TRANSPORT OF DIPHTHERIA TOXIN FRAGMENT A ACROSS MAMMALIAN CELL MEMBRANES

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Received February 23,1977

SUMMARY : The role of the hydrophobic region of diphtheria toxin B moiety in fragment A membrane traversal has been studied using crm45. This molecule, a serologically related diphtheria toxin protein, contains a normal enzymic fragment A and the hydrophobic domain of the toxin B chain but lacks a C-terminal polypeptide needed for specific cell binding. Relatively high concentrations of crm45 are required to inhibit protein synthesis in cells however, after the loss of its hydrophobic region crm45, which still contains an active fragment A,becomes almost non-toxic. It seems thus that the non-polar peptide found in crm45 or toxin facilitates the transport of the hydrophilic fragment A across the plasma membrane.

INTRODUCTION

Diphtheria toxin (MW 62000), inhibits protein synthesis in eucaryotic cells. The molecule, a single polypeptide chain, is split by mild proteolysis into two fragments (A and B) linked by a disulfide bridge. In order to exert its lethal activity, diphtheria toxin must transport across the plasma membrane its 21150 dalton N-terminal hydrophilic fragment A. This polypeptide catalyses then the cleavage of NAD followed by the transfer of its ADP-ribose moiety on the elongation factor 2 (EF2) which is thereafter inactivated (1). The process whereby, this enzymically active fragment reaches the cytosol, requires, the presence of fragment B (40850 dalton) of the toxin molecule (1). Entry of fragment A proceeds by binding first of the B subunit to, a yet unidentified, specific membrane receptor. Although all eucaryotic EF2 are inactivated by fragment A (2), some animal species (such as rats and mice) are highly resistant to toxin because their cells do not bind this protein (3). It is clear however, that if toxin binding is requisite a second

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ISSN 0006-291X

event must occur between B fragment and cells, to facilitate the entry of the hydrophilic fragment A across the lipid membrane.

We have recently shown (4) that toxin fragment B carries a hydrophobic region as membrane proteins do (5). Based on this finding, we have proposed a model (4) in which we postulated that fragment B, by forming a channel across the lipid bilayer, enables the hydrophilic A toxin chain to cross the membrane. In an attempt to support this hypothesis, the serologically related toxin protein crm45 has been used in this study. This molecule (6), a prematurely terminated protein, is ten thousand times less toxic, than toxin itself. It does not bind to cells because it lacks a 17000 dalton carboxy terminal polypeptide of the toxin B fragment, needed for receptors recognition (6-3). Nevertheless crm45 contains a normal enzymic fragment A and its B45 moiety carries the hydrophobic region found on the toxin B subunit (4). Crm45, which inserts into detergent micelles (4), should interact with plasma membranes provided molecules would be close enough to the phospholipid bilayer. Therefore, crm45 can be used to investigate the specific role of the B45 non-polar region, in the process of fragment A membrane traversal which takes place after receptors binding.

In the present study, it is shown that using relatively high concentrations of crm45 (to increase interactions between cell membranes and the mutant toxin), protein synthesis is inhibited in cells. However after the loss of its hydrophobic region, crm45 becomes almost non toxic.

MATERIALS AND METHODS

Cell culture : L-929 mouse cells were maintained in monolayers on MEM medium supplemented with 5% calf serum.

Radioactive products : An aqueous solution of ring-labeled [^{3}H] Triton-X 100 (0,93 g/ml), a product of Rohm and Haas Co. Philadelphia, was a gift of S. Clarke. The specific activity was determined in July 1976 as 125 cpm/µg. The preparation did not contain free tritium (4). Uniformely labeled [^{14}C] L-leucine (280 mCi/mM) was from CEA Saclay, France. Labeled [^{14}C] NAD (adenine 7-14C) (14 mCi/mM) was a gift of D.M. Gill.

Source of crm45 : Crm45 isolated from culture filtrates of Corynebacterium C_7 (β_{45}), was a gift of B. Vernon and A.M. Pappenheimer Jr. The protein ran as a single band on polyacrylamide gel electrophoresis.

Assay of crm45 enzymic activity : The method described by Gill and Pappenheimer was followed (7) : crm45 was diluted between 0.5 to 8 µg/ml in 10 mM sodium phosphate buffer pH 7.2 containing 0,1 % serum-albumin to prevent denaturation. Of each dilution 10 µl were added to 100 µl of a standard assay mixture (0.05 M dithiotreitol, 0.05 M Tris

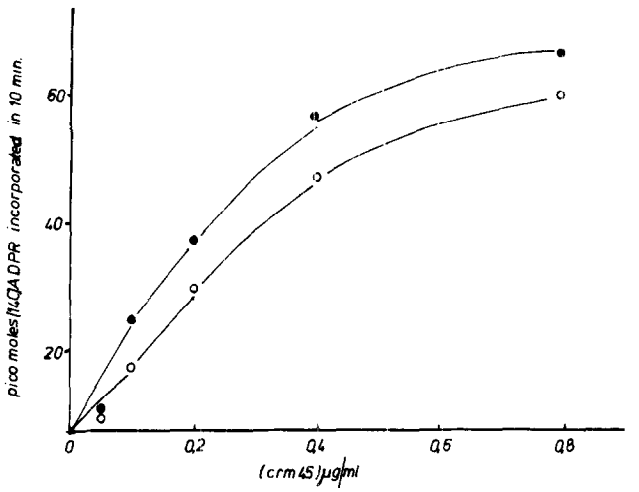

Fig. 1 : Enzymic activity of heated and unheated crm45
o—o heated crm45 ●—● unheated crm45
(ADPR : Adenosine diphosphoribose)

