

Penetration of protein toxins into cells

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AB toxins deliver their enzymatically active A domain to the cytosol. Some AB-toxins are able to penetrate cellular membranes from endosomes where the low pH triggers their translocation. One such toxin is diphtheria toxin and important features of its translocation mechanism have been unraveled during the last year. Other toxins depend on retrograde transport through the secretory pathway to the ER before translocation, and recent findings suggest that these toxins take advantage of the ER translocation machinery normally used for transport of cellular proteins. In addition, the intracellular targets of many of these toxins have been identified recently.

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Abbreviations

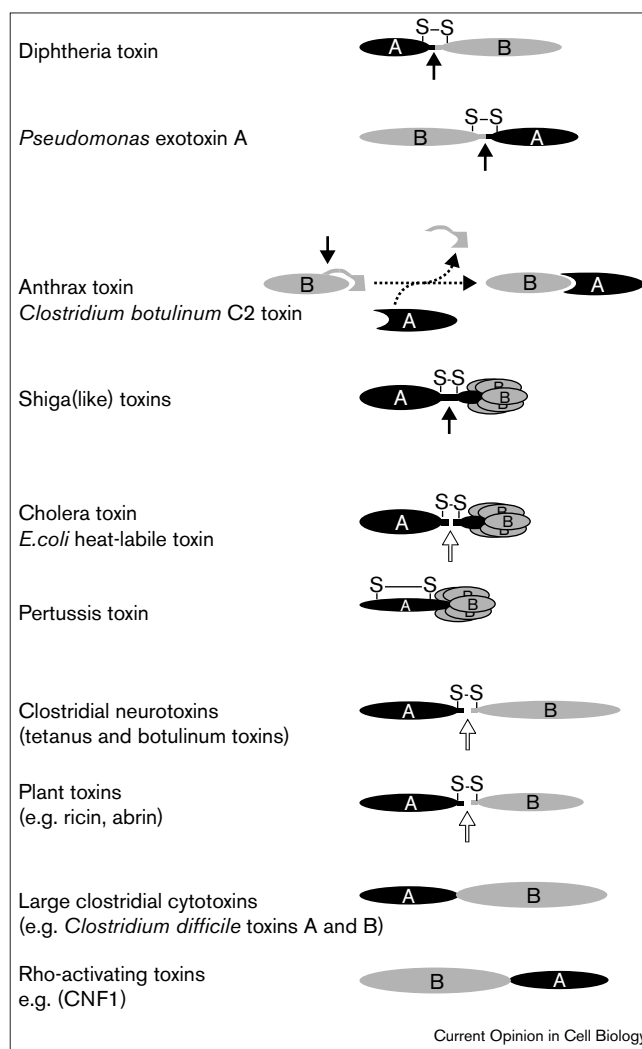
CNF1	cytotoxic necrotizing factor 1
EF	edema factor
GPI	glycosylphosphatidylinositol
LF	lethal factor
PA	protective antigen
SNAP-25	synaptosomal-associated membrane protein of 25 kDa
TGN	trans Golgi network
VAMP	vesicle-associated membrane protein

Introduction

A number of proteins from plants and bacteria are highly toxic to mammalian cells because of their ability to enter the cytosol and attack essential constituents (for a review see [1]). The majority of these toxins are referred to as AB-toxins because of their structural organization (summarized in Figure 1) [1]. The A moiety generally has enzymatic activity and modifies a cellular target upon entry into the cytosol (Table 1) [1], which leads to cell death or other effects on cellular physiology. The B moiety, consisting of one or more subunits, binds the toxin to cell-surface receptors and can also play a role in the translocation of the A moiety to the cytosol. Examples of intracellular targets are ribosomes, actin, small GTP-binding proteins like Rho, and heterotrimeric G-proteins [1]. A recent discovery is the finding that the anthrax toxin lethal factor cleaves mitogen-activated protein kinase kinase (MAPKK) [2,3]

