Ribosome-Inactivating Proteins: Entry into Mammalian Cells and Intracellular Routing

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Abstract: To catalytically-modify ribosomes *in vivo,* ribosome-inactivating proteins produced by plants must enter susceptible mammalian cells in order to reach their substrates in the cytosol. This review primarily focuses on the biosynthesis, mechanism of cell entry and intracellular trafficking of ricin, the most thoroughly studied ribosome-inactivating protein in this respect.

Key Words: Ricin, Depurination, Ribosome, Endoplasmic Reticulum, Retograde Transport, Retro-Translocation.

INTRODUCTION

Amongst the family of plant ribosome-inactivating proteins (RIPs) [1], those that have evolved a mechanism for efficiently entering mammalian cells are the two ch ain or type II RIPs. Of these, the most thoroughly studied is ricin [2], the major focus of this review. Other type II RIPs from plants are structurally and functionally equivalent to ricin, so it is assumed that their pathway into target mammalian cells will be the same. While the absence of a cell binding (B) polypeptide in the case of single chain type I RIPs renders these molecules significantly less cytotoxic than the type II RIPs [1], type I RIPs have been shown on occasions to possess inherent cytotoxicity [3-5], implying that they too are able to enter mammalian cells to some extent. Putative entry mechanisms for type I RIPs are largely unknown however, and therefore will receive only limited coverage here.

RICIN – THE PARADIGM FOR CELL ENTRY

Ricin is a member of the A-B family of plant and bacterial proteins that are potently cytotoxic to mammalian cells [2]. These toxins consist of a catalytically active A polypeptide or fragment associated with one or more cell binding B polypeptides. In the case of ricin, the A polypeptide (RTA) is a RNA *N*-glycosidase that catalyses the cleavage of a specific adenine residue [6] from within a universally conserved 12 nucleotide stretch present in the large ribosomal RNA that is known as the α-sarcin/ricin loop (SRL) [7]. This target adenine is part of a tetraloop (GAGA) within the SRL that is crucial for the binding of elongation factors [8]. Ricin-treated ribosomes containing a depurinated SRL are therefore no longer able to synthesise protein. It has also become evident that intact ricin can modify non-ribosomal nucleic acid substrates *in vitro* and in human endothelial cells, allowing it to be considered a polynucleotide:adenosine glycosidase [9,10]. While the physiological relevance of other activities ascribed to RIPs is currently unclear (reviewed in 11), it is evident that the specific depurination of 28S rRNA and the resulting inhibition of protein synthesis is the primary mechanism of ricin cytotoxicity to mammalian cells. The B polypeptide of ricin (RTB), in common with the B subunits of all other type II RIPs studied to date, is a galactose-specific lectin that is responsible for binding ricin to cell surface glycoproteins and glycolipids that have terminal *N*-acetyl galactosamine or β-1,4-linked galactose residues [12]. Mammalian cells have many potential surface binding sites for ricin: HeLa cells, for example, have 3 x 10^7 binding sites per cell. In the ricin holotoxin, RTA is covalently linked to RTB by a single disulphide bond, and both RTA and RTB are *N*-glycosylated [2,12].

RICIN BIOSYNTHESIS

There are several isoforms of ricin including ricin D, ricin E and the closely related *Ricinus communis* agglutinin (RCAI), which are encoded by a small multigene family of approximately eight members [13]. At least three members of this lectin gene family are non-functional [10]. Expression of the genes is both developmentally regulated and tissue specific. Ricin is synthesised in the endosperm cells of maturing castor oil (*Ricinus communis*) seeds [11], and it is stored within the protein storage vacuoles of mature seeds in common with the other major storage proteins [14-16]. In the mature dry seeds, ricin and RCA account for around 5% of the total seed protein. When the seeds germinate the storage proteins and ricin/RCA are rapidly degraded, disappearing entirely after a few days of postgerminative growth.

Although ricin is a heterodimer, its individual RTA and RTB subunits are initially synthesised together in the form of a single precursor [17]. Cloning the ricin precursor gene confirmed that it encoded a preproprotein of 576 amino-acyl residues comprising a 35 residue N-terminal extension which includes, but does not entirely consist of an endoplasmic reticulum (ER) signal peptide, joined to the 267 residues of RTA. This in turn precedes a 12 residue linker and the 262 residue RTB [18]. During ricin biosynthesis, the N-terminal signal sequence co-translationally directs proricin into the lumen of the ER. Segregation of proricin is accompanied by several co-translational modifications, including signal peptide cleavage, core-glycosylation at one or two sites in RTA and at two sites in RTB, and the introduction of five intrachain disulphide bonds. Four disulphide bonds form loops within RTB, whilst the fifth forms what will