HCl pH 8 about 0.1 µM transferase 2 (EF2) obtained from rabbit reticulocytes and 1 µM [^{14}C] NAD). The mixture was incubated 10 min. at 37ºC and then cold 5 % TCA was added to stop the reaction. The precipitates were collected on glass fiber filters washed and counted.

Triton-X 100 binding study : The method of Clarke was followed (5) : sucrose gradients 5-20 % wt/vol were prepared in 10 mM sodium phosphate buffer pH 7.2 containing 0.05 % [^{3}H] Triton-X 100 (above the critical micelles concentration CMC). Gradients were poured into 5 ml tubes and stored 4 hrs at 4ºC before use. Samples of 200 µl (2 mg of protein per ml in 10 mM sodium phosphate buffer pH 7.2), containing an excess of [^{3}H] Triton-X 100 were layered on the top of the gradients which were centrifuged for 17 hrs at 57000 rpm at 5ºC in a SW65 rotor using a Spinco L4. After centrifugation 23 fractions of 200 µl each were collected from the bottom of the tubes and 25 µl were counted by liquid scintillation. Protein concentration was determined by the Lowry method (8) using 100 µl aliquots diluted in 400 µl phosphate buffer to avoid precipitation of detergent.

Cell toxicity assay : Confluent monolayers (10^6 cells/Petri dish) were incubated with different concentrations of crm45 for 24 hrs at 37ºC. A pulse of [^{14}C] leucine (90 µCi/ml) was then given. One hour later the medium was discarded and 5 % TCA was added. The cells were scraped off washed on glass fiber filters, dried and counted.

RESULTS AND DISCUSSION

Selective denaturation of crm45 hydrophobic domain

Heating crm45 at 100ºC for 10 min. does neither coagulate the protein (6) nor, as it has been already shown for toxin fragment A (1) does

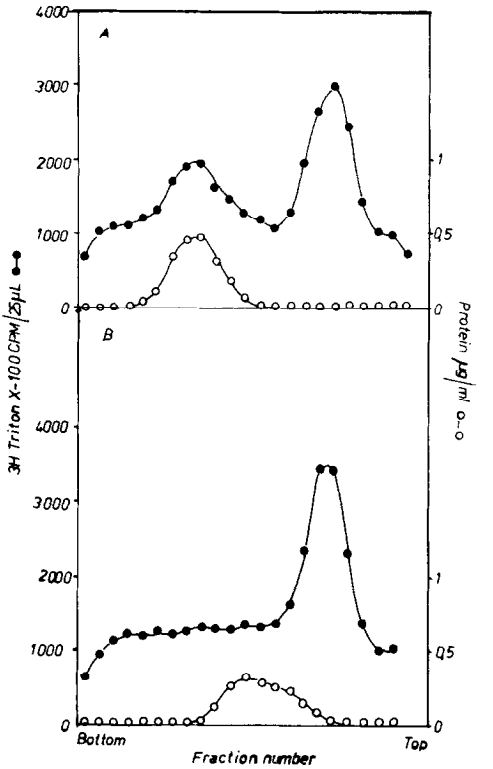

Fig. 2 : Binding of [^{3}H] Triton-X 100 by heated and unheated crm45
Panel A : unheated crm45 Panel B : heated crm45
●—● radioactivity o—o protein concentration

it inactivate its enzymic activity. Fig. 1 shows indeed that heated crm45 is as efficient as crm45 in ADP-ribosylation activity.

Using a detergent binding assay we have previously shown (4) that toxin fragment B, crm45 and its B45 fragment share a common hydrophobic region, located on the N-terminal portion of the B toxin chain. Furthermore, it was shown that crm45 binds Triton-X 100 only when the detergent reaches its CMC (4). According to this result, crm45 behaves as many membrane proteins (5) and becomes readily inserted into Triton-X 100 micelles. A typical detergent binding experiment using crm45 is shown in Fig. 2A. A large peak of radioactivity is associated with the protein peak equivalent to 0.5 mg of Triton-X 100 per mg of crm45. On the other hand, heated crm45 (as shown in Fig. 2B) does not bind measurable amount of detergent.

