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Single mutation in the A domain of diphtheria toxin results in a protein with altered membrane insertion behavior

Valerie W. Hu [a] and Randall K. Holmes [b]

[a] *Department of Biochemistry and* [b] *Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814-4799 (U.S.A.)*

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The insertion of the A domain of diphtheria toxin into model membranes has been shown to be both pH- and temperature-dependent (Hu and Holmes (1984) J. Biol. Chem. 259, 12226–12233). In this report, the insertion behavior of two mutant proteins of diphtheria toxin, CRM197 and CRM9, was studied and compared to that of wild-type toxin. Results indicated that both CRM197 and CRM9 resembled toxin with respect to the pH-dependence of binding to negatively-charged liposomes at room temperature. However, CRM197 differed from toxin with respect to both the pH- and temperature-dependence of fragment A insertion; fragment A_{197} inserts more readily into the bilayer at 0 °C and low pH or at neutral pH and room temperature than does wild type fragment A under these same conditions. This result indicates that the single amino acid substitution in the A domain of CRM197 facilitates entry of fragment A_{197} into the membrane, suggesting that CRM197 may be conformationally distinct from native toxin. In fact, the fluorescence spectra of CRM197 and wild-type toxin as well as their respective tryptic peptide patterns indicate that, at pH 7, CRM197 more closely resembles the acid form of wild-type toxin than the native form of toxin. These data suggest that CRM197 may be naturally in a more 'insertion-competent' conformation. In contrast, the mutation in the B domain of CRM9 which results in a 1000-fold decrease in binding affinity for plasma membrane receptors apparently does not cause a change in either the insertion of fragment A_9 or the lipid-binding properties of CRM9 relative to toxin.

Introduction

Diphtheria toxin (M_r 58390) can be readily cleaved into two fragments, A (M_r 21145) and B (M_r 37240) by trypsin or endogenous proteases [1–4]. Intoxication of cells by diphtheria toxin involves a multistep process which is initiated by fragment B-mediated binding of toxin to specific cellular receptors, endocytosis of bound toxin, acidification of the endosomal vesicles, followed by delivery of fragment A to the cytosol where it inhibits protein synthesis by catalyzing the NAD-linked ADP ribosylation of elongation factor 2 [5–11]. The mechanism by which fragment A crosses the endosomal membrane is still unclear. Based on the observations that whole toxin and, in particular, fragment B of toxin can form ion-conducting channels in black lipid membranes upon

Abbreviations: 12-APS-GlcN, 12-(4-azido-2-nitrophenoxy)stearoyl[1-^{14}C]glucosamine; LUVs, large unilamellar vesicles; SDS, sodium dodecyl sulfate.

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Correspondence: V.W. Hu, Department of Biochemistry, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4799, U.S.A.

exposure to low pH [12–16] and that toxin channels may in fact be as large as 18–22 Å in diameter [12,17], it has been proposed that fragment A crosses the membrane through fragment B channels [12,18]. Alternatively, it has been suggested that fragment A passes directly through the plasma (or endosomal) membrane by an undefined mechanism [19,20].

We and others have recently reported that both fragments A and B are labelled by membrane-embedded, radioactive, photoactivatible lipid probes, implying that fragment A (as well as B) is in direct contact with the lipid phase of the membrane and is not totally sequestered within a hydrophilic fragment B pore [17,21,22]. Furthermore, we observed that the insertion of fragment A into the membrane was both pH- and temperature-sensitive [21]. In this report, we present data on two mutants of diphtheria toxin: CRM197, which has a single amino acid substitution in the A domain [23], and CRM9, which has a B domain mutation affecting toxin binding affinity to specific cellular receptors [24]. The most striking finding of this study is that, unlike the A domain of native toxin, insertion of the A domain of CRM197 is not inhibited by high pH or low temperature. This finding is discussed in light of an earlier report [25] that CRM197 exhibits a higher affinity for toxin receptors than does wild-type toxin.

