How Ricin Reaches its Target in the Cytosol of Mammalian Cells

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Abstract The cytotoxic plant protein ricin comprises a lectin B chain that binds promiscuously to glycolipids and glycoproteins at the surface of mammalian cells, disulphide-coupled to a toxic A chain which depurinates target ribosomes. To find these cytosolic targets, the A chain has to cross a biological membrane, which is not a simple task for a folded protein. The secretory pathway of eukaryotic cells is reversible and ricin can take advantage of this to move from the plasma membrane, via the Golgi, to the ER whose membrane is crossed to gain access to the cytosol. Since membrane traversal is preceded by an unfolding step, there is a clear requirement for cytosolic re-folding of ricin to gain a catalytic conformation. This final step for ricin is accomplished after triage by cytosolic chaperones, underlining the central role of these in cellular protein folding.

1 Introduction

The cytotoxic heterodimeric plant protein ricin is laid down in the developing endosperm of the seeds of the castor oil plant, *Ricinus communis*, where it constitutes up to 5% of the dry mass of the seed protein (Harley and Beevers 1986). Its B chain (RTB) is a lectin, interacting with terminal non-reducing galactose residues (exposed $\beta 1 \rightarrow 4$ linked galactosyls) (Olsnes et al. 1974); thus RTB binds promiscuously to many glycolipids and glycoproteins at the surface of mammalian cells. RTB acts as a delivery agent for the catalytic toxic A chain (RTA), which depurinates target ribosomes (Endo et al. 1987). To find

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J.M. Lord and M.R. Hartley (eds.), *Toxic Plant Proteins*, Plant Cell Monographs 18,
DOI 10.1007/978-3-642-12176-0_11, © Springer-Verlag Berlin Heidelberg 2010

these cytosolic targets, RTA has to cross a biological membrane, which is not a trivial task for a folded protein.

2 Cytotoxicity Model

The model for intoxication of target mammalian cells by ricin is deceptively simple (Fig. 1a). Ricin holotoxin binds cell-surface receptors and is then internalised and trafficked in a retrograde manner, via early/sorting endosomes and the Golgi apparatus, to the endoplasmic reticulum (ER), where RTB and RTA are separated. Free RTA is then recognised as an unstable or misfolded protein and is exported (retrotranslocated/dislocated) to the cytosol for its destruction, using pre-existing cellular mechanisms for clearing misfolded proteins from the ER. However, instead of being destroyed, at least a proportion of cytosolic RTA gains an active catalytic conformation that depurinates the target ribosomes. Some details of this model are known, but many remain obscure.

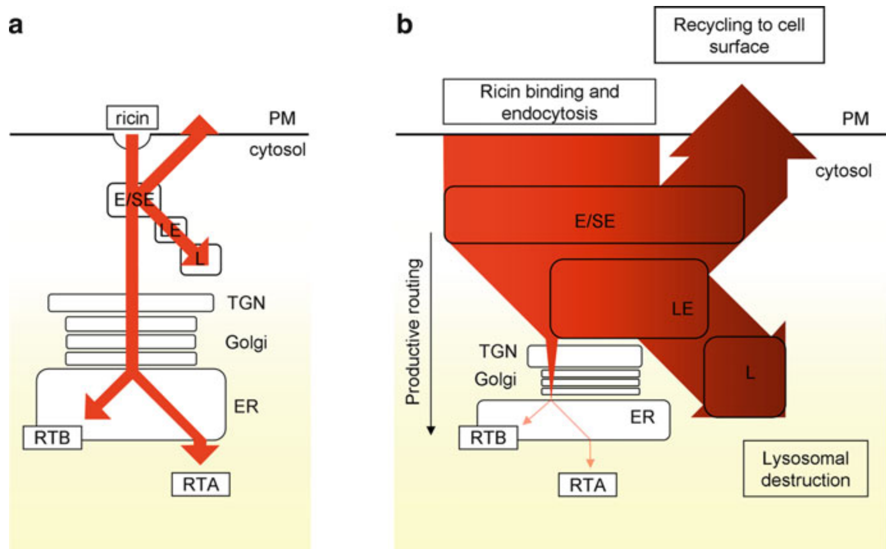

Fig. 1 Ricin trafficking schemes. (**a**) Receptor-bound ricin at the cell surface (plasma membrane, PM), is taken up by endocytosis into early/sorting endosomes (*E/SE*). From here proceed multiple routes – a recycling path back to the cell surface, a destructive track via the late endosome (*LE*) – lysosome (*L*) pathway, and a productive cytotoxic route via the *trans*-Golgi network (*TGN*) and the Golgi stack to the endoplasmic reticulum (*ER*). Here the A (*RTA*) and B (*RTB*) chains of ricin are separated reductively, and free RTA dislocates, crossing the ER membrane to enter the cytosol. (**b**) These routes are not equal: when the width of the *arrows* is adjusted to represent the approximate flow of ricin through these pathways, it becomes clear that the majority of ricin traffics endosomally, and the major cytotoxic route implied in *a* is utilised by only a small proportion of the ricin bound initially at the cell surface

