Volume 215, number 1, 73–78 FEB 04584 May 1987

Diphtheria toxin and its mutant *crm* 197 differ in their interaction with lipids

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Received 19 January 1987; revised version received 9 February 1987

The interaction of diphtheria toxin and its enzymatically deficient mutants *crm* 176 and *crm* 197 with liposomes has been studied by turbidity measurement and hydrophobic photolabelling with photoactivatable phosphatidylcholines. Diphtheria toxin and *crm* 176 at neutral pH bind to the surface of lipid bilayers while *crm* 197 also appears to interact with the fatty acid chains of phospholipids. All proteins undergo a change in conformation over the same range of acidic pH and become able to insert in the lipid bilayer. The tighter lipid interaction of *crm* 197 may account for its higher cell association constant. The possibility is discussed that the binding of diphtheria toxin to cells is mediated by both a protein receptor and an interaction with the head group of phospholipids.

Diphtheria toxin; Toxin mutant; Lipid interaction

1. INTRODUCTION

Diphtheria toxin (DT) is the most studied member of a group of protein toxins that kill cells by blocking their protein synthesis [1].

The protein is secreted as a single inactive polypeptide chain of 58.342 kDa by pathogenic strains of *Corynebacterium diphtheriae* [2,3]. DT is activated by trypsin which cleaves the protein into two fragments: A, an ADP-ribosylase (21.164 kDa), and B (37.194 kDa) that is considered to be responsible for binding of the toxin to sensitive cells [4,5].

Cell intoxication involves three main steps: binding, crossing of the membrane and target modification. Only the third point has been clarified at the molecular level: it is now known that protein synthesis is blocked by specific ADP-ribosylation of elongation factor 2 that causes its detachment from ribosomes [6]. Despite numerous studies little is known about DT binding and translocation; an as yet unidentified receptor protein is involved [7] and the toxin-receptor complex is thought to enter a coated pit and then an endosome [8,9]. There is strong evidence that the low pH of the vesicle lumen is sufficient to allow the toxin to translocate its enzymatic activity to the cytoplasm [10].

Several mutants of diphtheria toxin that cross-react immunologically with it (and hence are called *crm*) but are only partially toxic or completely non-toxic have been isolated. Among these mutants, *crm* 176 and *crm* 197 have been recently sequenced [11,12]. As shown in fig.1, they have a single amino acid replacement in the enzymic A chain. While *crm* 197 is totally inactive, *crm* 176 preserves part of its ADP-ribosylase activity. *crm* 197 shows very unusual cell association properties with a

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

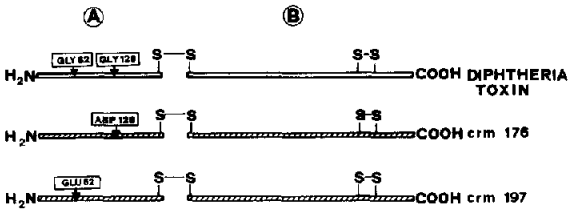

Fig.1. Schematic structure of diphtheria toxin showing its two-chain structure, position of disulfide bridges and the single amino acid substitutions present in the A chain of mutants *crm* 176 and *crm* 197.

much higher binding constant to cells than the native DT [13]. It has also been proposed that this mutant should be used for a safer and simpler new vaccine against diphtheria [14].

Here we compare the interaction of DT, *crm* 176 and *crm* 197 with liposomes by two sensitive techniques for monitoring lipid-protein interactions [15,16]. The present findings provide an explanation for the membrane binding property of *crm* 197 and suggest that, in addition to a protein, lipids are also involved in the association of diphtheria toxin to cells.

2. EXPERIMENTAL

2.1. *Materials*

DT, *crm* 176 and *crm* 197 were prepared from culture filtrates of the appropriate *C. diphtheriae* strain as reported by Rappuoli et al. [17,18]. The toxins were nicked by trypsin treatment [19]. Proteins were dissolved in 10 mM sodium phosphate, 125 mM NaCl (pH 7.2) at concentrations between 1.4 and 9.6 mg/ml and stored at −80°C.