Materials and Methods

Chemicals. Dimyristoylphosphatidylcholine was obtained from Calbiochem, cholesterol from Applied Science Laboratories, and dicetyl phosphate from Sigma. The photoprobe 12-APS-GlcN was synthesized by published procedures [26]. Its specific activity was 278 Ci/mol.

Toxins. Completely nicked diphtheria toxin was obtained from Calbiochem. The purification of partially-nicked toxin (Dc202) and the mutant CRM197 (fully-nicked form) has been previously described [27]. Isolation of the mutant CRM9 which has a 1000-fold lower binding affinity for endogenous receptors than wild-type toxin has been reported [24]. CRM9 was cleaved by trypsin (15 min, 37 °C) before use in experiments. There was no significant difference in the amount of endogenous nucleotides associated with these protein samples since the A_{260}/A_{280} ratios obtained for toxin, CRM197, and CRM9 were 0.716, 0.795, and 0.737, respectively. As reported previously, nucleotide-free toxin has an A_{260}/A_{280} value of 0.6 whereas nucleotide-bound toxin has an A_{260}/A_{280} ratio of 0.9 [28,29].

Vesicle preparation. Dimyristoylphosphatidylcholine, cholesterol, and dicetyl phosphate were dissolved in chloroform/methanol in a lipid molar ratio of 4 : 3 : 0.8, respectively, and the solvent was removed under vacuum. Large unilamellar vesicles (LUVs) were then prepared by the reverse phase ether evaporation method [30]. Vesicles were stored under a N_2 atmosphere and in a refrigerator until used.

Photolabeling studies. 2 μl of 12-APS-GlcN (200000 cpm) in ethanol were added to 200–250 μg of vesicle lipid in approx. 200 μl and the samples were incubated for 15 min at 37 °C under red light. An appropriate amount of acid was added to lower the pH to 4.5 or 3.6 for certain experiments and 20–22 μg of toxin or mutant protein was added. Samples were incubated either on ice or at room temperature (about 23 °C) for 15–30 min before a 5 sec irradiation with a high intensity ultraviolet light lamp operating at 366 nm. After irradiation, acidic samples were immediately neutralized to minimize further binding of toxin to vesicles. The vesicles were collected by centrifugation in a SW 50.1 rotor operating at 40000 rpm for 15 min. The membrane pellets were solubilized in reducing SDS-sample buffer [31] and run either on 10% or on 10–15% gradient SDS-polyacrylamide gels.

Gel electrophoresis and electroblotting methods. Samples were electrophoretically separated on 10% or on 10–15% gradient SDS-polyacrylamide slab gels with a 2.5% acrylamide stacking gel, using a 0.1% SDS/Tris/glycine running buffer [31]. Following electrophoresis, the protein bands were transferred from the gel to nitrocellulose paper using a Hoefer electroblot apparatus operating at 1 A for 2 h and 0.3 A overnight using transfer buffer previously described [32]. The blotting procedure was used to overcome gel cracking occasionally observed when drying high-percentage polyacrylamide gels. After blotting, the gels were routinely treated with Coomassie blue to verify complete transfer of proteins under these transfer

conditions. The blots were stained for protein with Amido Black, dried and then placed on Kodak X-O-Mat AR X-ray film for detection of radiolabeled protein. To determine the amount of radioactivity in a given protein band, the appropriate band was cut from the blot and counted in Beckman Ready-Solve scintillation fluid in a Packard Tri-Carb liquid scintillation spectrometer.

Generation of tryptic fragments of acid-pulsed and untreated toxin and CRM197. 1 μl of 0.2 M citric acid was added to 10–20 μg of CRM197 or native toxin in 30 μl of Tris buffer at pH 7.2. The final pH was approx. 4.0. After a 10 min incubation at room temperature, the pH was adjusted to approx. 7.0 with 0.2 M NaOH. These acid-pulsed protein samples and untreated (pH 7.2) controls were cleaved with trypsin (10 min, room temperature) using a toxin/trypsin molar ratio of approx. 100 : 1. The enzymatic reaction was stopped by the addition of 50 μl of reducing sample buffer [31] followed by boiling for 2 min. The boiled samples were run on a 10–15% gradient SDS-polyacrylamide gel.