3 Cell Entry

3.1 Cell Surface Events Remain Cryptic

Upon cell binding, the bulk of ricin is slowly internalised via clathrin-coated pits, but if coated pit formation at the cell surface is arrested then ricin cytotoxicity is unaltered, even though there is a 50% reduction in overall ricin internalisation (Moya et al. 1985; Sandvig et al. 1988). Thus productive (i.e. cytotoxic) routing of ricin does not appear to depend upon the recruitment of receptors to clathrin-coated pits. Furthermore, interfering with caveolar function makes little difference to ricin toxicity, suggesting that productive routing is caveolae-independent (Simpson et al. 1998). This invokes either a third endocytotic route or suggests that ricin intoxication involves multiple productive receptors that can enter cells via multiple mechanisms, so that the effect of inhibiting individual entry pathways makes little overall difference.

Favouring this latter interpretation is the promiscuous, low affinity, high capacity binding of ricin to cells (Sandvig et al. 1976; Spooner et al. 2004). To date, the identity of any ricin receptors that are required for cytotoxicity is unknown. The majority are likely to be proteinaceous, since manipulating cellular levels of glycosphingolipids does not measurably affect ricin toxicity, suggesting that ricin receptors are not glycolipid in nature (Spilsberg et al. 2003). However, cells which are unable to synthesise complex *N*-glycans owing to loss of *N*-acetylglucosaminyltransferase I bear glycans on their glycoproteins that are trapped at a defined stage prior to galactose modification (Reeves et al. 2002) and are only protected from a ricin challenge by a factor of ~20-fold (Crispin et al. 2009). This suggests that ~5% of productive ricin routing occurs via a non-protein, presumably glycolipid, targeted route.

This lack of knowledge about cell surface events that lead to ricin intoxication contrasts strongly with the knowledge of internalisation of the bacterial Shiga (-like) toxin (STx) and cholera toxin (CTx), both of which cross-link cognate lipid receptors, forcing membrane curvature and invagination (Römer et al. 2007; Windschiegl et al. 2009) and STx further regulates its own entry by stimulating activity of Syk kinase (Lauvrak et al. 2006).

For ricin, uncertainty of events at the cell surface means that trafficking pathways productive for ricin cytotoxicity cannot be studied by examining the behaviour of known receptors.

3.2 Retrograde Trafficking

Despite some confusion about cell surface and very early endocytic events, retrograde trafficking pathways for ricin appear to merge at or near the early endosome. A level of uncertainty also exists for some early endosome events, which may

indicate confounding cell-type specific effects. Thus the role of the small GTPase dynamin acting as a scission agent at the early endosome is not clear, with expression of mutant dynamin having either no effect on ricin toxicity in COS7 cells (Simpson et al. 1998), or else protecting HeLa cells about tenfold from ricin challenge (Llorente et al. 1998). Nevertheless, a number of cellular molecules required for ricin toxicity have been identified.

At least a portion of an endosomal trafficking toxin must avoid destruction by the lysosomes. This process is efficient for STx (Fuchs et al. 2007) but very inefficient for ricin (van Deurs et al. 1988), suggesting that access to a retrograde sorting complex is difficult for ricin (Fig. 1b). A candidate for this difficult step is the access to retromer components (Fig. 2a). Mammalian retromer mediates retrograde transport between the endosome and *trans*-Golgi network (TGN), and is associated with tubular–vesicular structures that spread from early endosomes or from intermediates in the maturation pathway from early to late endosomes (Arighi et al. 2004). These may constitute part of the tubular endosomal network (TEN) that sorts and recycles cargoes. Retromer appears to sequester membrane cargo proteins from vacuolar endosomal membranes into retrograde transport intermediates, thereby preventing default delivery to lysosomes. The retromer coat comprises a dimer of