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1389-5575/04 $45.00+.00

ultimately become the interchain disulphide bond in the holotoxin. The core-glycosylated, disulphide cross-linked proricin then is then transported via the Golgi to the storage vacuoles [19, 20]. This intracellular transport is accompanied by several poorly characterised post-translational modifications, including the Golgi enzyme-mediated modifications to the oligosaccharide side chains which result in the trimming of some sugar residues and addition of other sugar molecules, notably fucose to RTA [21]. The 12 residue linker carries a sequence-specific vacuolar targeting signal – LIRP (using the single letter amino acid nomenclature), whose function can be destroyed by mutation to LGRP. Interestingly, this signal more closely resembles the targeting determinant of proteins destined to the lytic vacuoles in vegetative tissues (NPIR), than the exclusively C-terminal signals that have previously been described for storage vacuole proteins [22]. Once proricin reaches the vacuole, endoprotease(s) remove the residual 9 residue propeptide as well as the intramolecular linker, to generate the mature, disulphide-linked heterodimer [23].

Proricin is therefore the form in which the RTA and RTB polypeptides are initially synthesised and subsequently transported to the vacuole. Recombinant proricin has been produced and its properties examined [24]. It is an active prolectin containing functional sugar binding sites. In contrast, proricin lacks any *N*-glycosidase activity, consistent with reports that in mature holotoxin, RTA must be reductively separated from RTB in order to have activity [25, 26]. It appears that in both the dimeric ricin holotoxin *and* in proricin, the RTB moiety can sterically obstruct the active site of RTA. When proricin is proteolytically cleaved and reduced, catalytic activity against ribosomes is observed. This perhaps explains why *Ricinus* cells are able to synthesise large amounts of ricin even though conspecific ribosomes are susceptible to the action of RTA [27]. Since proricin is segregated into the ER lumen, the compartment from which reduced RTA would normally enter the cytosol during ricin intoxication (see below), segregation as a precursor may also ensure that the RTA moiety is in a form that is unable to retrotranslocate to the cytosol. Indeed, if plant cells are forced to express ER-targeted RTA by itself, it becomes glycosylated and yet is toxic to ribosomes [28, 29]. Pulse-chase analysis revealed that most of this RTA was degraded in a proteasomal-dependent fashion in the cytosol, and, when proteasomes are inhibited, a deglycoslyated intermediate could be detected. Clearly, under conditions where RTA is made as a solo subunit, it can exit the ER to reach the cytosol and, although most is degraded in a process that involves deglycosylation and proteasomes, some can escape this process to damage ribosomes [29]. Synthesis as a precursor therefore allows the generation of an inactive protein that is also unable to retrotranslocate to the cytosol. In this way, it is compelled to leave the ER by anterograde transport to protein storage vacuoles, where the active holotoxin is ultimately generated.

Since the endosperm cells of *Ricinus* show no signs of ribosome damage whilst storing large amounts of ricin, it can be deduced that RTA does not escape from the low pH vacuoles to reach the cytosol. The generation of active ricin only after inactive proricin has reached the storage vacuoles,

