FEBS *Letters*

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Review

Protein toxins from plants and bacteria: Probes for intracellular transport and tools in medicine

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ARTICLE INFO

ABSTRACT

A number of protein toxins produced by bacteria and plants enter eukaryotic cells and inhibit protein synthesis enzymatically. These toxins include the plant toxin ricin and the bacterial toxin Shiga toxin, which we will focus on in this article. Although a threat to human health, toxins are valuable tools to discover and characterize cellular processes such as endocytosis and intracellular transport. Bacterial infections associated with toxin production are a problem worldwide. Increased knowledge about toxins is important to prevent and treat these diseases in an optimal way. Interestingly, toxins can be used for diagnosis and treatment of cancer.

1. Introduction

A large number of bacterial toxins, such as Shiga toxin produced by *Shigella dysenteriae*, Shiga-like toxins produced by *Escherichia coli*, diphtheria toxin, *Pseudomonas* exotoxin A, and cholera toxin consist of two moieties, one that binds to the cell surface and another, enzymatically active part, that enters the cytosol and exerts the action (for review, see [1–7]). With exception of cholera toxin, all of the above-mentioned toxins inhibit protein synthesis. Similarly, a number of plant toxins have two moieties, one that binds to the cell surface and another that enters the cytosol, and like Shiga and Shiga-like toxins, they inhibit protein synthesis by removing one single adenine from the 28S RNA of the 60S ribosomal subunit [1,2]. This group of toxins comprises ricin, abrin, modeccin, viscumin and volkensin, all found in different plants [2]. The schematic and crystallographic structures of Shiga toxin and ricin are shown in Fig. 1.

Bacterial toxins are still a problem in connection with infectious diseases, and *E. coli* as well as other bacteria producing Shiga-like

toxins seem to be an increasing health problem also in modern societies [8–10]. These toxin-producing bacteria can be found associated with various types of food, especially in meat, but also on vegetables, in juices, water, etc. Ingestion of such toxin-producing bacteria can cause diarrhea, hemolytic uremic syndrome (HUS) and kidney failure, especially in children and in the elderly. Plant toxins may cause intoxication by accident, but can also be used for criminal purposes [11–13]. Although the toxins thus serve as a health threat in their original form, promising studies are being performed where their properties of binding to cells and penetrating into the cytosol are exploited to bring epitopes into cells, and in cancer diagnosis and therapy (for review, see [3,14–16]). One such product is currently on the market, a diphtheria conjugate binding via interleukin 2 to leukemia cells [17].

Studies of toxin transport have also, as described below, thrown light on a number of cellular processes such as the different endocytic mechanisms, and pathways of intracellular transport. More information about the role of toxin domains in receptor binding and membrane penetration will provide us with insight of how we can exploit the toxins for our benefit.

2. Binding of plant and bacterial toxins to cell surface receptors

Plant and bacterial toxins bind to a large variety of cell surface receptors, some toxins have strict requirements for binding and intoxicate only certain cell types [18]. For instance, Shiga toxin
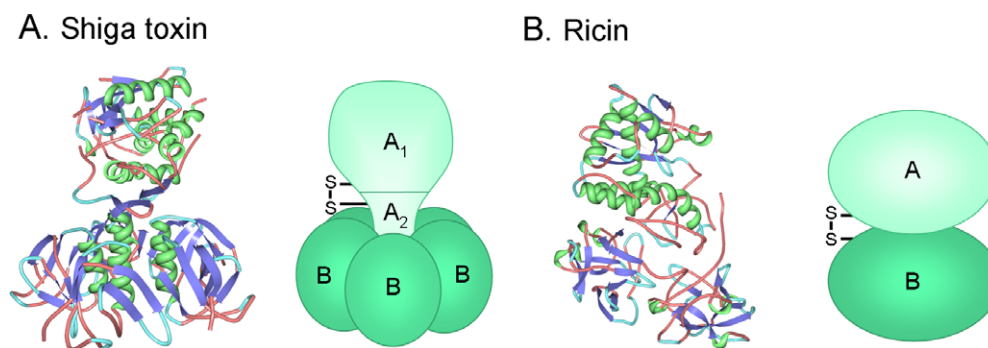
A. Shiga toxin

B. Ricin

Fig. 1. Structural and schematic models of Shiga toxin and ricin. (A) Stx (PDB protein data bank: 1DM0) consists of an A-moiety of ∼32 kDa, non-covalently attached to a B-moiety composed of five 7.7 kDa B-chains. During intracellular toxin transport the A-moiety is cleaved by furin into the enzymatically active A$_1$ fragment (∼27 kDa) and the carboxyl terminal A$_2$ fragment, which remain linked by a disulfide bond. (B) Ricin (PDB protein data bank: 2AAI) is composed of a binding moiety and an enzymatically active A-moiety (each of ∼30 kDa) linked together by a disulfide bond. The structure images were prepared by PDB ProteinWorkshop 3.6 [109].

and Shiga-like toxins bind to the neutral glycosphingolipid Gb3, which is expressed on only a few normal cell types, but on several types of cancer cells, a feature that may be exploited for imaging of cancer, and for therapeutic purposes (see below). Studies of the interaction of Shiga toxin with Gb3 reveal that the pentamer of B subunits in the toxin can bind up to 15 molecules of Gb3 [19]. However, these binding sites do not have equal affinity for the carbohydrates of Gb3 and may play different roles for the attachment of the toxin to the cell surface (a model is shown in [20]). Furthermore, toxin binding to Gb3 is unusually complex, as it is dependent on the type of fatty acid in the Gb3 molecule to which the toxin binds, and binding may occur more easily when there is a mixture of different Gb3 species [6,21]. Moreover, binding can be dependent on membrane phospholipids as well as the density of Gb3 and the cholesterol content of the membrane. It should be noted that although both Shiga toxin/Shiga-like toxin 1 (only one amino acid residue is different from Shiga toxin) and Shiga-like toxin 2 bind to Gb3, their dependency on Gb3 species (different N-amidated fatty acids) for binding differ. This may be important for their different toxicities on cells in culture and their different effects in humans. However, why Shiga-like toxin 2 seems more important for disease is not understood.

Importantly, the experimental system used to study toxin–Gb3 interactions, such as toxin binding to Gb3 on thin layer chromatograms, binding to liposomes or to cells, may give different results [6]. This is perhaps not so surprising since membrane constituents and fluidity are important for accessibility of the toxin to Gb3 and the ability of the toxin to crosslink Gb3 in the membrane. Changes in localization of Gb3, such as its localization to lipid rafts, would of course only occur in membranes, and due to the difference in membrane composition, one might also expect that results obtained from studies of liposomes/lipid membranes would differ from those obtained with cells. Also, in intact cells the toxin might affect lipid rafts and the cellular cytoskeleton in a way not obtainable in artificial membranes [22–24]. Interestingly, even liposomes and planar lipid membranes seem to differ [20,25]. It was recently found that Shiga toxin-induced tubule formation in liposomes occurred when the liposome contained either a semisynthetic C22:1 Gb3 or Gb3 purified from porcine kidney [25], but not when it contained Gb3 with C22:0. However, in planar lipid membranes C22:0 behaved like porcine Gb3, whereas Gb3 with C22-1 did not [20]. The reason for this apparent discrepancy is not obvious.

In contrast to Shiga toxin, the plant toxin ricin binds to and intoxicates most cell types (for review, see [2]). This plant toxin binds to both glycoproteins and glycolipids with terminal galactose, and there is no evidence for one type of productive receptor responsible for retrograde transport and intoxication. Clearly not only glyco-

sphingolipids can mediate toxicity, as cells are intoxicated with ricin even when glycosphingolipid synthesis is inhibited [26–28].

Both in the case of bacterial and plant toxins one may, by substituting the whole subunit responsible for binding with another receptor-binding protein, change the binding specificity to direct the toxins to certain cell types (for review, see [14,16,29–33]). However, one might want to keep part of the binding subunit if that improves the ability of the "new" toxin to penetrate into the cytosol and intoxicate the cell. For instance, studies of diphtheria toxin revealed that the B-chain, and even more specifically a portion of the B-chain is important for penetration of this toxin through the membrane [34]. Diphtheria toxin conjugates with altered binding specificities therefore contain this portion of the original molecule [17].

3. Endocytic uptake of protein toxins

The different protein toxins exploit a variety of the endocytic mechanisms that exist in a cell, and they might even increase their own uptake by signaling through kinases that affect molecules involved in endocytosis, by crosslinking surface molecules and by inducing changes in membrane curvature (for review, see [3,7,35–37]). While at the cell surface or after endocytic uptake, the toxins can become cleaved and activated by proteases such as furin that cycles between the cell surface, endosomes and the Golgi apparatus [38,39].

The view of today, that there are different endocytic pathways [35,36] (Fig. 2) is partly due to early studies with toxins. During many years, a quite general view was that endocytosis (with the exception of macropinocytosis and phagocytosis) occurred through clathrin-coated pits. However, experiments with ricin suggested that this toxin could enter by clathrin-independent endocytosis [40,41]. Both when clathrin-dependent uptake was blocked by removal of clathrin with hypotonic shock and potassium-depletion [40], or by "freezing" the coat at the cell surface by acidification of the cytosol [41], ricin was still endocytosed, demonstrating that it was taken up independently of clathrin. However, one argument was that when clathrin was no longer at the cell surface, perhaps the rest of the machinery normally working together with clathrin was sufficient to mediate uptake, and perhaps acidification caused some abnormal cell behavior. Such arguments are of course valid and could serve as a warning even today when siRNA allows us to remove molecules of interest and one might get upregulation of compensatory mechanisms. It was not until the finding that mutant dynamin blocked clathrin-dependent endocytosis whereas fluid was still taken up [42] that it was generally accepted that
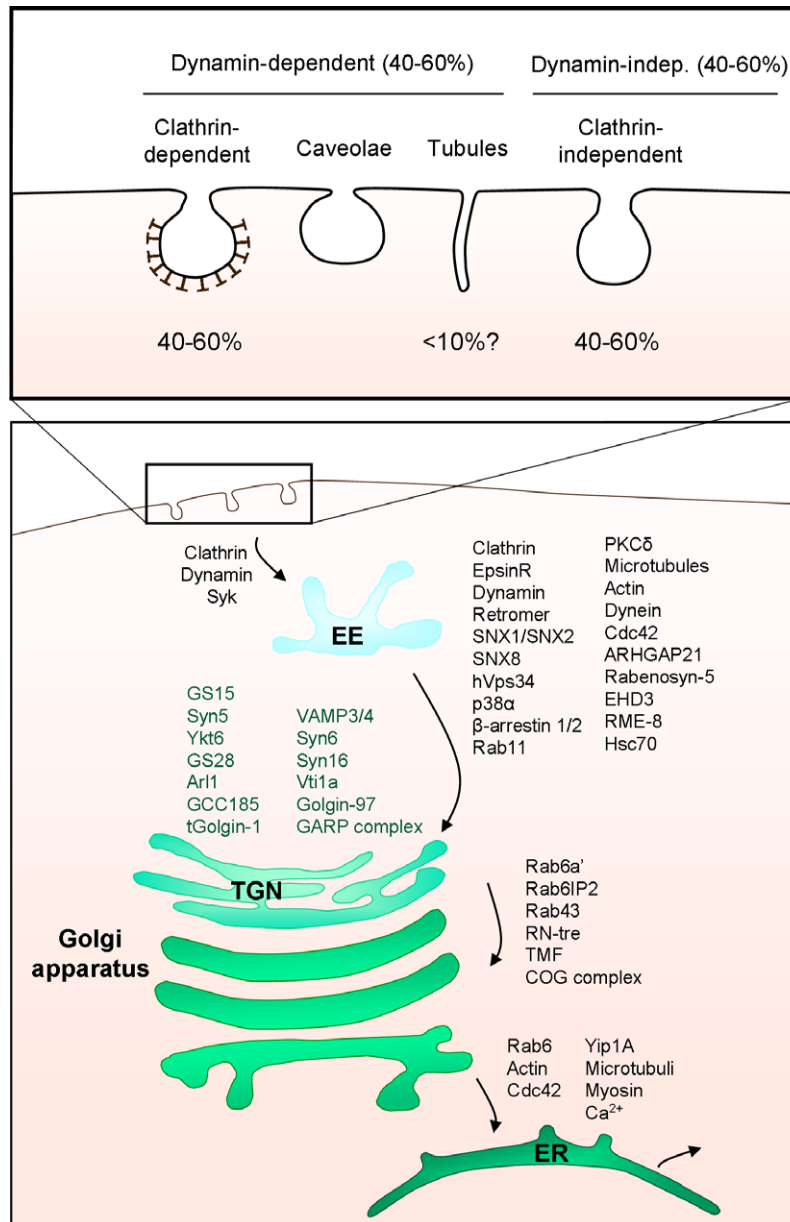
Fig. 2. Endocytic uptake and retrograde transport of Shiga toxin. The top panel shows an estimation of the amount of Shiga toxin entering by different pathways. See text for details. The lower panel shows an overview of components reported to be involved in the intracellular transport pathway of Shiga toxin. Upon internalization the toxin is sorted from the early endosomes (EE) into the retrograde pathway leading to the *trans*-Golgi network (TGN), and further to the endoplasmic reticulum (ER). From the ER the enzymatically active A_1 fragment is translocated to the cytosol where the 28S rRNA is targeted. The components highlighted in green are reported to be involved in the fusion of incoming vesicles at the TGN. For reviews see [3,4,7,56].

endocytosis as such is more complicated than previously acknowledged. It turns out that there are many challenges for studies of endocytosis. It was found that when mutant dynamin blocked clathrin-dependent endocytosis, fluid was not only internalized, but it was reported that fluid uptake was actually upregulated by other pathways [43]. Thus, the studies of today where we knockdown certain molecules by siRNA over a time-frame of 2–5 days or express mutant molecules at a level that leads to interaction with mechanisms normally not affected, certainly may induce upregulation of compensatory mechanisms that could give misleading information about the normal state of the cell.

