Minireview

Transport of protein toxins into cells: pathways used by ricin, cholera toxin and Shiga toxin

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Abstract Ricin, cholera, and Shiga toxin belong to a family of protein toxins that enter the cytosol to exert their action. Since all three toxins are routed from the cell surface through the Golgi apparatus and to the endoplasmic reticulum (ER) before translocation to the cytosol, the toxins are used to study different endocytic pathways as well as the retrograde transport to the Golgi and the ER. The toxins can also be used as vectors to carry other proteins into the cells. Studies with protein toxins reveal that there are more pathways along the plasma membrane to ER route than originally believed. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Ricin; Cholera toxin; Shiga toxin; Endocytosis; Golgi apparatus; Clathrin

1. Introduction

A large group of bacterial and plant toxins bind to cells by one moiety whereas another moiety with enzymatic activity enters the cytosol and exerts the action (for review, see [1–5]). Most of these toxins have to be endocytosed before translocation to the cytosol. Several bacterial toxins, such as diphtheria toxin and anthrax toxin, enter the cytosol in response to the low pH found in endosomes, whereas other toxins, such as the plant toxin ricin, and the bacterial toxins cholera and Shiga toxin (their structures are shown in Fig. 1) are transported not only to endosomes, but retrogradely through the Golgi apparatus and to the endoplasmic reticulum (ER) before the enzymatically active part enters the cytosol (Fig. 2). This complicated intracellular routing makes the toxins useful as tools to study the pathways involved in this journey.

Several of the bacterial toxins are still a threat to human health [1,2,6–9]. Knowledge about the action of toxins provides us with new possibilities to prevent and cure infectious diseases. For instance, Shiga-like toxins secreted by *Escherichia coli* seem to be an increasing problem even in developed countries, and the toxin can cause renal failure and death, especially among children [6,8]. Knowledge about the toxin and its receptor has recently been used to create bacteria with Shiga toxin receptor mimics [10] and to design soluble molecules that might interfere with toxin binding [8,10], two approaches that might be used in therapy.

The toxins are also used as components in targeted drug treatment, for instance in cancer therapy. Since the toxins block protein synthesis very efficiently (about 2000 ribosomes are destroyed per minute after entry of one ricin molecule), directing toxins to cells for selective destruction is being tried, and promising clinical trials are being performed [11,12]. Also, a number of the protein toxins have been used as vectors to bring into cells proteins or epitopes that are then presented at the cell surface by MHC class I [13–17]. The toxins can therefore also be used for vaccination purposes.

In this article we will concentrate on the pathways used by ricin, cholera and Shiga toxin to gain access to the cytosol, and we will discuss the differences and similarities between the behavior of these toxins when exploiting the cell machinery on their way into cells.

2. Endocytosis of ricin, cholera and Shiga toxin

The plant toxin ricin which binds to both glycoproteins and glycolipids with terminal galactose and therefore binds all over the cell surface can be used as a membrane marker that is internalized by all endocytic mechanisms operating in a given cell [3,5]. Thus, ricin has proven valuable to study regulation of endocytic mechanisms. Different types of endocytosis can operate simultaneously, and an overview of such mechanisms is presented in Fig. 3. Several of the endocytic mechanisms are susceptible to cholesterol depletion. Not only do caveolae disappear upon removal of cholesterol from the plasma membrane (or upon addition of cholesterol-binding drugs such as filipin) [18,19], but also uptake from clathrin-coated pits [19–21] is inhibited after removal of cholesterol with methyl-β-cyclodextrin. Only flat clathrin-coated membrane areas can be visualized after such treatment. It is not known whether cholesterol is required in the invaginated clathrin-coated pit as a structural component, or whether it could be involved in signalling necessary for formation of this structure. Recent data reveal that even macropinocytosis is critically dependent on membrane cholesterol. When the level of cholesterol is reduced, membrane recruitment of activated Rac, actin reorganization and ruffling do not occur [22].

