An Interaction between Ricin and Calreticulin That May Have Implications for Toxin Trafficking*

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Here we demonstrate that ricin is able to interact with the molecular chaperone calreticulin both in vitro and in vivo. The interaction occurred with ricin holotoxin, but not with free ricin A chain; and it was prevented in the presence of lactose, suggesting that it was mediated by the lectin activity of the ricin B chain. This lectin is galactose-specific, and metabolic labeling with [3H]galactose or treating galactose oxidase-modified calreticulin with sodium [3H]borohydride indicated that Vero cell calreticulin possesses a terminally galactosylated oligosaccharide. Brefeldin A treatment indicated that the intracellular interaction occurred initially in a post-Golgi stack compartment, possibly the trans-Golgi network, whereas the reductive separation of ricin subunits occurred in an earlier part of the secretory pathway, most probably the endoplasmic reticulum (ER). Intoxicating Vero cells with ricin whose A chain had been modified to include either a tyrosine sulfation site or the sulfation site plus available N-linked glycosylation sites, in the presence of Na235SO4, confirmed that calreticulin interacted with endocytosed ricin that had already undergone retrograde transport to both the Golgi and the ER. Although we cannot exclude the possibility that the interaction between ricin and calreticulin is an indirect one, the data presented are consistent with the idea that calreticulin may function as a recycling carrier for retrograde transport of ricin from the Golgi to the ER.

Ricin is a member of a large family of protein toxins produced by certain plants and bacteria that damage or kill mammalian cells by catalytically modifying target substrates in the cytosol (1, 2). In the case of ricin, the modification catalyzed is the removal of a specific adenine residue from a highly conserved loop present in 28S rRNA (3). The adenine residue removed by ricin action is crucial for the binding of elongation factors, and as a consequence of the toxin-mediated depurination is the cessation of protein synthesis, leading inevitably to cell death.

Structurally, ricin is a disulfide-linked heterodimer in which the rRNA N-glycosidase (termed the ricin A chain (RTA)1) is covalently joined to a galactose-specific lectin (the ricin B chain (RTB)) (4). For ricin to act, RTA must enter cells to encounter ribosomes in the cytosol. Cell entry is mediated by RTB, through which the toxin binds to cell-surface galactosides. Some of the surface-bound ricin then enters cells by endocytosis and is initially delivered to the endosomal system (5). Before endocytosed ricin can act, however, RTA must traverse an intracellular membrane to reach the ribosomes. This membrane translocation step is now known to be achieved by a proportion of the endocytosed toxin that undergoes intracellular transport beyond endosomes. Initially, the toxin is transported to the trans-Golgi network (TGN) and then, via retrograde vesicular transport through the Golgi stack, to the lumen of the ER (6). Translocation of free RTA into the cytosol from the ER possibly occurs because the toxin is perceived as a candidate for ER-associated protein degradation (7, 8). Clearly, however, a proportion of translocated RTA must escape the subsequent proteolytic degradation to then be capable of intoxicating the cell.

Electron microscopic analysis of the cellular fate of endocytosed ricin first showed that a proportion reaches the TGN (9). This raises the question of how ricin achieves retrograde transport from the TGN to the ER lumen. Several bacterial toxins also follow this trafficking route, and structural features of some of these toxins have provided an explanation of how this transport step is achieved. Pseudomonas aeruginosa exotoxin A, cholera toxin, and Escherichia coli heat-labile enterotoxin have the tetrapeptide sequence -Lys-Asp-Glu-Leu (KDEL), or a functionally related homolog, at the C terminus of the translocated catalytic polyepitide (10–12). It has been shown that cholera toxin and P. aeruginosa exotoxin A utilize the KDEL receptor, which recycles between the TGN and the ER to retrieve escaped ER lumen resident proteins from all Golgi compartments (13, 14), to undergo retrograde Golgi-to-ER transport (15, 16).

