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Role of Lipids in the Retrograde Pathway of Ricin Intoxication

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The plant toxin ricin binds to both glycosphingolipids and glycoproteins with terminal galactose and is transported to the Golgi apparatus in a cholesterol-dependent manner. To explore the question of whether glycosphingolipid binding of ricin or glycosphingolipid synthesis is essential for transport of ricin from the plasma membrane to the Golgi apparatus, retrogradely to the endoplasmic reticulum or for translocation of the toxin to the cytosol, we have investigated the effect of ricin and the intracellular transport of this toxin in a glycosphingolipid-deficient mouse melanoma cell line (GM95), in the same cell line transfected with ceramide glucosyltransferase to restore glycosphingolipid synthesis (GM95-CGlcT-KKVK) and in the parental cell line (MEB4). Ricin transport to the Golgi apparatus was monitored by quantifying sulfation of a modified ricin molecule, and toxicity was studied by measuring protein synthesis. The data reveal that ricin is transported retrogradely to the Golgi apparatus and to the endoplasmic reticulum and translocated to the cytosol equally well and apparently at the same rate in cells with and without glycosphingolipids. Importantly cholesterol depletion reduced endosome to Golgi transport of ricin even in cells without glycosphingolipids, demonstrating that cholesterol is required for Golgi transport of ricin bound to glycoproteins. The rate of retrograde transport of ricin was increased strongly by monensin and the lag time for intoxication was reduced both in cells with and in those without glycosphingolipids. In conclusion, neither glycosphingolipid synthesis nor binding of ricin to glycosphingolipids is essential for cholesterol-dependent retrograde transport of ricin. Binding of ricin to glycoproteins is sufficient for all transport steps required for ricin intoxication.

Key words: cholesterol, endocytosis, glycosphingolipids, rafts, ricin

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In the present article we have investigated the importance of glycosphingolipids (GSL) for retrograde transport and action of ricin. Ricin can bind to GSL and glycoproteins with terminal galactose (1), and recent data reveal that lipid rafts which are enriched in GSL are important for endocytosis and intracellular transport (2). Thus, the possibility existed that GSL and not glycoproteins were required for ricin intoxication.

Ricin consists of two polypeptides joined by a disulfide bridge [for review see (3,4)]. In addition to the B-subunit which binds to terminal galactose, ricin has an enzymatically active A-subunit which inactivates ribosomes by depurination of a specific adenine residue on the 28 S rRNA. In order for ricin to intoxicate cells, the toxin first binds to the cell surface, then it is endocytosed, and subsequently it is transported retrogradely in a cholesterol-dependent manner to the Golgi apparatus and then to the endoplasmic reticulum (ER), before the A-subunit is translocated to the cytosol, presumably through the Sec61p protein complex (3–5). In this study, toxin transport in a mouse melanoma cell line unable to synthesize GSL due to lack of ceramide glucosyltransferase has been compared with ricin transport in cells where the enzyme has been back-transfected (6,7). Furthermore, we have studied whether there is a cholesterol-dependency of Golgi transport of ricin also in the cells without GSL.

Lipid rafts present both in the plasma membrane and in intracellular organelles seem to play an important role in endocytosis of surface-bound ligands and in intracellular sorting [for review see (2,8)]. The rafts are enriched not only in GSL but also in cholesterol (2), and by lowering or increasing the cholesterol level in the cells, transport processes dependent on lipid rafts can be affected. By removing cholesterol, for instance by using methyl-β-cyclodextrin (mβCD), segregation of GSL to rafts will be abolished, and their transport into the cell or between cellular organelles might be affected (2). The uptake of some ligands but not others is inhibited by reduced cholesterol (8–10). Whether such differences reflect the association of the receptor with lipid rafts, or whether it is due to a direct requirement for cholesterol in the formation of a vesicle is often not known. Disruption of rafts by removal of cholesterol might also inhibit membrane recruitment of cytosolic proteins involved in transport (11,12).

Transport from endosomes to the Golgi apparatus of protein toxins such as ricin might be dependent on GSL in lipid rafts. Endosome to Golgi transport of the plant toxin ricin was shown to be strongly inhibited both by decreasing and by increasing the level of cholesterol (1,8), and the same

was later found to be the case for the GSL-binding toxins cholera toxin (13) and Shiga toxin (14). One might therefore speculate that although ricin is able to bind to glycoproteins, GSL could be essential for mediating transport to the Golgi apparatus. In the case of Shiga toxin it was reported that association of the toxin–GSL receptor complex with lipid rafts was required not only for Golgi transport, but also for retrograde transport to the ER. This transport seems to occur by a COPI-independent, Rab6-dependent transport route (14,15). Similarly to ricin, Shiga toxin does not contain a KDEL-sequence that can facilitate retrograde transport mediated by COPI-dependent transport (1,3,4). Thus, in analogy with Shiga toxin, the possibility existed that also retrograde transport of ricin and therefore also toxicity of ricin, would require binding to GSL and their presence in lipid rafts. It should be noted that GSL might not only be important as toxin-binding sites, but GSL synthesis could be essential for recruitment of cytosolic factors (e.g. coat proteins) required for a certain transport route or for signaling affecting transport (6,12). Thus, even retrograde transport of ricin bound to glycoproteins could require GSL synthesis. Although the intracellular transport of ricin has been studied quite extensively, it was still an open question whether GSL were essential.