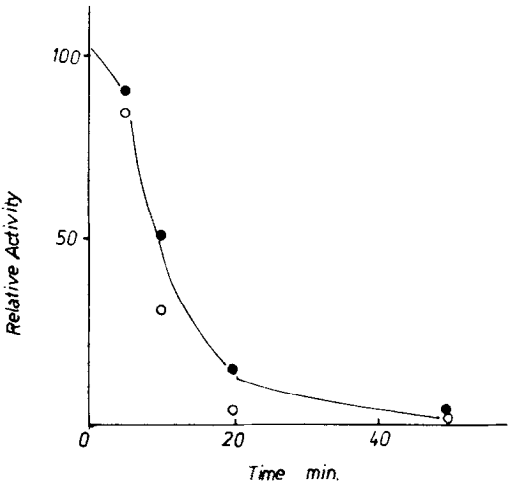

Fig. 3 : Effect of trypsin on the enzymic activity of heated and unheated crm45
Heated and unheated crm45, 2 mg/ml in sodium phosphate 10 mM pH 8 were incubated with trypsin 15 µg/ml at 37ºC for periods up to 50 min. At time indicated, samples were withdrawn and diluted 4000 times in phosphate buffer 10 mM pH 7.2 and assayed for ADP-ribosylation as described in methods.
o—o heated crm45 ●—● unheated crm45

The difference observed in sedimentation between unheated and heated crm45 is due to the insertion of 3 crm45 molecules per Triton-X 100 micelle (4) increasing thus slightly crm45 velocity.

In addition, heated crm45 does not behave differently than unheated crm45 when treated by a proteolytic agent such as trypsin (Fig. 3). The same decay in the enzymic activity is observed in both cases, indicating that fragment A of heated crm45 has refolded in a native conformation.

On the basis of the data here presented, we can assume that heating crm45 at 100ºC for 10 min. destroys the hydrophobicity of B45, while the enzymic activity of its A fragment is unchanged.

Inhibition of mouse L-cells protein synthesis by crm45

Mouse L-cells lack toxin receptors (1,3) and crm45 does not recognize specific binding sites (3). Uptake of this protein in such cells should therefore be realized only through the bulk movement of extracellular fluid into endocytotic vesicles. In these conditions, provided some molecules escape from lysosomal degradation, cell toxicity of crm45 should be identical with or without its hydrophobic region. It is clearly

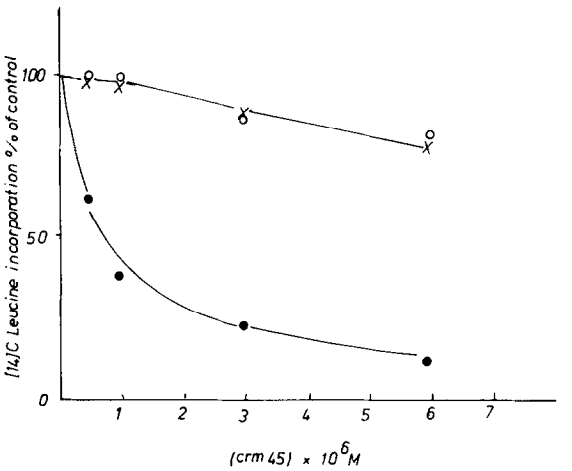

Fig.4 : Comparative toxicity of crm45 heated and unheated on L-929 mouse cells
o—o heated crm45 ●—● unheated crm45
x—x unheated crm45 in the presence of 400 µg/ml ammonium chloride.

shown in Fig. 4 that this is not the case. Crm45 incubated for 24 hrs with mouse L-cells can inhibit almost totally protein synthesis using relatively high doses of crm45 (more than 10^{-6}M). It must be recalled that less than 10^{-12}M diphtheria toxin is required to block protein synthesis in sensitive cells in 24 hrs (9). After heating, although crm45 retains a normal enzymic activity, less than 20 % inhibition of protein synthesis is observed (Fig. 4).

Fragment A from crm45 enters cell in a similar way than diphtheria toxin A chain. As shown in Fig. 4 ammonium chloride which has been repeatedly used to block only the specific entry of diphtheria toxin in sensitive cells (10,11), prevents most of the toxic expression of crm45.

Our study suggests that after stochastic interactions between crm45 molecules and cells, insertion of this molecule within the membrane lipid takes place through its hydrophobic region. This mechanism is successfull in achieving the penetration of fragment A into the cell cytoplasm. On the other hand upon heating, crm45 behaves like fragment A alone and is merely taken up by endocytotic vesicles. As pointed out by others (11,12) this way of internalization is almost uneffective in transporting fragment A in an active biological state into the cytosol.

One could argue that heating crm45 brings about some structural changes of fragment A, which remains active enzymically. However, this fragment could become more sensitive to proteolytic degradations, thus explaining its low cell toxicity. This possibility appears unlikely since no difference in protease sensitivity was found between heated and unheated crm45.

Our data strongly suggest that toxin B fragment is divided into two different structural regions. Each have separated but complementary roles in the process of entry of fragment A into the cytoplasm. The function of the carboxy-terminal polypeptide of B fragment is likely to bind specific cell receptor in order to bring into close proximity the molecule with the lipid membrane. The amino-terminal moiety of toxin B fragment contains a hydrophobic region which insert itself into the phospholipid bilayer and through which fragment A traverses the membrane to reach the cytosol.

ACKNOWLEDGMENTS : I would like to thank Dr Paul Tournier in whose laboratory this work was done and Dr J. Alouf for critical reading of the manuscript.

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