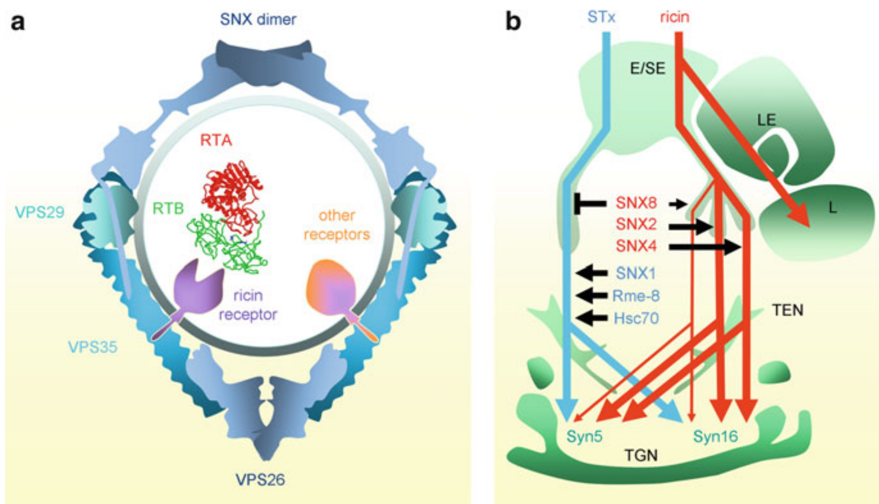

Fig. 2 A model for endosomal sorting of ricin. (**a**) A diagrammatic cross-section through a tubular portion of an early/sorting endosome, showing the arrangement of the retromer coat proteins (SNX, VPS29, VPS35 and VPS26). It is likely that ricin receptors are bound by VPS35, allowing them to enter the tubular portions of the endosome and gain access to the retrograde transporting TEN. (**b**) Sorting events at the early endosome can be complex. STx (*blue arrow*) gains efficient entry to TEN and subsequently the TGN by utilising a retromer complex containing SNX1 and the chaperones Hsc70 and its partner the DnaJ containing protein Rme-8. Ricin (*red arrows*) enters TEN inefficiently, via multiple routes, but the majority is not sorted in the retrograde direction. Two TGN docking mechanisms are currently identified, using SNARE complexes containing either syntaxin 5 or syntaxin 16

different sorting nexin combinations (typically SNX1, SNX2, SNX5 and SNX6) and a Vps26–Vps29–Vps35 trimer (Bonifacino and Hurley 2008). Early endosome trafficking also requires phosphoinositide (PI)-3 kinase activity, that generates the PI(3)P lipid that is bound by the PX domain of sorting nexins. To date, hVps34 is the only identified kinase that phosphorylates PI in the third position to produce PI (3)P. Since access to retromer-controlled retrograde transport intermediates would be an advantage for a protein toxin, it is not too surprising that roles for SNX2, SNX4 and hVps34 have been elucidated in the transport of ricin from early endosomes to the TGN (Skånland et al. 2007). What is surprising is that SNX2 and SNX4 appear to control separate retrograde routes, pointing to some complexity of trafficking events at the early endosome. Presumably, the interaction with retromer is indirect, in that the cargo being sorted is the (ricin-bound) ricin receptor, rather than ricin itself.

Choice of retrograde routes by toxins is not universal (Fig. 2b) – for examples, STx achieves efficient retrograde routing by utilising SNX1 (Bujny et al. 2007; Popoff et al. 2007); CTx has only a small requirement for SNX1 (Bujny et al. 2007), so it may utilise other components; and the subAB toxin that traffics from the plasma membrane to the ER uses neither SNX1 nor SNX2 to access the TGN (Smith et al. 2009), emphasising the complex manner in which eukaryotic cells sort and traffic endogenous components. Sorting nexin 8 has unusual roles – it has a weak promoting effect for ricin transport but inhibits retrograde transport of STx (Dyve et al. 2009). STx transport also requires RME-8, the receptor mediated endocytosis-8 protein, that interacts with SNX1 and the molecular chaperone Hsc70 (Popoff et al. 2009). In contrast, retrograde transport of ricin appears to be independent of Hsc70 function (Spooner et al. 2008a).

From the retrograde endosomal transport intermediates, ricin is transported to the TGN, and early studies, using immuno-gold labelling and electron microscopic techniques, demonstrated that this is inefficient, with only ~5% of cell-surface bound ricin reaching this compartment (van Deurs et al. 1988). Fusion of transport intermediates with target membranes is mediated by SNARE complexes. Ricin transport specifically requires SNARE complexes localised to early endosomes and the TGN. One complex comprises syntaxin 5, Ykt6, GS15 and GS28: the other complex comprises VAMP3 or VAMP4, syntaxin 16, syntaxin 6 and Vti1a (Amessou et al. 2007). This requirement for at least two separate SNARE-controlled docking systems points to multiple transport routes between endosomes and the TGN. A similar argument can be made for roles of multiple sorting nexins in ricin transport (Skånland et al. 2007).

When ricin is reconstituted from a recombinant RTA with a sulphatable tag and native plant RTB and this is used to intoxicate mammalian cells, [^{35}S]-labelled RTA can be immunoprecipitated from the cytosol (Rapak et al. 1997). Thus at least a proportion of ricin traffics from the TGN via the *trans*-Golgi cisternae, where the relevant sulphotransferase resides (Spooner et al. 2008b) and presumably on from there via the Golgi stack to the ER.

The evidence that the Golgi stack is an important conduit for retrograde transport is extensive. For some proteins there is a critical dependence on binding KDEL

receptors which cycle between the TGN and the ER via the Golgi cisternae (Miesenbock and Rothman 1995) in a COP1-dependent manner which typifies retrograde transport in the classic secretory pathway. *Pseudomonas* exotoxin A, which bears a KDEL-like sequence at its C-terminus, can utilise this route (Smith et al. 2006) as can subAB toxin (Smith et al. 2009). However, STx traffics in a COP-1 independent manner, instead requiring the small GTPase Arl1 (Tai et al. 2005) and its effector the Golgi tethering factor golgin-97 (Lu et al. 2004; Tai et al. 2005) and its targeting co-factor ARFRP1 (Shin et al. 2005). It also requires the Golgi tethering factors golgin-245 (Yoshino et al. 2005) and GCC88 (Lieu et al. 2007), the conserved oligomeric Golgi COG complex (Zolov and Lupashin 2005), the Golgi-associated retrograde protein GARP (Perez-Victoria et al. 2008) and the TGN tethering factor GCC185 (Derby et al. 2007). Roles for the Golgi docking and fusion promoter Rab6a′ (Girod et al. 1999; Mallard et al. 1998; White et al. 1999), for its RabGAP Rab6IP2 (Monier et al. 2002) and for Rab11 (Wilcke et al. 2000) have also been established. *Pseudomonas* exotoxin A can also utilise a Rab6-dependent route (Smith et al. 2006), and subAB toxin also utilises this and the COG complex (Smith et al. 2009), remarkable examples of toxins with single cognate receptors taking advantage of multiple retrograde trafficking options through the Golgi stack. Finally, sub-cellular microsurgery to remove the Golgi stack halts retrograde transport of the STx B chain to the ER (McKenzie et al. 2009).

In contrast to this wealth of detail for other toxins, the evidence that ricin proceeds from the *trans*-Golgi cisternae through the Golgi stack is much scarcer. Simultaneous blocking of the COPI-mediated and the Rab6a′-controlled routes through the Golgi do not affect ricin toxicity, suggesting that there is a third, uncharacterised route from the TGN to the ER (Chen et al. 2003). Transport of ricin through the Golgi stack may not even be unidirectional, since ricin intoxication is governed by Rab1, a small GTPase that controls vesicular traffic in the (opposite) anterograde direction, between the ER and the Golgi (Simpson et al. 1995a). Consistent with this, ricin can be co-immunoprecipitated with calreticulin, an ER chaperone that recycles between the ER and the Golgi (Day et al. 2001) that may act as a fortuitous delivery agent to the ER. Overall, then, the manner of ricin's progress through the Golgi stack remains somewhat mysterious.

3.3 Ricin Is Delivered to the ER

Utilising a ricin holotoxin whose A chain is modified by addition of sulphation and glycosylation motifs allows biochemical tracking of ricin to the ER, where the glycosylation motifs become core-*N*-glycosylated (Rapak et al. 1997). Furthermore, a holotoxin with a KDEL retrieval sequence appended to the C-terminus of RTA is more toxic than native holotoxin (Tagge et al. 1996; Wales et al. 1992; Wesche et al. 1999), consistent with increased ER delivery by forcing at least a proportion of Golgi-delivered ricin through the efficient COPI-dependent retrograde route.

3.4 Ricin Is Reduced to its Constituent Chains in the ER

The disulphide bond linking RTA and RTB is occluded, since, in the absence of a denaturant and heat, high concentrations of the small molecule reducing agent DTT are required to separate the two chains of ricin in vitro (Emmanuel et al. 1988; Simpson et al. 1995c). Furthermore, the ER is a relatively oxidising environment that favours the formation, not the scission, of disulphide bonds. These considerations immediately invoke a protein or proteins capable of remodelling the structure of ricin holotoxin to open the interface between the two chains and expose the interchain disulphide bond, allowing reductive cleavage. Protein disulphide isomerase (PDI) possesses these qualities, in that it acts both as a chaperone and as a disulphide exchanger (Ferrari and Söling 1999; Klappa et al. 1997), and also is found predominantly in the ER.

The tiny amounts of ricin that reach the ER (Fig. 1) impose severe experimental difficulties, particularly if a chaperone interaction is suspected, since recombinant tagged RTA versions are not suitable because they may force chaperone interactions. Despite these difficulties, evidence has accumulated that PDI is responsible for the reductive separation of RTA and RTB (Fig. 3). In vitro, on microsomal membranes and in crude cell extracts, PDI can reduce ricin in the presence of thioredoxin, thioredoxin reductase and NADPH (Bellisola et al. 2004). Supporting this, auranofin, an irreversible inactivator of thioredoxin reductase, protects cells against ricin, but not against challenge with pre-reduced ricin, suggesting that activation of PDI by thioredoxin reductase has some physiological significance for ricin intoxication.

