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Targeting ricin to the ribosome

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ABSTRACT

The plant toxin ricin is highly toxic for mammalian cells and is of concern for bioterrorism. Ricin belongs to a family of functionally related toxins, collectively referred to as ribosome inactivating proteins (RIPs), which disable ribosomes and halt protein synthesis. Currently there are no specific antidotes against ricin or related RIPs. The catalytic subunit of ricin is an *N*-glycosidase that depurinates a universally conserved adenine residue within the sarcin/ricin loop (SRL) of the 28S rRNA. This depurination activity inhibits translation and its biochemistry has been intensively studied. Yet, recent developments paint a more complex picture of toxicity, with ribosomal proteins and cellular signaling pathways contributing to the potency of ricin. In particular, several studies have now established the importance of the ribosomal stalk structure in facilitating the depurination activity and ribosome specificity of ricin and other RIPs. This review highlights recent developments defining toxin–ribosome interactions and examines the significance of these interactions for toxicity and therapeutic intervention.

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1. Introduction

Ricin is a naturally occurring protein found in castor beans (*Ricinus communis*) that has a long and storied notoriety due to its potent toxicity (Olsnes, 2004). Castor beans are cultivated for oil production (Olsnes, 2004; Mutlu and Meier, 2010). Castor oil has been used in pharmaceutical and industrial applications and in the synthesis of biodiesel fuels (Mutlu and Meier, 2010). However, ricin remains at high levels in the castor cake after oil extraction, which limits use of this by-product and causes health problems (Mutlu and Meier, 2010). Ricin belongs to a family of functionally and structurally related toxins of plant and bacterial origin that are collectively referred to as ribosome inactivating proteins (RIPs) due to their ability to depurinate the α-sarcin/ricin loop (SRL) of the large rRNA and inhibit protein synthesis (Endo et al., 1987; Endo and Tsurugi, 1987; Lord et al., 1994). Family members include the plant toxins pokeweed antiviral protein (PAP) (from *Phytolacca americana*), trichosanthin (TCS) (from *Trichosanthes kirilowii*), abrin (from *Abrus precatorius*), saporin (from *Saponaria officinalis*), gelonin (from *Gelonium multiflorum*), and Shiga toxins (Stx) found in the bacterial species *Shigella dysenteriae* and Shiga-toxigenic *Escherichia coli* (de Virgilio et al., 2010). These Shiga toxin-producing pathogens are associated with severe gastrointestinal disease in humans and are responsible for significant morbidity and mortality world-wide (Paton and Paton, 1998; Bergan et al., 2012). The potent toxicity of ricin and other RIPs is being exploited for the development of targeted cancer therapies; yet this same property is feared as a formidable weapon of bioterrorism (Olsnes, 2004; Musshoff and Madea, 2009; de Virgilio et al., 2010). Ricin is a category B bioterrorism agent and to date, there are no US Food and Drug Administration-approved vaccines or therapeutics for either treatment or protection against ricin or other RIPs. Various therapeutic strategies against RIPs have been investigated, the majority of which have involved either inhibition of enzymatic activity (Yan et al., 1997; Miller et al., 2002; Mandal et al., 2008; