Membrane Translocation and Channel-Forming Activities of Diphtheria Toxin Are Blocked by Replacing Isoleucine 364 with Lysine

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A mutant of diphtheria toxin in which Ile-364 was replaced by Lys was at least 500-fold less toxic to Vero cells than the parental toxin. Its ability to undergo low-pH-triggered translocation across the plasma membrane was greatly diminished, as was its ability to form ion-conductive channels. In addition, the mutant toxin was inactive in the pH-dependent killing of Escherichia coli.

Diphtheria toxin (DT) consists of a single 58-kDa polypeptide chain, which can be proteolytically cleaved and reduced by thiols into two fragments, A and B (12). The A fragment (DTA; M_r, 21,150) inhibits protein synthesis in most mammalian cells by catalytically inactivating elongation factor 2 (12). The B fragment (DTB; M_r, 37,240) binds to specific cell surface receptors (12) and facilitates the translocation of DTA through the endosomal membrane into the cell cytoplasm (for a review of translocation, see reference 8). To probe the mechanism by which DTB promotes the pH-dependent translocation of fragment A across membranes, we have constructed and characterized a DT mutant, DT-I364K, in which Ile-364 was replaced by lysine. Ile-364 lies within a highly hydrophobic region of fragment B and was shown in the recently reported crystallographic structure of DT to reside within a long apolar α-helix (TH9) (5).

Mutant DT-I364K was constructed by directed mutagenesis of a pseudo-wild-type parental toxin (pwt-DT), in which Glu-148 had been replaced by Ser (10). For mutagenesis, the EcoRI-KpnI fragment of pTarB (2), encoding 90% of the DTB fragment sequence, was cloned into M13mp18 and mutagenized (4). The mutagenized sequence was then subcloned into the ClaI and SphI sites of pDO1, a plasmid encoding pwt-DT under control of the inducible tac promoter (10). DT-I364K was expressed in Escherichia coli JM103, and the bacterial periplasmic fraction was prepared by osmotic shock (10).

The cytotoxicity of the DT-I364K mutant for sensitive mammalian cells was then measured by assaying its ability to inhibit protein synthesis in Vero cells (10). The DT-I364K mutant showed no cytotoxic activity at concentrations of up to 5×10^{-9} M, conditions in which pwt-DT displayed complete inhibition of protein synthesis. We estimated its cytotoxicity to be at least 500-fold lower than that of pwt-DT (not shown). Control experiments showed that pwt-DT and DT-I364K are equally able to compete with ^{125}I-labeled wild-type toxin for binding to cell surface receptors (9); 50% inhibition of binding was observed with about 200 ng of unlabeled competitor protein per ml (data not shown). In addition, ADP-ribosyltransferase activity, a property of free
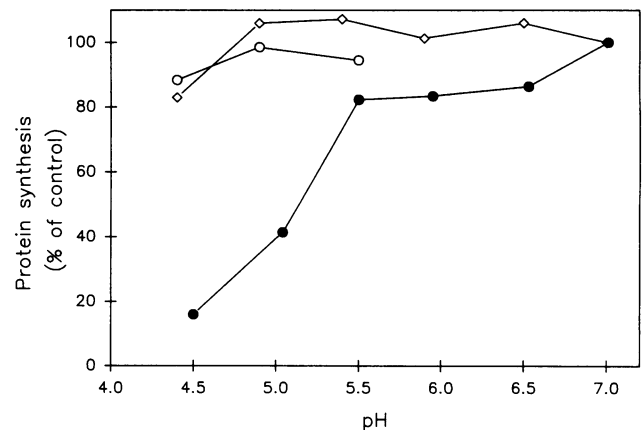

FIG. 1. pH-mediated entry of DT-related protein into Vero cells. Protein synthesis is expressed as a percentage of the radioactivity incorporated into cells when pulsed at neutral pH. ◇, Vero cells without toxin; ●, DT-E148S; ○, DT-I364K.
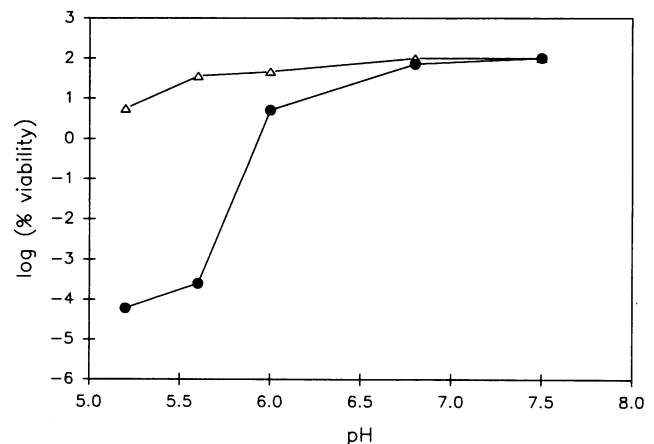