Excess RTB at the site of reduction of ricin should act as a dead-end receptor for newly liberated RTA, and therefore protect cells against ricin challenge. When RTB is expressed in the ER of mammalian cells, this is precisely what happens (Spooner et al. 2004). ER-targeted RTB is retained by a thiol anchor in the ER for some time before disposal, and breaking this bond in vivo by treating cells with DTT reverses the protective effect against ricin challenge of RTB expression. This thiol anchor is a mixed disulphide between RTB and PDI, suggesting that PDI can both make and break bonds in ricin confirmed in vitro using glutathione reduced PDI. Free RTB in the ER has two fates – a proportion is an ERAD substrate and is degraded by the cytosolic proteasomes whilst the remainder is secreted (Spooner et al. 2004). The fate of RTA, though, is much more interesting.

3.5 RTA Unfolds in the ER

For CTx, interactions with PDI also result in the unfolding of its A chain (CTxA), making it susceptible to trypsin cleavage (Tsai et al. 2001). A model has been presented where unfolded CTxA is then released from PDI by reduced Ero1p at the ER membrane as a prelude to dislocation (Tsai and Rapoport 2002). For ricin and

Fig. 3 A model for ER events. (**a**) Treatment of ricin with reduced PDI does not result in increased trypsin sensitivity, suggesting that, unlike CTX, ricin is not substantially unfolded by PDI interactions. *Lane 1*, BSA-treated ricin; *lane 2*, ricin treated with reduced PDI. After treatment, samples were incubated with trypsin and subjected to reducing SDS-PAGE and immunoblotting, probing with anti-RTA antibodies. (**b**) Ricin entering the ER lumen is reductively separated into its constituent RTA (*red*) and RTB (*green*) chains by reduced protein disulphide isomerise (*PDI*). A proportion of RTB is removed from the ER by ERAD and the rest by secretion. The A chain undergoes structural alteration at or near the ER membrane, and partially inserts, with the majority of the inserted region being derived from the hydrophobic stretch of amino acids towards the C-terminus of free RTA. It thus presents to the ER quality control mechanisms as a misfolded membrane-associated protein, stimulating membrane extraction and dislocation to the cytosol as an ERAD substrate. The Hsp90 chaperone GRP94 may play in role in maintaining solubility of RTA during the membrane extraction/dislocation preparation steps

RTA, interactions with PDI do not cause obvious protease sensitivity (Fig. 3a), suggesting that unfolding of RTA occurs by a different mechanism.

A patch of hydrophobic amino acids near the C-terminus of RTA is occluded by RTB until revealed by reduction of the holotoxin in the ER. Adding positively charged residues near this site reduces ricin toxicity (Simpson et al. 1995b), suggesting that this patch might normally interact with membrane lipids. When fused to the N-terminus of other proteins, this region can act as a signal sequence, directing these nascent proteins into the ER (Chaddock et al. 1995), so this region, at least in this context, can enter the lipid bilayer of the ER. After Triton-X114 extraction, RTA (but not ricin) partitions in the detergent phase, suggesting that also in the context of native RTA structure, this region can interact with lipid membranes (Day et al. 2002). Furthermore, in the presence of a negatively charged phospholipid RTA undergoes spontaneous structural alterations, leading to a model where lipid-induced partial unfolding of RTA allows its recognition as an ERAD

substrate (Day et al. 2002). A major caveat of this is that the negatively charged phospholipid that was used is not found physiologically, but this has been addressed recently, using liposomes generated from phosphatidyl serine, a physiologically relevant lipid, to promote identical changes in RTA structure (Mayerhofer et al. 2009).

When RTA is presented to microsomal membranes saturated with a lipophilic quenching agent, sophisticated experiments that use RTA tagged with fluorophores in different positions around the molecule demonstrate that the interaction with ER membranes is not random (Mayerhofer et al. 2009): fluorophores in some positions are quenched, showing membrane insertion, whilst fluorophores in other positions are not quenched. Thus RTA inserts into membranes in a precise, ordered manner (Fig. 3b). While the majority of amino acids from RTA that insert into the membrane are clustered at or near the C-terminal hydrophobic patch, there is a curious exception – a positively charged amino acid (arginine) on a loop near the N-terminus also inserts into the lipid bilayer. When a signal peptide is released from the translocon into the lipid environment of the mammalian ER membrane, its hydrophobic core forms a strong anchor in the membrane and its removal requires signal peptide peptidase to cut the signal peptide into two halves which can be extracted with ease. Perhaps insertion of a hydrophobic RTA-derived structure that includes a positively charged amino acid reduces the strength of the interaction with the ER membrane, facilitating extraction of RTA as a prelude to dislocation.