The mechanisms for the different clathrin-independent ways of endocytosis are now being characterized in a number of laboratories, but several details are still lacking, not only about the molecules involved, but also about the relative importance of the pathways when it comes to uptake of fluid as well as uptake of lipid-binding ligands such as Shiga toxin and cholera toxin, which can be taken in by clathrin-dependent as well as dynamin-independent pathways ([44–46] and see below). Also, in most cases non-polarized cells are investigated, and we know that for instance in polarized cells the clathrin-independent uptake of ricin is under differential regulation at the apical and the basolateral pole [1]. In MDCK cells caveolae are, for unknown reasons, found only at the basolateral side, whereas clathrin-independent endocytosis at the apical pole is under regulation by signaling pathways that do not affect the basolateral uptake. Again, studies with ricin have been useful for elucidation of these mechanisms, and uptake of ricin by apical clathrin-independent endocytosis turns out to be regulated by protein kinase C, protein kinase A, cyclooxygenase, and calmodulin (for review, see [1]). Furthermore, the uptake was found to be RhoA-dependent [47].

As described above, ricin enters the cell by different endocytic mechanisms. What about the Gb3-binding Shiga toxin? One might think that since this toxin has only one type of receptor, only one endocytic mechanism could be used. However, this is not the case, and Shiga toxin can to a certain extent even mediate its own up-take. It was recently shown that the toxin can induce membrane tubules that pinch off in a dynamin-dependent manner [25], and it was described that these tubules seem to originate from caveolar structures [48]. However, this uptake mechanism may not contribute to any large extent to the total endocytic uptake [48] (Fig. 2). On the other hand, Shiga toxin can enter both by clathrin-dependent endocytosis (in spite of being bound to a glycolipid) and by clathrin- and dynamin-independent endocytosis. In fact, Shiga toxin induces an activation of Syk which in turn leads to clathrin-phosphorylation [49,50], and Syk seems to be important for endocytic uptake of the toxin. How, and if, clathrin-phosphorylation is important for Shiga toxin internalization is not yet clarified, and it is possible that phosphorylation of other molecules involved in endocytosis might be essential. In that connection, it is interesting to note that in Ramos cells Shiga toxin can activate Src [50], a kinase that was recently published to be involved in transferrin induced signaling leading to dynamin and cortactin phosphorylation and increased clathrin-dependent uptake of transferrin [51]. The clathrin- and dynamin-independent uptake of Shiga toxin is responsible for 40–60% of toxin uptake in Hela and BHK cells [45,52] (Fig. 2), but the mechanism(s) involved remain to be characterized. The fraction of toxin entering by different endocytic mechanisms is likely to be cell-type dependent as the Gb3 level and fatty acid composition of cellular membranes differ (Table 1). Probably the extent of Gb3 in rafts might determine signaling induced by the toxin as well as the ability of the membrane to form invaginations of different types. Studies from our own lab revealed that also the A-moiety of the toxin can affect the internalization [52]. With increasing toxin concentrations there is an A-chain dependent increase in toxin uptake by clathrin-dependent endocytosis. Thus, studies of toxin internalization may not give the same results as obtained when only the B-moieties are used, and one should keep in mind that the A-chain might affect transport also at a later step on the way to the cytosol.

4. Endosome-to-Golgi transport of Shiga toxin and ricin

4.1. Proteins involved in sorting of toxins to the Golgi apparatus

Protein toxins behave quite differently also when it comes to retrograde transport from endosomes to the Golgi apparatus. Not only are several of the toxins, such as ricin and Shiga toxin quite resistant to proteolytic enzymes, but they do in addition avoid lysosomal enzymes by being transferred directly from early endosomal compartments in the direction of the Golgi apparatus [53,54]. Microscopical studies revealed that Shiga toxin was rapidly transported to the Golgi apparatus from an early endosomal compartment [53], and ricin transport to the Golgi was found to be Rab9 independent as cells with inducible synthesis of dominant negative Rab9 showed no reduction in ricin transport to the Golgi [54]. On the other hand, although there are similarities between requirements for endosome-to-Golgi transport of these two toxins, there are many differences, suggesting that there may be more than one pathway between endosomes and the Golgi apparatus [3,4,7,27,55,56]. It is important to note that the involvement in Shiga toxin transport of some molecules such as clathrin, is more easily demonstrated at low toxin concentrations [45,57]. This suggests that the machinery for efficient Golgi transport may differ from what one can observe at higher toxin concentrations, which may allow the toxin to reach the Golgi apparatus via other, less efficient mechanisms. Some of the apparently contradictory results reported in the literature may result from the use of different toxin concentrations and of course from cell-to-cell differences.