Results

Effect of ricin on protein synthesis in mouse melanoma cells with and without glycosphingolipids

To investigate a possible role of GSL as binding sites for ricin or as necessary membrane constituents along the pathway used by ricin on its journey to the cytosol, we have measured the toxic effect of ricin on the following cell lines: (a) cells without ceramide glucosyltransferase and as a consequence of this without GSL (GM95 cells) (7); (b) the same cells transfected with ceramide glucosyltransferase with an ER retention signal (GM95-CGlcT-KKVK) (6); (c) a mock-transfected cell line (GM95-mock); and (d) the parental cell line (MEB4). GM95 and GM95-mock do not have detectable levels of GSL while GM95-CGlcT-KKVK have a GSL synthesis close to wild-type levels (6).

The results from 3-h toxicity assays are shown in Figure 1(A). The GM95 cells were actually more sensitive to ricin than the parental cell line MEB4, possibly due to clonal differences in the MEB4 cells. However, the important point is that the sensitivity of the GM95-CGlcT-KKVK cells was about the same as that of the cells without GSL (GM95). The mock-transfected cells were somewhat more sensitive to ricin. Results obtained after long-time incubation (18-h) were in agreement with the 3-h assay (Figure 1B). These results suggest that GSL are not essential for any of the steps involved in ricin transport to the cytosol. Due to the possible clonal differences in the mother cell line, MEB4, the rest of this study focuses on the GM95 cell lines.

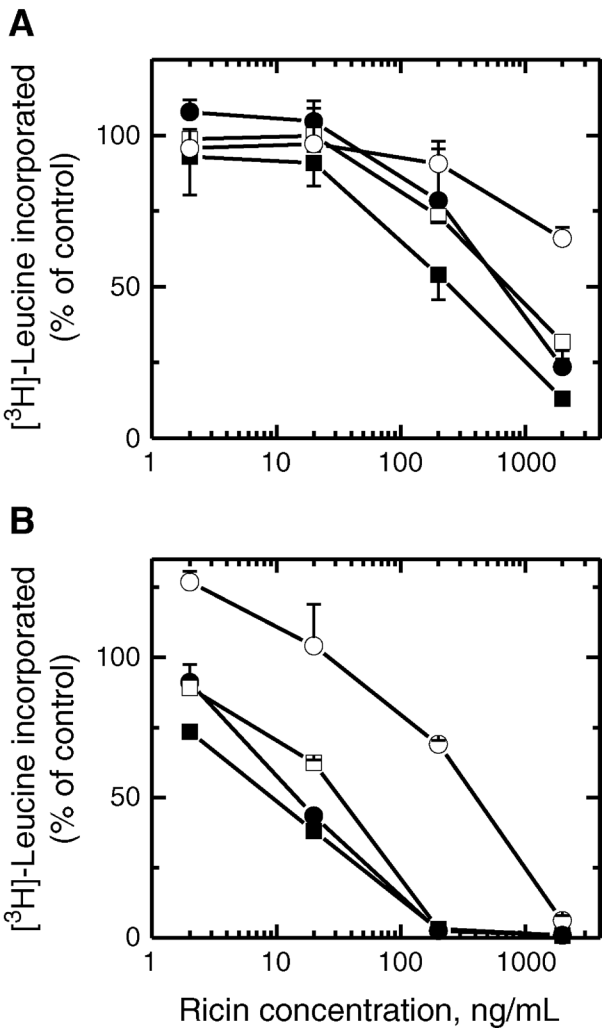

Figure 1: Toxicity of ricin in cells with and without GSL. GM95 (●), GM95-CGlcT-KKVK (□), GM95-mock (■) and MEB4 (○) cells were exposed to increasing amounts of ricin for 3 h (A) and 18 h (B) before the ability to synthesize protein was determined as described in Materials and Methods. The error bars show standard deviations between triplicates (A) and deviations between duplicates (B) of a typical experiment.

The pattern of GSL found in GM95-CGlcT-KKVK is similar to that found in MEB4. In both cases the ganglioside GM_3 is the GSL expressed at the highest level (6). GM_3 does not have a terminal galactose and is therefore not expected to bind ricin. To transform GM_3 to lactosylceramide, which can bind ricin, we treated the cells with neuraminidase. This enzyme removes sialic acid from both GSL and glycoproteins. The neuraminidase treatment resulted in a 3-fold increase in ricin binding in both GM95 and GM95-CGlcT-KKVK cells (Figure 2A vs. 2B). However, both before and after neuraminidase treatment the binding of ricin to the different cell lines was the same (Figure 2). Also, even after such treatment, there were no large differences in the toxicity of ricin on these cell lines (Figure 3). The increase