Commonly, an AB-toxin is synthesised in an inactive form that is activated by proteolytic processing (Figure 1, [4]). Some toxins are cleaved by the producing organism (e.g. cholera toxin, ricin, clostridial neurotoxins) at a region between two cysteine residues [4]. In other cases (e.g. diphtheria toxin, Shiga toxin and *Pseudomonas* exotoxin A),

Figure 1



Structural organization of AB-toxins. Proteolytic cleavage of the toxin is required, in many cases, for activity, and such cleavage often occurs in the region between the A (black) and the B (gray) subunits. Either the toxin has been cleared by a protease from the plant or the bacterium that produces the toxin (indicated by open arrows) or a protease (usually furin) from the target cell cleaves the toxin (indicated by closed arrows). Proteolytic cleavage often results in a toxin with an enzymatically active part linked to the rest of the molecule by a disulfide bond. However, the cleavage of the anthrax toxin B moiety by furin leads to the dissociation of part of the molecule and exposure of a site where the A moiety binds. In the cases where no cleavage is indicated, it is possible that cleavage takes place.

such processing is performed by furin, which is expressed by the target cell [4]. Other toxins (e.g. anthrax toxins and *Clostridium botulinum* C2 toxin) depend on proteolytic processing of the B moiety to expose a site that then binds the A moiety non-covalently [4]. In this article, we describe strategies employed by AB-toxins when entering cells, emphasizing recent discoveries in the field.

Table 1

Mode of action of some AB toxins.

Toxin	Enzymatic activity	Cellular target(s)
Diphtheria toxin	ADP-ribosyl transferase	EF-2
<i>Pseudomonas</i> exotoxin A	ADP-ribosyl transferase	EF-2
Anthrax edema toxin	Adenylate cyclase	cAMP-modulated proteins
Anthrax lethal toxin	Zinc endoprotease	MAPKK
<i>Clostridium botulinum</i> C2 toxin	ADP-ribosyl transferase	G actin
Shiga toxin	N-glycosylase	28S rRNA
Cholera toxin	ADP-ribosyl transferase	Heterotrimeric G-protein
Pertussis toxin	ADP-ribosyl transferase	Heterotrimeric G-protein
Clostridial neurotoxins	Zinc endoprotease	VAMP/synaptobrevin, SNAP-25, syntaxin 1
Plant toxins (ricin, abrin etc.)	N-glycosylase	28S rRNA
<i>Clostridium difficile</i> toxins A and B	Glucosyl transferase	Rho proteins
Cytotoxic necrotizing factor 1 (CNF1)	Deamidase	Rho proteins

Toxin receptors at the cell surface and their role in penetration and intoxication

The AB-toxins, in many cases, bind to specific receptor molecules at the cell surface. For instance, the receptor for diphtheria toxin is the uncleaved precursor of the heparin-binding EGF-like growth factor [5]; the receptor for *Pseudomonas* exotoxin A is the α 2-macroglobulin receptor [6]; Shiga toxin binds to the glycolipid Gb3 [7] and cholera toxin binds to GM1 [8]. In the case of plant toxins, like ricin, they usually bind to carbohydrates [9], regardless of whether they are attached to lipids or proteins.

The receptor, in addition to providing binding sites at the cell surface, has several other functions in the intoxication process. Firstly, if the receptor is internalized efficiently, for instance from clathrin-coated pits (see below), the toxin is also rapidly taken up. Importantly, receptor expression might be under regulation by growth factors/cytokines. This is the case for the Shiga toxin receptor (Gb3) [9], and it seems to play an important role in hemolytic uremic syndrome (HUS) caused by *Escherichia coli* that synthesize Shiga-like toxins and cause food poisoning [10]. Secondly, the toxin receptor is important for targeting of the toxin to the organelle before it enters the cytosol. An example is the Shiga-toxin receptor, where the lipid composition of the receptor is essential for retrograde toxin transport [9,11]. A third point is that the receptor may play a direct role in toxin penetration through the membrane. In the case of diphtheria toxin, the intact receptor seems to play an important role in efficient translocation across biological membranes. If the transmembrane or cytoplasmic domain of the receptor is replaced by a GPI (glycosylphosphatidylinositol) anchor, the efficiency of toxin translocation across the membrane is considerably reduced [12]. Point mutations in the receptor also appear to inhibit translocation [13].

Endocytosis and intracellular transport of protein toxins

Although exceptions exist where a toxin seems to penetrate directly from the cell surface into the cytosol

(e.g. *Bordetella pertussis* invasive adenylate cyclase) [14], most toxins are endocytosed, although by different mechanisms [9,15], before translocation to the cytosol. Uptake from the cell surface can take place through clathrin-dependent and -independent mechanisms. For example, cells expressing a mutant form of dynamin, which blocks uptake from clathrin-coated pits and caveolae, are protected against diphtheria toxin bound to the wild-type receptor [16,17] but are not protected against diphtheria toxin bound to an engineered, GPI-anchored form of the receptor [17]. This result, in combination with other data, suggests that clathrin-dependent endocytosis is essential for efficient intoxication by diphtheria toxin bound to the wild-type receptor [16,17].

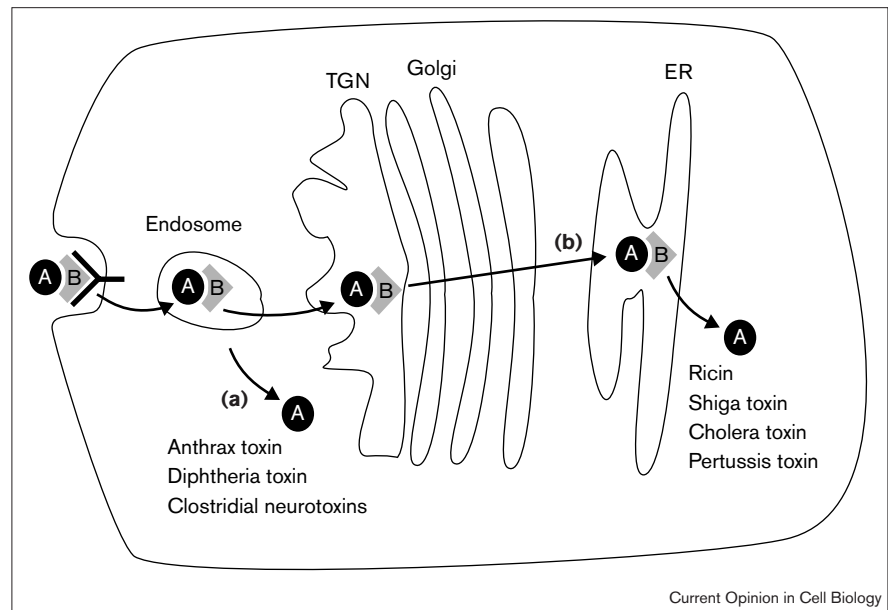
In contrast to ricin, which is endocytosed by all available mechanisms [15], Shiga toxin (despite being bound to a glycolipid receptor [9]) and *Pseudomonas* exotoxin A [18], are internalized from clathrin-coated pits. Also, a fraction of cholera toxin is internalized by this pathway [19]. Although most toxins need to be endocytosed before translocation to the cytosol, in many cases the mechanism by which this occurs has not been investigated.

Several toxins are able to enter the cytosol from acidic endosomes, whereas others seem to be transported to the Golgi apparatus and the ER, before translocation takes place (Figure 2). These toxins, from both acidic endosomes and the Golgi and ER, might circumvent late endosomes and use a more direct pathway to the Golgi apparatus. The exact pathway is not known [15,20•,21], but it seems to be dependent on the GTP-binding protein dynamin [22].

