Commentary

Probing Pores with Peptide Plugs: Topology of Membrane-inserted Diphtheria Toxin

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Extracellular proteins are made in the cytosol and transported out of the cell. Certain proteins travel the opposite way, from the outside to the cytosol. Most of these proteins are toxins made by bacteria and plants, but evidence is accumulating that certain growth factors can do the same. Toxins that enter the cytosol can, according to current knowledge, be roughly classified into two main groups, those that enter from endosomes and those that enter from the endoplasmic reticulum (Alouf and Freer, 1999). In both cases, the travel to the cytosol is initiated by binding of the toxin to cell surface receptors, followed by endocytosis.

Diphtheria toxin, the protein responsible for the tissue damage in diphtheria, is the most famous and best studied of these toxins. Like many other toxins acting inside cells, the active form consists of two polypeptides linked by a disulfide bond. Because the toxin is synthesized by the bacterium as a single chain protein that is subsequently cleaved, the two parts are referred to as the A and the B fragments. The B fragment consists of two functional parts that form separate domains in the crystallized protein, the receptor-binding R domain and the T domain (transmembrane) that is involved in translocation of the catalytic A fragment (or C domain) to the cytosol. The A fragment is an enzyme that ADP-ribosylates elongation factor 2 in the cytosol, leading to protein synthesis inhibition and, consequently, to cell death. Upon endocytosis of the toxin-receptor complex, the low pH in the endosome triggers unfolding of the toxin molecule, leading to insertion of the T domain into the endosomal membrane and concomitant translocation of the A fragment to the cytosol. The process can be induced already at the cell surface when cells with bound toxin are exposed to low pH.

The T domain is different from the rest of the molecule in that it consists almost entirely of α-helices. In this respect, it resembles certain pore-forming toxins such as colicin A and *Bacillus thuringiensis* δ-endotoxin (Parker and Pattus, 1993). In fact, as was first detected in black lipid films (Kagan et al., 1981) and later confirmed in living cells (Eriksen et al., 1994), diphtheria toxin also has the ability to form ion-conducting channels in biological membranes. Expression and periplasmic secretion of diphtheria toxin in *Escherichia coli* caused the bacteria to die at acidic pH, due to channel formation in the inner membrane (O'Keefe and Collier, 1989). A screen for toxin mutants that allowed the survival at low pH identified the residue Glu349 as pivotal for the membrane insertion of the toxin's T-domain (O'Keefe et al., 1992).

There has been much speculation as to whether the ion-conducting channel formed by the T domain also can accommodate the A fragment, which could be translocated across the membrane through a hydrophilic channel, much the same way as export proteins are translocated from the cytosol to the lumen of the endoplasmic reticulum. The toxin must be extensively unfolded before translocation (Falnes et al., 1994) similarly to proteins that are transported into the endoplasmic reticulum. Also, there is a qualitative correlation between the potency of toxins with mutations in the T domain and their ability to form channels, but specific mutations in the T domain that reduce the channel-forming ability of the toxin by several orders of magnitude do not give much reduction in toxicity (Falnes et al., 1992). Thus, the exact role of the toxin channel remains unclear.

So why does the toxin form a channel? Proteins evolve in a modular way and it is conceivable that diphtheria toxin has evolved from a toxin that once formed lethal pores in cellular membranes. The pores made by *Bacillus thuringiensis* δ-endotoxin and other toxins that are lytic to eukaryotic cells have conductances of several hundreds and even thousands of picosiemens per pore (Slatin et al., 1990; Benz et al., 1994; Maier et al., 1996; Menzl et al., 1996; Korchev et al., 1998), whereas the diphtheria toxin channel has a conductance of only 10–30 pS at physiological salt concentrations. Perhaps the exceptionally toxic A fragment (one molecule in the cytosol kills the cell) was once bestowed onto a pore-forming toxin. As a result, the channel forming property became superfluous and later atrophic, while the T domain's ability to insert into membranes was developed into an efficient translocation machinery for the A fragment. In that case, the channel-forming property of the T domain could be considered as a faint memory, which the toxin retains from bygone times when it was a mighty

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pore former capable of opening cells up for deadly fluxes of ions and other solutes. The channel formed by diphtheria toxin B fragment allows only a trickle to pass, a flux that does not do much harm to the cells even when the toxin is inserted into the plasma membrane at low pH, and the channel is eventually closed or inactivated by the cell. Whatever role the channel may play in the intoxication process, in the hands of Alan Finkelstein and his colleagues it is an exceptionally useful tool for studying the insertion of the T domain into membranes and translocation of the A fragment across them.

Diphtheria Toxin Translocation from the Channel's Perspective

Kagan et al. (1981) first demonstrated that diphtheria toxin forms channels in black lipid membranes at low pH. Later experiments showed that a small part of the toxin molecule is sufficient to form the channels, namely two adjacent α-helices, TH8 and TH9, linked by an acidic loop, thus forming a hairpin that is able to insert into the membrane at low pH (Silverman et al. 1994). Although these two helices alone form channels with the same properties as those formed by the whole T domain, the efficiency of channel formation is very much lower than when the whole T domain is used. Additional parts of the molecule must therefore strongly facilitate the insertion of the channel into the membrane.