FIG. 2. Viability of E. coli cells harboring plasmids encoding DT-related proteins when plated at various pHs. ●, DT-E148S; △, DT-I364K.
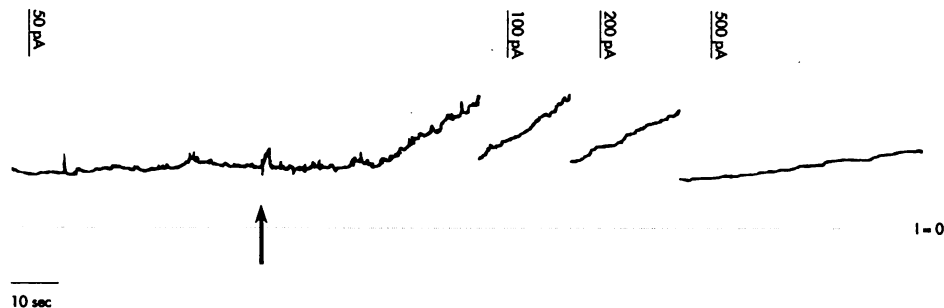
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FIG. 3. Comparison of channel-forming activities of DT-I364K and DT-E148S in a planar lipid bilayer. Seven minutes before the section of record shown here was recorded, a membrane was formed at symmetric pH 4.7; 50 ng of DT-I364K was added to the cis compartment, causing no change in membrane conductance over 1 min. Then the trans pH was raised to 7.4, causing a gradual increase in membrane conductance. Six minutes later, the current had reached about 55 pA, as seen at the beginning of the record shown here. At the arrow, 5 ng of DT-E148S was added to the cis compartment. Within 2 min, the membrane conductance increased more then 10-fold, as shown on the figure. Note that the vertical jumps on the record reflect the indicated changes of scale. The membrane voltage was held at +40 mV throughout the experiment.

DTA (3), was unaltered by the I364K mutation, as expected (data not shown).

We used the system of Sandvig and Olsnes (13) to study translocation of the mutant toxin across the plasma membrane. Cytotoxicity is induced in this protocol by a 15-min pulse at low pH, and protein synthesis was measured 20 h after the pH pulse (Fig. 1). As reported previously for wild-type DT (13), pHs below 5.5 induced strong inhibition of protein synthesis by pwt-DT (5×10^{-9} M). In contrast, negligible cytotoxic activity was observed with the same concentration of DT-I364K, even at pHs as low as 4.4.

E. coli was transformed with plasmids containing mutant or pwt-DT toxin genes, and the cells were grown to an optical density at 595 nm of 0.7 and spread on agar plates at various pHs (11). The following day, the colonies were counted, and their number was plotted as a function of the pH (Fig. 2). Below pH 6.0, the number of colonies expressing the pwt-DT was drastically reduced (11). As shown in Fig. 2, DT-I364K showed markedly impaired activity in this assay, even at pH 5.2. Western immunoblots showed that DT-I364K and the control toxin were expressed at similar levels, implying that the observed difference in activity was not due to a dosage effect (data not shown).

To explore the basis for the functional defect in translocation, we studied the ability of DT-I364K to form channels in planar asolectin bilayers (1, 6). The bathing solutions on both sides of the membrane contained 1 M NaCl, 2 mM $MgCl_2$, 1 mM EDTA, and 5 mM citrate, pH 4.7. All reported voltages refer to the cis (toxin-containing) compartment; the trans side is considered to have zero potential. The channel-forming activity of the I364K mutant was found to be greatly reduced relative to that of pwt-DT; 5 ng of pwt-DT induced a 10-fold greater conductance in 2 min than did 50 ng of DT-I364K in 7 min (Fig. 3). The conductance (50 pS) and the ion selectivity (cation selective at pH 5.5) of the DT-I364K channels were identical to those formed by pwt-DT (data not shown).

The current work originated from a proposal by Falmagne et al. (7) that the region between residues 346 and 371 formed an amphipathic α-helix that might interact with membranes and conceivably might form part of the ion-conductive channels formed after membrane insertion by DT, DTB, or other toxin fragments containing this region. To probe this hypothesis, we replaced a centrally located hydrophobic residue, Ile-364, with Lys and characterized the resulting mutant toxin. This mutation dramatically diminished its cytotoxicity while causing no change in receptor-binding activity or ADP-ribosylation activity. Direct evidence that the mutation blocked translocation came from experiments involving pH-dependent translocation across the plasma membrane. In this experiment, even a pH as low as 4.4 failed to induce detectable cytotoxicity.

Supportive data for the notion that the I364K mutation affects translocation came from experiments showing that DT-I364K is impaired in the ability to form ion-conductive channels in artificial lipid bilayers. In addition, DT-I364K within the periplasm of E. coli was deficient in pH-dependent killing of these bacteria. This is consistent with the hypothesis that the lethal activity of the toxin for these bacteria depends on its ability to insert into and permeabilize the bacterial inner membrane.

The location of residue 364 within the recently published crystallographic structure of DT (5) is consistent with the hypothesis that the mutation impedes membrane insertion. Ile-364 is centrally located within a long hydrophobic α-helix (TH9), which is part of a helical hairpin, including helices TH8 and TH9 and the short connecting loop (TL5). The hydrophobic nature of this hairpin has led to the proposal that the hairpin forms a pH-dependent membrane insertion motif. The properties of two other DT mutants support this notion. Thus, mutation of Glu-349 (10) or Asp-352 (14), two acidic residues within TL5, to Lys has been found to inhibit membrane translocation and channel formation. The role of the channel in the cytotoxicity of DT remains unexplained. In both the DT-I364K mutant and the DT-E349K mutant (unpublished data), there is a correlation between impairment of channel formation and loss of translocation activity. However, channel formation may merely be an indicator of insertion of DT in a transmembrane mode, the latter being necessary for translocation. Further work will be necessary to determine whether the channel per se serves as a direct conduit for transfer of DTA across lipid bilayers.

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