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Minireview

Endocytosis and intracellular sorting of ricin and Shiga toxin

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Abstract

Ricin and Shiga toxin belong to a group of protein toxins with targets in the cytosol. These toxins consist of one moiety that binds the toxin molecule to cell surface receptors, and another enzymatically active moiety that enters the cytosol after endocytic uptake of the toxin. The toxins are of current interest in relation to disease and the construction of immunotoxins. Moreover, they have proven useful to investigate mechanisms of endocytosis and to follow intracellular pathways of transport. Some of the recent results obtained with ricin and Shiga toxin are discussed.

Key words: Endocytosis; Ricin; Shiga toxin; Golgi apparatus; Endoplasmic reticulum

1. Introduction

The current research on protein toxins with intracellular targets is of considerable interest for several reasons. A number of the protein toxins are produced by bacteria and are important for severe diseases caused by these organisms. This is, for instance, the case for diphtheria toxin, although vaccination of the population has helped to control this disease, and for pseudomonas toxin, tetanus toxin, botulinum toxin, and Shiga toxin [1–4]. Shiga toxin is not only produced by *Shigella dysenteriae*, which is the infective agent in dysenteria, but Shiga-like toxins are also produced by *E. coli*, giving rise to infections and disease and being a serious health problem in several countries [1,2]. Knowledge about the toxins and their action on cells is important for the understanding of these diseases. Furthermore, protein toxins have long been used to construct immunotoxins and other toxin conjugates in attempts to find more efficient drugs in the therapy of cancer and other diseases [5]. Another important point is that the protein toxins are very attractive tools in modern cell biology, for instance, with respect to the study of protein translocation across membranes [1,6–9], protein internalization by endocytosis, sorting along the endocytic pathway, and exocytosis [3,4].

In this article we will focus on the internalization and intracellular transport of the plant toxin, ricin, and the bacterial toxin, Shiga toxin, two of the toxins studied by our groups. The schematic structure of these proteins is shown in Fig. 1. Ricin, which binds to both glycoproteins and glycolipids with terminal galactose and therefore binds to a large number of different molecules at the cell surface (3×10^7 binding sites per cell (HeLa cells)), has proven useful as a tool to follow membrane traffic in general [10–12]. On the other hand, Shiga toxin, a toxin which binds to certain glycolipids (Gb_3) [13] and is internalized from clathrin-coated pits [14,15], has given us insight into a new pathway in the cell, a pathway that leads all the way from the cell surface, through the Golgi apparatus and to the endoplasmic reticulum from where the toxin may enter the cytosol [7].

2. Mechanisms of endocytosis

Structural studies with ricin reveal that this molecule is bound all over the cell surface. This can be shown by EM, using ricin–gold conjugates or ricin bound to the enzyme, horseradish peroxidase (HRP) [10,11]. The endocytic uptake of ricin is relatively slow compared to e.g. the uptake of transferrin, a ligand which, after binding to its receptor, is internalized via clathrin-coated pits and vesicles. Based on EM studies with a ricin–gold conjugate it was clear that ricin also, at least in part, was located in clathrin-coated pits. This, however, did not exclude the possibility that a significant fraction of endocytosed ricin could be internalized by another mechanism. Depletion of cells for potassium in combination with a hypotonic chock strongly inhibited receptor-mediated internalization of LDL in human fibroblasts by removing clathrin-coated pits, or by preventing their formation [16]. Using a similar assay, we found that potassium depletion prevented internalization of transferrin, whereas ricin was still endocytosed, although at a reduced rate [11]. Also, the toxin was still able to intoxicate cells [11,17]. Another way to interfere with the function of clathrin-coated pits is to acidify the cytosol. When the cytosolic pH falls below 6.5 the clathrin-coated pits become paralyzed at the cell surface; they can no longer

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pinch off and form clathrin-coated vesicles [11,18]. This allowed us to look for other mechanisms of endocytosis that might not be affected by this treatment. Interestingly, in acidified cells ricin was still internalized at rates corresponding to 40–80% of the control value. Thus, in addition to the clathrin-dependent mechanism of endocytosis, a clathrin-independent mechanism is responsible for internalization of some molecules like ricin (see Fig. 2).

By using ricin we have provided evidence for independent regulation of clathrin-independent endocytosis. In some cells it can be reduced by cytochalasin D [11], whereas in A431 cells clathrin-independent endocytosis can be up-regulated by epidermal growth factor (EGF) and TPA, a tumor promotor and activator of protein kinase C [11,19a]. Under these conditions there is no increase in the transferrin uptake, which takes place from clathrin-coated pits [11,19a]. Recently we have also obtained evidence for the presence of clathrin-independent endocytosis both on the apical and the basolateral side of polarized MDCK cells [19b]. Interestingly, physiological stimuli, like cAMP, seem to give a selective increase in clathrin-independent endocytosis at the apical side of these cells, suggesting an important function for this process in a polarized epithelium.