In contrast to ricin, cholera toxin and Shiga toxin bind to a defined receptor structure. Cholera toxin is a pentavalent toxin (Fig. 1) that binds to the glycolipid GM1, before it is

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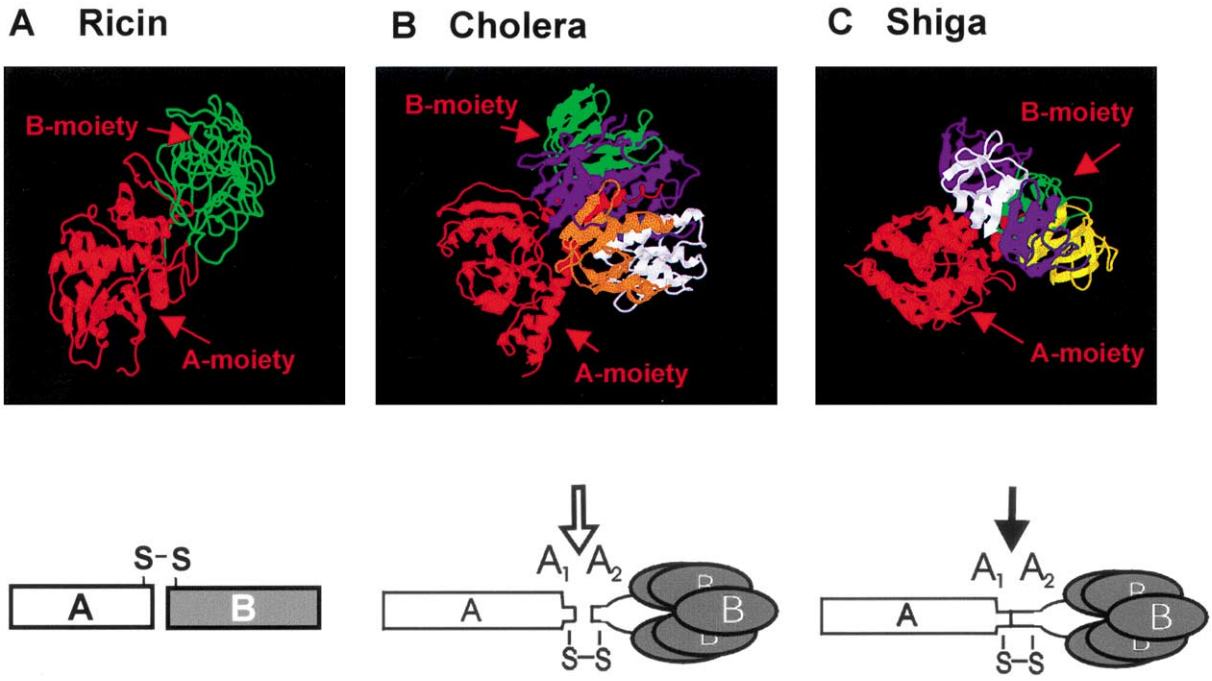


Fig. 1. Structures of ricin (A), cholera toxin (B), and Shiga toxin (C). At the top are shown the crystallographic structures of ricin (PDB protein data bank: 2AA1), Shiga toxin (PDB protein data bank: 1DMO) and cholera toxin (1XTC). Below are shown the schematic structures: ricin is not processed by the target cells; cholera toxin A fragment is cleaved by the bacteria producing the toxin (arrow); Shiga toxin A fragment is processed by the target cells (arrow). The enzyme that normally cleaves Shiga A is furin [45].