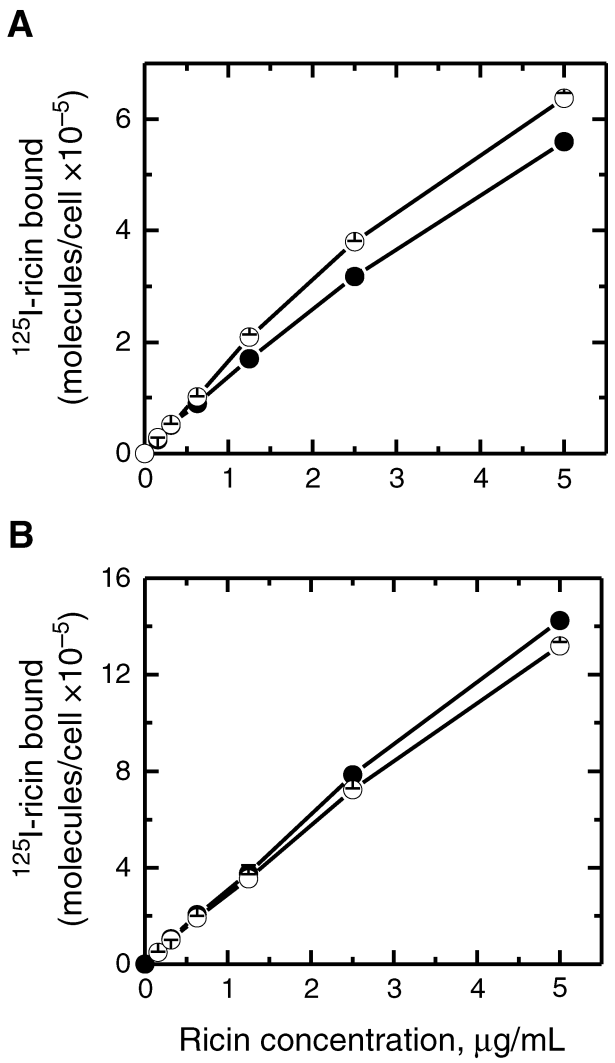

Figure 2: Binding of ^{125}I-ricin with and without neuraminidase treatment. GM95 (●) and GM95-CGlcT-KKVK (○) cells were incubated without (A) and with (B) neuraminidase (0.01 U/mL; Roche) for 30 min at 37 °C before the cells were chilled to 4 °C and incubated with ^{125}I-ricin for 1 h and the cell-associated radioactivity determined as described in Materials and Methods. The error bars show deviations between duplicates of a typical experiment.

in sensitivity to ricin was approximately proportional to the increased binding.

Conditions that normally sensitize cells to ricin: Do they function in cells without glycosphingolipids?

It has previously been shown that low concentrations of monensin (0.01–0.1 μM) can sensitize both single cells (16, 17) and whole animals (mice) (18) to ricin. As shown in Figure 4(A), monensin had an unusually strong sensitizing effect on the mouse melanoma cells, but again the shift in toxicity was about the same in the cell lines here studied, indicating that also this phenomenon is independent of GSL synthesis. Importantly, the drug brefeldin A which disrupts the Golgi apparatus protected the cells against ricin both in the absence and in the presence of monensin, indicating that transport through the Golgi apparatus was required in all cases (results not shown).

The possibility existed that the carbohydrate part of newly synthesized glycoproteins was required for retrograde transport of ricin in cells unable to synthesize GSL. However, addition of tunicamycin, which inhibits addition of carbohydrate to asparagine in newly formed proteins, greatly sensitized all the cell lines tested to ricin (Figure 4B). Similar results were obtained when protein synthesis was inhibited altogether by addition of cycloheximide during the incubation with ricin (results not shown). The toxin was still able to enter the cytosol and inactivate ribosomes. It should be noted that in these experiments cycloheximide was removed before the ability of these cells to synthesize proteins was tested.

Since ricin A-chain is a glycoprotein in itself, one could imagine that cellular lectins could bind the A-chain and function as retrograde carriers or be involved in translocation of the A-chain to the cytosol in the ER. However, when toxicity of recombinant ricin-A chain (which is not glycosylated) reconstituted with its B-chain was measured in a 5-h leucine incorporation assay, the toxicity was similar to that found with native ricin (results not shown).

Endosome to Golgi transport of ricin

In theory, opposite effects on the different transport steps of ricin from the cell surface to the cytosol might lead to a lack of effect on toxicity, even though individual steps such as endosome to Golgi transport of ricin could be changed. Due to the strong dependency on cholesterol of the transport of ricin (8) as well as of other toxins (13,14) between endosomes and the Golgi apparatus, we have investigated this step separately by incubating the cells with a modified ricin molecule with a sulfation site in the A-chain (19). Since sulfation is a *trans*-Golgi modification (20), one can use incorporation of radioactively labeled sulfate into ricin to monitor arrival in the Golgi apparatus and to quantify this transport step. Such modified ricin was added to the different cell lines studied here, and as shown in Figure 5(A,B), sulfation of ricin was as efficient in the cells that were devoid of GSL as in those cells that had the ability to synthesize GSL. In the mock-transfected cell line sulfation was about 2 fold more efficient, a result that is in agreement with the short-term (3-h) toxicity data (Figure 1A). Control experiments showed that the level of radioactive sulfate incorporated into cellular proteins was similar in the different cell lines (data not shown). Also, there were no significant differences in sulfation of ricin in the presence of monensin for the GSL-deficient cells and the control cells (Figure 5C,D). The presence of monensin increased the sulfation of ricin A-sulf-1 to the same extent in the different cell lines (4–10 times in different experiments). An increased ricin sulfation is in agreement with