Fluorescence spectroscopy. Protein fluorescence was measured using an SLM 8000 spectrofluorometer. Emission spectra (from 300 to 400 nm) were obtained at pH 7.4 in 0.01 M Tris/2 mM NaCl, and at pH 4.0 after acidification of the protein sample with 0.1 M HCl. Emission slits of 1 nm nominal bandpass were used. Excitation was at 280 nm with excitation slits set at 8 nm. The step size was 0.5 nm. Emission maxima were determined from corrected spectra. No differences in excitation maxima were observed.

Results

Insertion of fragment A_{197} into membranes is independent of pH and temperature

Toxin, CRM197 and CRM9 were added to probe-containing vesicles at different pH values and at different temperatures. After a 15–30 min incubation period, the samples were irradiated and the membrane pellets collected. Fig. 1 shows the Western blots and corresponding autoradiograms of these membrane samples after SDS-PAGE. Inserted peptide domains are identified as radioactive bands on the autoradiogram. The amido black-stained blots show that binding of toxin and both mutant proteins to vesicles is greatest at low pH and room temperature (lanes 1, 1′). Although binding of all proteins is substantially decreased at neutral pH (lanes 3, 3′), it is only slightly reduced at low temperature (lanes 2, 2′). These data indicate that the lipid binding characteristics of both mutants are not significantly different from that of wild-type toxin. However, the autoradiograms show that CRM197 differs substantially from wild-type toxin with respect to the dependence of fragment A insertion on temperature and pH. While the insertion of fragment A (but not fragment B) of wild-type toxin is inhibited at 0 °C (compare lanes 1 and 2 or 1′ and 2′ of the autoradiograms for Dc202 or DT, respectively), the insertion of fragment A of CRM197 is only slightly reduced at low temperature or at neutral pH relative to the amount of CRM197 bound. The stained blots show that the lack of radiolabel in fragment A of wild-type toxin at 0 °C is not due to the lack of transfer of fragment A to the blot. The insertion of fragment A of CRM9, like that of wild-type toxin, is strongly inhibited by low temperature. At neutral pH, too little CRM9 is bound to assess insertion accurately. The differences in fragment A insertion behavior shown in Fig. 1A are quantitatively expressed in Table I as the ratio of radioactive counts in fragment A to the counts in fragment B, which is labeled under all conditions tested. In contrast to the temperature- and pH-dependence of fragment A insertion, fragment B insertion is dependent primarily on the amount of toxin bound to the vesicles.

The fluorescence emission maximum of CRM197 is red-shifted with respect to that of wild-type toxin

Table II shows that, at pH 7.4, the fluorescence emission maximum of CRM197 is 4 nm higher than that of wild-type toxin. This red-shift in the emission maximum of CRM197 relative to wild-type toxin is consistent with greater exposure of buried tryptophan residues in the mutant protein. Interestingly, the magnitude and direction of the shift are similar to that observed upon exposure of wild-type toxin to low pH (Table II and Ref. 28). Upon acidification of CRM197, a small but additional red-shift is observed, suggesting a movement towards an even more open conformation.