An outstanding question concerns how ricin, which lacks a C-terminal KDEL or related sequence, achieves Golgi-to-ER transport for its subsequent translocation into the cytosol? Our earlier work indicates that the lectin activity of RTB may be important in this regard (17–20). Primary sequence analysis and x-ray crystallographic studies have shown that RTB forms two distinct globular domains with identical folding topologies (17, 18). Each domain can bind a galactose residue (18). We have shown that mutationally abrogating one or other of these

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1 The abbreviations used are: RTA, ricin A chain; RTB, ricin B chain; TGN, trans-Golgi network; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; BFA, brefeldin A.
two galactose-binding sites does not abolish either the lectin activity of RTB or the cytotoxicity of the ricin holotoxin (19, 20). Simultaneous abrogation of both sites, however, does predictably abolish lectin activity and significantly reduces cytotoxicity (20). We have also shown that the ricin mutant unable to bind galactose did not kill macrophages, even though the mutant toxin (which was N-glycosylated) could still bind to mannose receptors and was endocytosed by these cells (20). An explanation that could account for these findings is that wild-type ricin normally has to be bound, or transferred, to a galactosylated component that itself undergoes retrograde transport, e.g., an endogenous KDEL-containing protein capable of recycling between the TGN and the ER (21). This prompted us to examine which, if any, Golgi/ER protein(s) might bind ricin holotoxin. Rat liver calreticulin is a potential candidate in that it contains a C-terminal KDEL motif and is galactosylated (22). The data presented here indicate that calreticulin interacts directly or indirectly with ricin both in vitro and in vivo. As such, it is a candidate for a Golgi-to-ER recycling cellular protein that might be opportunistically used by ricin to reach the ER lumen, an essential transport step in the entry process of this protein.

**EXPERIMENTAL PROCEDURES**

**Materials—** Antibodies against calreticulin, calnexin, BIP (immunoglobulin heavy chain-binding protein), and protein-disulfide isomerase were purchased from Stressgen Biotech Corp. (Victoria, British Columbia, Canada), and antibody against Rab5 was from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against RTA, RTB, and fibroblast growth factor intracellular binding protein were raised in rabbits by Enhanced Chemiluminescence Western Blotting. Antibodies against calreticulin, calnexin, BiP (immuno- globulin heavy chain-binding protein), and protein-disulfide isomerase antibodies in a total volume of 1 ml of ER resuspension buffer. After centrifugation at 150 µl aliquots, and immunoprecipitated with anti-calreticulin, anti-calnexin, or anti-protein-disulfide isomerase antibodies in the presence of toxin. Where appropriate, cells were pretreated with brefeldin A (BFA; 10 µg/ml) for 1 h prior to adding toxin.

**Calreticulin Labeling—** Vero cells were washed twice with PBS and then lysed in lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride and 200 units/ml aprotinin. The cleared lysate was incubated for 1 h with 200 µCi of [35S]methionine or washed twice with sulfate/serum-free minimal essential Eagle's medium for suspension cells (SMEM) (BioWhitaker UK, Ltd., Wokingham, United Kingdom) and incubated for 4 h in the presence of 200 µCi of Na2[35S]SO4. Following labeling, cells were incubated for up to 4 h in the presence of toxin. Where appropriate, cells were washed twice with PBS and then lysed in lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride and 200 units/ml aprotinin. The cleared lysate was incubated for 1 h with 200 µCi of [35S]methionine or washed twice with sulfate/serum-free minimal essential Eagle's medium for suspension cells (SMEM) (BioWhitaker UK, Ltd., Wokingham, United Kingdom).

**Preparation of Cell Extracts—** Cells were washed twice with PBS and then lysed in lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride and 200 units/ml aprotinin. The cleared lysate was incubated for 1 h with 200 µCi of [35S]methionine or washed twice with sulfate/serum-free minimal essential Eagle's medium for suspension cells (SMEM) (BioWhitaker UK, Ltd., Wokingham, United Kingdom).