Insertion into the membrane is also a temperature-dependent process, with membrane binding evident at low temperatures, but with structural changes increasing as the temperature approaches 37°C (Mayerhofer et al. 2009). Purified RTA in vitro aggregates and rapidly becomes insoluble at 45°C, but even at 37°C (the physiological temperature for intoxication of mammalian cells) it is relatively unstable (Spooner et al. 2008a). Thus the driving force for RTA unfolding appears to be thermal instability of RTA released from RTB, coupled with an ordered insertion into the ER membrane.

3.6 Chaperone Interactions in the ER

From its partially buried state in the ER membrane, RTA must be extracted and interact with a dislocation mechanism that can transfer it across the ER membrane into the cytosol. The partial unfolding of RTA to permit membrane entry may also allow recognition by ER molecular chaperones. For the ER-trafficking STx, interactions of the A chain have been noted with the ER Hsp70 chaperone BiP (Falguières and Johannes 2006) and its Hsp40 co-chaperone ERdj3, whose over expression protects cells from toxin challenge (Nakanishi et al. 2004; Yu and Haslam 2005). It is likely that these chaperones promote solubility of the STx A chain, so aiding its recognition as an ERAD substrate, in the same way that ERAD of many misfolded proteins in yeast is aided by DnaJ domain-containing Hsp40 co-chaperones that interact with the ER luminal chaperone Kar2p/BiP (Nishikawa

et al. 2001). To date, though, no clear role for BiP and its co-chaperones has emerged for ricin.

There is a hint of a role, though, for the ER Hsp90 GRP94 chaperone (Fig. 3b), since *N*-ethylcarboxamidoadenosine, a GRP94 inhibitor that does not affect cytosolic Hsp90 (Rosser and Nicchitta 2000) protects cells slightly from ricin (Spooner et al. 2008a). This may suggest a role for GRP94 in preparing RTA for dislocation, consistent with its ability to direct the null Hong Kong variant of α1-anti-trypsin to ERAD (Christianson et al. 2008).

Manipulating expression levels of EDEM, the ER degradation enhancing α-mannosidase I-like protein, alters the sensitivity of cells to ricin: overexpression protects cells from ricin, but knockdown reduces dislocation of RTA and so also protects (Slominska-Wojewodzka et al. 2006). Co-immunoprecipitation of RTA and EDEM suggested a direct role for EDEM, perhaps as a transporter of RTA to the dislocation machinery. Subsequently, the yeast homologue of EDEM, Htm1p, has been shown to retain mannosidase activity, which generates an exposed $\alpha 1 \rightarrow 6$ mannose residue on glucose-trimmed *N*-glycans of misfolded proteins (Quan et al. 2008). These constitute signals for ERAD recognition, delivering the glycoprotein to the lectin Yos9p and from there to the Hrd3p subunit of an E3 ubiquitin–ligase complex required for dislocation of ERAD substrates with lesions in their luminal domains. Since ricin reconstituted with recombinant RTA contains a non-glycosylated RTA, but is as toxic as the native plant holotoxin (which contains glycosylated RTA), the role of EDEM becomes less clear. It may be that the EDEM target is RTB, which is glycosylated. However, the effects may be caused indirectly. CHO cells up-regulated for ERAD can display a CTx/*Pseudomonas* exotoxin A/ricin-resistant phenotype by increasing the coupling efficiency between toxin dislocation and toxin degradation (Teter et al. 2003). Down-regulation of ERAD can produce the same multitoxin-resistant phenotype, attributed here to decreased retrotranslocation (Teter and Holmes 2002). Thus, manipulating the rate of ERAD steps influences the cytotoxicity of ER-trafficking toxins, but by multiple possible mechanisms. Such indirect effects may explain, in part, the effect of manipulating expression levels of EDEM.

3.7 The Dislocation Process for RTA Remains Mysterious

RTA can be co-immunoprecipitated from cells with Sec61α (Slominska-Wojewodzka et al. 2006; Wesche et al. 1999), a component of the translocon through which nascent proteins enter the ER, and which has been implicated in the dislocation of a number of ERAD substrates in both yeast (Scott and Schekman 2008; Willer et al. 2008) and mammalian systems (Imai et al. 2005; Koopmann et al. 2000; Wiertz et al. 1996). Supporting evidence that the translocon constitutes part of the dislocation system comes from expression of RTA in the yeast ER (Simpson et al. 1999). However, in mammalian cells, a functional requirement remains to be demonstrated. To date, no other molecules required for RTA dislocation have been identified,

although a number of candidates, such as derlins, have been shown to have no obvious functions in this process (Slominska-Wojewodzka et al. 2006). Dislocation of RTA, then, remains a cryptic process.