1-Palmitoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycero-3-[^{3}H]phosphocholine (PC I, spec. act. 2.6 Ci/mmol) and 1-myristoyl-2-[12-amino(4-*N*-3-nitro-1-azidophenyl)]dodecanoyl-*sn*-glycero-3-[^{14}C]phosphocholine (PC II, spec. act. 174 Ci/mol) were prepared as described [20]. Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidic acid (DPPA) and soybean asolectin were from Sigma. DPPC was purified by acetone treatment and asolectin as described earlier [21].

2.2. *Methods*

Small unilamellar vesicles (SUV) of DPPC-DPPA (9:1, w/w) or asolectin were prepared by pulsed ultrasonic dispersion of lipid films in 125 NaCl, 10 mM sodium phosphate, 10 mM sodium citrate, 1.5 mM EDTA, pH 7.4 (citrate-phosphate buffer) with a Branson B 30 sonifier under a nitrogen atmosphere for a total sonication time of 15 min. Undispersed lipids and particles were removed by 15 min centrifugation at 40000 × *g*. The lipid vesicles were used after overnight incubation at 45°C for DPPC-DPPA and at 18°C for asolectin.

Liposome turbidity was measured as the change in absorbance at 334 nm in an Eppendorf photometer equipped with a water thermostatted and stirred cuvette.

Hydrophobic photolabelling and radioactivity determinations were performed as in [21] except for the use of a citrate-phosphate buffer instead of Tris-acetate. Electrophoresis was performed according to Laemmli [22] in 15% polyacrylamide slab gels.

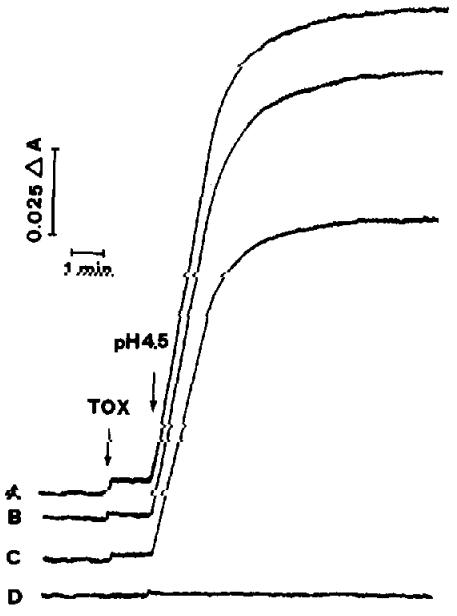

Fig.2. Effect of *crm* 197 (A), DT (B) and *crm* 176 (C) or nothing (D) on the turbidity of SUV of DPPC-DPPA. The toxin (20 μg) was added to 2 ml of a stirred lipid suspension (200 μM) in 125 mM NaCl, 10 mM Na-citrate, 10 mM Na_nPi, 1.5 mM EDTA, pH 7.4, thermostatted at 45°C. pH was lowered to 4.5 by addition of an appropriate amount of 8.5% phosphoric acid. Turbidity was determined as the absorbance change at 334 nm.

3. RESULTS

The change in turbidity of phospholipid vesicles induced by a protein is a very sensitive parameter for monitoring the occurrence of lipid-protein interactions as has been shown for colicin A and diphtheria toxin [15,23]. Here we have used this assay to compare the effects of DT, *crm* 176 and *crm* 197 on liposomes. In most experiments SUV of DPPC-DPPA were used because they give very reproducible results, but similar findings were obtained with other liposomes containing negative lipids, for instance asolectin.

Fig.2 shows that *crm* 197, more than DT and *crm* 176, induces a very small but significant change in turbidity implying that it causes a small change in dispersion state of the lipid vesicles.