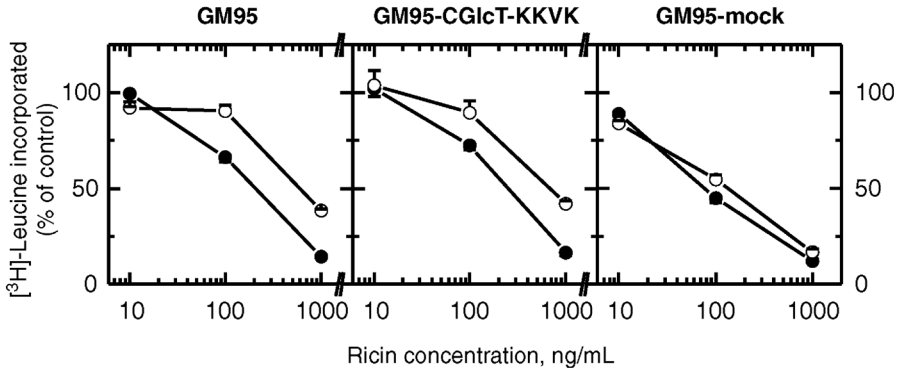

Figure 3: Effect of neuraminidase on ricin toxicity. GM95, GM95-CGlcT-KKVK and GM95-mock cells were incubated without and with neuraminidase (0.01 U/ml; Roche) for 30 min at 37 °C before ricin was added and the cells were incubated for 3 h (with and without neuraminidase). The ability to synthesize protein was then determined as described in Materials and Methods. (●): Neuraminidase added and (○): no neuraminidase added. The error bars show deviations between duplicates of a typical experiment.

the observed increase in toxicity in the presence of monensin (Figure 4). Experiments with ^{125}I-labeled ricin demonstrated that the amounts of ricin bound (Figure 2A), endocytosed and recycled (Figure 6A,B) were equal in these cells under the conditions studied. One can therefore conclude that the efficiency of endosome to Golgi transport of ricin is not dependent on GSL.

Kinetics of transport to the Golgi apparatus and intoxication with ricin

To further explore whether the pathways of intoxication and their properties are identical in the cell lines studied here, we added a high concentration of ricin (3 μg/ml) to the cells and measured the rate of intoxication under different conditions. We studied the rate both with and without sensitizing concentrations of monensin at different temperatures. Lack of GSL synthesis might have affected the ricin transport at high or low temperature. As shown in Figure 7, the rate of ricin intoxication was identical in the two cell lines at 37 °C whether or not monensin was present, and the same was the case when the temperature was increased to 42 °C or lowered to 24 °C (data not shown). Interestingly the cells were rapidly intoxicated in the presence of monensin. There was a strong reduction in the lag time between addition of toxin and observable protein synthesis inhibition. Also, when the kinetics of transport of ricin to the Golgi apparatus was measured by quantification of sulfation of ricin A-sulf-1 after short time there was no difference in cells with or without GSL (Figure 5E), even in the presence of monensin (Figure 5F).

Cholesterol depletion inhibits endosome to Golgi transport of ricin bound to glycoproteins

We have previously found that transport of ricin from endosomes to the Golgi apparatus is reduced in HeLa cells when cholesterol is extracted (8). Also, cholesterol depletion inhibits Golgi transport of the two GSL-binding toxins, cholera toxin (13) and Shiga toxin (14). Thus, in the case of ricin the possibility existed that the effect of cholesterol depletion was connected to Golgi transport of toxin bound to GSL in lipid rafts. We therefore investigated whether cholesterol depletion would reduce the Golgi transport of ricin also in cells without GSL both in the absence and in the presence of monensin. As shown in Figure 8, this was indeed the case. In fact, the reduction of sulfation caused by mβCD treatment was equally strong in cells with and without GSL, regardless of whether monensin was present.

Discussion

We here demonstrate that ricin is transported retrogradely to the Golgi apparatus and intoxicates cells equally well in cells unable to synthesize GSL as in cells with production of this group of lipids. Thus, binding of ricin to glycoproteins is sufficient for all transport steps from the cell surface to the cytosol, and GSL synthesis is not required for other processes; for instance, for adsorption of coat proteins, along this pathway. Moreover, we demonstrate that the level of cholesterol is important for endosome to Golgi transport of ricin even in cells without GSL.

