by dialysis, enzymatically active toxin will form quantitatively (21). A fragment was prepared from natural diphtheria toxin as previously described (23), mixed with B fragment made by in vitro transcription and translation (3 μg of A fragment/100 μl of translation mixture), and A and B fragments were allowed to associate by overnight dialysis against dialysis buffer.

Analytical Gel Electrophoresis in the Presence of SDS-PAGE—SDS-PAGE (10% acrylamide, 0.27% N,N'-methylene-bis-acrylamide) was carried out as earlier described (22). After electrophoresis the gel was fixed for 30 min in 25% methanol, 4% acetic acid, and then incubated for 30 min in 1 M Na-salicylate, 2% glycerol, pH 5.8. Kodak XAR-5 film was exposed to the dried gel in the absence of intensifying screen at ~80°C.

Assay for Toxin Binding to Cells—Vero cells were incubated with reticulocyte lysate containing [³⁵S]methionine labeled toxin or B fragment for 20 min at 25°C, washed four times in HEPES medium, and lysed for 10 min on ice in lysis buffer. The cell lysate was transferred to an Eppendorf tube, nuclei were removed by centrifugation, and cellular protein was precipitated for 30 min on ice in the presence of 5% trichloroacetic acid. After centrifugation, the trichloroacetic acid pellet was washed in ether and subjected to SDS-PAGE.

Pronase Protection Experiments—Toxin was bound to Vero cells by incubating [³⁵S]methionine labeled translation mixture containing 1 mM unlabeled methionine and 10 μM monensin with cells for 20 min at 25°C. The unbound toxin was washed away, and the cells were incubated for 5 min at 25°C with 0.5 μg/ml TPCK-treated trypsin, in order to nick surface-bound toxin. This nicking step was omitted from experiments described elsewhere. After a 2-min incubation to pH 4.8 at 37°C in MES-glucuronate buffer, the cells were incubated for 8 min at 37°C with 10 mg/ml Pronase E in HEPES medium. The cells, which now were detached from the plastic, were transferred to an Eppendorf tube, and pelleted by centrifugation. After washing with PBS medium containing HEPES 1 mM NEM and 1 mM PMSF, the cells were lysed for 10 min in lysis buffer on ice, and nuclei were removed by centrifugation. Cellular protein was precipitated with 5% trichloroacetic acid for 30 min on ice, and pelleted by centrifugation.

FIG. 1. Hypothetical model for the membrane insertion of diphtheria toxin B fragment (from Ref. 16). The negatively charged amino acids that have been replaced by lysines are indicated by boxes.
The pellet was washed with ether and subjected to SDS-PAGE under non-reducing conditions.

**Measurement of $^{22}$Na$^+$ Influx**—Reticulocyte lysate containing translated protein was incubated with Vero cells for 20 min at 25 °C. The lysate was removed, and MES-gluconate buffer with 1 mM amiloride, 50 μM ouabain, and 1.6 μCi/ml $^{22}$NaCl was added. The cells were incubated for 8 min at 37 °C, and rapidly washed four times in ice-cold PBS. The radioactivity associated with the cells was extracted with 5% trichloroacetic acid and measured by scintillation counting.

**Measurement of Cytotoxicity of Endocytosed Toxin**—Vero cells were incubated overnight in DMEM medium with increasing amounts of toxin-containing reticulocyte lysate. The cells were allowed to incorporate radioactivity for 30 min at 37 °C in HEPES medium with 4 μCi/ml [3H]leucine and no unlabeled leucine. After a 10-min wash, followed by a brief wash in 5% trichloroacetic acid, the cells were dissolved in 0.1 M KOH, and the radioactivity was measured.

**Measurement of Low pH-induced Cytotoxicity**—Vero cells were incubated with toxin-containing reticulocyte lysate for 1 h at 4 °C. Unbound toxin was washed away, and translocation was induced by exposing the cells to MES-glucanate buffer, pH 4.8, for 5 min at 37 °C. The cells were incubated overnight in DMEM growth medium in the presence of 15 mM NH₄Cl, to avoid entry of toxin from endosomes to the cytosol (8). The incorporation of radioactive leucine and the subsequent steps were performed in the same way as in the experiment described above.

