Minireview

Endocytosis and intracellular transport of ricin: recent discoveries

Kirsten Sandvig[a,*], Bo van Deurs[b]

[a] *Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, Oslo, Norway*
[b] *Structural Cell Biology Unit, Department of Medical Anatomy, The Panum Institute, University of Copenhagen, Copenhagen, Denmark*

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Abstract The plant toxin ricin has proven valuable as a membrane marker in studies of endocytosis as well as studies of different intracellular transport steps. The toxin, which consists of two polypeptide chains, binds by one chain (the B-chain) to both glycolipids and glycoproteins with terminal galactose at the cell surface. The other chain (the A-chain) enters the cytosol and inhibits protein synthesis enzymatically. After binding the toxin is endocytosed by different mechanisms, and it is transported via endosomes to the Golgi apparatus and the endoplasmic reticulum before translocation of the A-chain to the cytosol. The different transport steps have been analyzed by studying trafficking of ricin as well as modified ricin molecules.

Key words: Ricin; Endocytosis; Endosome; Golgi apparatus; Endoplasmic reticulum

1. Introduction

Ricin belongs to a group of plant and bacterial protein toxins that bind to cells and inhibit protein synthesis enzymatically after entry of part of the toxin into the cytosol [1]. This group of toxins includes diphtheria toxin, Shiga toxin, *Pseudomonas* exotoxin A, abrin and modeccin. The enzymatically active moiety of ricin is able to inactivate about 2000 ribosomes per minute, and the entry of a few molecules into the cytosol will therefore have a dramatic effect on the cell [2]. Thus, toxicity serves as a very sensitive test system to monitor entry of toxin into the cytosol. The extreme toxicity of molecules such as ricin is also the basis for their use in construction of target-specific toxins. In these molecules either the enzymatically active subunit or a larger part of the toxin molecule is coupled to an antibody directed against certain cell types or to another cell surface binding molecule such as transferrin. Clinical studies of such hybrid toxins now show promising results [3,4].

Ricin has proven useful to study different endocytic processes [1,5,6]. Since it binds to both glycoproteins and glycolipids with terminal galactose, it labels the cell surface and will be endocytosed by any structure that pinches off (Fig. 1). In the case of endocytosis studies, ^{125}I-labeled ricin as well as horseradish peroxidase (HRP)-labeled ricin are useful markers. Moreover, by using these modified ricin molecules and cell fractionation as well as electron microscopy, we have also studied the transport of ricin from endosomes to the Golgi apparatus in non-polarized as well as in polarized cells. Recently, ricin molecules containing a sulfation site that can be modified in the *trans*-Golgi network (TGN) have proven valuable to quantify entry into the Golgi apparatus [5,7]. Furthermore, ricin molecules with both a sulfation site and glycosylation sites can be used to monitor retrograde transport through the Golgi apparatus and to the endoplasmic reticulum (ER), and ricin can therefore be used not only to study endocytosis but also to investigate transport between different organelles of the cell [5,7]. As discussed below, studies of toxin transport are not only useful to characterize already known transport routes in a cell, but they can also reveal new pathways.

2. Endocytic uptake of ricin

Early studies with ricin revealed that although the well-characterized clathrin-dependent endocytosis clearly is involved in uptake of ricin into endosomes, ricin can also be internalized by clathrin-independent mechanisms (for review, see [1]). Thus, acidification of the cytosol which inhibits formation of clathrin-coated vesicles from clathrin-coated pits in all cell types tested so far [8], as well as potassium depletion which removes clathrin-coated pits from the membrane in some cell types [9,10], blocked uptake of transferrin, whereas ricin endocytosis continued. More recent evidence for clathrin-independent endocytosis comes from studies where cells with inducible expression of mutant dynamin, which inhibits clathrin-dependent endocytosis, were used. In these cells both fluid and ricin were endocytosed upon expression of mutant dynamin [5,11]. Also, studies of cells expressing a dominant-negative fragment of clathrin heavy chain [12], thereby interfering with clathrin-dependent uptake, revealed the presence of clathrin-independent endocytosis.