endocytosed [7]. The toxin can in many cell types be visualized in caveolae, which have been suggested to be involved in endocytosis of this toxin in different cell types. This may be the case in some cells such as endothelial cells, where there is evidence for a dynamin-dependent fission of caveolae [23,24]. However, cholera toxin is endocytosed also in cells without caveolae [25,26], and recent data have revealed that a large fraction of cholera toxin can be taken up by clathrin-dependent as well as clathrin- and caveolae-independent endocytosis in different cell types [25,27,28]. Actually, the toxin that is visualized in caveolae could be retained in these structures without being internalized, and this might be the reason for the high concentration of toxin–receptor complexes in caveolae. In fact, caveolae have in some cells been shown to be quite stable structures that do not pinch off unless they are stimulated to do so [29]. It should be noted that although cholera toxin binds GM1, the toxin can not automatically be used to determine the normal distribution of this lipid. The pentavalent binding, as well as the ability of the toxin to cause signalling, might very well affect the distribution of the toxin–receptor complex. Toxin-induced relocalization of GM1 has actually been demonstrated in different cell types [30,31]. Thus, the fact that genestein, a tyrosine kinase inhibitor, inhibits internalization of the cholera toxin B-subunit [32] does not necessarily mean that the endocytic mechanism used by cholera B is affected by genestein, it could also mean that the movement of the toxin into a given pathway involves kinase activity. In agreement with this idea is the finding that genestein inhibits uptake of cholera toxin in normal BHK cells, but after expression of antisense to clathrin heavy chain and complete inhibition of the clathrin-dependent pathway, it does not longer inhibit [25]. One interpretation of this finding is that there is a requirement for tyrosine kinase activity in the aggregation of cholera toxin into clathrin-coated pits, a process that is currently not understood.

Shiga toxin which like cholera toxin has five subunits (B) that bind to a glycolipid receptor, Gb3, is also endocytosed from clathrin-coated pits, although in some cells other endocytic mechanisms can be involved in endocytosis of the intact toxin or its binding subunits [2,9,28,33]. It should be noted that Shiga toxin can activate tyrosine kinases such as Lyn [34] and Yes [35] in some cell types. However, the mechanism behind the aggregation of Shiga toxin in clathrin-coated pits, and the possible cell type-dependent differences have not yet been elucidated. The extent of localization of Shiga toxin–receptor complexes to lipid rafts seems to be cell-dependent [36], and could be one factor giving rise to differences in endocytosis and intracellular sorting of this toxin. Interestingly, Shiga toxin and cholera toxin have been reported to colocalize in the same lipid rafts [37], but it is not known whether this is a general phenomenon and whether this reflects the original localization of GM1 and Gb3 in these cells. Thus, future studies designed to elucidate these mechanisms will provide us with information about the toxins and their exploitation of the cellular machinery, as well as about the properties of glycolipids and their localization in rafts.

3. Transport of ricin, cholera and Shiga toxin from endosomes to the Golgi apparatus

How do toxins move from endosomes to the Golgi apparatus? Studies of intracellular transport have revealed that there is more than one pathway leading from endosomes to the Golgi apparatus [33,38–41]. One well characterized pathway is the Rab9-dependent pathway from late endosomes to the Golgi apparatus, a pathway that is involved in transport

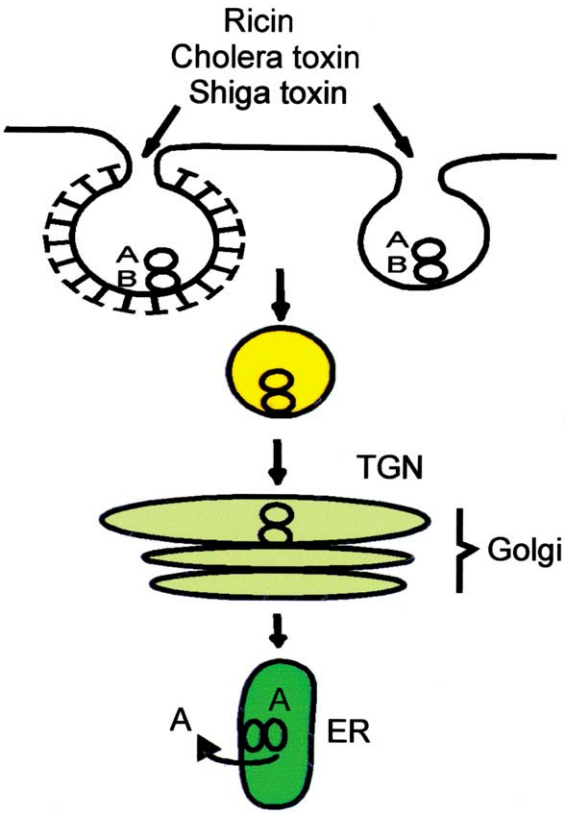


Fig. 2. The plant toxin ricin and the bacterial toxins cholera toxin and Shiga toxin all bind to cells before they are endocytosed, transported retrogradely to the Golgi apparatus and the ER, and then the enzymatically active subunit is translocated to the cytosol.