**Measurement of Cytosolic pH**—The cytosolic pH was estimated by measuring the distribution of [14C]DMO across the plasma membrane as previously described (11).

**Variability of the Data Presented**—Many of the experiments described here must be performed rapidly in order to be successful, and it is difficult to do them with many parallels. Therefore, the standard deviation of each point is not shown. The presented data represent typical experiments, and in the following, when a difference between a mutant and the wild-type protein is described, a difference of the same magnitude was observed in several experiments.

**RESULTS**

**Formation and Stability of Mutant Toxin**—To study the effect of changing negatively charged amino acids into positively charged ones, we chose 7 residues in putatively membrane inserting or associating regions (Asp²⁶⁵, Asp³¹⁸, Glu³⁶², Asp⁴⁰³, Glu⁴⁰³, Asp⁴¹³, Gln⁴⁴⁵), and exchanged them with lysines by site-directed mutagenesis, generating seven mutants containing a single mutation each. The mutant toxin genes were placed behind a T3 RNA polymerase promoter and transcribed and translated in vitro. For safety reasons the plasmids that have been introduced into E. coli carry the mutation DT-Ser-148 which strongly reduces the enzymatic activity of the A fragment (19). If not otherwise mentioned, the toxins described in the following all carry this mutation, and the term “wild-type toxin” therefore refers to a toxin with a wild-type B fragment.

To test the possibility that the mutations introduced could have altered the conformation of the protein, we studied the protease sensitivity of the translation products, as conformational changes often lead to increased susceptibility to proteases. We tested the sensitivity both to proteinase K and trypsin, and the results of the proteinase K assay are shown in Fig. 2. The toxin was nicked into A and B fragments already at the lowest concentration of proteinase K, and Fig. 2 shows the degradation pattern of the B fragment when full-length toxin is exposed to increasing concentrations of proteinase K and analyzed by reducing SDS-PAGE. The mutants D403K, E413K, and D442K were all approximately 3-fold more sensitive to proteinase K than the wild-type toxin, and the sensitivity to proteinase K of the mutants D318K and E362K was also slightly increased. All the mutants showed wild-type sensitivity to trypsin (data not shown), implying that the proteinase K assay is a more sensitive indicator of conformational changes.

**Binding of Mutant Toxins to Vero Cells**—Fig. 3A shows the ability of the mutant toxins to bind to cells. Except for D392K, which showed a strongly reduced binding, all the mutants appeared to possess wild-type binding affinity. When an excess amount of unlabeled diphtheria toxin was present, the binding of the mutant toxins was abolished, indicating that the binding is mediated by specific receptors (data not shown).
A Fragment Translocation and B Fragment Insertion of Mutant Toxins—When diphtheria toxin is bound to cells, a subsequent exposure to acidic medium leads to translocation of the A fragment to the cytosol (7,8), and insertion of the B fragment into the plasma membrane (9). After this, the entire A fragment and a 25-kDa polypeptide derived from the B fragment are protected against externally added Pronase. Such experiments were performed with the mutant toxins, as shown in Fig. 3B. No protected fragments could be observed in the case of the mutant D392K, probably because of the low binding affinity of this mutant. The amount of protected A fragment is significantly reduced in the case of the mutants D295K and D318K. There appears to be less protected 25-kDa fragment than A fragment, and this is often observed when using in vitro translated toxin. This may be caused by some inhibitory component in the reticulocyte lysate, preventing complete digestion of the B fragment by the Pronase.

Cytotoxicity of Mutant Proteins—We tested to see if the mutant B fragments were able to form active toxin with enzymatically active A fragment. Since cloning of the gene for whole active toxin is considered hazardous, we took advantage of the finding that active, full-length diphtheria toxin can be reconstituted by combination of fragment B made in vitro with wild-type A fragment isolated from diphtheria toxin (21). All the mutant B fragments described here were able to reconstitute with A fragment. The cytotoxicity of the mutants was investigated by measuring their ability to inhibit protein synthesis in Vero cells, using two slightly different approaches. In the first approach, in the following denoted “overnight toxicity,” cells were incubated overnight with the toxin present in the growth medium, allowing the toxin to be continuously endocytosed. In the second method, termed “pulse-toxicity,” toxin was allowed to bind to cells, unbound toxin was washed away, and the translocation of receptor-bound toxin was induced by a brief exposure to low pH. Subsequently, the cells were incubated overnight in the absence of toxin, to allow the translocated toxin time to express its effect on protein synthesis. The results are shown in Fig. 4.

The mutants E362K and D442K both displayed wild-type overnight toxicity (Fig. 4A), whereas the toxicity of the other mutants was reduced when measured by this assay. The results for the mutant D392K are not shown, but the observed reduction in toxicity corresponded approximately to the reduction in binding.

Additionally, in the pulse-toxicity assay (Fig. 4B) the mutants E362K and D442K displayed wild-type toxicity, but in this assay even D403K and E413K appeared to be as toxic as wild-type toxin. In this assay the least toxic mutants were D295K and D318K, showing approximately a 10-fold reduction in toxicity. The cytotoxicity is a measure of how much A fragment has been translocated to the cytosol, and the results of the pulse-toxicity experiments correlate well with the amount of protected A fragment observed in the Pronase protection experiments (Fig. 3B).

Properties of Mutant B Fragments—When bound to cells and exposed to low pH, nicked diphtheria toxin induces the formation of cation-selective channels in the plasma membrane (11). The B fragment alone is approximately 100-fold more efficient than full-length toxin in forming cation-selective channels in the plasma membrane (12). We therefore decided to use the B fragment to test the mutants for this ability.

A mutant B fragment lacking the internal disulfide bridge has been shown to behave as wild-type toxin in the presence of A fragment, whereas the mutant B fragment alone appeared to fold incorrectly (23). Conceivably, the mutant B fragments tested here could be unable to fold correctly in the absence of the A fragment, and we first examined the protease susceptibility of the B fragments both with trypsin and proteinase K. The results are shown in Fig. 5. Trypsin and proteinase K gave, in principle, the same qualitative results, but the sensitivity to proteinase K varied more between the different B fragments than did the trypsin sensitivity. When comparing the stability of the mutants essentially the same picture was obtained as with full-length toxin (Fig. 2), except for D403K and D392K, which were relatively less stable as B fragments than as whole toxin. The reason for this could be that the A fragment is shielding protease-sensitive sites when these mutations are present in the full-length toxin.

The mutant B fragments were tested for their ability to bind to cells and to insert into the plasma membrane (Fig. 6).
The binding of the mutant D392K was considerably reduced, whereas the mutant D403K, which was very sensitive to proteinase K, was degraded in the presence of cells. It is therefore difficult to conclude whether the binding of this mutant B fragment is reduced, or if the apparent reduction in binding is solely due to cell-mediated degradation of the protein. When cells with bound B fragment were exposed to low pH followed by Pronase treatment all the mutant B fragments, with the exception of D392K and D403K, gave rise to the same amount of 25-kDa fragment as the wild-type B fragment.

The effects of the mutations on the channel forming ability were examined by measuring to what extent the mutant B fragments were capable of permeabilizing Vero cells to $^{22}\text{Na}^+$. The results are shown in Fig. 7. Since the binding affinity to an Eppendorf tube and washed with cold HEPES medium with 5% trichloroacetic acid, and analyzed by non-reducing SDS-PAGE. The gel in panels A and B were exposed to x-ray film for 18 h and 11 days, respectively.

The observation above that the mutants D295K and D318K are reduced, it is difficult to assess to what extent the channel forming ability as such is affected by the mutation, and the data for this mutant are not included in Fig. 7. However, the reduction in channel formation appeared to be of the same order of magnitude as the reduction in binding, indicating that the channel forming ability as such is not affected. The mutants E362K, E413K, and D442K were all as efficient as the wild-type B fragment in permeabilizing the plasma membrane. The channel forming ability of the mutant D403K appears to be reduced by approximately a factor of 100, but this could also be a result of cell-mediated degradation of the protein, as observed in the binding experiments. In fact, the full-length toxin carrying the mutation D403K, which is relatively less protease sensitive (see Fig. 2), showed wild-type channel forming ability (data not shown).