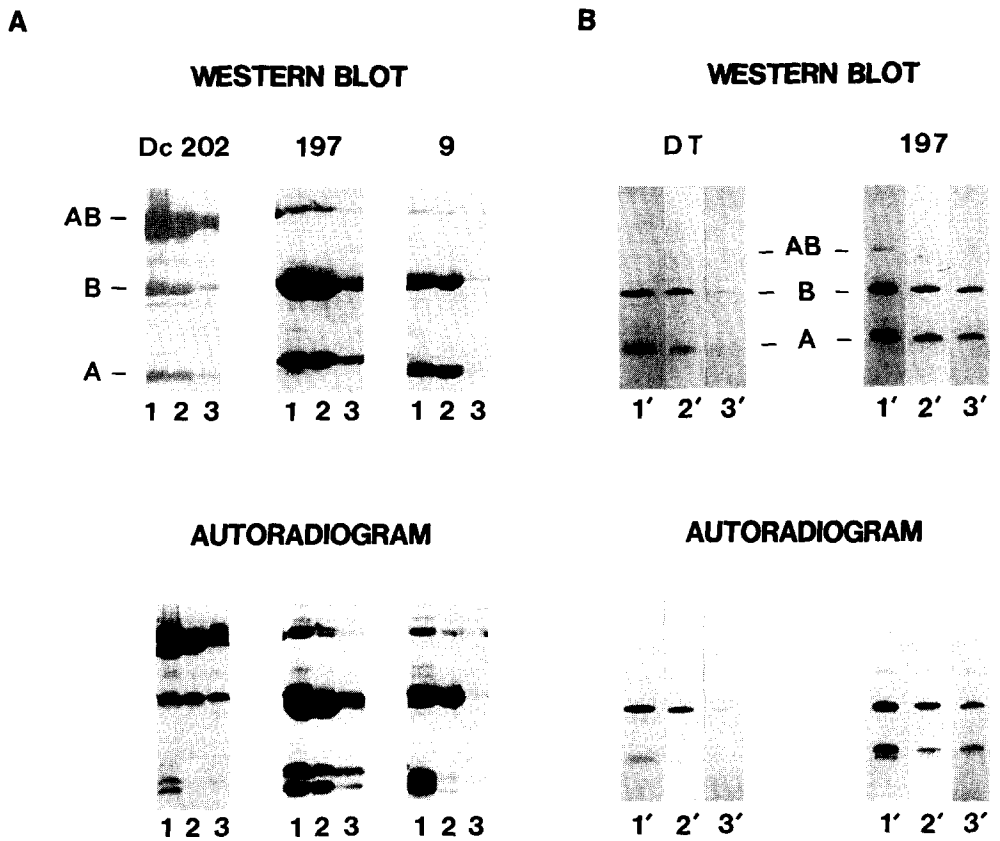

Fig. 1. The effect of pH and temperature on the binding and insertion of toxin, CRM197, and CRM9. (A) 22 μg of partially-nicked toxin (Dc202), CRM197 or CRM9 were incubated with vesicles containing 12-APS-GlcN (200000 cpm) in 0.15 M NaCl (250 μg of total lipid) for 30 min under the following conditions: (1) pH 3.6, room temperature; (2) pH 3.6 0 °C; (3) pH 6.7, room temperature. The pH was adjusted to 3.6 by the addition of isotonic barbital/acetate buffer, at pH 3.6. Samples were run on 10% SDS-polyacrylamide gels. The amido black-stained Western Blots of the gel samples (top) and the corresponding autoradiograms (bottom) are shown. On these gels, multiple fragment A bands are clearly resolved on the autoradiogram but not on the blot. These multiple bands may arise either from trypsin cleavage at several closely-spaced susceptible arginine sites near the COOH-terminus of fragment A or from cleavage at the arginine or lysine residues at positions 170, 172, or 173 in addition to cleavage at the C-terminus of fragment A. Though both radiolabeled bands fall within the diffusely-stained unresolved band on the blot, it does appear that the smaller of the bands labels with higher efficiency. (B) 20 μg of fully nicked toxin (DT) or CRM197 were incubated with vesicles (200 μg total lipid and 200000 cpm of 12-APS-GlcN) for 15 min under the following conditions; (1′) pH 4.5, room temperature; (2′) pH 4.5, 0 °C; (3′) pH 7.2, room temperature. Vesicles were prepared in 0.1 M Tris/20 mM NaCl (pH 7.2) and the pH was adjusted to 4.5 by the addition of an appropriate amount of 0.2 M citric acid. Samples were run on 10–15% gradient SDS-polyacrylamide gels. Amido black-stained blots are shown above and the corresponding autoradiograms below.

By comparison, the emission maximum of CRM9 is identical to that observed for wild-type toxin at pH 7.4, while the acid-induced shift is not quite as large. These data suggest that the conformation of CRM197 may be similar to that of acidified toxin. In support of this interpretation, the CD spectrum of CRM197 at pH 7.4 more closely resembled that of acidified toxin than that of toxin at neutral pH (data not shown). These results are in agreement with the published CD spectra of CRM197 [25] and acidified toxin [21,28] which is distinct from that of toxin at neutral pH [21,28].