A large number of endosomal proteins involved in Golgi transport of toxins are now known [4,7,58–60]. Common to both Shiga toxin and ricin is the importance of the PI3 kinase Vps34, involved in production of PI3P which serves as a docking site for many endosomal proteins, including sorting nexins (SNXs) [61]. Toxin transport have been found to be affected by SNX1, SNX2, SNX4 and SNX8, but differences between the toxins are observed [55,59,62–64]. The less characterized of these SNXs, SNX8, was recently found to be endosome-associated, and curiously, knockdown of SNX8 increases Golgi transport of Shiga toxin while ricin transport is slightly reduced [55]. The possible association of SNX8 with SNX1/SNX2-dependent transport needs to be investigated. Also cytoskeletal elements are reported to be involved in endosome-to-Golgi transport of toxins [23,24], and Rab-proteins and v-/t-SNAREs have been characterized as parts of this machinery [4,7,58]. Furthermore, it was recently reported that knockdown of the proteins RME-8 or Hsc70 affects retrograde transport of both Shiga toxin B subunit and the cation-independent mannose-6-phosphate receptor [65]. However, in spite of all the details we now have, it is still not clear how the many molecules involved can coordinate endosomal functions including sorting to the Golgi. For instance, are there parallel pathways, to which extent does sorting go via the recycling compartment, and how does the requirement for dynamin relate to the role of SNXs in Golgi transport?

4.2. Involvement of kinases in regulation of endosome-to-Golgi transport

Not only is there a large number of proteins and protein complexes involved in sorting in the direction of the Golgi apparatus, but the transport, at least that of Shiga toxin can be regulated by kinases. When protein kinase C delta is downregulated by siRNA, or blocked by addition of chemical inhibitors, transport of Shiga

Table 1
Percent of different fatty acid chain length of Gb3 species in various cell types.

Chain length	HEp-2[a]	HeLa[b]	HeLa[c]	XF-498[d]	SF-539[d]	SKOV3[d]	SKVLB[d]	HBMEC[e]	EA.hy 926[e]
24:1	50	8	30	7	9	15	1	30	30
Other 24	11	42	32	32	23	12	1	26	40
22	20	35	12	11	9	8	2	6	10
20	<3	–	–	1	1	2	1	–	–
18	<3	4	4	23	30	28	52	–	–
16	16	6	19	20	26	28	42	38	20

[a] Raa et al. [27]; MS analyses.
[b] Smith et al. [79]; MS analyses.
[c] Falguieres et al. [80]; MS analyses; numbers given are our estimates of the data shown for the plasma membranes in their Fig. 7.
[d] Arab and Lingwood [81]; fatty acid methyl esters quantified with chromatography following hydrolysis of Gb3.
[e] Muthing et al. [83]; the numbers given are our estimates based on the MS spectra presented in their Fig. 5.

toxin to the Golgi is reduced in HeLa cells [66]. Similarly, also when the kinase p38α is deleted by siRNA, the Golgi transport of Shiga toxin is reduced, and again, also inhibitors of this enzyme reduce the transport [67,68]. Interestingly, none of these enzyme-activities seem to be required for endosome-to-Golgi transport of the plant toxin ricin. The targets for these kinases are still not known. However, it has been found that Shiga toxin induces formation of a complex between p38 and arrestin [67], and there is a toxin-induced increase in endosomal association of both p38 and arrestin [67]. Interestingly, the increase in endosomal localization of arrestin is inhibited upon knockdown of p38, and together these results suggest that arrestin may modulate the effect of p38. Indeed, we found that downregulation of arrestin increased Golgi transport of Shiga toxin [67], but again, we do not have a complete understanding of the mechanisms involved.