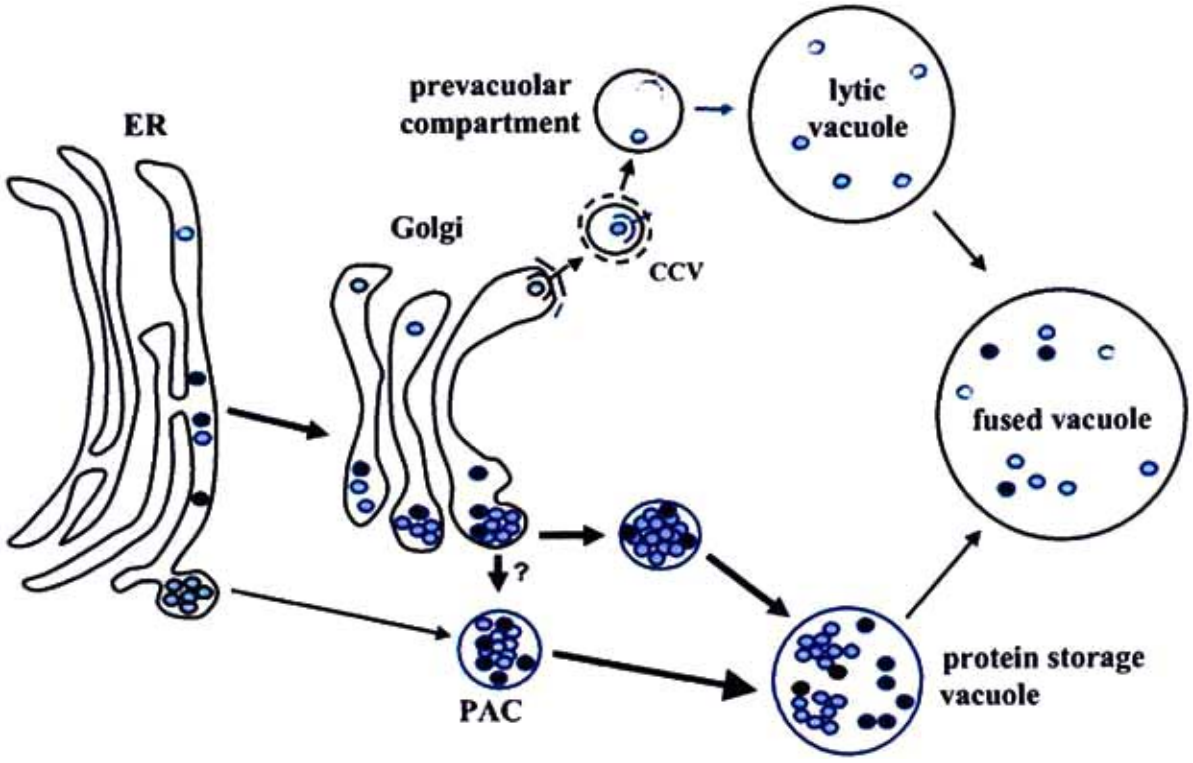

Fig. (1). ***Model for the targeting of proricin to plant vacuoles.*** Indicated are endoplasmic reticulum (ER), clathrin coated vesicles (CCV), and precursor accumulating vesicles (PAC). Within *Ricinus communis* seeds, proricin (•) is transported from the ER to protein storage vacuoles through the Golgi and then possibly via PAC (thick arrows). It is hypothesized that other storage proteins either follow a similar route or may aggregate within the ER to be transported exclusively via PAC in a Golgi-independent manner. The receptor-mediated route to lytic vacuoles via CCV is also indicated. In older cells, lytic and storage vacuoles may fuse.

compartments from which RTA cannot escape to the cytosol either directly or indirectly, enables the seeds to safely synthesise and store large quantities of a potent toxin that is presumed to be there as a deterrent to predators. The site of synthesis of proricin and its intracellular transport in maturing *Ricinus* endosperm cells is illustrated schematically in Figure **(1)**.

RICIN ENTRY INTO MAMMALIAN CELLS

After binding galactosides on the mammalian cell surface, ricin is taken into the cell by endocytosis. The precise endocytic route may be influenced by the nature of the surface molecule to which the toxin has bound, and since ricin promiscuously binds to many different surface glycoproteins, it isn't perhaps surprising to find that it can enter by both clathrin-dependent and clathrin-independent endocytosis [30-32]. Whatever the identity of the earliest vesicles, the pathways converge at the level of early endosomes from where most internalised ricin appears to be either recycled to the cell surface or delivered via late endosomes to lysosomes, presumably for degradation (see early steps in Figure **(2)**). However, raising the pH of endosomes/lysosomes does not protect against ricin whilst treating cells with the ionophore monensin, a reagent that influences Golgi structure and function, does [reviewed in 33]. These data suggested a requirement for the Golgi apparatus in ricin intoxication. Indeed, ~5% of internalised ricin can be visualised within the *trans*-Golgi network (TGN) [34] and transport there appears to be independent of Rab 9 [35, 36], a GTPase associated with the classical pathway from late endosomes to TGN. Uptake to the TGN has also been directly observed for Shiga toxin and cholera toxin, two glycolipid-binding toxins. Direct transport of Shiga toxin from early endosomes to the TGN has been particularly well characterised [37], where routing may occur when receptor/toxin complexes are recruited into lipid microdomains [38], commonly referred to as lipid rafts. Indeed, this direct pathway has recently been shown to require the GTP-binding Rab6a' isoform and specific early endosome v-SNAREs (VAMP3 and VAMP4) and putative TGN-localised t-SNAREs (Vti1a, syntaxins 6 and16) [39].

Toxins reaching the TGN (including ricin, and a number of bacterial proteins such as Shiga toxin, cholera toxin, pertussis toxin and Pseudomonas exotoxin A) then appear to undergo retrograde transport through the Golgi stack to reach the ER lumen. Appearance in the ER has been readily visualised using microscopy techniques for both Shiga toxin and cholera toxin [40, 41]. Such transport would normally occur to retrieve material via COP-I-coated carrier vesicles. By interfering with the formation of such coated vesicles, or with the COPI-dependent Golgi to ER cycling of KDEL receptors, cells have shown an increased resistance to the bacterial toxin *Pseudomonas* exotoxin A (PE) [42]. Surprisingly however, functional disruption of classical COPI vesicle transport did not affect the entry of Shiga toxin [43, 44]. In contrast, a dominant negative mutant of Rab6-GDP that had no effect on the COPI-dependent cycling of the early Golgi marker, ERGIC-53, had a significant effect on the flow of Shiga toxin from the Golgi to ER. The physiological significance of the COP1-independent pathway uncovered by studies using bacterial toxins remains unclear, as does its relationship with the COP1 pathway and the nature of the transport intermediates. Only one study has been made with ricin, where a block in COPI function did not protect cells from intoxication [45].