**RESULTS**

**Ricin Interacts with Calreticulin in Vitro**—The productive intoxication pathway for ricin requires retrograde transport from the TGN to the ER lumen, from where RTA translocates into the cytosol (6, 25). To achieve this retrograde trafficking, ricin possibly interacts with a cellular component(s) normally capable of recycling between the Golgi and the ER. To examine whether any Golgi/ER components could interact with ricin in...
vitro via the RTB lectin activity, we passed solubilized rat liver microsomal preparations down an immobilized ricin column. Since the galactose-binding activity of RTB seems to be essential for optimal ricin cytotoxicity (20, 26), any lectin-active components were eluted from the column with lactose. Immunoprecipitation followed by ECL Western blotting showed that the lactose eluate contained calreticulin (Fig. 1A). The lactose eluate did not contain calnexin, protein-disulfide isomerase, or BiP, but we cannot exclude the possibility that these chaperones bind to ricin in a non-lectin association. Free RTA or ricin holotoxin was next added to the microsomal extracts; calreticulin was immunoprecipitated; and any interaction between calreticulin and toxin was demonstrated by probing reducing gels of the immunoprecipitates with anti-RTA antibodies. As shown in Fig. 1B, RTA was detected only in calreticulin complexes containing ricin rather than free RTA, suggesting that the toxin was interacting with the chaperone via its B chain. The addition of either mannose or Ca\(^{2+}\) to the microsomal extracts did not prevent the association of toxin and calreticulin, whereas the addition of lactose did (Fig. 1C). This suggests that the association with RTB required a galactosylated oligosaccharide on calreticulin (Fig. 1C).

**Immunoprecipitation of Ricin from Extracts of Intoxicated Vero Cells**—Before looking for an interaction between ricin and calreticulin in vivo, we determined that we could visualize endocytosed ricin and, in particular, its reduced subunits in extracts from Vero cells treated with toxin. After incubation with toxin for the times indicated in Fig. 2, the cells were washed with 0.1 M lactose and lysed, and toxin was recovered from extracts by immunoprecipitation with either rabbit anti-RTA or anti-RTB antibodies and visualized on nonreducing gels by Western blotting using sheep anti-RTA or anti-RTB antibodies. As expected, the amount of total internalized ricin remained relatively constant over the time course due to toxin recycling between the medium and endosomes, whereas free subunits, released after intracellular cleavage within the ER or cytosol, accumulated in a time-dependent fashion. The relative accumulation rates for free RTA (Fig. 2A) and RTB (Fig. 2B) were approximately equal, suggesting that neither subunit was rapidly degraded following release from holotoxin or that both subunits were degraded to similar extents. The time course for the appearance of reduced toxin was consistent with the observed time course for cytotoxicity at the ricin concentration used, which showed that protein synthesis was significantly inhibited within 30 min and was almost abolished by 60 min (data not shown).

**Accumulation of Free Toxin Subunits Can Be Prevented by Preincubation of Cells with Brefeldin A**—Ricin toxicity is abolished by preincubating cells with BFA (27, 28) presumably by preventing passage of ricin through the Golgi stack to the ER, the site of toxin entry into the cytosol. Preincubation with BFA also blocks the sulfation of ricin containing an A chain with an introduced tyrosine sulfation site (designated RCA-sulf1 (6)), a modification that is trans-Golgi-specific (29). In the proposed model for cellular intoxication by ricin, the lectin activity of RTB is required for efficient retrograde transport of holotoxin to the ER (21). Hence, it would be expected that the reductive separation of RTA and RTB would also be prevented by BFA treatment. Cells were intoxicated with ricin for 2 h in the presence or absence of BFA, and extracts were immunoprecipitated using either anti-RTA or anti-RTB antibodies. Fig. 3 shows that preincubation with BFA had only a small effect (typically a 15% reduction) on the total amount of ricin internalized, but almost completely abolished the appearance of the free toxin subunits, suggesting that reduction normally occurs in an early (pre-TGN) compartment of the secretory pathway such as the endoplasmic reticulum. This is consistent with the earlier observation that some of the RTA-sulf2 in ricin holotoxin (RTA-sulf2 is the ricin A chain modified to have overlapping N-glycosylation sites at the C terminus (6)), as well as reduced RTA-sulf2, becomes N-glycosylated during cell entry (6). This shows that endocytosed ricin can reach oligosaccharyltransferase in the ER.

**Ricin Interacts with Calreticulin in Vivo**—It has been proposed that the retrograde transport of ricin from the Golgi to the ER might be mediated, at least in part, by the KDEL retrieval system (21). This model proposed that ricin might bind to a recycling KDEL-tagged glycoprotein because of the lectin activity of RTB, allowing ricin to “hitch a lift” to the ER as the KDEL protein is retrieved by KDEL receptors.