4.3. Modulation of membrane lipids and effect on transport to the Golgi apparatus

Lipid rafts, membrane areas rich in cholesterol and glycosphingolipids with long, saturated fatty acids, are found both in the plasma membrane, in endosomal structures, and in the Golgi apparatus [69]. Rafts may serve as signaling platforms and are a prerequisite for structures such as caveolae [70,71]. However, cholesterol is also essential to obtain a certain thickness of the membrane and to ensure low permeability to ions, and membrane cholesterol can be essential to anchor molecules such as annexins to the membrane [72]. A common way to reduce membrane cholesterol in order to study its role for a certain process is to add methyl-β-cyclodextrin which extracts cholesterol from the membrane. Several years ago we found that addition of cyclodextrin reduces transport of ricin from endosomes to the Golgi apparatus [73], and similarly, this was found to be the case also for Shiga toxin transport to the Golgi apparatus [74]. These results lead to the suggestion that lipid rafts might be important for toxin transport from endosomes to the Golgi apparatus. However, although it might be the case for a toxin such as Shiga toxin that binds to a glycosphingolipid, this does not seem to be the case for toxins in general. It is important to note that in the case of ricin, toxin transport to the Golgi (measured as sulfation of a genetically modified toxin molecule) and retrograde transport to the cytosol (measured as intoxication), occurs equally well in cells unable to synthesize glycosphingolipids [28]. In these studies, a mouse melanoma cell line lacking glucosylceramide transferase (GM95 cells) as well as a back-transfectant (GM95-CGlcT-KKVK) were used. However, these cells do contain normal amounts of sphingomyelin, and since cholesterol depletion could inhibit Golgi transport also in glycosphingolipid-deficient cells, one might argue that ricin bound to glycoprotein receptors found in lipid rafts were important for Golgi transport and retrograde transport. To investigate lipid requirements in more detail, also cells with a general defect in sphingolipid synthesis were investigated. A CHO-cell line (LY-B cells) lacking serine palmitoyl transferase and the mutant with back-transfection of the enzyme, as well as the mother cell line were studied [26]. When the cells without serine palmitoyl transferase are incubated without serum for 2 days the sphingolipid level is reduced more than 70%. Interestingly, Golgi transport of ricin was increased in cells with reduced levels of sphingolipids suggesting that rafts might even restrict transport of ricin to the Golgi apparatus. Further retrograde transport of ricin to the endoplasmic reticulum (ER) and the cytosol, measured as toxicity, was also increased. Interestingly, cholesterol depletion could still reduce the transport between endosomes and the Golgi apparatus in cells depleted for sphingolipids, suggesting that cholesterol depletion might affect association of endosomes with cytoskeletal elements or other proteins of importance for transport or fusion. Addition

of sphinganine to the cells unable to synthesize this intermediate reversed the effect on ricin transport. Moreover, an inhibitor of serine palmitoyl transferase, myriosin, sensitized cells to ricin. Of interest in this connection is the finding that most of the ricin that was transported to the Golgi apparatus (sulfated ricin) was not associated with detergent-resistant membranes. Thus, in the case of ricin transport, there is no evidence for a requirement for lipid rafts for retrograde transport. Actually, also other protein toxins, such as abrin, modeccin and *Pseudomonas* exotoxin A had a larger toxic effect on LY-B cells lacking sphingolipids than on the back-transfectant with normal sphingolipids [26] suggesting that also their retrograde transport actually works better at low levels of sphingolipids.

In the case of Shiga toxin the type of fatty acids present in Gb3 may be important not only for cell surface binding and endocytosis, but also for Golgi transport [3,6,27]. The first observation of retrograde transport of Shiga toxin was made after incubation of A431 cells with butyric acid which changes the glycosphingolipid content in cells, and which in A431 cells allowed us to visualize Shiga toxin not only in the Golgi apparatus but also in the ER and the nuclear envelope [75,76]. Different types of experiments indicated that the changed transport was associated with a change in lipid composition. This was the first time that a cell surface-bound molecule was found to be transported all the way from the cell surface to endosomes, the Golgi, the ER and the nuclear envelope. Since then, similar transport has been demonstrated also for other toxins, such as cholera toxin and ricin, and for native cellular proteins [3,4,77,78].

We recently found that when synthesis of new glycosphingolipids was inhibited for a short time by PDMP or by fumonisin B1 in HEp-2 cells, there was a slight inhibition of endocytosed toxin (less than 40%) under conditions when Golgi transport was reduced more than 10 times [27]. During the incubation with inhibitors there was a selective disappearance of glycosphingolipids with certain fatty acids, the fastest reduction (probably due to sorting to lysosomes and degradation) was found for (glyco)-sphingolipids with the N-amidated fatty acid 16:0. However, whether this fatty acid somehow facilitates endosome-to-Golgi transport still needs to be investigated. Interestingly, under conditions where reduced Shiga toxin transport to the Golgi was seen, there was also a redistribution of SNX1 and SNX2, away from the Golgi apparatus. Since SNX1 and SNX2 are involved in Shiga transport to the Golgi, these findings are likely to be related to the observed change in toxin transport. Perhaps the lipid changes somehow alter the curvature of membranes and association with molecules containing BAR-domains [61]. Interestingly, PDMP and fumonisin B1 had no effect on ricin transport. Although ricin transport to the Golgi has been found to be partly SNX2-dependent [60] it is also SNX4-dependent [67], and reduction of ricin transport by one pathway may allow the toxin to be transported by another route. The effects obtained upon addition of inhibitors of glycosphingolipid synthesis could be cell line dependent as it was reported that in the HeLa cells studied by Smith et al. [79] such inhibitors lead to inhibition of Shiga transport at a stage after arrival to the Golgi apparatus.