To date, endocytosed ricin has not been directly visualised by microscopy in the ER lumen despite numerous attempts to do so. The conclusion that retrograde transport of ricin to the ER *is* a prerequisite for cellular intoxication is therefore based on biochemical and inhibitor studies. Several early studies demonstrated that ricin cytotoxicity required a brefeldin A (BFA)-sensitive step [46-48]**.** In most cell types,

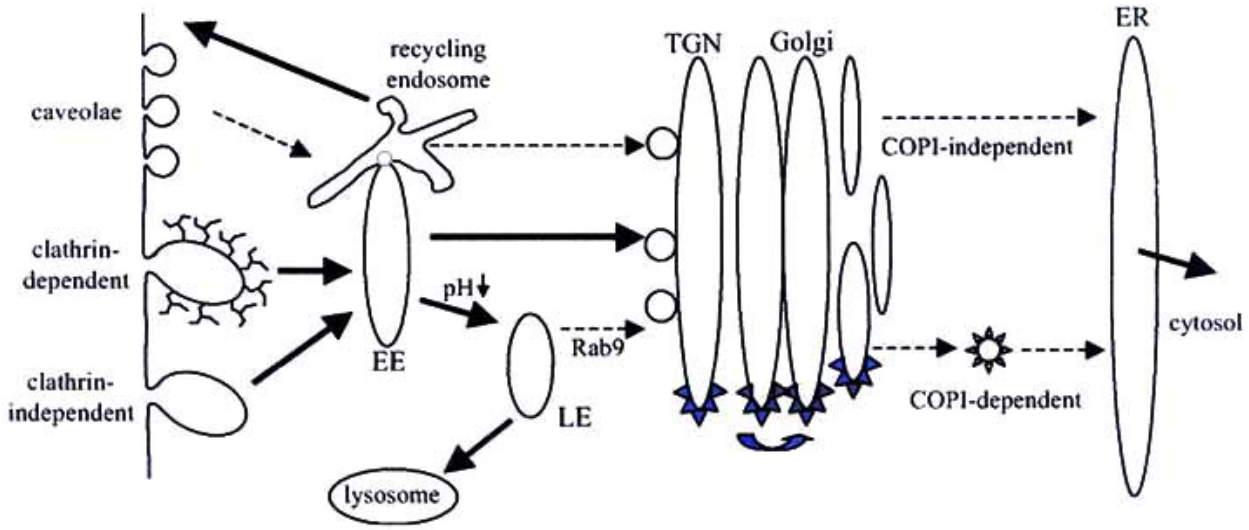

Fig. (2). ***Schematic representation of the endocytic uptake of ricin into mammalian cells.*** The intracellular transport routes followed by ricin are illustrated by bold arrows and, where uncertainties remain, by dashed arrows. Indicated are early endosomes (EE), late endosomes (LE), recycling endosomes (or endocytic recycling compartment), trans Golgi network (TGN), Golgi and endoplasmic reticulum (ER).

BFA interferes with a membrane bound GTP exchange factor leading to a redistribution of Golgi contents to the ER. Ilimaquinone, a drug that causes Golgi vesiculation in some cell types, can also protect against ricin [49]. Transient expression of *trans* dominant negative GTPases that regulate vesicle transport steps in the early secretory pathway partially protected [50], whilst the introduction of a C-terminal ER retrieval signal (KDEL) into RTA increased the cytotoxicity of both reconstituted holotoxin [51, 52] and of free RTA [53, 54]. This apparent increase in potency suggested an encounter of incoming toxin with Golgi-to-ER cycling KDEL receptors. Taken together, the simplest interpretation of these data is that ricin must travel through the Golgi before it gains access to its ribosomal substrates in the cytosol (shown schematically in Figure **(2)**).

Perhaps the most convincing evidence for retrograde trafficking through the Golgi to the ER came from experiments where standard molecular biology methods were used to fuse a tyrosine sulphation signal and *N*-glycosylation sequon onto the C-terminus of RTA [55]. The modified recombinant RTA was purified from *E. coli* and presented to cells labelled with $^{35}SO_4^{2-}$. The small amount of toxin reaching the Golgi therefore became selectively labelled since tyrosyl sulphotransferase is located in this compartment. Furthermore, a fraction of the ^{35}S-labelled RTA was subsequently core glycosylated, a modification that signified a post-translational encounter with ER oligosaccharyl transferase. ^{35}S-labelled RTA was also found in the cytosol, strongly suggesting that toxin does indeed translocate the ER membrane. It should be noted that plant-derived RTA is naturally *N*-glycosylated at two positions [25,26]. While these internal native sites are not glycosylated when RTA is produced in *E. coli,* neither are they efficiently glycosylated when the folded protein reaches the ER lumen of mammalian cells. However, the supplementary glycosylation sites can become glycosylated, possibly because they are appended as part of a non-structured extension at the C-terminus of the protein.