The clathrin-coated vesicle and it's components have been studied in considerable detail [20]. The vesicles responsible for clathrin-independent endocytosis have so far not been isolated, and much less is known about this process. It seems likely that clathrin-independent endocytosis comprises more than one process since in some systems it gives rise to large vesicles [21], whereas in HEp-2 cells the pre-endosomal vesicles formed by clathrin-independent endocytosis has a diameter of 95 nm, i.e. somewhat smaller than the clathrin-coated vesicles in these cells [22]. On the other hand, caveolae, small omega-shaped structures (50–80 nm) at the cell surface, do not seem to be responsible for clathrin-independent endocytosis [23,24]. The main point, however, is that ricin is internalized by both clathrin-dependent and clathrin-independent mechanisms, and also the clathrin-independent pathway can lead to intoxication of the cells [10,11,17]. In HEp-2 cells both pathways seem to lead to the same, transferrin receptor-enriched endosome compartment [25]. However, although the pathways may merge in several cell types, this is not always the case. In ruffling A431 cells clathrin-independent endocytosis seems to give rise to vesicles that are able to fuse with each other and not with transferrin receptor-containing endosomes with low pH [26]. Also, when desmosomes are internalized by clathrin-independent endocytosis, the structures are not transferred to vesicles containing markers for late endosomes or lysosomes [27].

In contrast to ricin, Shiga toxin binds to a much more limited repertoire of cell surface molecules, namely neutral glycosphingolipids with terminal galα1–4gal [13].

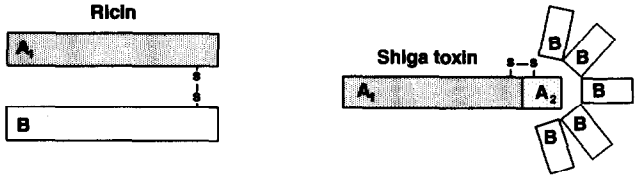


Fig. 1. Schematic structure of ricin and Shiga toxin.

Since endocytosis from clathrin-coated pits seems to require a cytoplasmic signal [20], and since it has been suggested that both tetanus toxin and cholera toxin, which both bind to negatively charged glycolipids, are endocytosed by clathrin-independent endocytosis [28], we suspected that Shiga toxin would be slowly internalized by clathrin-independent endocytosis. However, experiments with immunogold detection of cell surface-bound Shiga toxin revealed that this toxin, surprisingly, is internalized predominantly or exclusively via clathrin-coated pits [14,15]. This conclusion was supported by the finding that acidification of the cytosol prevented the endocytic uptake of the toxin. Aggregation of Shiga toxin in clathrin-coated pits may be due to interactions between the neutral glycolipid receptor and membrane or coat proteins, interactions strong enough to keep the glycolipid-receptor in clathrin-coated pits after cross-linking of the receptors by the multivalent toxin.

Once internalized by clathrin-dependent endocytosis Shiga toxin is delivered to endosomes which we assume, although we have not performed direct immunogold double labelings at the EM level, are identical to those reached by internalized ricin. At least, Shiga-HRP conjugates are delivered to an endosomal compartment with normal appearance in the EM [14,15].

3. Transport to the trans-Golgi network

From the endosomal compartment endocytosed molecules may be (i) recycled back to the cell surface from where they came, (ii) delivered to lysosomes for degradation, (iii) transcytosed to the opposite surface in polarized epithelia, or (iv) routed to the trans-Golgi network (TGN) (Fig. 2; for review, see [29]). We will not discuss the first two possibilities in the present review. However, transcytosis is relevant in relation to the application of e.g. immunotoxins. When ricin is internalized in polarized MDCK cells grown on filters, the toxin is actually transcytosed in an active form; it is still capable of inhibiting protein synthesis in other cells [30].