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**Fig. 1. Rat liver calreticulin binds to immobilized ricin.** A, ER extracts were passed over an immobilized ricin-agarose column, which was then washed with ER resuspension buffer until no further protein was present in the wash fractions. Bound material was eluted from the column using 100 mM lactose in ER resuspension buffer. The presence of calreticulin was detected by SDS-PAGE and Western blotting. Lane 1, ER extract; lane 2, first collected wash fraction; lane 3, final wash (fraction 20); lane 4, lactose eluate. B, ricin (5 μg) or RTA (10 μg) was added to ER extracts, and calreticulin was recovered by immunoprecipitation. The immunoprecipitates were subjected to reducing SDS-PAGE, and coprecipitated RTA was visualized by Western blotting. Lane 1, extract supplemented with RTA; lane 2, extract supplemented with ricin. C, ricin-treated ER extracts were immunoprecipitated using anti-calreticulin antibodies, and coprecipitated RTA was visualized by Western blotting using anti-RTA antibodies as described for B, either in the absence of additives (lane 1) or in the presence of mannose (lane 2), lactose (lane 3), or calcium (lane 4).

**Fig. 2. Appearance of free ricin subunits in cells endocytosing the toxin.** Vero cells were incubated with ricin (10 μg/ml) for various times before being washed twice with PBS containing 100 mM lactose and once with PBS alone. After lysing with Triton X-100, cleared lysates were treated with antibodies to RTA (A) or RTB (B). Precipitated proteins were separated by nonreducing SDS-PAGE. Cells were harvested immediately after the addition of ricin or after the times indicated.
dates for such a carrier include the abundant chaperones of the ER, several of which have putative N-glycosylation sites (Grp94, calreticulin, calnexin, protein-disulfide isomerase, etc.). Vero cells were intoxicated with ricin and lysed in the presence of Triton X-100, and the lysates were immunoprecipitated with selected anti-chaperone antibodies. Immunoprecipitated proteins were analyzed by Western blotting using anti-RTA antibodies (Fig. 4A). Controls showed that ricin was immunoprecipitated by either anti-RTA or anti-RTB antibodies, but not by irrelevant antibodies (anti-Rab5). As expected, a small amount of free RTA was also precipitated by anti-RTA antibodies. Anti-calreticulin antibodies also coprecipitated ricin (but not free RTA), but no toxin was seen in complexes using antibodies against calnexin or protein-disulfide isomerase (Fig. 4A) or BiP (data not shown). Coprecipitation of ricin with calreticulin was prevented in the presence of 0.1 mM lactose, which had little or no effect on the direct immunoprecipitation of ricin with either anti-RTA or anti-RTB antibodies (Fig. 4B). This observation is consistent with earlier findings that RTB plays an intracellular role during the intoxication process, in addition to its initial role in binding holotoxin to the target cell surface (20).

In keeping with this, Vero cells were treated with either free recombinant or native (glycosylated) RTA (which enters via fluid-phase uptake (30)) or free RTA for 4 h, washed, and then homogenized. Anti-RTA or anti-RTB antibodies precipitated the appropriate intracellular toxin subunit, whereas only RTB coprecipitated when calreticulin was precipitated with anti-calreticulin antibodies (Fig. 5). Although equal concentrations of RTA and RTB were added to cells, the relative amounts of RTA and RTB precipitated predictably confirmed that the cellular uptake of RTB was much more efficient. This is because uptake of RTB is receptor-mediated, whereas free A chains enter cells in the fluid phase.

To test that calreticulin was able to interact with ricin that had already been transported to the Golgi complex, where tyrosylsulfotransferase is found, the coprecipitation experiment was repeated, but using cells that had been intoxicated with ricin containing RTA-sulf1 (RTA that had been modified to contain a tyrosine sulfation site (6)) in the presence of Na$_2^{35}$SO$_4$. Fig. 6A shows that $^{35}$S-labeled ricin holotoxin was immunoprecipitated by anti-RTA, anti-RTB, or anti-calreticulin antibodies and that the interaction was again abolished in the presence of lactose.