Although ricin can be visualised in the Golgi (Figure **(3)**), it has never been observed by microscopy in the ER lumen, even where putative translocation mutants have been used that ought to accumulate in this compartment. Bearing in mind the multiplicity of surface binding sites for ricin, early uptake by both clathrin-dependent and -independent pathways, and the probable RTB-mediated interaction with secretory glycoproteins that would return a significant proportion of ricin to the cell surface, it is anticipated that very little internalised toxin actually reaches the ER. It might therefore be rationalised that such a small amount would be effectively "lost" to microscopy in the relatively large volume of this organelle. Nevertheless, the fraction that does successfully negotiate the endomembrane system to reach this site is clearly very effective in accomplishing membrane translocation to inactivate ribosomes.

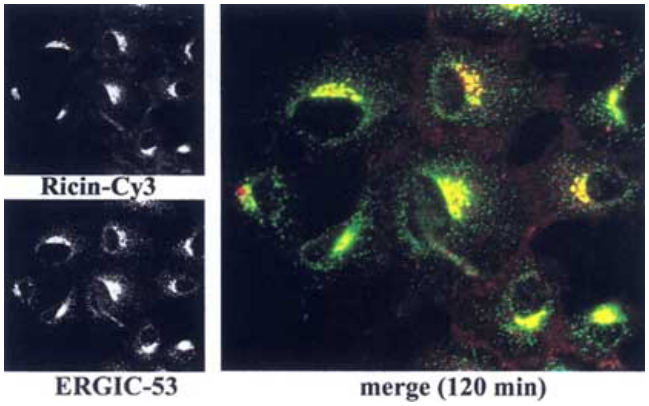

Fig. (3). ***Ricin can be tracked only to the Golgi using microscopy techniques.*** Ricin can be readily detected in the Golgi of most sensitive cells, here after 120 min uptake of a ricin-Cy3 conjugate into Vero cells. Co-localisation with the Golgi marker ERGIC-53 is shown. Longer incubations do not reveal an ER distribution of the fraction reaching this organelle by retrograde transport.

Because of a steric block of the active site, for RTA to act it must be reduced from RTB [25,26]. Interchain disulphide bond reduction may potentially occur in the cytosol, or within the ER lumen catalysed by protein disulfide isomerase (PDI), as is the case for cholera toxin [56-58] and Pseudomonas exotoxin A (PE) [59]. For ricin however, the site of reduction has never been formally demonstrated. However, it has been reported that a ricin holotoxin containing a non-reducible covalent linker between the subunits remained potently cytotoxic to mammalian cells, suggesting that both subunits may translocate to the cytosol [60]. Implicit in this finding is the presumption that a downstream proteolytic step in the cytosol must liberate a catalytically active fragment to inactivate ribosomes. By contrast, co-expression of ricin subunits to the ER lumen of transformed plant cells has strongly supported the view that free RTA is competent for retro-translocation by itself [28, 29]. In such cells, the toxic effect observed when newly synthesised RTA is retro-translocated across the plant cell ER can be greatly reduced when both RTA and RTB are simultaneously expressed. In this scenario, heterodimers are formed in the ER lumen that appear unable to retrotranslocate [29]. These data therefore support the hypothesis that in circumstances where holotoxins are endocytosed to the ER from the outside of the cell, reduction must occur prior to the membrane translocation of a free RTA subunit.

MEMBRANE TRANSLOCATION OF RICIN

It has been hypothesised [61] that non pore forming toxins such as ricin and other type II RIPS that undergo retrograde vesicular transport to the ER, do so in order to get into the cytosol via translocation machineries e.g. abundant peptide transporters (TAP) [62] and/or protein translocases (Sec61) [63], that are uniquely present in the membrane of this organelle. However, TAP-independent cells have been shown to be as sensitive to ricin as parental TAP-positive cells [64]. Rather more attention has been paid to the Sec61 complexes, more familiarly associated as the conduits for nascent protein import, as the possible channels for toxin export to the cytosol. Since the discovery that tightly gated Sec61 channels can operate to transport proteins in the reverse direction, this idea has been rendered more plausible. So in what process do such channels normally operate to retro-translocate proteins?

Misfolded proteins or orphan subunits do not accumulate in the ER lumen. In eukaryotic cells, an elaborate system of

molecular chaperones and associated proteins assist in the folding of nascent proteins. Some of these serve as timers whilst others somehow couple recognition of a terminally defective or orphan protein with the retro-translocation machinery in the ER membrane for ejection to, and eventual degradation within, the cytosol. This quality control pathway is known as ER-associated protein degradation (ERAD). Genetic and biochemical evidence now support a role for Sec61 complexes in the retro-translocation of ERAD substrates (for a recent review see [65]). Tightly coupled to the process of physical dislocation is the deglycosylation of glycoproteins and the ubiquitination of internal lysyl residues to mark the dislocated ERAD substrate for recognition and degradation by proteasomes.