However, retrograde transport of toxins through the Golgi apparatus to the ER may take place through more than one mechanism. Some toxins have a KDEL sequence (e.g. cholera toxin, *Pseudomonas* exotoxin A), which might bind to KDEL receptors and facilitates retrograde transport by a COPI-dependent mechanism [9,23]. However, other toxins do not have such a sequence (e.g. ricin, Shiga

Figure 2

Intracellular transport of protein toxins. The toxin binds to a cell surface receptor (Y shaped) through its B moiety (gray). The toxin A moiety (black) is, in some cases (a), translocated to the cytosol from endosomes, whereas in other cases (b), further transport, most probably all the way to the ER, is required for translocation. The fate of the toxin receptor has not been addressed in this cartoon.



toxin, modeccin) [9] and may use a different, perhaps Rab6-dependent, retrograde pathway to the ER [20^{**},21].

The translocation process

How soluble, hydrophilic proteins are able to cross the barrier presented by the hydrophobic membrane is a fundamental problem in biology, and the protein toxins have developed different strategies to solve it. Some protein toxins appear to carry with them the machinery required for translocation, whereas others are strictly dependent on cellular components.

Diphtheria toxin – a paradigm for toxin translocation

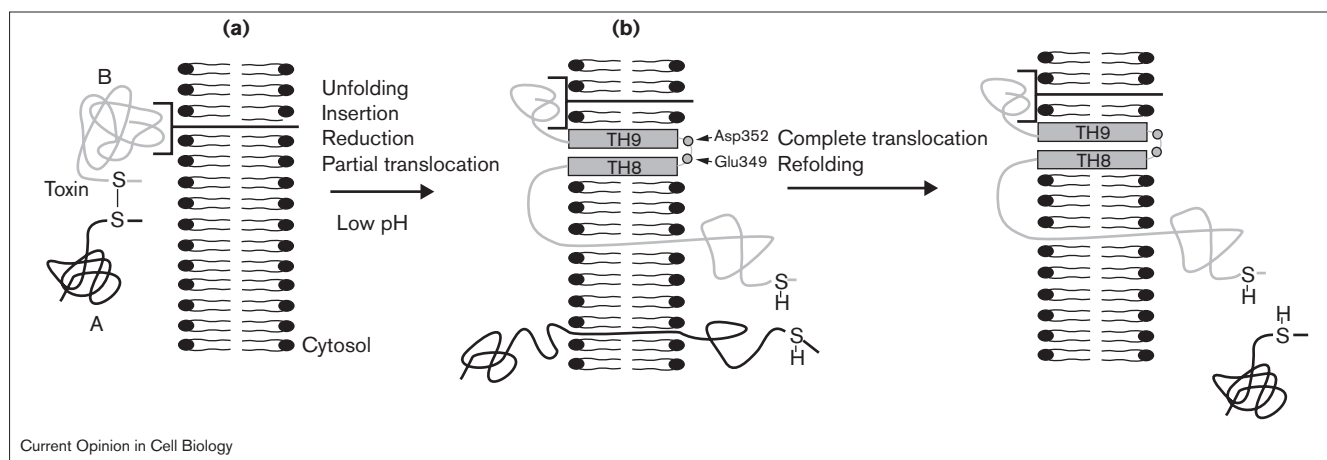
The mechanism of toxin penetration of a cellular membrane is best understood in the case of diphtheria toxin, which requires low endosomal pH for translocation. Both the A and the B moieties of the toxin unfold at low pH [24], leading to the exposure of hydrophobic domains and an increased tendency to interact with membrane lipids [25,26]. Also, when diphtheria toxin is bound to the cell surface, and subsequently exposed to low pH (thereby mimicking the conditions in the endosome), an immediate translocation of the A moiety to the cytosol is induced [27,28]. Thus, the low pH encountered by diphtheria toxin in the endosome triggers the translocation of the A moiety to the cytosol. Introduction of disulfide bonds at several locations in the A fragment blocked this translocation [29], indicating that unfolding is a prerequisite for penetration into the cytosol. However, although the translocation of the mutant A moieties was blocked, the disulfide bond connecting the A and the B chains was still reduced upon exposure of the toxin to acidic pH. This indicates that reduction takes place at an early stage in the translocation process [30], most likely through

exposure of the interfragment disulfide bond to the reducing milieu of the cytosol (Figure 3).