TABLE I

RELATIVE INSERTION OF FRAGMENTS A AND B AS A FUNCTION OF pH AND TEMPERATURE

To determine the amount of radioactivity in fragments A and B of the respective proteins, the appropriate bands on the amido black-stained blot were cut, immersed in scintillation fluid and counted in a scintillation counter.

Protein	pH	Temp.	Corrected cpm [a]		Count ratio A/B
			fragment A	fragment B	
Wild-type	3.6	RT [b]	94 [c]	120	0.78
Wild-type	3.6	0 °C	−2	80	~ 0
Wild-type	6.7	RT	−9	47	~ 0
CRM197	3.6	RT	1370	1392	0.98
CRM197	3.6	0 °C	676	863	0.78
CRM197	6.7	RT	223	316	0.71
CRM9	3.6	RT	990	1110	0.89
CRM9	3.6	0 °C	137	671	0.20
CRM9	6.7	RT	– [d]		

[a] Counts – background (47 cpm).
[b] RT, room temperature.
[c] Counts for wild-type fragments are low because much of the toxin is still unnicked in these samples in comparison to the mutant samples in which the proteins are almost totally nicked.
[d] Binding is too low to obtain accurate numbers above background.

Tryptic peptide analysis of CRM197 and wild-type toxin also reveals structural differences

Because the fluorescence emission spectra of CRM197 at neutral pH resembled that of toxin at acidic pH, we compared the tryptic peptides of CRM197 with that of toxin at both neutral and acidic pH. As shown in Fig. 2, CRM197 is more sensitive than toxin to trypsin at pH 7.2 and all of the CRM197 fragments at pH 7.4 correspond to fragments of acid-pulsed CRM197. Furthermore, while there is good correlation between the fragments of acid-pulsed wild-type toxin and those of CRM197, some differences exist, as noted by the arrows. There is no degradation of fragments A and B of wild-type toxin or CRM197 at either pH in the absence of trypsin (Fig. 1B and additional data not shown). Since CRM197 differs from toxin in only one amino acid [22], these results indicate greater exposure of trypsin-sensitive sites in CRM197 at neutral pH, and lend additional support for the idea that CRM197 bears conformational similarities to acid-pulsed wild-type toxin.

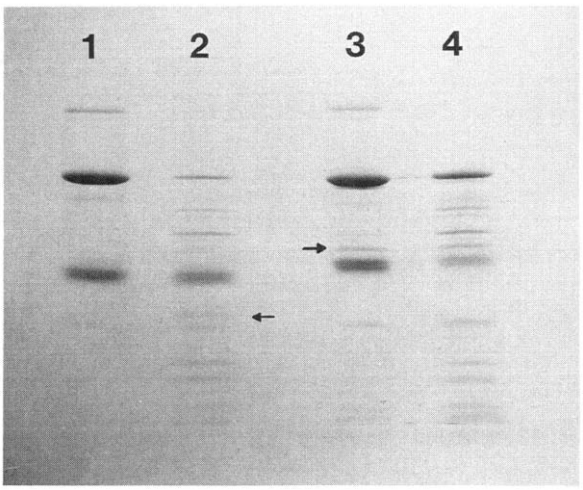

Fig. 2. Tryptic peptides of acid-pulsed and untreated toxin and CRM197. Toxin and CRM197 were treated with trypsin for 10 min at room temperature in 0.1 M Tris/20 mM NaCl (pH 7.2). Acid-pulsed forms were generated by preincubation of toxin or CRM197 at pH 4.0 for 10 min at room temperature. These samples were neutralized before addition of trypsin. The Coomassie blue-stained gel lanes contain: (1) toxin, (2) acid-pulsed toxin, (3) CRM197, (4) acid-pulsed CRM197. Arrows point to distinct peptides generated from toxin and CRM197.