The possibility existed that ricin binding to GSL or their presence could be essential for retrograde toxin transport or for translocation of the toxin from the ER to the cytosol. In the case of Shiga toxin, which binds to GSL (Gb_3), the presence of the toxin-receptor complex in lipid rafts is required for the retrograde transport (14), but as shown here, ricin transport to the Golgi apparatus and intoxication occurs as efficiently without GSL as in their presence. One could also imagine that presentation of the toxin molecule to chaperones (21) or to the Sec61p complex (5,17) in the ER might occur most efficiently in the presence of GSL, but this is apparently not the case. That lipid domains are present in the ER and the Golgi apparatus is supported not only by the experiments with Shiga toxin, but also from

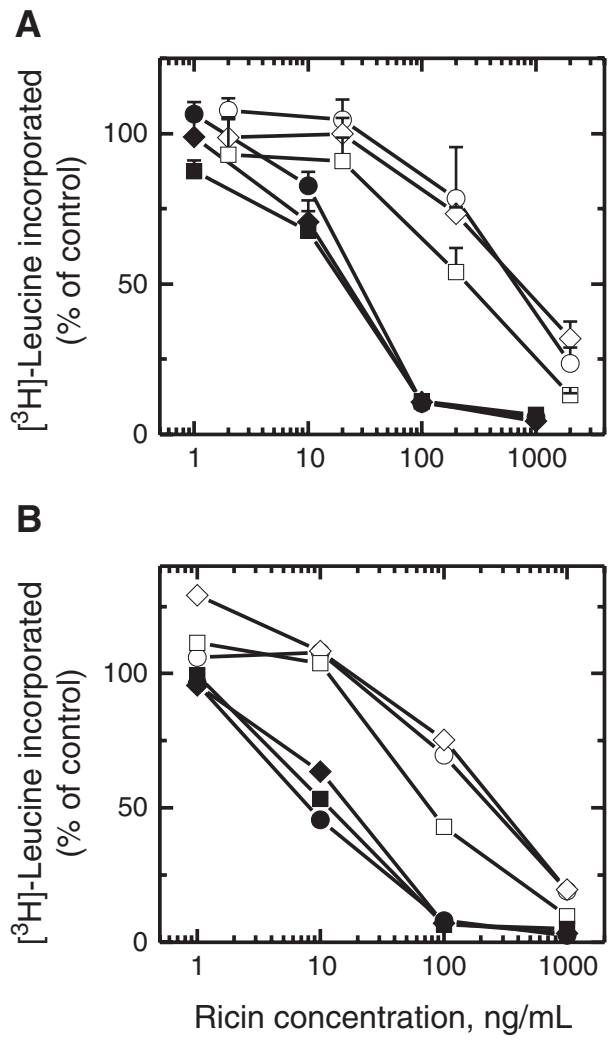

Figure 4: Effect of monensin and tunicamycin on ricin toxicity. GM95 (●,○), GM95-CGlcT-KKVK (◆,◇) and GM95-mock (■,□) cells were incubated with and without monensin (A; 0.1 μM) or tunicamycin (B; 5 μM) for 30 min at 37 °C before the cells were exposed to increasing amounts of ricin for 3 h (with and without monensin or tunicamycin present). The ability to synthesize protein was then determined as described in Materials and Methods. Filled symbols: monensin or tunicamycin added, and open symbols: no drug added. In A the error bars show standard deviations between triplicates and in B deviations between duplicates which are <10%.

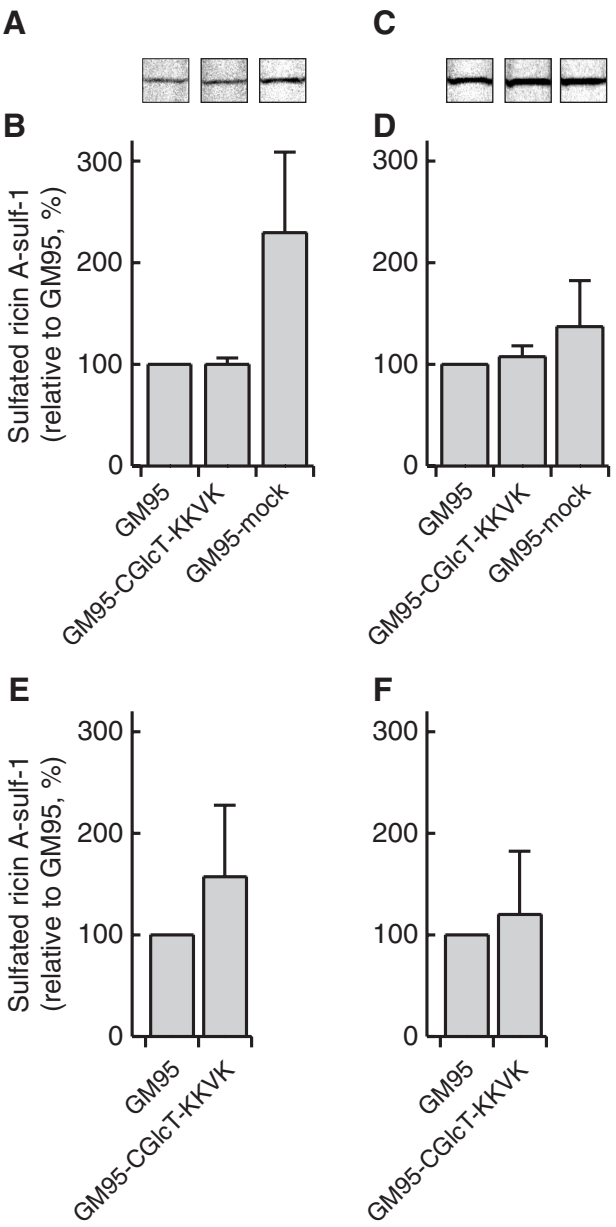

Figure 5: Sulfation of ricin A-sulf-1 in cells with and without GSL in the presence and absence of monensin. Autoradiograms of sulfated ricin A-sulf-1 in a representative experiment without monensin (A) and with monensin (C) after 3 h incubation with ricin sulf-1. The bars show quantification of sulfation from autoradiograms of four independent experiments without monensin (B) and with monensin (D) under the same conditions. E: After 2 h with ricin sulf-1 without monensin (n = 2). F: After 1 h with monensin (n = 5). The data were corrected for protein concentration in the cell lysate before plotting. The experiments were performed as described in Materials and Methods.

studies performed on yeast where lipid rafts seem to be present in vesicles emanating from the ER (22).