To test that calreticulin remained in contact with ricin until it reached the ER (the location of oligosaccharyltransferase), coprecipitations were also performed using extracts from cells that had been intoxicated with ricin containing RTA-sulf2 (a recombinant RTA modified to contain overlapping C-terminal N-glycosylation sites (6)) in the presence of Na$_2^{35}$SO$_4$. Fig. 6B shows that immunoprecipitation with either anti-calreticulin or anti-ricin antibodies yielded both non-glycosylated RTA and newly glycosylated RTA (6). (To clearly see the gel mobility difference between RTA and glycosylated RTA, reducing SDS-PAGE was used in this case.) Significantly less RTA or glyco-

![Image](Image 363x613 to 500x729)

**Fig. 3.** Brefeldin A treatment prevents the release of free ricin subunits. Vero cells were preincubated in the presence (+) or absence (−) of 10 μg/ml BFA for 1 h prior to being incubated with ricin for a further 2 h and subsequently treated exactly as described in the legend to Fig. 2. αA and αB indicate immunoprecipitation with anti-RTA and anti-RTB antibodies, respectively.

![Image](Image 122x670 to 225x729)

**Fig. 4.** Ricin specifically coprecipitates with calreticulin. A and B, Vero cells were incubated with ricin for 4 h, washed, and lysed as described in the legend to Fig. 2 and treated with a range of immobilized antibodies in the absence (A) or presence (B) of 100 mM lactose. Precipitated proteins were separated by nonreducing SDS-PAGE and visualized by Western blotting using sheep anti-RTA antibodies. αA, anti-RTA antibody; αB, anti-RTB antibody; αCNX, anti-calnexin antibody; αCRT, anti-calreticulin antibody; αRab, anti-Rab5 antibody; αPDI, anti-protein-disulfide isomerase antibody. In A and B, the upper arrows indicate ricin, and the lower arrows indicate RTA.

![Image](Image 368x213 to 495x376)

**Fig. 5.** RTB coprecipitates with calreticulin. Vero cells were incubated with 5 μg of glycosylated RTA (αRTA; lanes 1 and 2), recombinant RTA (rRTA; lanes 3 and 4), or RTB (rRTA lanes 5 and 6) for 4 h prior to washing and lysis in Triton X-100. Cleared lysates were treated with immobilized antibodies, and the precipitated proteins were analyzed by SDS-PAGE. Lanes 1 and 3, anti-RTA antibody (αA); lane 5, anti-RTB antibody (αB); lanes 2, 4, and 6, anti-calreticulin antibody (αCRT).

![Image](Image 372x450 to 491x513)

**Fig. 6.** Calreticulin binds to ricin that has reached the Golgi complex and the ER. A, Vero cells were labeled with Na$_2^{35}$SO$_4$ for 4 h prior to the addition of ricin containing RTA-sulf1. After incubation for a further 4 h, cells were washed, lysed, and immunoprecipitated with anti-RTA (αA), anti-RTB (αB), or anti-calreticulin (αCRT) antibodies in the absence or presence of 100 mM lactose. Precipitated proteins were analyzed by nonreducing SDS-PAGE and visualized by fluorography. The upper arrow indicates $^{35}$S-sulfated ricin, and the lower arrow indicates $^{35}$S-sulfated RTA. B, the same experiment was repeated, but using reconstituted ricin containing RTA-sulf2. In this case, precipitated proteins were analyzed by reducing SDS-PAGE, αCNX, anti-calnexin antibody; αA and A, glycosylated RTA and non-glycosylated RTA, respectively.

sylated RTA was precipitated by anti-calnexin antibodies, but the data shown in Fig. 6B indicate that limited interaction with calnexin may also have occurred.

To ensure that the interaction between calreticulin and ricin was not simply due to post-lysis binding events, time course
Brefeldin A reduces the coprecipitation of ricin with calreticulin. A, Vero cells were intoxicated with ricin for the indicated times, washed, lysed, and immunoprecipitated with immobilized anti-calreticulin antibodies. Immunoprecipitates were analyzed by nonreducing SDS-PAGE and visualized by Western blotting using anti-RTA antibodies. B, Vero cells were incubated in the presence (+) or absence (−) of BFA (10 μg/ml) for 1 h prior to the addition of ricin and incubation for a further 2 h. Cells were washed, lysed, immunoprecipitated, and analyzed as described for A.

experiments were performed. The amount of ricin internalized remained reasonably constant over a 4-h time course (see Fig. 2). In contrast, the amount of internalized ricin that co-immunoprecipitated with calreticulin increased steadily over the first 2 h and then remained relatively constant for the remaining 2 h of the assay (Fig. 7A), perhaps suggesting that a steady state had been reached. It would be expected that BFA pretreatment would also reduce any specific interaction between ricin and calreticulin since it would prevent recycling between the ER and the Golgi complex. Fig. 7B shows that BFA treatment did significantly reduce (by ~75%) the co-immunoprecipitation of ricin with calreticulin, whereas it had only a small effect on the amount of ricin internalized (~15% reduction) (Fig. 3).