Clathrin-independent endocytosis can clearly be different from uptake which might occur from caveolae, the small cholesterol-rich invaginations reported to pinch off and form vesicles in endothelial cells and some other cell types [13–17] (Fig. 1). Thus, there is clathrin-independent endocytosis in lymphocytes [18] which do not have caveolae [19], and there is a highly regulated form of clathrin-independent endocytosis on the apical side of polarized MDCK cells [20–22], in which all of the caveolae are found at the basolateral side [23]. The role of this interesting polarizaton of caveolae is not known. It should be noted that mutant dynamin has been reported to inhibit formation of vesicles also from caveolar structures [14,15], and the clathrin-independent endocytosis of ricin occurring in HeLa cells expressing mutant dynamin is therefore clearly independent of these structures. Also, the HeLa cells

*Corresponding author. Fax: (47) 22508692.
E-mail: ksandvig@radium.uio.no

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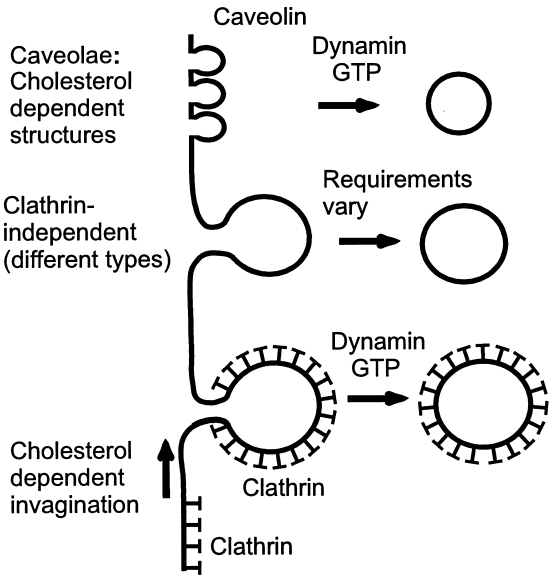

Fig. 1. Surface structures proposed to be involved in endocytosis.

with inducible synthesis of mutant dynamin have few or no caveolae that could have contributed to the uptake of ricin.

Caveolae are covered by caveolin on the cytosolic side of the membrane, and there is evidence that the two isoforms caveolin-1 and caveolin-2 are required for their formation [24]. It is an old observation that caveolar structure and function are dependent on membrane cholesterol [25]. Upon addition of filipin or other drugs that form complexes with cholesterol there are no invaginated caveolar structures at the cell membrane [26], and also, when cholesterol is removed by addition of cyclodextrin [27], caveolae are removed [6,28]. Remarkably, it turns out that the invagination of clathrin-coated pits is also dependent on cholesterol [6]. When HEp-2 cells, MDCK cells, A431 cells or NIH3T3 cells are treated with methyl-β-cyclodextrin to selectively remove cholesterol from the membrane, not only are caveolae affected, but also transferrin endocytosis is strongly inhibited. Detailed morphological studies of the HEp-2 cells revealed that after cyclodextrin treatment few invaginated clathrin-coated pits could be seen, suggesting that there is an inhibition of invagination of this structure. The concentration of the transferrin receptor in the clathrin-coated areas of the membrane was the same in control cells and in cyclodextrin-treated cells. Interestingly, the flattened coated pits in cholesterol-depleted cells were not distributed at random but appeared in microdomains of two to four, suggesting that preexisting clathrin assemblies on the membrane act as nucleation sites for the binding of clathrin and formation of new coated pits (Fig. 2). It is, however, not clear whether cholesterol is required in the clathrin-coated invaginated structure or whether it is required for signaling involved in inducing formation of this structure. Importantly, endocytosis of ricin continues in cells where the function of clathrin-coated pits and caveolae has been perturbed by extraction of cholesterol with cyclodextrin, providing yet another demonstration of endocytosis which is independent of both clathrin and caveolae, and which functions with a lower concentration of cholesterol in the membrane than the two other pathways.

What sort of mechanisms are involved in clathrin- and caveolae-independent endocytosis? The data so far suggest that there is more than one mechanism involved. In addition to macropinocytosis (formation of relatively large vesicular structures where the vesicle formation is dependent on PI-3 kinase [29]) which we will not go further into in this short review, we have already discussed that there is clathrin-independent endocytosis of ricin which is independent of dynamin. It has, however, been reported that the clathrin-independent endocytosis occurring after induced exocytosis in some cell types is dependent on dynamin [30]. Clathrin-independent endocytosis can be differentially regulated at the apical and the basolateral side of polarized cells [20–22]. However, the physiological role of such a regulation is not known. In polarized MDCK cells apical clathrin-independent endocytosis can be upregulated both by activation of protein kinase A and C, by heterotrimeric G proteins and by calmodulin antagonists without any concomitant effect on the basolateral endocytosis. Remarkably, recent results demonstrate that the apical clathrin-independent endocytosis, in contrast to other endocytic processes described so far, functions in the presence of GTPγS, and that it is regulated by Rho proteins (Ø. Garred, K. Rodal, B. van Deurs and K. Sandvig, submitted). Whether there is a similar regulation at the basolateral side is not yet known. Also in other cells a regulation of the clathrin-independent uptake has been shown. In HeLa cells expressing mutant dynamin there seems to be an upregulation of the clathrin-independent endocytosis [31,32]. Impressively, the total fluid and ricin uptake in these cells seems to end up at the original level after this upregulation. It is unknown how the cells are able to control the level of membrane taken in under