The first direct evidence that ricin might subvert the ERAD pathway to facilitate export from the ER came from studies in which RTA was expressed in either yeast or plant cells and was co-translationally delivered into the ER lumen by virtue of an appropriate N-terminal signal sequence [66 ,29]. In pulse-chase experiments, newly synthesised, radiolabelled RTA present in the ER lumen was rapidly degraded in both systems. In yeast, the rate of RTA degradation was significantly reduced in mutants defective in either functional retro-translocation via Sec61p complexes, or with defective proteasomal degradation [636]. In contrast, the rate of RTA degradation was unaffected in ubiquitination mutants, implying that the degradation was not facilitated by ubiquitination [66]. In plant cells, the rate of degradation of glycosylated (ER) RTA was significantly reduced when cells were treated with a specific proteasome inhibitor [29], a correlation that supported the idea of RTA dislocation to cytosolic proteasomes. The involvement of Sec61 complexes in ricin retro-translocation in mammalian cells has been shown by co-immunoprecipitation of the Sec61 alpha subunit with the post-translationally core-glycosylated RTA variant that had been endocytosed to the ER as part of a holotoxin [49]. Interestingly, Chinese hamster ovary cell lines with genetic defects in ERAD also exhibited increased resistance to ricin intoxication [67]. Taken together, these data strongly suggest that RTA present in the ER lumen is perceived as an ERAD substrate and exported to the cytosol.

To be toxic to ribosomes the dislocated RTA must avoid degradation by proteasomes. Since treating cells with proteasomal inhibitors sensitises them to ricin (typically by 2-3 fold) [52, 64], it would appear that a significant proportion of the exported toxin is normally degraded. However, since cells are quite clearly killed when proteasomes are active, cell death must be caused by a fraction of retro-translocated RTA that somehow becomes uncoupled from the sequence of steps leading to complete degradation. How might this be achieved? The first inkling came from the observation that RTA, in common with the A subunits of all other toxins believed to access the cytosol from the ER, has an unusually low lysine content [68]. In contrast, type I RIPs, such as momordin, that are thought not to reach the ER should they become taken into cells by fluid phase uptake but whose overall protein fold is similar to RTA, have a more typical content of lysine (Figure 4). This led to the hypothesis that a paucity of internal lysine residues reduced the chance of polyubiquitination and subsequent ubiquitin-dependent proteasomal degradation of the toxin; a hypothesis that has now been experimentally verified [69]. The two internal lysyl residues in RTA are not normally targets for ubiquitination since their removal neither stabilised RTA nor affected the cytotoxicity of holotoxin. In contrast, when four additional surface lysines were introduced into RTA in a way that did not compromise the activity, structure, or stability of the toxin, degradation was potentiated and the lysine-enriched toxin was approximately 100-fold less potent to cells than native toxin. This phenotype was reversed when proteasomes were inhibited. Similar observations have been made when the lysine content of abrin A chain of the plant RIP abrin [69] and cholera toxin A chain [70] were modulated.

Other factors may also facilitate escape from the degradation pathway. Since folded proteins are not normally targets for ubiquitination, rapid refolding in the cytosol

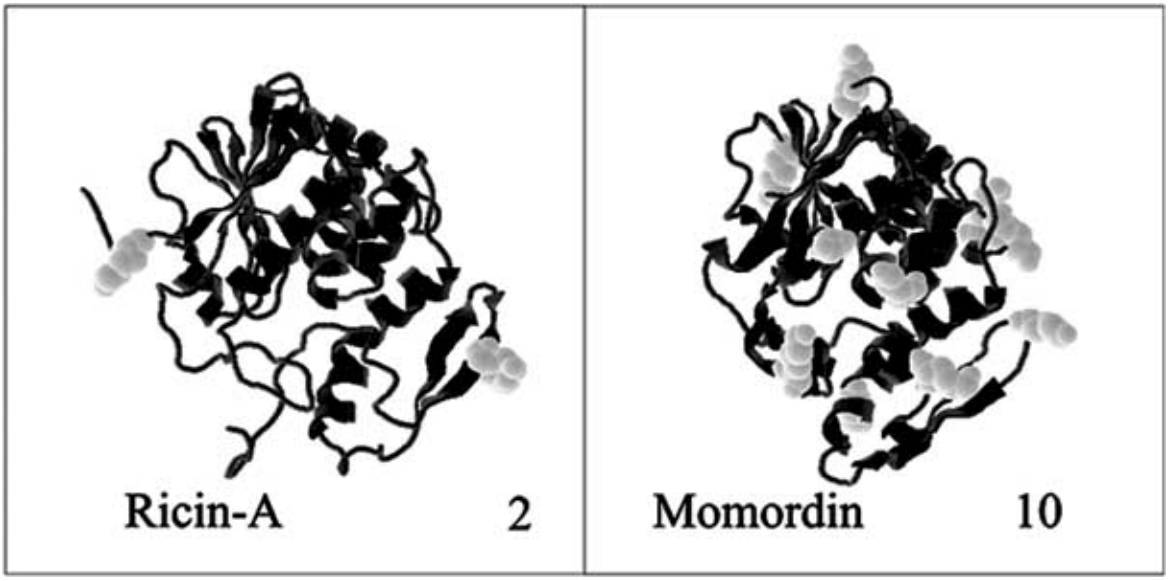

Fig. (4). ***Lysine content of ricin A chain and momordin.*** The similar backbone structures of ricin A chain and momordin (a type I RIP) and their differing content of lysyl residues is depicted.