So far there is not much known about the role of GSL in intracellular transport, but GSL-deficient cell lines are important tools to improve our understanding of their importance (12,23). Thus, it was recently shown that GSL are necessary for sorting of melanosomal proteins directly from the Golgi complex to melanosomes, whereas transport from the Golgi apparatus to endosomes was normal (6).

To increase the number of GSL that can bind ricin on the cell surface, the cells were treated with neuraminidase (24). Ricin binds to a heterogeneous group of receptors on the cell surface, but it is likely that not every molecule

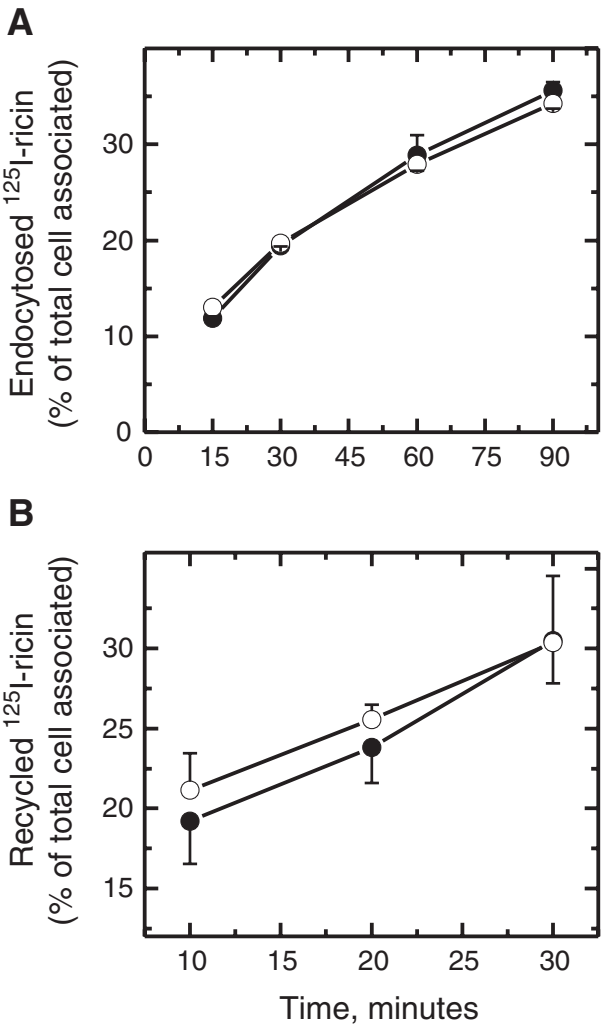

Figure 6: Endocytosis and recycling of ^{125}I-ricin. A. GM95 (●), and GM95-CGlcT-KKVK (○) cells were incubated with 120 ng/ml ^{125}I-ricin at 37 °C for the indicated time before the cell associated and total radioactivity was measured as described in Materials and Methods. In B the cells were preincubated with 30 ng/ml ^{125}I-ricin at 37 °C for 20 min before the cells were washed and the radioactivity in medium and the total cell associated radioactivity were measured after the indicated time as described in Materials and Methods. In A the error bars show deviations between duplicates, and in B standard deviations between triplicates.

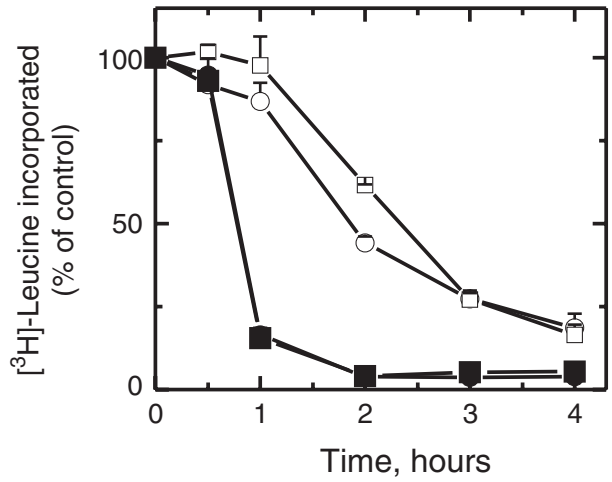

Figure 7: Kinetics of ricin intoxication with and without monensin. GM95 (●,○), and GM95-CGlcT-KKVK (■,□) cells were incubated with 3 μg/ml ricin in the presence and the absence of monensin (0.1 μM) for the indicated time before the ability to synthesize protein was determined as described in Materials and Methods. Filled symbols: monensin added, and open symbols: no monensin added. The error bars show deviations between duplicates.

that binds ricin is able to mediate ricin toxicity. It is thus possible that a subgroup of ricin binding sites is important for ricin intoxication. As shown in this work, GSL originally present in the mouse melanoma cell line here studied, or those created by neuraminidase treatment do not seem to constitute such subgroups. Clearly, binding of ricin to glycoproteins is sufficient for all transport steps from the cell surface to the cytosol.