of the mannose-6-phosphate receptors [42,43]. Also, transport of furin, an enzyme responsible for the cleavage and activation of several protein toxins [44], including Shiga toxin [45], seems to be transported from the cell surface through late endosomes and to the Golgi apparatus [40]. However, transport to the Golgi apparatus of ricin [33,41], Shiga toxin [33], Shiga toxin B-subunit alone [38,46], as well as the Golgi marker TGN38 [39,40] seems to occur from an earlier endosomal compartment. This has been shown partly by microscopy, and partly by selective inhibition of the Rab9-dependent pathway by inducible synthesis of dominant negative mutants of Rab9 that will inhibit transport of the mannose-6-phosphate receptors to the Golgi. Evidence for transport of ricin from early endosomes to the Golgi and not from late endosomes by a Rab9-independent pathway was provided by experiments demonstrating that ricin transport to the Golgi also occurs independently of Rab7 [33]. When using microscopy to investigate routing of toxins, it is important to be aware of the fact that toxins can travel through a certain organelle without being visible, possibly due to a high exit rate compared to the entry rate into a given organelle. Another possibility is that the number of toxin molecules per membrane area becomes low due to a large membrane content of the organelle. For instance, ricin is transported to the ER, but has never been visualized by microscopy in this location.

How are toxins and other molecules transported from early endosomes to the Golgi apparatus? There may be more than one pathway: Shiga toxin B-chain transport has been reported to be dependent on the small GTP-binding proteins Rab11 and Rab6a′ [46], whereas ricin transport seems to occur independently of Rab11 [41]. Perhaps ricin, due to its ability to bind different types of molecules, can be transported by more mechanisms than a toxin such as Shiga toxin. Also, cholera toxin is transported rapidly to the Golgi [28,47], but again, the pathway is not well characterized. It has been suggested that this toxin can pass through endosomes that are different from the ones that contain transferrin, but the authors also suggested that the toxin might be rapidly sorted away from transferrin [28]. Detailed kinetic studies are required to determine whether lack of colocalization could be due to retention of the toxin in certain membrane domains or rapid segregation from other markers. Although the organelles and the regulatory mechanisms involved are not yet well characterized, different factors important for toxin transport to the Golgi have been found. The v/t-SNARE system seems to be involved both in the transport of TGN38 and Shiga B [46]. In the case of Shiga toxin, the lipid composition of the receptor seems to be important for transport of the toxin to the Golgi apparatus [2,9]. Also the cholesterol content of the cell may regulate the fraction of different toxins such as ricin [33,48], cholera toxin [49] and Shiga toxin B-subunit [36] transported in the direction of the Golgi apparatus. Furthermore, signalling via protein kinase A and Ca^{2+}/calmodulin is important for this transport of ricin to the Golgi [3]. Studies of toxin transport to the Golgi apparatus might provide us with knowledge about new pathways previously not characterized.

4. Retrograde toxin transport to the ER and translocation to the cytosol of the enzymatically active part of the toxin

One well characterized retrograde transport system in the Golgi apparatus involves the formation of COPI-coated vesicles which are responsible for transport of proteins with