It has been suggested that diphtheria-toxin entry involves a synchronous release of a large quantity of A chain molecules into the cytosol, possibly through rupture of endosomes, which results in the immediate inhibition of protein synthesis [31]. However, recent studies indicate that at low toxin concentrations a single toxin molecule can enter a cell and optimal protein synthesis inhibition requires prolonged action of the toxin in the cytosol [32,33^{*}].

Diphtheria toxin is able to form cation-selective channels at low pH both in the plasma membrane of cells [34,35] and in lipid membranes [36]. The toxin B moiety consists of an amino-terminal, α -helical transmembrane/translocation (T) domain and a carboxy-terminal receptor-binding (R) domain with a β -sheet structure. A part of the T domain, comprising two helices (denoted TH8 and TH9) and the loop connecting them, can form a cation channel with the same characteristics as that formed by the entire T domain in planar lipid bilayers [37] (Figure 3). Although the efficiency of channel formation by the TH8/9 helical hairpin alone was low, this observation indicates that TH8/9 comprises the minimal channel-forming entity. The exact role, if any, of channel formation in translocation of the A moiety to the cytosol remains unclear, as Lanzrein *et al.* [12] presented evidence that channel formation may not be strictly required. An important step in T-domain insertion is the low pH-induced protonation of residues Glu349 and Asp352 at the tip of the TH8/9 helical hairpin, thereby neutralizing the charge of these residues and facilitating their translocation across the lipid bilayer, concomitant with the membrane insertion of the two helices [38] (Figure 3). A

Figure 3



A model of diphtheria toxin translocation. **(a)** In the first step of the translocation process, the toxin molecule unfolds in response to low pH, leading to **(b)** membrane insertion of part of the T domain of the B moiety and a partial translocation of the A fragment to the cytosol. The insertion of the T domain is thought to be initiated by the protonation of the acidic residues Glu349 and Asp352 at the tip of the TH8/9 helical hairpin. The amino-terminal part of the T domain and the

carboxy-terminal part of the A fragment are translocated across membrane, and the disulfide bond connecting them is reduced, most probably by the reducing milieu in the cytosol. The final step is the translocation of the remainder of the A fragment to the cytosol, where it refolds. The model is intended to integrate some of the data obtained in cells [29,30] and in lipid membranes [38,40,41**].

recent study showed that the T domain interacts with proteins in a 'molten globule' state (e.g. the unfolded A moiety), suggesting that the T domain may work as a chaperone in the translocation process [39**].

The channel-forming properties of the T domain in planar lipid bilayers have proved useful for elucidating the mechanism of toxin translocation. Senzel *et al.* [40] found that an oligopeptide containing a hexahistidine stretch was able to close the channel from the *trans* side (the side opposite of where the protein was added) when fused to the amino terminus of the T domain, indicating that the amino terminal part of the T domain is translocated across the lipid bilayer. Remarkably, using channel closure as a marker for membrane translocation, it was demonstrated that the A fragment also crossed the membrane in this system [41**]. Thus, the toxin itself provides sufficient machinery for translocation of the A moiety across the lipid bilayer, but as discussed, the receptor seems to modulate the translocation process in the living cell.

The translocation of other toxins

The translocation of surface-bound toxins to the cytosol upon exposure to low pH would provide convincing evidence that a toxin enters the cytosol from endosomes. Such low pH-induced translocation has been demonstrated for the anthrax toxins [42] and the cytotoxic necrotizing toxin 1 (CNF1) [43], as well as for diphtheria toxin.