might be an effective strategy, as has been shown for the cholera toxin A fragment [70]. Partially unfolded RTA can refold in the presence of ribosomes *in vitro* [71]. Whether this refolding capacity of substrate ribosomes occurs *in vivo* is unclear, but it is an attractive idea since the restoration of toxin to its biologically active conformation would be a "suicidal" act resulting in ribosome inactivation.

FACTORS THAT MIGHT INFLUENCE RETROTRANSLOCATION OF RTA.

How might a stable, globular protein such as RTA be initially perceived as a retro-translocation substrate by the cell? It is well established that free RTA can interact with biological or artificial membranes, whereas RTB or RTA as part of a holotoxin do not. Observations of crystal structures confirm that the hydrophobic C-terminus of RTA is exposed in the free subunit but is masked by RTB in the holotoxin. Assuming that reduction of holotoxin occurs within the ER lumen, the presence of such a patch may make interaction with chaperones or ER lipids more likely. Such interactions may then lead to the dislocation step itself. However, no chaperones have ever been implicated in ricin toxicity, other than a B-chain-mediated interaction of holotoxin, but not free A chain, with the chaperone calreticulin [72] (see below). Several reports have indicated that ricin and its subunits interact with lipid vesicles, but no clear consensus has been reached [73-77]. It has recently been observed that RTA can interact with negatively charged phospholipid vesicles *in vitro,* in which there is major structural change in the protein and a destabilisation of the lipid bilayer [78]. Although it is tempting to speculate about a specific ER lipid-RTA interaction, this awaits experimental testing.

A ROLE FOR RTB IN CELL ENTRY?

Apart from its obvious and obligatory role in binding ricin holotoxin to the cell surface, early studies with ricin-antibody conjugates (immunotoxins) revealed a second potential role for RTB, this time in relation to intracellular events. When the B chain galactose binding sites were chemically modified to minimise non-target cell toxicity, uptake of immunotoxins by alternative receptors on target cells still occurred, but toxicity was reduced to the same extent as it was with non-target cells [e.g. 79, 80]. This suggested that intracellular binding of ricin, involving B chain and a galactose-containing receptor, was important in facilitating toxin uptake. High mannose glycosylated RTB mutants have been generated in which each or both of the two sugar binding pockets were inactivated by amino acid substitution. These were reassociated with RTA for an assessment of their potency under conditions when cell entry was mediated either by interactions of the RTB sugar binding sites with galactose-containing receptors or via the RTB glycans and the mannose receptors of macrophages [81]. Following uptake by galactose-containing receptors, only the double site mutant showed no detectable potency. Using lactose to block uptake by galactose-containing receptors, internalisation became possible only because of an interaction between the glycans of RTB with surface mannose receptors. In the presence or absence of lactose, once again, only the mutant lacking both galactose-binding pockets was ineffective. These findings were consistent with a model in which the galactose binding activity of RTB has an intracellular role, possibly in effecting a concentration of toxin in a cell compartment or in facilitating routing to or through the Golgi.

What are the likely intracellular ricin receptors? This remains unresolved, for although the ER molecular chaperone calreticulin has been recognised as a binding partner of holotoxin *in vivo* [72], calreticulin-deficient cell lines are as sensitive to ricin as normal cells (unpublished data). The interaction of a terminally galactosylated calreticulin with incoming holotoxin rather than free subunits does however imply that it may be relevant to the Golgi-ER transport step of toxin uptake since the KDEL-containing ER chaperone presumably cycles through the Golgi where it is modified by galactosyl transferase and from where ricin may hitch a ride. The model that emerges is one in which ricin exploits certain cycling proteins through a lectin interaction between the B chain and a terminal galactose on the inadvertent carrier. Most galactosylated proteins in the secretory pathway that might encounter ricin would be predicted to sweep toxin out of the cell, and it is intriguing to note that inhibition of glycosylation increases sensitivity of cells to ricin [82]. Although the effect of such inhibition might be connected to the anticipated induction of the unfolded protein response in the ER, it may also be explained by the loss of secretory glycoproteins whilst cycling glycoproteins are retained. The probability this pool is more readily accessible to toxin under these conditions would therefore be predicted. However, much of this is speculation and remains to be experimentally resolved. Quite how many glycoproteins continuously cycle from the late Golgi to the ER having been modified by galactosyl transferase is unknown, but it *is* increasingly recognised that Golgi enzymes as well as cargo receptors do indeed undergo iterative cycling [83].