Previously, we found that less acidic conditions are required for insertion of the B fragment into the membrane to form 25-kDa protease-protected fragment than those required to form cation channels and allow translocation of the A fragment (12). We have found that the pH dependence of the B fragment of E362K to insert into the membrane and form 25-kDa protected polypeptide was identical to that of the wild-type B fragment (data not shown). It therefore appears that the ability of wild-type B fragment to form cation channels was half-maximal at pH ~ 5.5, whereas the corresponding value for E362K was pH ~ 5.8 (Fig. 8). The mutants D413K and D442K showed wild-type behavior.

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The observation above that the mutants D295K and E318K which had the lowest capability of mediating A fragment.
The artificial translocation system used above is assumed to mimic translocation from acidic endosomes, which is the natural entry route. Translocation of the mutant E362K should therefore require less extensive acidification of the endosomes than wild-type toxin. To test this, we took advantage of the finding that ammonium chloride raises the endosomal pH, since NH₄⁺ is membrane permeant, and can act as a proton acceptor. Ammonium chloride added to the external medium inhibits the toxic effect of diphtheria toxin (8). From the above experiments it was therefore predicted that the cytotoxicity of the mutant E362K should be more resistant to inhibition by ammonium chloride than wild-type toxin. The results in Fig. 10 show that with the mutant E362K, approximately twice as high concentrations of ammonium chloride were required to inhibit toxicity than with wild-type toxin.

**DISCUSSION**

In the present work we have attempted to alter the properties of diphtheria toxin B fragment by changing negative to positive amino acids. The mutants were analyzed with respect to the effect on (a) gross conformation, measured as protease resistance; (b) receptor binding; (c) membrane insertion at low pH; (d) cation channel formation; and (e) ability to support translocation of the toxin A fragment into cells. We obtained four mutants that are informative. One mutant, E362K, exhibited an altered pH profile, whereas the mutant D92K showed a strong reduction in its ability to bind to cells. The mutations D295K and D318K were both found to drastically reduce the channel forming ability of the diphtheria toxin B fragment without affecting the binding and membrane insertion of the protein, while the translocation efficiency was reduced.

The residue Asp₂⁹⁷ could be involved in the initial interaction of the toxin with its receptor since change of this residue to lysine strongly reduced the binding ability without introducing gross conformational changes as measured by protease sensitivity tests. We have the impression that cation channel formation and ability to translocate A fragment of this mutant B fragment is reduced to the same extent as the binding, suggesting that only the ability of the mutant to bind to the toxin receptor is reduced.

It has been shown that an inwardly directed proton gradient is required for A fragment translocation (24) and for the translocation also possessed very little channel forming ability suggested that there is a coupling between cation channel formation and A fragment translocation. We reasoned that if this is the case, the mutant E362K, which is able to conduct cations at a higher pH value than the wild type B fragment, might also be able to mediate A fragment translocation at a higher pH value. We therefore exposed cells to identical amounts of toxin reconstituted from enzymatically active A fragment and either wild-type or E362K-mutated B fragment. After binding of the reconstituted toxins, the cells were exposed to different pH values and then incubated overnight in medium containing NH₄Cl to prevent translocation from endosomes. Finally, the ability of the cells to incorporate [³H]leucine was measured. The results showed that the curve for the mutant E362K was shifted ~0.2 pH units toward the right as compared to the wild-type toxin (Fig. 9). Clearly, this mutant is able to support A fragment translocation at a higher pH value than wild-type toxin.

**FIG. 8.** pH dependence of ²²Na⁺-uptake induced by wildtype and mutant B fragments. *In vitro* translated B fragment (5 × 10⁻¹¹ M) was bound to Vero cells for 20 min at 25 °C and then removed. MES-glucuronate buffer, of different pH values, containing 1 mM amiloride, 50 μM ouabain, and 1.6 μCi/ml ²²NaCl, was added and, after 8 min at 37 °C, the cells were rapidly washed four times in ice-cold PBS. The radioactivity associated with the cells was extracted with 5% trichloroacetic acid and measured. The results are expressed as percent of the maximal uptake after subtraction of the background level.