Anthrax toxin consists of either of two A moieties: the edema factor (EF) or the lethal factor (LF). These factors associate in a mutually exclusive manner with a common B moiety, the protective antigen (PA), to form edema toxin

and lethal toxin. To expose the binding site for LF or EF, PA must be cleaved by furin [44] (Figure 1), and this cleavage also induces the formation of a PA heptamer. Each molecule in the PA heptamer associates with one molecule of EF or LF [45*]. Upon exposure to low pH, the effector moieties translocate to the cytosol, whereas the PA oligomer inserts into the membrane and forms cation-selective channels [42]. As with diphtheria toxin, the role of the channel formation is not known, and unfolding of the A moieties appears to be a requirement for translocation to the cytosol [46*].

The entry of several AB-type toxins, including diphtheria and anthrax toxins, into cells is inhibited by compounds elevating the pH of acidic endosomes [9]. However, this does not necessarily mean that the toxin enters the cytosol directly from endosomes. The toxic effect of *Pseudomonas* exotoxin A, even when furin-cleaved, is blocked by pH-elevating drugs [47,48]. This toxin contains an ER-retention-like sequence that is absolutely required for its toxicity [49], and the low pH in the Golgi [50,51] may be important for intoxication. On the other hand, lack of low pH-induced translocation from the plasma membrane does not necessarily imply that a toxin is not translocated from endosomes; the translocation process may require factors that are only present in endosomes. In the case of the clostridial neurotoxin tetanus toxin, toxicity is inhibited by bafilomycin A1, which elevates endosomal pH [52]. It is likely that this toxin enters the cytosol from endosomes, as the toxic effect is not abrogated by brefeldin A, which disrupts the Golgi apparatus and inhibits the toxic effect of several toxins that are believed to enter the cytosol from the ER after transport through the Golgi apparatus [9].

Ricin, Shiga toxin, cholera toxin, and pertussis toxin seem to enter the cytosol via the Golgi and the ER. These toxins may take advantage of the existing translocation machinery in the ER membrane [53], such as the Sec61 translocon [54]. The ER-to-cytosol translocation is best understood in the case of ricin. When a signal for ER-associated glycosylation was introduced into the ricin A chain and the resulting toxin incubated with cells, a substantial fraction of the toxin transported to the Golgi apparatus was glycosylated [55]. Glycosylated molecules could also be recovered from the cytoplasmic fraction, indicating that the molecules had passed through the ER. Interestingly, part of the glycosylated ricin could be coimmunoprecipitated with anti-Sec61 antibodies [56*]. Also, when the ricin A chain was expressed and imported into the ER in yeast, mutations in the proteasome and mutations in Sec61 inhibited protein export out of the ER and caused a reduction in the degradation of the ricin A chain [57]. These findings indicate that ricin is translocated out of the ER by the machinery that is used for the disposal of misfolded proteins [54] and the toxic effect is provided by ricin molecules that are able to avoid degradation. This is further substantiated by the finding that the toxic effect of ricin is potentiated by the proteasome inhibitor lactacystin [56*]. The observation that the introduction of a disulfide bond into the ricin A fragment inhibits the toxic effect [58] suggests that unfolding of the toxin is required for translocation to the cytosol.

Conclusions

Over the last couple of years, considerable progress has been made in unraveling the mechanisms of toxin entry into cells. Recent discoveries indicate that several toxins either have the ability to penetrate membranes under certain conditions or to exploit translocation systems already present in the cell for traversing the cellular membranes. The low pH in endosomes triggers membrane penetration of several toxins, such as the anthrax and diphtheria toxins. The toxin receptors may be important for this translocation process. Other toxins, for example ricin, are transported all the way to the ER, where they utilize the translocation machinery of this organelle to enter the cytosol. However, many important questions remain to be answered, and for some toxins virtually nothing is known about their penetration mechanism. One of the major challenges for future research will be the identification of the cellular components involved in such processes.

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