Cell type dependent differences in intracellular transport should be expected, as the cellular lipid compositions vary. The overview of cell lines in Table 1 reveals large differences in the Gb3 species. One should be careful in interpreting small differences between these numbers, as different analytical methods were used. A quantitative standard for MS analysis of Gb3 species has not been available until very recently, and MS data have been restricted to estimating the ratio between different Gb3 species, but not to determine the exact concentration of these species. The remarkable large differences reported for HeLa cells in two different studies (Table 1) should be noted [79,80]. One may wonder if these

differences are due to different media or the serum used. Interestingly, similar data for Gb3 species in the plasma membranes and internal membranes of HeLa cells were reported [80], whereas the detergent-resistant fraction showed a 7% higher content of fatty acid 16:0 and a 10% lower content of fatty acid 24:1. There are several indications in the literature that Gb3 species with N-amidated fatty acid 16:0 is important for the toxicity of Shiga toxin. Arab and Lingwood [81] analyzed the Gb3 composition of two astrocytoma cell lines (XF-498 and the multi drug resistant SF-539) and two ovarian cell lines (SKOV3 and its multi drug resistant variant SKVLB). When comparing the drug resistant and drug sensitive cell line pairs, it was found that the drug resistant cell type was at least 1000 times more sensitive to Shiga toxin than the drug sensitive counterpart. The data in Table 1 show that the most sensitive astrocytoma cell line (SF-539) and the most sensitive ovarian cell line (SKVLB) both contained a higher fraction of the 16:0 fatty acid than the less sensitive corresponding cell lines (i.e. the astrocytoma cell line XF-498 and the ovarian cell line SKOV3). It was reported that SF-539 expressed slightly lower levels of Gb3 than XF-498 as analyzed with HPLC [81], whereas binding of Shiga toxin to thin layer chromatograms showed more binding to SF-539 than to XF-498 cells [82]. As discussed elsewhere, toxin binding does not necessarily correlate with the Gb3 level. Remarkably, the most sensitive ovarian cell (SKVLB) was reported to contain very low amounts of N-amidated fatty acids with 20 carbons or more (approx. 5% of total Gb3). HBMEC (human brain microvascular endothelial cells) and EA.hy 926 (a cell line derived by fusing human umbilical vein endothelial cells (HUVECs) to the lung epithelial cell line A549) seem to contain rather similar composition of Gb3 species (Table 1). For these cell lines, the much higher total amount of Gb3 species (as revealed by orcinol staining of thin layer chromatograms) in the EA.hy 926 cells compared to the HBMEC cells may explain the 1000x higher sensitivity of the EA.hy 926 cells to Shiga toxin [83,84].

In agreement with the idea that the types of fatty acids present in the membrane lipids are important for Shiga toxin transport, incubation of cells with poly-unsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) for 2 days was found to protect against Shiga toxin [85]. The protection was mainly due to reduced endosome-to-Golgi transport, but also endocytosis of the toxin was inhibited. In contrast to the protective effect of PUFAs against Shiga toxin, there was a sensitization to ricin and *Pseudomonas* exotoxin A. Clearly, not only proteins involved in transport to the Golgi vary for the different toxins, also various types of lipids have a differential effect on toxin transport.

5. Retrograde transport of Shiga toxin and ricin to the ER and the cytosol

Transport pathways of cellular proteins and lipids through the Golgi apparatus have been debated for decades, and even today, it is not clear to which extent different molecules are transferred by vesicles, by maturation of cisterns, or by tubular connections [86]. One of the best studied ways to bring molecules from the Golgi to the ER is via COPI-coated vesicles after binding to the KDEL-receptor. However, neither Shiga toxin nor ricin have such a sequence (KDEL), and Shiga toxin transport has been found to be COPI-independent, but dependent on actin and microtubules, Cdc42 and Yip1A [87–91–93]. Also, ricin transport to the ER seems to occur independently of COPI-coated vesicles [94]. This is in contrast to the toxin *Pseudomonas* exotoxin A which seems to depend on empty KDEL-receptors for its retrograde transport [95]. If these receptors are saturated by overexpression of the protein lysozyme-KDEL, toxicity of *Pseudomonas* exotoxin A is abolished. Also, when

the KDEL-receptor is redistributed out of the Golgi by activation of Src, cells are protected against *Pseudomonas* exotoxin A [96]. These findings illustrate the ability of the toxins to exploit different mechanisms. Importantly, it was recently shown that molecules that bind the KDEL-receptors can induce signaling which again can affect transport through the Golgi apparatus [97]. Some of the toxins might therefore affect also the transport through the Golgi apparatus. Clearly, constructs such as the Shiga B-subunit with a KDEL-sequence are not likely to follow the route normally used by Shiga toxin. A KDEL-containing molecule is most likely transported by the COPI-mechanism, and in addition it might change transport through signaling.

Translocation of toxins from the ER and into the cytosol can occur after reduction of the internal disulfide bond connecting the enzymatically active part of the toxin with the rest of the molecule, and several ER chaperones and components have been described to be of importance for translocation to the cytosol. For instance, HEDJ/ERdj3 and BIP [98] were found to be important for Shiga toxin transport, and EDEM seems to be involved in ricin transport [99]. Although both ricin [78] and Shiga toxin [98] were reported to form a complex with Sec61p, its role in translocation is not yet clarified.