To further demonstrate a specific interaction between calreticulin and ricin, it was important to show that immunoprecipitates of ricin from intoxicated cells also coprecipitated calreticulin. Vero cells were metabolically labeled with [3H]galactose for 4 h prior to intoxication with ricin for a further 4 h. Lysates were immunoprecipitated with anti-RTA, anti-RTB, or anti-calreticulin antibodies; the pellets were washed and boiled in 1% SDS; and samples were diluted and re-immunoprecipitated with anti-calreticulin antibodies. Fig. 8 shows that in the absence of toxin treatment, calreticulin was precipitated only when the primary antibody was anti-calreticulin. In contrast, when cells had been intoxicated with ricin, calreticulin could be precipitated by both anti-RTA and anti-RTB antisera (Fig. 8).

Calreticulin from Vero Cells Is a Galactose-containing Glycoprotein—The interaction between ricin and calreticulin described here is mediated by the toxin B chain and is prevented by lactose. This requires that Vero cell calreticulin is glycosylated and that the glycosyl moiety contains terminal galactosyl residues. Vero cells were therefore incubated with [3H]galactose for 24 h prior to immunoprecipitation of lysates with anti-calreticulin antibodies. Fig. 9A shows that [3H]galactose was incorporated into calreticulin. It could not be excluded, however, that the [3H]galactose was being metabolized in the cells and that 3H was being incorporated into calreticulin in another form. To test further if Vero cell calreticulin is galactosylated, we took advantage of a biochemical assay to selectively label proteins with terminal galactose (31). Proteins present in a Vero cell homogenate were treated with the enzyme galactose oxidase and then with sodium [3H]borohydride to bind any oxidized terminal galactose residues. Immunoprecipitation with anti-calreticulin antibodies showed that a band corresponding in molecular mass to calreticulin was labeled after such treatment (Fig. 9B, lane 1).

This band was not obtained in immunoprecipitates with antibodies against fibroblast growth factor intracellular binding protein (Fig. 9B, lane 2) or ricin (lane 3) or in the absence of galactose oxidase treatment (Fig. 9C). For comparison, Vero cells labeled with [35S]methionine were lysed and subjected to
immunoprecipitation with anti-calreticulin antibodies. The [35S]methionine-labeled calreticulin seen in lane 4 of Fig. 9B had the same migration rate as the [3H]borohydride band in lane 1. The other bands seen in lane 1–3 could be due to labeling of the polyclonal rabbit antibodies used or other galactosylated proteins that nonspecifically interact with the antibodies. Taken together, the data indicate that at least a fraction of the calreticulin expressed in Vero cells contains a terminal galactose residue.

**DISCUSSION**

The data presented here show that ricin can interact with calreticulin in vitro and in vivo. Interaction in vivo did not appear to be an artifact caused by homogenizing the cells since (i) the level of interaction was shown to increase with time over a period when the total intracellular ricin pool did not alter significantly and (ii) BFA treatment of cells significantly reduced the extent of this interaction without significantly affecting ricin uptake.

The interaction could take one of several forms, although a direct protein-protein interaction is unlikely because of its lactose-dependent nature. An interaction of the sugar-binding site of calreticulin with glucose residues present on RTA and/or RTB is also unlikely since the glycans present on native RTA are complex and endoglycosidase H-resistant (32). Also, since both toxin subunits have undergone ER-to-vacuole transport with associated Golgi processing of their glycans in the producing plant (33), the toxin subunits are not monogalactosylated, a requirement for any interaction with the sugar-binding site of calreticulin. Furthermore, the interaction we observed involves native ricin, whereas calreticulin is known to interact with partially folded proteins. This, together with the data presented here, supports an interaction between the galactose-specific binding sites of RTB and galactosyl groups present on calreticulin. This contention is further supported by the fact that calreticulin interacts with ricin, but not free RTA, indicating that the association occurs via RTB, and that the interaction is abolished in the presence of lactose, which is known to block the biological activity of the RTB lectin.