4 Recovery of Activity in the Cytosol

The conserved Sec61 (mammals, yeast) or SecY (bacterial) heterotrimeric membrane protein complex forms a protein-conducting channel, allowing polypeptides to be transferred across or integrated into membranes. Structural studies of the bacterial SecY translocon show that the translocation pore is narrow, so large structures are unlikely to be extruded (Van den Berg et al. 2004). This leads to the notion that dislocated substrates are to a large extent unfolded, and enter the cytosol in a vulnerable non-native conformation. Thus a dislocated toxin with cytosolic targets might be expected to refold in the cytosol.

In principle, acquisition of a catalytic conformation by dislocated RTA could be accomplished by multiple mechanisms. RTA carefully unfolded by heat to a molten globule state can regain activity when presented to ribosomes in vitro, leading to a model whereby dislocated RTA can undergo substrate (ribosome)-mediated re-folding (Argent et al. 2000). An alternative might be by spontaneous re-folding, proposed for dislocated CTxA chain (Rodighiero et al. 2002). However, in vivo, the Hsc70 inhibitor deoxyspergualin protects cells from ricin, and overexpression of Hsc70 co-chaperones alters the cytotoxic response, demonstrating a vital role for the molecular chaperone Hsc70 in the activation of cytosolic RTA (Spooner et al. 2008a). Since Hsc70 prevents heat-inactivation of RTA, then one role for Hsc70 might be to prevent aggregation of vulnerable, unfolded dislocated RTA in the cytosol.

Dihydrofolate reductase (DHFR) fusions with RTA or with Class I major histocompatibility complex heavy chain still act as ERAD substrates, even when the DHFR domains are stabilised into fully folded structures by addition of a folate analogue (Beaumelle et al. 1997; Tirosh et al. 2003). Thus either the Sec61 channel can be modified to accommodate a folded DHFR domain for dislocation, (but not for translocation into the ER), or a different channel is used for dislocation. Since folded domains can dislocate, dislocated RTA may still have considerable structure. In vitro, RTA shows a degree of thermal instability at 37°C which is reduced in the presence of Hsc70 (Spooner et al. 2008a), so the role of Hsc70 may be to stabilise RTA in the cytosol, perhaps by masking the C-terminal hydrophobic patch implicated in membrane associations.

Furthermore, there is a chaperone triage of RTA in the cytosol (Fig. 4) that includes interactions with the chaperone Hsp90, but only after previous interactions with Hsc70 (Spooner et al. 2008a). RTA possesses only two lysyl residues, a remarkable shortage when compared with the number of lysines found in other non-cytotoxic RIPs. This led to the proposal that ricin traffics to the ER in order to allow the A chain to somehow disguise itself as a substrate for ERAD, the