In typical lipid bilayer experiments, the cis compartment to which the toxin is added has an acidic pH as in endosomes. This is necessary to induce a conformational change required for insertion. The pH in the trans compartment is neutral, mimicking the conditions in the cytosol. Working on the topology of the inserted toxin, Senzel et al. (1998) expressed the T domain of the toxin with an NH_2-terminal hexahistidine tag for purification purposes. When the protein was studied for channel-forming properties, it was observed that the channel opened as expected at positive voltages. However, at negative voltages it rapidly closed, a property not found in molecules without the hexahistidine tag. The tag that contains an arginine residue in addition to the histidines has a weak positive charge under the conditions used, and when it reaches the mouth of the channel by diffusion it could be trapped by the negative potential and plug the channel. At negative voltages, this would only occur if the hexahistidine tag is on the trans side of the membrane (opposite the chamber where the T domain was added, the cis side). When the tag is attached to the NH_2 terminus of the T domain, it may act like a ball on a chain, similarly to how the inactivation gate of the *Shaker* potassium channel is thought to be regulated. As Senzel et al. (1998) stated, it is also possible that the tag acts outside the channel as such by inducing a conformational change that closes the channel.

The channel closure could be relieved by adding Ni^{2+} ions, which bind to the hexahistidine tag on the trans side of the membrane, or trypsin, which cleaves the tag off. Addition to the cis side did not have this effect. Furthermore, when a cysteine residue in the vicinity of the hexahistidine tag was biotinylated, it was observed that streptavidin, which binds to biotin, interfered with channel closure when added to the trans, but not to the cis, side of the membrane. Taken together, the results indicate that when the T domain inserts into the membrane in response to low pH, its NH_2-terminal region is translocated to the trans side.

The team then went on to study translocation of toxin A fragment across the membrane, the decisive step in the transport of the toxin to its target in the cytosol. They also found that when the hexahistidine tag was attached to the NH_2 terminus of the A fragment (the COOH terminus of which was joined to the T domain by a disulfide bond), the channel was closed at negative voltages (Oh et al., 1999). When a membrane-impermeable reducing agent was added to the trans solution, the block was eliminated, indicating that the disulfide between the A fragment and the T domain had been reduced and that the A fragment with the tag had therefore diffused away from the channel. Addition of the reducing agent to the cis compartment did not have any effect. This indicates that the disulfide bond had been translocated to the trans side.

In addition to the trick with hexahistidine, Finkelstein and colleagues also applied an approach they had earlier used with great success in studying the insertion of colicin 1a in artificial membranes (Slatin et al., 1994). In elegant experiments, they demonstrated that a large part of the colicin molecule is translocated back and forth across the membrane depending on the membrane potential and that this translocation is required for opening and closure of the channel.

In those experiments, they placed cysteine residues at defined positions in the protein and then bound biotin covalently to the cysteines. The modified protein was allowed to interact with membranes under different voltage conditions, and then streptavidin was added to one or the other compartment. If the biotin (and therefore the cysteine residue) was on the side where streptavidin was added, the relevant part of the colicin became unable to flip across the membrane as it was unable to carry the bulky streptavidin molecule across the membrane. When streptavidin was bound to the protein loop in the trans position, the channel stayed open irrespective of the voltage, whereas when it was bound to the loop at the cis side before translocation, a positive voltage was unable to open the channel.

In the case of diphtheria toxin, two A fragment mutants were constructed, each of which contained a single free cysteine residue. This residue was reacted with biotin, and then membrane insertion was induced. In

both cases, binding of streptavidin to the A fragment in the trans position prevented the rapid closure of the channel by the histidine tag (Oh et al., 1999). Therefore, not only the NH_2 terminus, but also the rest of the A fragment must be on the trans side of the membrane.

Topology of the Inserted T Domain

From the experiments mentioned above, it is clear that the insertion of the channel-forming helices somehow pulls the NH_2 terminal part of the T domain and the whole A fragment across the membrane. However, the NH_2 terminus of the T domain is located at a distance from the channel-forming hairpin helices. Furthermore, the NH_2-terminal region is highly hydrophilic, containing 26 charged residues within an amino acids stretch of 63 residues total. The unusual ability of the T domain to translocate the A fragment across the membrane raised questions about how the T domain inserts into the membrane. Senzel et al. (2000) applied the channel blocking properties of the hexahistidine tag as a tool to probe the topology of the membrane-inserted T domain. Initially, they established that free hexahistidine peptide (at fairly high concentrations) closed the channel at negative voltages when added to the trans side of the membrane and at positive voltages when the peptide was added to the cis side. In both cases, reversal of the potential reopened the channel.