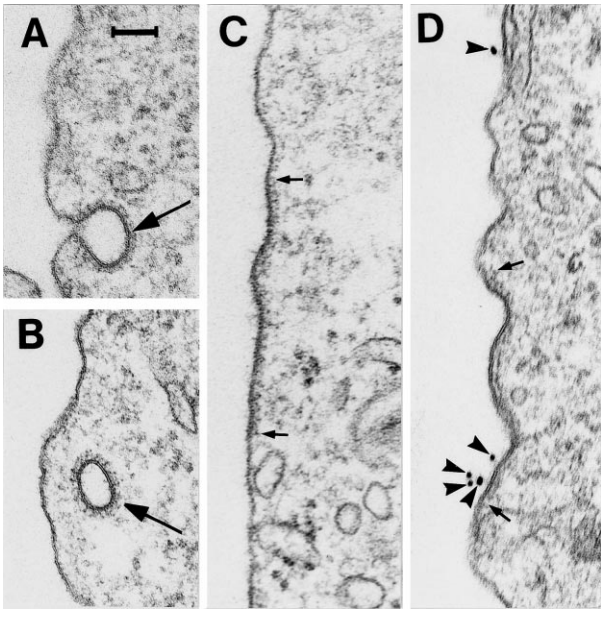

Fig. 2. Effect of methyl-β-cyclodextrin on clathrin-coated pits. Arrows in A and B show a deeply invaginated clathrin-coated pit and a coated vesicular profile as seen in control cells. C shows a flat (non-invaginated) region of the plasma membrane with clathrin assembly (between small arrows) following cholesterol extraction with methyl-β-cyclodextrin. D shows a flattened area of the plasma membrane associated with clathrin (between small arrows) from another cyclodextrin experiment where the cells were labeled with immunogold [6] to demonstrate that transferrin receptors (arrowheads) are still present in the flattened clathrin-coated pits found in cells after cholesterol extraction. Bar, 100 nm.

the different conditions. It is therefore difficult to estimate the quantitative role of the different endocytic mechanisms. Conditions that interfere with one process, such as the ability of cytosolic acidification to inhibit uptake from clathrin-coated pits [8], might on the one hand also have some inhibitory effect on other endocytic processes, on the other hand one cannot exclude that the inhibition of one process upregulates the other. Anyway, cytosol acidification reduces ricin endocytosis to about 50% in a number of different cell types.

A large number of physiologically important molecules are known to enter by clathrin-dependent endocytosis (for review, see [33]), but there are also physiologically important molecules entering by clathrin- and caveolae-independent endocytosis. The first example published was the uptake of interleukin-2 in lymphocytes [18]. Now there are other examples as well: angiotensin [34], M2 muscarinic receptors [35] as well as D2 dopamine receptors (in contrast to D1 dopamine receptors) [36] are reported to be internalized by a dynamin-independent endocytic process. The appearance of physiologically important molecules entering by this mechanism (or these mechanisms) might facilitate the investigation of the process as such. We would like to stress that also the clathrin-dependent endocytosis might turn out to consist of more than one mechanism [37] and be differentially regulated [38]. Also with respect to the regulation of clathrin-dependent endocytosis there seem to be differences in its regulation at the apical and basolateral side in a polarized cell [38].