ENTRY OF TYPE I RIPS INTO MAMMALIAN CELLS

Single chain type 1 RIPs and the free A subunits of type II RIPs can be cytotoxic at high concentrations. Initially it was thought that the single chain toxins might enter cells via passive mechanisms such as fluid phase uptake [84]. However, different cell types vary considerably in their sensitivities to particular type I RIPs, even though fluid phase endocytosis should be occurring in all cell types. This observation, coupled to the organ-specific toxicity of type I RIPs, suggests that specific mechanism(s) occur to permit their uptake. Significant experimental evidence has implicated the α2-macroglobulin receptor (α2-MR), also known as the low density lipoprotein receptor-related protein (LRP), as the cell surface receptor responsible for the uptake of at lease some type I RIPs [85]. The α2-MR/LRP is a large cell surface receptor expressed in certain cell types and is exploited by *Pseudomonas* exotoxin A [86]. α2-MR/LRP consists of a 515 kDa heavy chain responsible for ligand binding, and a non-covalently associated 85 kDa light chain which contains the transmembrane and cytoplasmic domains. When present on cells, the α2-MR/LRP mediates the cellular uptake and subsequent degradation of proteinases, including the tissue-type plasminogen activator

(tPA) and the urinary-type plasminogen activator (uPA), plus other proteinase-inhibitor complexes. uPA is a serine protease that activates plasminogen to plasmin, which then degrades proteins of the extracellular matrix. uPA binds with high affinity to its receptor uPAR. The catalytic activity of uPA is eliminated by interaction with plasminogen activator inhibitors (PAIs). While active uPA bound to uPAR is stable at the cell surface, the uPA-PAI complex is internalised and degraded. Internalisation of the uPA-PAI complex via uPAR requires both components of the uPA-PAI complex to make contacts with the α2-MR/LRP. Hence uPAR is internalised along with the uPA-PAI complex and the α2-MR/LRP. α2-MR/LRP binds ligands other than α2-M-proteinase complexes: prominent amongst these other ligands is the α2-MR associated protein (RAP).

Ippoliti and colleagues [87] synthesised a conjugate linking human uPA and the type I RIP saporin, from *Saponaria officinalis*. UPA-saporin was potently and specifically cytotoxic to cells expressing uPAR and α2-MR/LRP, whereas cells devoid of uPAR and α2-MR/LRP were resistant. It was shown that some cell types, such as the α2-MR/LRP-negative T lymphocytes and Jurkat cells, are resistant to saporin, whereas other cell types, such as α2-MR/LRP-positive macrophages, are very sensitive. These findings prompted the suggestion that expression of the α2-MR/LRP was necessary for cells to be susceptible to intoxication by saporin. This, and other studies that have shown an interaction of both saporin and free RTA with α2-macroglobulin, suggest that saporin can enter at least some cell types by an endocytic mechanism mediated by α2-macroglobulin and the α2-MR/LRP system [88, 89 reviewed in 90]. This conclusion was further strengthened when trichosanthin was also found to interact with, and enter cell via, the low density lipoprotein receptor family members [91]. However, this emerging story has been complicated by the finding that toxins binding to the urokinase receptor are cytotoxic without requiring simultaneous binding to the α2-MR/LRP [92], and that the sensitivity of LRP-positive and negative cells to saporin can be similar [93].

Overall, and in contrast with ricin, little is known about the intracellular pathways followed by type I RIPs and the compartment(s) from which they enter the cytosol has not been elucidated. Productive uptake of free RTA and its subsequent toxicity is completely sensitive to brefeldin A and partially sensitive to the expression of *trans*-dominant negative GTPases that regulate biochemical complexes mediating anterograde and retrograde transport between the ER and the Golgi [94]. These and other data indicate when cells are treated with high doses of RTA, a proportion *can* reach an early compartment of the secretory pathway in at least some cell types before translocation occurs [95]. However, when 36 type I RIPs are compared, the average number of lysyl residues is 18.6 per polypeptide whilst the average number of lysyl residues from a set of seven A chains from cytotoxic type II RIPs is just 2.3 per polypeptide [69]. It is not known whether the correlation between degradation avoidance and the low lysine content of toxins known to retro-translocate the ER into the immediate vicinity of the ubiquitin-proteasome machinery means that a type I toxin with a more typical lysine makeup is more likely to translocate from different cellular compartments, but it remains a possibility. A study of the uptake of gelonin has suggested that this RIP, when internalised by pinocytosis, is released from endosomes and lysosomes [96,97], although the mechanism by which this is achieved is completely unknown. While the precise details of the cellular entry pathway of most type I RIP into mammalian cells remain to be elucidated, it seems clear that the absence of a cell-binding B polypeptide does not constitute an obligatory block on entry: type I RIPs apparently parasitise normal endocytic uptake processes, even though their scope is significantly less than their type II RIP counterparts.

ACKNOWLEDGEMENTS

Work at Warwick University is supported by grants from the UK Biotechnology and Biological Sciences Research Council and The Wellcome Trust.

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