6. Uptake of nanoparticles in cells: effect on transport of toxins

Protein toxins or rather their binding subunits can be used to direct nanoparticles to cells and to mediate their endocytosis. In a recent study we found that the ricinB:quantum dots (QDs) were effectively endocytosed, whereas the Shiga:QDs were internalized more slowly than Shiga toxin. Neither the Shiga:QDs nor the ricin:QDs were routed to the Golgi apparatus as would be the case for Shiga toxin and ricin, but they accumulated within late endosomes. Fig. 3 shows the intracellular localization of ricinB:QD bioconjugates in HeLa cells. Moreover, the toxins can also be used to test whether the internalization of nanoparticles affect normal transport. Remarkably, it turns out that internalization of quantum dots (QDs) may increase Golgi transport of Shiga toxin whereas it
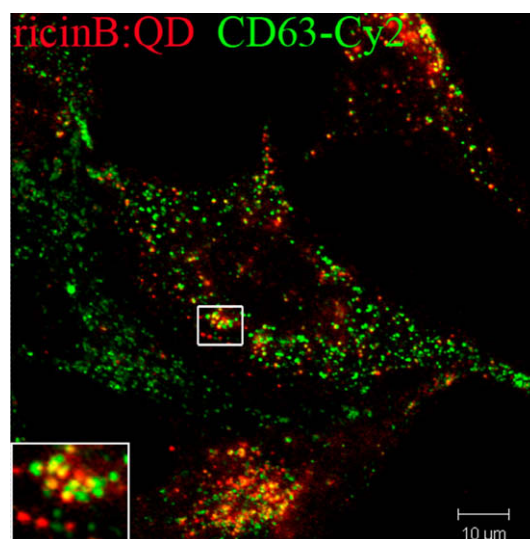

Fig. 3. Intracellular localization of ricinB:QD705 bioconjugates in HeLa cells. The QD bioconjugates were internalized by the cells for 3 hours at 37 °C. The cells were then fixed and prepared for immunofluorescence microscopy imaging by labeling them with antibodies against the lysosomal marker CD63, followed by the corresponding secondary antibody-Cy2 conjugate. The image shows the QD bioconjugates in red and CD63 stained with the Cy2 fluorophore in green. Yellow color indicates colocalization. The ricinB:QDs display partial colocalization (~30%) with CD63 after 3 h of internalization.

reduces retrograde ricin transport [100]. Thus, such studies of toxin transport can be useful to examine the consequences of particle internalization. In fact, this type of information is highly needed before intravenously injected nanoparticles can be commonly used in medicine. Future studies are required to learn if particles actually can be used to follow toxins on their retrograde journey.

7. Use of protein toxins in medicine

It is a very old idea that toxins can be directed to for instance cancer cells [18,101], either by coupling them to antibodies or by using ligands such as interleukins to direct the toxins to certain cell types [14,16,30,102]. In spite of many trials, the only approved product so far is a diphtheria toxin conjugate where interleukin 2 serves as the binding moiety [103–105]. However, as methodology is improving and more becomes known about entry mechanisms and the role of different parts of the toxin molecules, new improved drugs can be produced, and clinical trials are ongoing. The toxins may be used to bring in epitopes that produce an immune response leading to protection against cancer cells, and the binding part of Shiga toxin has been used to transport a prodrug against colon cancer to the ER where it is released by the reducing conditions and then transported to the cytosol [3]. Moreover, the toxin B-subunit can, following conjugation to porphyrins, also potentiate the toxic effect of these chemicals (for review, see [3]). Furthermore, the toxin can be used as a vector to bring DNA into cells [106]. Interestingly, the Shiga toxin receptor, Gb3, is to a certain extent overexpressed on cancer cells (compared to normal cells) [15,107,108], and (Engedal, N., Skotland, T., Torgersen, M.L. and Sandvig, K., submitted), and in the case of colon cancer cells it seems to be associated with the ability to metastasize [108]. This has raised the idea of using Shiga toxin (or its B subunit) for diagnosis of cancer cells, and the principle has been shown to work in murine models [3]. Thus, the future use of protein toxins in diagnosis and therapy might prove to be a useful aspect of these interesting molecules.

8. Conclusion

As discussed in the current review, studies of protein toxins are useful for several purposes. The toxins are still a problem in connection with infectious diseases, and they may be a threat as bioterror weapons. However, they serve as excellent tools to study cellular mechanisms, and increased knowledge about the toxins may give us new products for use in medicine, both when it comes to diagnosis and therapy of diseases such as cancer.

Acknowledgements

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