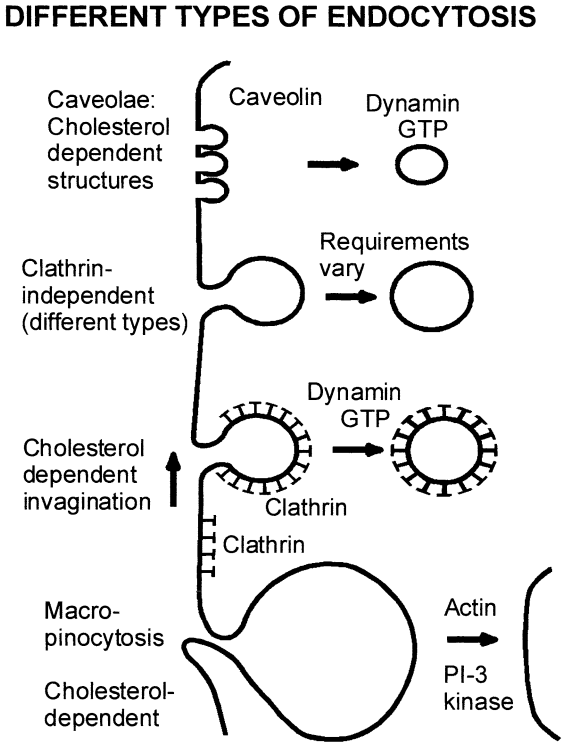


Fig. 3. Endocytic mechanisms operating in cells.

the KDEL sequence, an ER retention signal [50,51]. This sequence becomes bound to so-called KDEL receptors which are distributed throughout the entire Golgi apparatus [51]. Some protein toxins that contain a KDEL or a KDEL-like signal such as *Pseudomonas* exotoxin A seem to be transported retrogradely by this mechanism since antibodies to the KDEL receptor or saturation of KDEL receptors by overexpression of lysosome-KDEL will protect cells against this toxin [52]. Also mutations of the KDEL sequence inhibit toxicity [53]. Cholera toxin has a KDEL sequence in its A-subunit, and mutations in this sequence cause a delay in the action of the toxin, but not a complete inhibition of its action, suggesting that retrograde transport can occur independently of the KDEL receptor [54]. Furthermore, the cholera B-chain, which lacks a KDEL sequence, can be seen in the various Golgi cisterns, demonstrating retrograde transport which occurs independently of the A-subunit [55]. Importantly, neither Shiga toxin nor ricin has a KDEL sequence but are nevertheless transported from the Golgi apparatus to the ER before translocation to the cytosol [3]. How does this occur? One possibility would be that they are transported in COPI-coated vesicles without being bound to the KDEL receptor. It has been shown that even resident Golgi enzymes are transported in these vesicles, a finding in agreement with the maturation model of the Golgi apparatus [56]. However, even when COPI-dependent transport is inhibited by antibodies to COPI, retrograde transport of Shiga toxin transport occurs [57,58]. It was recently published that this retrograde transport occurs by a Rab6-dependent pathway [57,58], which might also be involved in retrograde ricin transport. However, this has not yet been investigated.

After entry of the toxins to the ER, ER-resident chaperones and enzymes can be involved in facilitating reduction of the internal disulfide bonds and preparation of the toxin A-subunit for transport into the cytosol by the Sec61p complex. This protein complex is normally involved in transport of newly synthesized proteins from the cytosol into the ER, but has also been shown to be required for transport of misfolded proteins (even with carbohydrates added) back to the cytosol where they are then ubiquitinylated and degraded by proteasomes [59–61]. In the case of cholera toxin it was demonstrated that after arrival of the intact toxin to the ER, the enzyme protein disulfide isomerase is involved in the reduction of the disulfide bond of the A-subunit, thus releasing the active A1 subunit, which can then be transported to the cytosol [62]. It has however not been demonstrated whether the intact A might be translocated to the cytosol and then reduced. The Sec61p complex seems to be involved in translocation of several toxins. Ricin A-chain [63], cholera toxin [64] and *Pseudomonas* exotoxin A [65] can all be found associated with this protein complex, and ricin A-chain transport through the complex has been demonstrated [66]. The details in the translocation process and the molecules involved in reduction of the internal disulfide bonds in ricin and Shiga toxin have not yet been characterized. It has also been suggested that even the binding subunits (the toxin B-chains) might become translocated from the ER to the cytosol [13,67]. However, this has not yet been directly demonstrated.

5. Conclusion

Protein toxins exploit intracellular transport to move all the way from the cell surface to the ER before they are translocated to the cytosol where they exert their action. Knowledge about the mechanisms involved in toxin transport and membrane translocation can provide us with increased understanding of transport in general, and may improve our possibilities to use the toxins in therapy, both for vaccination purposes and in targeted cell elimination. Clearly more work is needed to understand the action of these fascinating molecules.

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