Different experimental approaches have indicated that at low pH DT becomes hydrophobic and inserts into the lipid bilayer [21,24–27]. As shown in fig.2, at pH 4.5 all toxins induce a very large change in turbidity that may be related to liposome aggregation and/or fusion. That both phenomena occur is evident from electron micrographs of negatively stained preparations of liposomes incubated with the toxins at pH 5 (not shown). These results are closely related to those obtained by Cabiaux et al. [23]; however, we found that on SUV containing a negatively charged lipid such as DPPA, DT shows an effect several fold higher than that on liposomes of pure phosphatidylcholine.

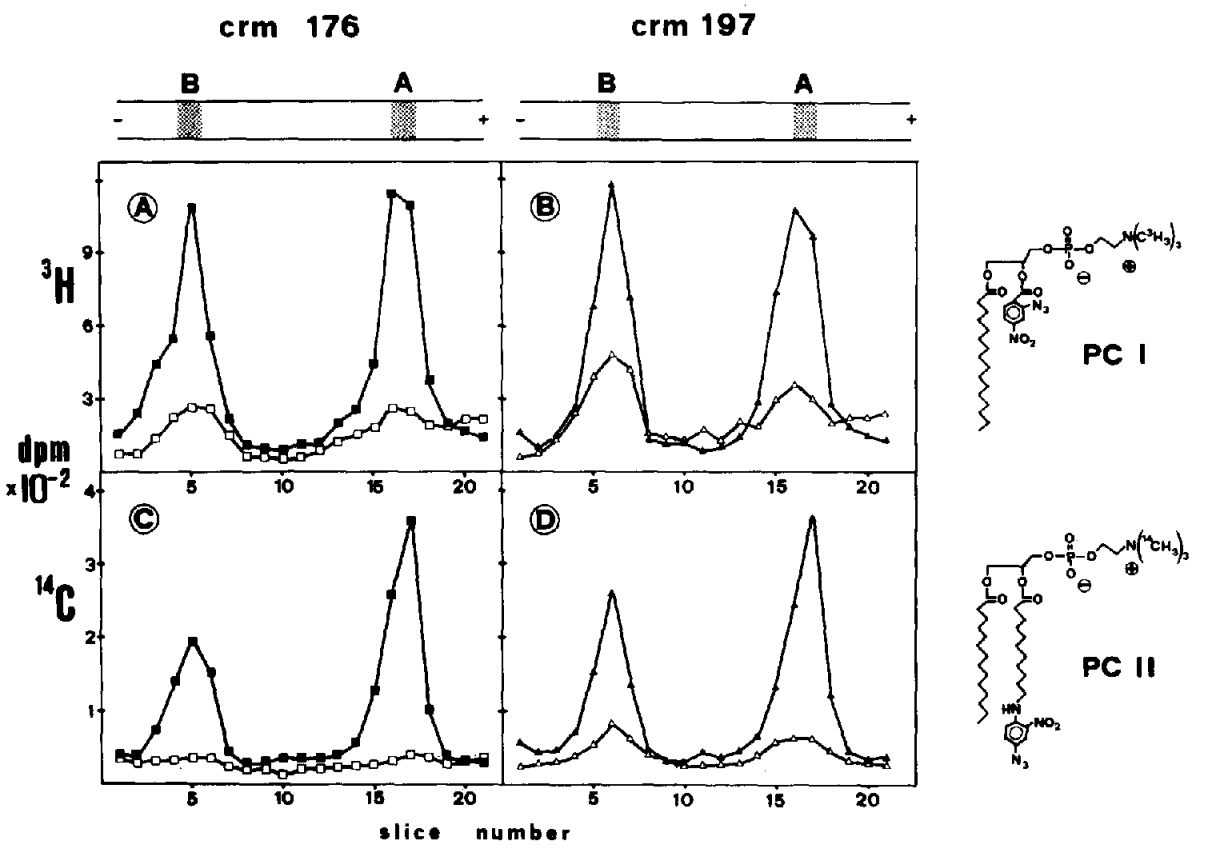

Fig.3. Hydrophobic photolabelling of *crm* 176 (A,C) and *crm* 197 (B,D) with purified soybean liposomes tagged with both PC I (A,B) and PC II (C,D) at neutral pH (open symbols) and at pH 4.5 (closed symbols). The top panel shows the profile of the Coomassie blue-stained gel of the toxins before slicing and counting; note that the A fragments of *crm* 197 have a lower electrophoretic mobility in SDS than the corresponding polypeptide of *crm* 176 (and of DT). On the right-hand side the structural formulae of PC I and PC II are shown with respect to their corresponding patterns of labelling. The lipid front is not shown here because slicing was terminated shortly after the protein bands.