Through maleimide chemistry, an oligopeptide containing hexahistidine was attached to T domain mutants that each contained a single cysteine residue. From the gating properties of the resulting channels, it was inferred to which side of the membrane the cysteine was localized. By studying a number of such single cysteine mutants with respect to the channel gating caused by the attached hexahistidine peptide, a new model for the membrane topology of the T domain was proposed (Fig. 1). In addition to the helices TH8-9, only one other helix, TH5, in the T domain assumed a transmembrane position. The most surprising result was that the entire hydrophilic and highly charged sequence was translocated to the trans side of the membrane. One could have feared that the attachment of a hexahistidine peptide to a cysteine residue that is transported to the trans side of the membrane when the channel is inserted could have prevented the channel insertion altogether, but that appears not to be a problem. The insertion model was supported by the results of experiments in which the single cysteine residues were biotinylated, and it was then studied how addition of streptavidin to the cis and trans sides of the chamber affected channel properties.

Relevance to Translocation in Living Cells

All this was done in a surprisingly simple in vitro system consisting of only toxin and lipids. This excludes the possibility that other proteins such as the toxin receptor play a necessary role in the translocation. But does the phenomenon here observed reflect what is going on when toxin enters living cells? Probably the mechanisms are similar. Also, in intact cells, the toxin forms channels with properties resembling those formed in the planar lipid membranes (Lanzrein et al., 1997). B fragment that had been inserted into the plasma membrane of cells was cleaved by externally added trypsin (Moskaug et al., 1991) at a site that is found to be located at the cis side in the artificial system. On the other hand, the cell experiments did not produce an NH_2-terminal fragment shielded from extracellular proteases, as would be predicted from the in vitro experiments. Whether or not this represents a real difference between insertion in the cellular and artificial systems remains to be elucidated.

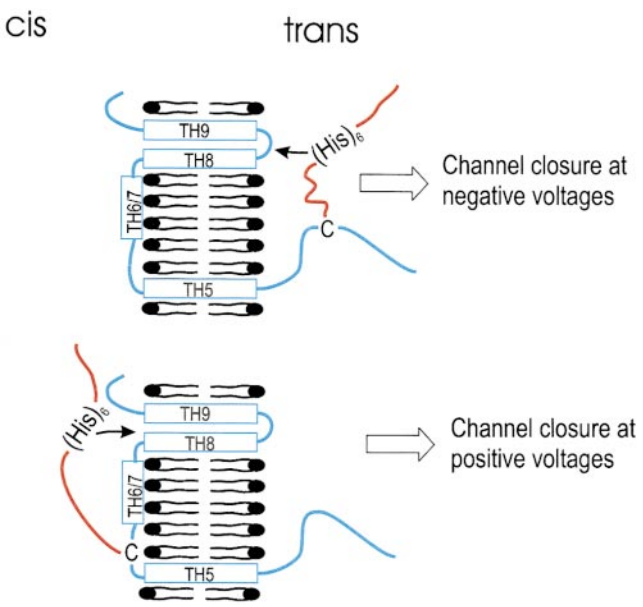

Figure 1. Attachment of hexahistidine peptide (red) to a single cysteine residue (C) in the T domain (blue) affects channel behavior.

The disulfide linking the A and B fragments appears to be reduced by exposure to the cytosol during translocation in living cells (Falnes and Olsnes, 1995). In the black lipid membrane system, it is reduced when a reducing agent is added to the trans solution that corresponds to the cytosol. The disulfide is therefore in both systems translocated across the membrane.

A remaining problem that could possibly be approached by the black lipid film system is the fate of the COOH-terminal receptor-binding domain of the B fragment, the R domain. When the B fragment is inserted into membranes of living cells, this domain is shielded from the action of extracellular proteases (Moskaug et al., 1991). In accordance with this, the R domain is inserted into liposomes at low pH (Querten-

mont et al., 1999). Is it translocated across the membrane? And, if so, what would be the reason for this, as the bilayer data indicate that the R domain is not necessary for translocation?

With all its virtues with respect to dissection of the translocation process, the in vitro system has some disadvantages as compared with studies on living cells. The bilayer system is extremely sensitive and able to detect a single channel in a membrane that is enormous in extent compared with the surface of a cell. This is probably the reason why the toxin receptor can be disposed of in this system, while in the living cell a measurable signal can only be obtained when the receptor provides up-concentration and correct positioning of the toxin on the membrane. Also, other molecules in the intact cells could play a role in making the translocation more efficient. An obvious possibility is that chaperones in the cytosol could be required for efficient translocation of the A fragment, perhaps by facilitating the refolding of the protein. Exposure to low pH induces the molten globule state of the A fragment, and it was recently found that the T domain is able to interact with proteins in this state (Ren et al., 1999). Possibly, therefore, the T domain acts as the toxins' own chaperone in helping the A fragment across the membrane.

Protein toxins acting inside cells have been useful tools in the elucidation of many biological processes, such as studies of G proteins, analysis of the mechanism of vesicular fusion in neurons and other cells, and in work on intracellular transport. The recent work from the Finkelstein laboratory on the membrane insertion of diphtheria toxin challenges current ideas of how proteins interact with membranes.

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