3. Transport of ricin from endosomes to the TGN

After endocytosis ricin is transported through the endosomal system to lysosomes, and the toxin can also be found in the Golgi apparatus [1]. As mentioned in Section 1, ricin transport to this organelle can be monitored by cell fractionation after incubation with ^{125}I-labeled ricin, by electron microscopy of cells labeled with HRP-ricin, or by sulfation of ricin modified to contain a sulfation site. Such studies have revealed that mutant dynamin inhibits ricin transport from endosomes to the Golgi apparatus suggesting that dynamin-2 or a dynamin-like molecule is involved in this transport step [5]. The studies also show that ricin transport to the Golgi apparatus occurs independently of low endosomal pH [1]. Thus there is no requirement for a low pH-dependent formation of any carrier vesicle which has been reported to be involved in transport from early to late endosomes in some cell types [39]. It should, however, be noted that transport from early to late endosomes may occur by maturation [40]. In for instance HEp-2 cells [41], internalized ligands can be transported to late endosomes in the presence of bafilomycin which will inhibit the endosomal proton pump and increase the pH in these compartments [41]. Not only is ricin transport to the Golgi apparatus occurring in the absence of low endosomal pH, ricin transport to the Golgi apparatus can actually be enhanced by addition of monensin which will abolish the low endosomal pH [1]. Furthermore, ricin transport to the Golgi apparatus can be regulated by cAMP [42] and by calmodulin inhibitors [22]. It should be noted that the amount of ricin entering the Golgi apparatus of polarized MDCK cells is differentially regulated depending on whether ricin is endocytosed from the apical or the basolateral pole of the cells [1,22,42]. These findings are in agreement with the idea that ricin might enter the Golgi apparatus by another route than the only route from endosomes to the Golgi apparatus that has been characterized so far, the Rab9-dependent transport from late endosomes [43]. It has recently been suggested that Shiga toxin [44] and TGN38 [45] move directly from the perinuclear recycling compartment to the TGN, without involvement of late endosomes. This conclusion was partially based on the finding that none of the molecules could be visualized in late endosomes (Fig. 3). However, this does not mean that they are not transported through this organelle at a concentration which is too low to be seen by microscopy. For instance, ricin has never been observed by microscopy in the ER, but by using the modified ricin that contains a glycosylation site, ricin localization in the ER can nevertheless be demonstrated by measuring glycosylation of ricin entering this compartment [5,7]. One way to study whether ricin is routed to the Golgi apparatus via the Rab9-dependent pathway from late endosomes is to express mutant Rab9 in an inducible manner to block the Rab9-dependent transport [43]. Results from such experiments support the idea that ricin actually enters the Golgi apparatus independently of Rab9, but at present one cannot say whether the Rab9-independent transport occurs from late endosomes or from an earlier compartment (T.-G. Iversen, A. Llorente, P. Nicoziani, B. van Deurs and K. Sandvig, submitted).

4. Retrograde transport of ricin to the ER and translocation to the cytosol

The first toxin found to be transported all the way from the cell surface, through endosomes and the Golgi apparatus and to the ER was the glycolipid binding bacterial toxin Shiga toxin [46,47]. Later on a similar retrograde transport was shown for cholera toxin [48] and ricin [7]. In the case of ricin it was demonstrated that toxin molecules with glycosylation sites that were modified in the ER were actually transported to the cytosol. Remarkably, in contrast to cholera toxin [49], neither ricin [50] nor Shiga toxin [51–53] has a KDEL sequence that might facilitate the retrograde transport to the ER. It is not known, and clearly a goal for future studies to investigate, whether the retrograde transport of these molecules occurs in the same way as those with a KDEL sequence. When finally arriving in the ER it is still unknown how the toxin molecules enter the cytosol. However, the recent studies of transport from the ER to the cytosol by the sec61 complex [54] make it tempting to suggest that toxins might exploit this mechanism to gain access to the cytosol. Once in the cytosol they can then exert their final action: both Shiga toxin and

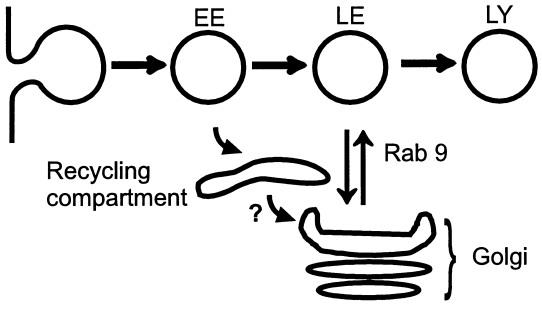

Fig. 3. Transport routes between endosomes and the Golgi apparatus. EE, early endosomes; LE, late endosomes; LY, lysosomes.

ricin inactivate the 60S subunit of the ribosomes [2] and thereby they kill the cells.

5. Conclusions

Studies of uptake and intracellular transport of ricin are useful to elucidate the various endocytic mechanisms. Clearly we need to learn more about the clathrin-independent mechanisms. Why do the cells have these mechanisms and why and how are they regulated? Studies of ricin transport in the retrograde directions to the Golgi and the ER can furthermore provide us with information about these transport steps, and might even reveal new pathways not easily discovered by studies of endogenous molecules. Investigations of protein toxins as such might also facilitate construction of molecules that could be beneficial in treatment of disease.

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