The extent of the turbidity change of SUV of DPPC-DPPA induced by DT, *crm* 176 and *crm* 197 in all cases reaches a plateau in the range 4.5–5.0; the pH values of half-maximal effect are reported in table 1.

To gain more information on the interaction of the toxins with the lipid bilayer hydrophobic photolabelling experiments were performed with two photoactive phosphatidylcholines, whose formulae are shown on the right-hand side in fig.3. They probe membrane regions at different depths [28]. Once activated by illumination, PC I reacts with protein segments intercalated with the polar head groups of phospholipids while photoactivated PC II reacts with protein residues embedded in the hydrophobic core of the membrane.

Fig.3 shows the distribution of radioactivity associated with the two fragments of *crm* 176 and *crm* 197 after incubation with asolectin vesicles at neutral and low pH. The profile of labelling of DT (not shown) is almost superimposable on that of *crm* 176. While DT and *crm* 176 at neutral pH are labelled only with the surface probe PC I, *crm* 197 reacts with both phospholipid reagents.

These findings indicate that DT and *crm* 176 interact at neutral pH with a negatively charged lipid bilayer and that this interaction is confined to the polar head group region. In addition to a surface interaction, *crm* 197 appears to expose part of its surface to the fatty acid chains of phospholipids and both fragments of *crm* 197 are involved in this interaction. However, we do not know whether the PC II labelling of *crm* 197 at neutral pH corresponds to its actual penetration in the hydrophobic core of the lipid bilayer or if it perturbs the bilayer architecture in such a way as to bring the fatty acid methyl terminus closer to the bilayer surface. Whatever the reason for the different *crm* 197 labelling, this finding indicates that an alteration in the A chain can be transmitted to the B chain in such a way as to influence the membrane interaction of this mutant toxin.

Table 1

pH values at which 50% of the maximal effects of DT and mutants on the change in turbidity of DPPC-DPPA SUV and on the hydrophobic photolabelling with photoreactive phosphatidylcholines are found

Fragments	Turbidity (A–B)	Hydrophobic photolabelling			
		PC I		PC II	
		A	B	A	B
DT	5.8	5.3	5.8	5.3	5.8
crm 176	5.8	5.2	5.7	5.2	5.7
crm 197	5.9	5.4	5.7	5.4	5.8

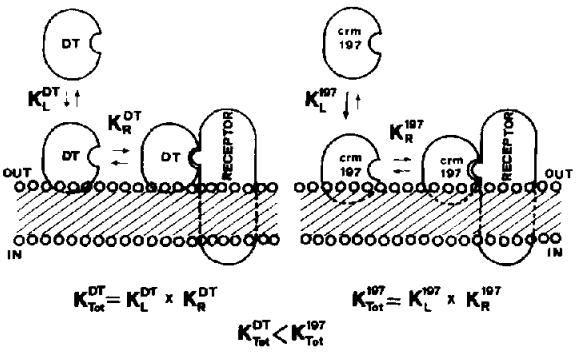

Fig.4. A schematic drawing illustrating the different lipid interaction of DT and *crm* 197 which may explain the different strength of interaction shown by these two proteins with the plasma membrane. The toxin is shown to bind to lipids first and then to move laterally with lipids to encounter a protein receptor to take into account different amount of cell surface occupied by lipids and by the protein receptor.