TABLE II

FLUORESCENCE EMISSION MAXIMA, λ_{max}, OF WILD-TYPE TOXIN, CRM197, AND CRM9 AT pH 7.4 AND pH 4.0

Fluorescence emission spectra (from 300 to 400 nm) of nicked toxin and mutant proteins CRM197 and CRM9 were obtained at pH 7.4 in 0.01 M Tris/2 mM NaCl and at pH 4.0 after acidification of the sample with 0.1 M HCl. Excitation was at 280 nm. The emission maxima are reported below.

Protein	λ_{max} at pH	
	7.4	4.0
Wild-type toxin	325 [a]	329
CRM197	329	331.5
CRM9	325	327.5

[a] The resolution was ± 1 nm.

Discussion

We have studied the membrane insertion requirements of two mutants of diphtheria toxin and have compared them with the requirements

for insertion of the two domains of wild-type toxin. CRM9, a protein that has a 1000-fold lower affinity than wild-type toxin for plasma membrane receptors [24] due to a mutation(s) that is/are believed to affect the receptor-binding region of the B domain (Phipps, T.J., Russell, L.M. and Holmes, R.K., unpublished results), closely resembles toxin both with respect to lipid binding and insertion of fragment A.

In contrast to CRM9, CRM197 differs from toxin with respect to insertion of fragment A, but not fragment B. Unlike the insertion of fragment A of toxin, insertion of A_{197} is not strongly pH- or temperature-dependent. The anomalous behavior of A_{197} is not related to the amount of endogenous nucleotide bound to CRM197 since there was no significant difference in the amount of bound nucleotides among the three proteins studied. Furthermore, additional studies show that the relative insertion of fragment A vs. fragment B does not differ for nucleotide-bound and nucleotide-free toxin (Hu, V.W. and Killeen, M.P., unpublished results). Thus, these results suggested that the conformation of CRM197 might be different from that of native toxin. This conformational variance is supported by fluorescence data as well as by peptide analysis of CRM197 and wild-type toxin at pH 7.2 and 4.0. In particular, the fluorescence emission maximum of CRM197 at neutral pH is similar to that of acidified toxin, suggesting an inherent conformational difference relative to wild-type toxin at neutral pH. A generally more open conformation for CRM197 is moreover indicated by its relatively higher sensitivity to trypsin at pH 7.2. We therefore postulate that the natural conformation of CRM197 resembles the conformation of acidified wild-type toxin, which facilitates fragment A_{197} insertion into membranes. In view of the fact that the mutation in CRM197 involves a Gly to Glu substitution at position 52 within the NAD-binding site on fragment A [23,33] which is proximal to the P-site on fragment B [34–38], it is possible that charge interaction between the Glu 52 and the cationic P-site might permit the concomitant insertion of fragment A_{197} with fragment B_{197} under conditions which normally exclude wild-type fragment A from the membrane.

In contrast to the results for insertion, the binding of both CRM197 and toxin to lipid vesicles is pH-dependent. These latter observations suggest that separate pH-dependent processes control binding of the toxin molecule with lipid membranes and fragment A insertion. Since fragment B of CRM197 is identical in primary structure to fragment B of wild-type toxin [23,33], it may be reasonable to assume that the pH-dependent binding is attributable to the same pH-dependent conformational change in the B domain of both molecules.

It has previously been reported that nicked CRM197 exhibits a much higher affinity for cellular toxin receptors than wild-type toxin [25] and, consequently, it has been suggested that fragment A of CRM197 plays a direct role in receptor binding. Our data show that the single amino acid substitution in CRM197 also alters the insertion behavior of the A domain of CRM197 relative to that of toxin. This altered behavior appears to be associated with a conformationally-distinct form of the molecule which may facilitate fragment A entry into membranes. Thus, although the direct involvement of fragment A_{197} in receptor binding cannot be ruled out, we suggest that the higher receptor affinity of CRM197 could alternatively be the result of the anchoring of A_{197} in the lipid bilayer upon binding of the B domain to cellular receptors at pH 7 or of an altered toxin conformation which might directly affect toxin binding affinity without actually involving direct participation of fragment A in the binding process.

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