Since there is evidence that ricin and Shiga toxin (and a number of other toxins as well) are transported to the trans-Golgi network as a necessary step on their way to the cytosol [7,29,31] this transport has been studied by several methods. By using EM one can visualize transport of toxin–HRP conjugates to the TGN, and quantifi-

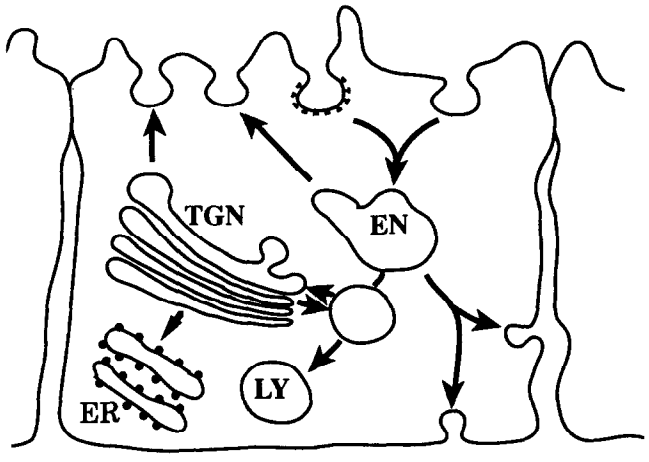


Fig. 2. Toxins are endocytosed both by clathrin-dependent and -independent pathways, and ricin and Shiga toxin are transported to the TGN. Shiga toxin is also transported through the Golgi apparatus and to the endoplasmic reticulum [7].

cation by immunogold-labeling shows that 5–10% of the endocytosed toxin actually enters the TGN in 1 h [29]. Similar results are obtained by using cell fractionation performed on cells incubated with radioactively labeled toxin [8,15]. It should be mentioned that gold-labeled ricin is not transported to the TGN [29]; this conjugate is exclusively routed to the lysosomes. Also, multivalent conjugates of ricin and HRP were similarly routed to lysosomes [29]. Thus, the valency clearly affects the routing, and it is therefore important to use monovalent conjugates.

There are several lines of evidence suggesting that the Golgi apparatus is important for intoxication. At lower temperature (below 18°C), when toxin transport to this organelle is inhibited [29,32], the cells are protected against ricin and Shiga toxin [29] but not against diphteria toxin, which is believed to enter the cytosol from endosomes upon acidification [33]. Also, hybridoma cells producing anti-ricin antibody are protected against the toxin, presumably because ricin meets the antibody in the Golgi apparatus [34]. Recent evidence for the involvement of the Golgi apparatus comes from the finding that brefeldin A, a drug that disrupts the Golgi apparatus, protects against several of the protein toxins, including ricin and Shiga toxin [8,35]. It might be argued that this protection could be due to inhibition of transport of newly synthesized proteins to another organelle. However, protein synthesis is not required for intoxication with ricin and Shiga toxin; rather, addition of cycloheximide to cells will make the transport to the cytosol more efficient [29]. Other results which support a role for the Golgi apparatus in the intoxication process are as follows: an amino acid sequence (KDEL) that is known as a retention signal for ER proteins was found to increase the toxicity of ricin A-chain [36]; and a similar sequence seems to be required for efficient intoxication with a related toxin, pseudomonas toxin [31,37]. Thus, although the exact location from where the toxins are translocated has not been determined, the Golgi apparatus seems to play a central role.

4. Retrograde transport from the Golgi to the ER

Speculations have been made that several of the protein toxins are transported to the ER before translocation to the cytosol [7,8,31,37]. However, not until recently was such transport demonstrated [7]. A431 cells normally bind and internalize Shiga–HRP, but they are resistent to the toxin. However, after treatment of the A431 cells with butyric acid these cells became sensitive to Shiga toxin. At the same time, transport of internalized Shiga toxin (Shiga–HRP) to all cisterns of the Golgi stack, the ER and even the nuclear envelope was observed [7], supporting the idea that transport through the Golgi apparatus to the ER may be important for intoxication, and for the first time demonstrating the existence of such a pathway (Fig. 2). More recently, a similar transport to the ER was demonstrated in cells originally very sensitive to Shiga toxin (Garred et al., submitted), showing that this pathway can operate also in non-treated cells. So far we have been unable to visualize transport of ricin to the ER. It is possible that so few molecules are transported to this location that the methods currently available are not sensitive enough to demonstrate such a transport. It should be noted that neither Shiga toxin [38,39] nor ricin [40] contain a KDEL sequence, however, the toxins may somehow interact with proteins with such a sequence.

5. Future directions

There are many important questions to be answered by future studies. How is clathrin-independent endocytosis regulated, and what are the roles of this pathway? Which sorting signals are required for retrograde transport of toxin through the Golgi apparatus to the ER? Furthermore, if the toxins are translocated to the cytosol from the ER, how does this occur? Is the translocation dependent on transport systems in the ER membrane, or some of the enzymes or proteins present in the ER lumen? What are the requirements for efficient translocation, and how much modification of the molecule can be tolerated by the translocation apparatus? These questions have to be answered to understand how the protein toxins exploit the cell machinery to reach the cytosol and kill the cells. Furthermore, the answers are important for further development of toxin conjugates designed to cure disease. Knowledge about the translocation machinery might also allow us to protect cells and organisms against the severe effects of the protein toxins.

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