It has been previously shown that at low pH DT becomes hydrophobic and penetrates the lipid bilayer in a process involving both fragments [21,25,26]. Also, *crm* 176 and *crm* 197 are able to undergo this low pH-induced conformational change and to enter into contact with the hydrocarbon chains of phospholipids as shown in fig.3. Table 1 reports the pH of the half-maximal effect for both the turbidity and hydrophobic photolabelling experiments. These values fall within the physiological pH range since they have been actually shown to be reached in endosomes in vivo [29].

The close similarity of the patterns obtained with the surface (PC I) and deeper (PC II) probes strongly suggests that DT, *crm* 176 and *crm* 197 at low pH become true integral proteins of the membrane with both their fragments exposing part of their surface to lipids. This is a necessary step in the membrane-crossing process which has been shown to require a pH gradient across the membrane to be accomplished [30].

4. DISCUSSION

The different membrane interaction of *crm* 197 with respect to DT and *crm* 176 that appears to involve both fragments A and B was rather unexpected on the basis of a single amino acid substitution, Glu for Gly residue at position 52 of the A chain [11]. That this single replacement is sufficient to cause a relevant structural difference between *crm* 197 and DT is also suggested by its higher trypsin sensitivity, and different CD spectrum [13,31] and mobility of the A chain in SDS-PAGE (cf. top panels of fig.3). The present findings suggest that an altered conformation of the A chain of DT can affect the structure of the B chain. This conclusion is supported by the different affinity of two B chain-specific monoclonal antibodies vs *crm* 197 and DT (Bigio, M., Rappuoli, R. and Ratti, G., in preparation). The present data provide further support in favour of Lory et al. [32] who suggested that the two chains of DT are closely associated and do not form separate domains. Moreover, if Gly 52 is a part of the NAD^+-binding site as suggested by its relevance for the enzymic activity of DT, these data would also agree with the idea that the NAD^+-binding site of DT is in a cleft between the two toxin fragments [32].

On the basis of the present results and of a model recently put forward to account for the membrane binding properties of tetanus and botulinum toxins [33], we can advance an explanation for the higher cell association constant of *crm* 197 with respect to DT [13]. As depicted in fig.4, DT is suggested to bind to the cell surface not solely via a protein receptor but also through an interaction with the polar head group of negatively charged phospholipids. Hence, its cell binding constant is the product of the two single binding constants: that for lipids and that for the protein receptor. This mode of binding can account for strong association of toxin to a sensitive cell even in the absence of a specific DT receptor. Such toxin-specific receptors are unlikely to exist because their presence would improve the efficiency of cell killing. Therefore, it may be assumed that there has been no evolutionary pressure to increase the cell ability to bind toxins; rather, it is believed that the toxin parasitizes a plasma membrane protein of as yet unknown function.

It should also be noted that binding of the toxin to lipid leads to an effective increase in its concentration on the membrane and hence to a much higher concentration of toxin-receptor complexes thus leading to a high efficiency of intoxication. Moreover, lipid binding can change the conformation of DT and hence its affinity for the protein receptor as suggested for the binding of tetanus and botulinum neurotoxins to polysialogangliosides [33].

The interaction of *crm* 197 with the phospholipid fatty acid chains, found here, is expected to confer additional strength on its binding to the lipid bilayer with respect to DT as shown in fig.4. This difference could well account for its stronger binding to cells reported in [13]. If this is the case the suggested purification of the DT receptor by affinity chromatography on a *crm* 197 column [13] may turn out to be unsuccessful.

The unspecific binding of DT to negative lipids may be relevant to its killing of insensitive cells which occurs in the micromolar range instead of the nanomolar range in the case of sensitive cells: it corresponds to a concentration step of the toxin on the membrane thus increasing its probability of being endocytosed even in the absence of a receptor protein present on sensitive cells.

ACKNOWLEDGEMENTS

We thank Drs R. Bisson and S. Massari for critically reading the manuscript and Professor G.F. Azzone for encouragement and support. This work was partially supported by a grant from Regione Veneto and is in partial fulfillment of the Doctorate degree of the University of Padova in Molecular and Cellular Biology and Pathology of E.P.

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