**FIG. 9.** pH profile of translocation of toxin reconstituted with wild-type B fragment and with the mutant E362K. Vero cells were incubated with toxin that had been reconstituted from A fragment and in vitro translated B fragment (2 × 10⁻¹¹ M) for 1 h at 4 °C. The cells were exposed to MES-glucuronate buffer of different pH values for 5 min at 37 °C, and subsequently incubated overnight at 37 °C in growth medium with 15 mM NH₄Cl, and then the ability of the cells to incorporate [³H]leucine was measured. The results are expressed as percent of the control level (no toxin, pH 4.8).

**FIG. 10.** Protective effect of NH₄Cl against toxin reconstituted with wild-type B fragment and with the mutant E362K. Vero cells were incubated overnight with reconstituted wild-type or mutant toxin (8 × 10⁻¹¹ M) and different concentrations of NH₄Cl. The next day the ability of the cells to incorporate [³H]leucine into trichloroacetic acid-insoluble material was measured.
formation of cation-selective channels (11), but not for membrane insertion of the B fragment (9). Likewise, an outwardly directed chloride gradient inhibits cation channel formation and the translocation of the A fragment, without affecting B fragment insertion (13). Similarly, we here find that B fragment mutants with reduced ability to conduct cations and mediate translocation of the A fragment do not show any reduction in the efficiency of plasma membrane insertion. In experiments where the pH profile of the membrane insertion of the B fragment was investigated (12, and present study), it was found that at pH 6.0, a substantial amount of 25-kDa fragment is inserted into the membrane. However, at this pH value, there is no cation channel formation, implying that the membrane insertion of the B fragment is not the limiting step in cation channel formation when the pH is increased.

From our previous data (16) we have indications that Asp^{205} and Asp^{1318} are located in transmembrane regions of the inserted B fragment and could therefore line the cation channel. It is therefore interesting that when each of these residues is altered to lysine the channel formation is essentially abolished.

The mutants D295K and D318K did not differ strongly from the wild-type B fragment in susceptibility to protease and it is thus unlikely that gross conformational changes are responsible for abolishment of the channel forming ability. The data therefore suggest that the residues Asp^{205} and Asp^{1318} are important for cation conductance when the B fragment is inserted into the plasma membrane. The two mutants D295K and D318K, which possessed none or very little cation forming ability, were also the least toxic ones among the six mutants that were able to bind to cells to the same extent as wild-type toxin. This suggests that the formation of cation-specific channels may be important for the translocation of the A fragment across the plasma membrane. It should be noted, however, that the reduction in toxicity of the mutants (∼10-fold), was less than that of channel formation by B fragment (1000-fold or more).

Additionally, in the case of the mutant E262K, the shift in the pH profile was larger in the cation channel formation experiments (0.3–0.4 pH units) than in the pulse-toxicity experiments (0.1–0.2 pH units). Together, this could mean that the cation channels are required for highly efficient translocation, but that translocation is not totally dependent on their formation. It cannot be excluded that the apparent coupling between cation conductance and toxicity is fortuitous and that the low pH-induced alterations in the B fragment required for translocation also induce cation channels without direct involvement of these in the translocation process. However, when the tight link between channel formation and translocation under a number of conditions is taken into account, this is considered less likely.

The present results demonstrate a close correlation between cation channel formation and ability to translocate fragment A at high efficiency. However, the functional link remains unknown. The possibility that the cation permeability is due to a channel formed to allow passage of the A fragment in a similar way as it has been proposed for translocation of proteins across the membrane of the endoplasmic reticulum (25) appears less likely, since the pore formed by diphtheria toxin B fragment is selective to monovalent cations (12), and a protein conducting pore would be expected to be too large to have such a selectivity. The present finding that mutants virtually devoid of channel forming ability are able to translocate A fragment at one-tenth of the wild-type efficiency argues against this interpretation. Possibly, ion fluxes occurring through a channel formed in close proximity to the A fragment may somehow facilitate the translocation process, thereby increasing its efficiency.

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