From the earlier observations that cholesterol is important for Golgi transport of toxins (8,13,14), one could speculate that the role of cholesterol was to concentrate toxin-GSL-receptor complexes on their way to the Golgi apparatus. However, as here demonstrated, this is not the case for ricin. So why is the cholesterol level important for endosome-to-Golgi transport of ricin? There are several possibilities: cholesterol could be important for recruitment of cytosolic factors involved in Golgi transport or for formation of membrane buds (9). Furthermore, since membrane cholesterol also can modulate the activity of ion channels (25) and enzymes (26), the possibility exists that an alteration in membrane cholesterol might have indirect effects on a certain transport process. Cholesterol in a compartment (perhaps the perinuclear recycling compartment) along the route to the Golgi apparatus could also be required to inhibit or slow down recycling of the toxins to the cell surface, perhaps thereby increasing the chance of being transported to the Golgi. Such cholesterol-dependent retention in endosomes has been demonstrated for the GPI-anchored folate receptor (27).

It is an old observation that low concentrations of monensin can sensitize cells to ricin and immunotoxins. As shown here, and as earlier suggested (17), transport of ricin to the Golgi apparatus is greatly increased in the presence of monensin. We here demonstrate that the time it takes from addition of toxin until inhibition of protein synthesis can be detected is strongly reduced, suggesting that the toxin is transported faster and/or more efficiently to the ER and to the cytosol. Why does monensin increase endosome-to-Golgi transport? The concentrations used are so low that they should not affect endosomal acidification. However, it has been reported that Golgi structure is affected by low concentrations of monensin (28). Somehow this change in

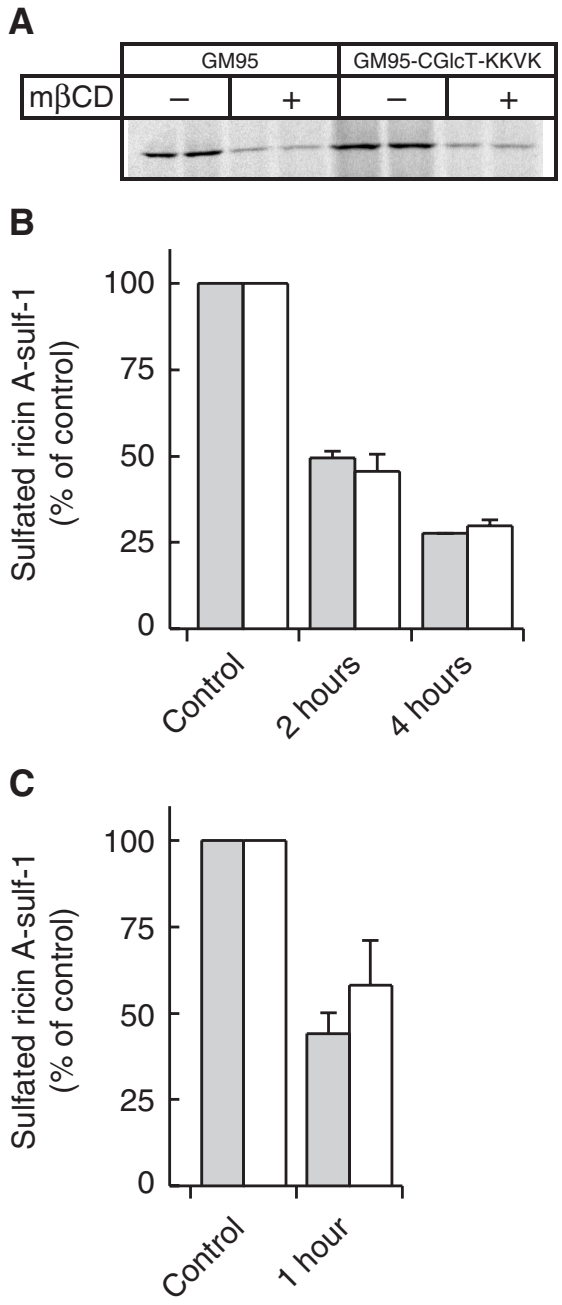

Figure 8: Effect of methyl-β-cyclodextrin (mβCD) on sulfation of ricin A-sulf-1 in cells with and without GSL. Autoradiogram of sulfated ricin A-sulf-1 with and without 10 mM mβCD as indicated after 2 h incubation with ricin sulf-1 (A). The experiment was performed as described in Materials and Methods. The bars show quantification of sulfation from duplicates on autoradiograms after 2 h and 4 h with ricin sulf-1 relative to controls (B) and after 1 h in the presence of 0.1 μM monensin (C). Grey bars represent GM95 and white bars represent GM95-CGlcT-KKVK. The error bars show deviations between duplicates.

the Golgi apparatus might facilitate fusion of incoming vesicles with the Golgi cisternae. Whatever the mechanism, it does not require GSL synthesis.