An interaction mediated by the sugar-binding sites of RTB would require that calreticulin is glycosylated and that the oligosaccharide contains a terminal galacose residue. Until recently, the glycosylation status of calreticulin has been unclear. Although the primary sequences of calreticulin isolated from several different species all contain a single putative N-glycosylation site, the search for an attached glycan has been inconclusive. Calreticulins from rat liver (34) and bovine liver (35) are known to be glycosylated; but in most species, including human, calreticulin was thought to be non-glycosylated (36). More recently, however, calreticulin from human myeloid cells has been shown to be glycosylated, despite the fact that neither tunicamycin treatment of the cells nor endoglycosidase digestion of calreticulin had any effect on the protein’s mobility on SDS-polyacrylamide gels (37). Since human glycosylated calreticulin has the apparent properties of a non-glycosylated protein, it is possible that other forms are also glycosylated, despite reports to the contrary. Here we were able to metabolically label calreticulin from Vero cells with [3H]galactose and confirm that Vero cell calreticulin contains an oligosaccharide with a terminal galactose residue by treatment with galactose oxidase followed by sodium [3H]borohydride, providing an explanation for its apparent ability to interact with RTB.

Calreticulin contains a N-terminal signal sequence and a C-terminal ER retrieval sequence (KDEL); and as expected, its major intracellular location is the lumen of the ER (36, 38). Calreticulin has been reported to be present in other cellular locations, however, including later compartments of the secretory pathway (39) and at the cell surface (40). It is tempting to suggest that the interaction between ricin and calreticulin that occurs in vivo is physiologically significant in that calreticulin functions as a retrograde carrier that transports ricin from the TGN to the ER. Although the present data do not provide unequivocal evidence for such a function, several features of the interaction are consistent with this idea. To act as a retrograde carrier, calreticulin would normally need to recycle between the ER and the trans-Golgi. This certainly seems to be the case for rat liver calreticulin since it is terminally galactosylated (22), a modification that occurs in the trans-Golgi cisternae (41). Likewise, we have shown here that Vero cell calreticulin is galactosylated, a feature that would allow its interaction with the RTB subunit of incoming ricin. The finding that BFA pretreatment of cells (to disrupt the Golgi stack, but not the TGN), significantly reduced, but did not abolish, the calreticulin-ricin interaction is also consistent with the TGN or a later secretory pathway compartment being the site where the interaction initially occurs. This supports the idea that a ricin-calreticulin interaction can occur before ricin reaches the ER rather than after it reaches this compartment and suggests that its primary purpose is the delivery of toxin to the ER rather than any subsequent events. The interaction in vivo is stable, and the fact that it does not involve free RTA implies that what we are seeing does not reflect events within the ER leading to membrane translocation where association with chaperones may be very transient and involve the translocating RTA subunit. By virtue of its C-terminal ER retrieval sequence, any calreticulin present in the TGN and interacting with incoming ricin could predictably be recycled to the ER lumen via KDEL receptors. These receptors are known to recycle between the ER and the Golgi and can retrieve proteins to the ER from the trans-most cisterna of the Golgi complex (14). In keeping with this, we have shown that over-expressing lysozyme-KDEL, which causes a relocation of the KDEL receptor from the Golgi complex to the ER (42), reduced the sensitivity of cells to ricin intoxication. This implies that ricin, at least in part, might indirectly utilize the KDEL retrieval system to undergo Golgi-to-ER retrograde transport. Because ricin is a galactose-specific lectin, it could presumably bind any appropriate galactosylated glycoprotein or glycolipid it might encounter intracellularly, and if any of these were to recycle between the TGN and the ER, they could potentially be parasitized by ricin for retrograde transport. However, it must be stressed that much of the data presented here could be explained by an indirect interaction between ricin and calreticulin, and we concede that the physiological significance of the interaction during toxin entry remains speculative. In any event, calreticulin is unlikely to be the exclusive carrier for ricin. Indeed, calreticulin-deficient cell lines remain sensitive to ricin (data not shown). We suggest that calreticulin is one of a number of as yet unidentified cellular components that can act in this way and that retrieval systems other than that involving the KDEL receptor may likewise be utilized. Calreticulin may be the preferred carrier, however, since if it enters the ER lumen in association with ricin, it would be well placed to guide toxin unfolding and/or some other event required for the subsequent RTA translocation across the ER membrane.

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