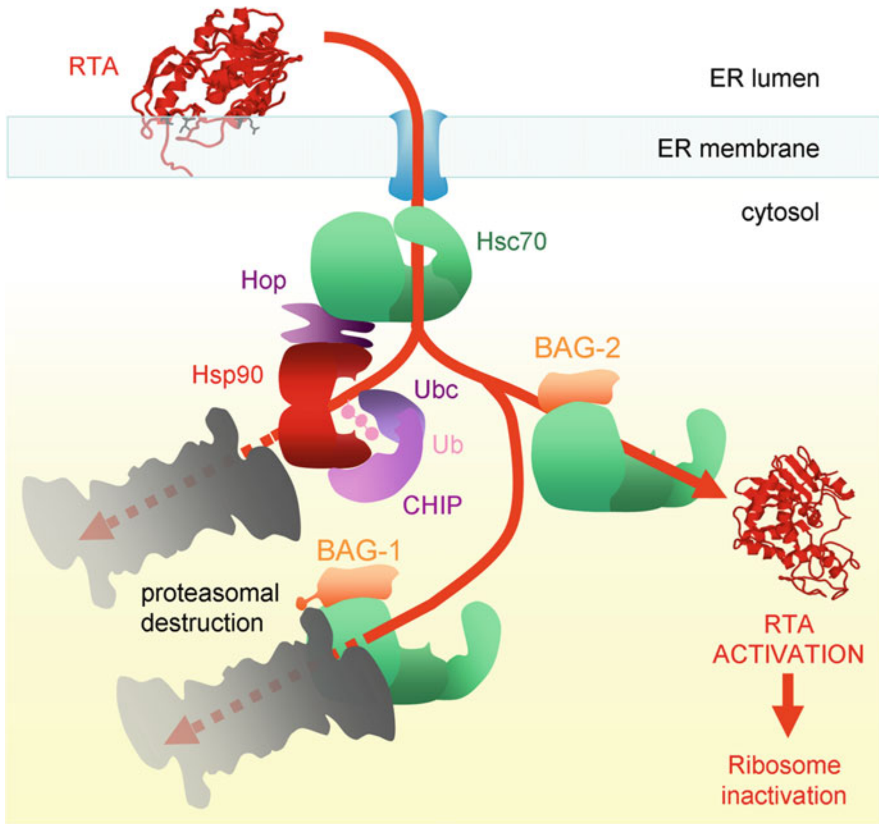

Fig. 4 RTA fate is determined after triage by cytosolic chaperones. RTA enters the mammalian cytosol by a dislocation mechanism that has yet to be elucidated, in a vulnerable non-native conformation. Interactions with the cytosolic chaperone Hsc70 are vital in the recovery of an active conformation. From Hsc70, multiple routes lead to inactivation: these appear to be controlled by ubiquitin (*Ub*) or ubiquitin-like signals that permit engagement with the cytosolic proteasomes. Direct passage to the proteasome occurs after ubiquitylation of RTA by CHIP (C-terminus of Hsc70 interacting protein) and associated ubiquitin conjugating enzymes (*Ubc*) following sequential interactions of RTA with Hsc70 and Hsp90 that are co-ordinated by the dual co-chaperone Hop (Hsc70-Hsp90 organising protein). Indirect routes probably also include "piggy-back" transport to the proteasome through CHIP-mediated ubiquitylation of Hsc70 and via CHIP auto-ubiquitylation (not shown). Uncoupling from the final destructive steps of ERAD requires release of RTA from Hsc70. Release by BAG family (BCL2-associated anathogene protein family) nucleotide exchange factors can lead to either inactivation or activation of RTA. BAG-1 interactions result in release of RTA at the proteasome, which is engaged by the interlaced ubiquitin-like domain of the nucleotide exchange factor. However, RTA release from Hsc70 by BAG-2, which has no ubiquitin-like domain, permits RTA to acquire a catalytically active structure which then inhibits protein synthesis ability by specifically depurinating a large ribosomal RNA

ER-associated protein degradation mechanism that clears misfolded and orphan proteins from the ER, dispatching them to the cytosol for proteasomal elimination (Hazes and Read 1997). Seen in this light, the reduction in lysyl residues may reduce the potential for polyubiquitylation, and so hamper processing by the proteasomal core. Experimental support for this was obtained by replacing some of the arginyl residues of RTA with lysyls, in positions where RTA activity and structure were not compromised. The resulting holotoxins were much reduced in toxicity, but inhibition of proteasomal activity increased the toxicity of lysine-rich ricin variants to close to normal (Deeks et al. 2002). Despite possession of only a low number of lysyl residues that reduce polyubiquitylation and subsequent proteasomal targeting (Deeks et al. 2002; Hazes and Read 1997), inhibition of proteasomal core activities sensitises cells to ricin (Deeks et al. 2002; Slominska-Wojewodzka et al. 2006; Wesche et al. 1999), so at least a proportion of RTA is degraded by proteasomes. Sensitivity to ricin correlates with ubiquitylation or ubiquitin-like signals (Spooner et al. 2008a). Thus overexpression of the Hsc70 nucleotide exchange factor BAG-1 (which has a ubiquitin-like domain that targets the BAG-1: Hsc70: client complex to the proteasome) protects cells from ricin challenge, and overexpression of the Hsc70-Hsp90 associated E3 ubiquitin ligase CHIP also protects cells. Furthermore, over expression of the dual co-chaperone Hop, that transfers RTA from Hsc70 to Hsp90, also protects cells, reflecting the ability of CHIP to interact with RTA and mark it for destruction only in the context of Hsp90 binding. Since replacement of the two lysyls in RTA with arginine residues does not increase the toxicity of ricin (Deeks et al. 2002), it is likely that cytosolic ubiquitylation of RTA is non-canonical, and does not occur on the two remaining lysine residues (Fig. 4).

5 Conclusions

The secretory pathway of eukaryotic cells is fully reversible and some plant protein toxins such as ricin can take advantage of this to move from the plasma membrane, via the Golgi and the ER to the cytosol. There are multiple retrograde routing steps which are utilised by different toxins. “Fast” routes through the Golgi can be utilised by the dedicated bacterial ER-trafficking toxins such as STx and SubAB. However, to a first approximation, ricin appears to be an endosomal trafficker and only a small proportion of cell-bound toxin can access retrograde routes to the ER, trafficking slowly and inefficiently through the Golgi stack. In the ER, reductive separation releases the active A chain from the inactive holotoxin. The ER-cytosol dislocation step is currently cryptic for these toxins, but since this is preceded by an unfolding step, there is a clear requirement for re-folding in the cytosol to gain a catalytic conformation. This final step for ricin is dependent upon cytosolic chaperones, underlining the central role of Hsc70 in cellular protein folding.

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