The data presented in the present article reveal that ricin binding to GSL is not essential for intoxication. Furthermore, although GSL synthesis can be strictly required for some intracellular transport steps (6), the complex pathway followed by ricin on its way to the cytosol functions as normal in cells without the ability to synthesize GSL. GSL does thus not seem to be a general requirement for retrograde transport.

Materials and Methods

Materials

Monensin was from Calbiochem (San Diego, CA, USA) and neuraminidase was from Roche Diagnostics (Mannheim, Germany). All other chemicals were from Sigma (St. Louis, MO, USA) and all cell culture reagents were from Gibco (Paisley, UK) unless otherwise stated.

Cell culture

GM95, GM95-CGlcT-KKVK, GM95-mock and MEB4 cell lines from Sprong et al. (6) were maintained in DMEM containing 10% FCS (Sigma, St. Louis, MO, USA), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin under standard cell culture conditions.

Measurement of ricin toxicity

Cells were seeded out in 24-well tissue-culture plates. Each well was washed once with MEM buffered with HEPES (HMEM) without leucine and incubated for 1 h before increasing concentrations of ricin were added. The cells were incubated for 3 or 18 h with the toxin. After a brief wash with leucine-free HMEM, the cells were pulsed with 1 μCi/ml [^{3}H]-leucine (New England Nuclear, Boston, MA, USA) in the same medium for 15–20 min. The cells were then washed twice with 5% (w/v) trichloroacetic acid for 10 min and subsequently dissolved in 0.1 M KOH. The radioactivity was then counted.

Binding of ^{125}I-ricin

Ricin was labeled with ^{125}Iodine as described by Fraker and Speck (29). Cells were seeded out in 24-well tissue-culture plates. Each well was washed twice with HMEM and treated with neuraminidase as indicated in the figure legends. The cells were then washed twice with ice-cold HMEM and incubated at 4 °C for 15 min before increasing concentrations of ricin were added. The cells were incubated for 1 h with the toxin and washed three times with ice-cold HMEM. The cells were dissolved in 0.1 M KOH and the radioactivity was counted.

Sulfation of ricin A-sulf-1

Ricin A-sulf-1 was produced and reconstituted with ricin B-chain (to ricin sulf-1) as described by Rapak et al. (19). Cells were seeded out in 100-mm dishes. The cells were washed twice in MEM without sulfate supplemented with 1 × MEM nonessential amino acids and 2 mM L-glutamine

and incubated overnight in the same medium with 0.2 mCi/ml $^{35}SO_4^{2-}$ (Amersham Pharmacia Biotech, Uppsala, Sweden). Ricin sulf-1 was added and the incubation was continued as indicated in the figure legends. The dishes were then washed twice with 0.1 M lactose in MEM and incubated for 5 min at 37 °C in the same solution. The cell culture dishes were placed on ice and washed immediately with ice-cold PBS. The cells were lysed in ice-cold lysis buffer [0.1 M NaCl, 10 mM phosphate, 1 mM EDTA, 1% (v/v) Triton X-100 and a protease inhibitor mixture; Complete; Roche Diagnostics, Mannheim, Germany]. The lysate was centrifuged at 2000 × g for 10 min at 4 °C and ricin sulf-1 in the supernatant was immunoprecipitated at 4 °C overnight with a rabbit polyclonal anti-ricin antibody bound to Protein A Sepharose CL-4B (Amersham Pharmacia Biotech, Uppsala, Sweden). The precipitate was washed twice with 0.35% Triton X-100 in PBS and subjected to SDS-PAGE (12% acrylamide) under reducing conditions. The dried gel was exposed for 48 h on a low energy storage phosphor screen (Molecular Dynamics, Sunnyvale, CA, USA) and scanned on a STORM 860 scanner (Molecular Dynamics). The bands were quantified with ImageQuant 5.0 (Molecular Dynamics). The total protein concentration in the lysate was determined with a BCA protein assay kit as described by the manufacturer (Pierce, Rockford, IL, USA).

Endocytosis of ^{125}I-ricin

Cells were seeded out in 24-well tissue-culture plates. Each well was washed twice with HMEM and incubated at 37 °C for 30 min before ^{125}I-ricin was added. The cells were incubated with the toxin as indicated in the figure and washed three times with ice-cold HMEM. The cells were dissolved in 0.1 M KOH, and the radioactivity was counted and the value used as total associated radioactivity. The endocytosed ricin was measured by incubating the cells with 0.1 M lactose in HMEM for 5 min at 37 °C, then washed twice in the same medium after the toxin incubation.

Recycling of ^{125}I-ricin

Cells were seeded out in 24-well tissue-culture plates. Each well was washed twice with HMEM and incubated at 37 °C for 30 min before ^{125}I-ricin was added. The cells were incubated for 20 min with the toxin before the cells were incubated for 5 min at 37 °C in 0.1 M lactose in HMEM and then washed twice in the same solution to remove surface-bound toxin. The cells were then incubated as indicated in the figure. Both the medium and cell-associated radioactivity was counted.

Acknowledgments

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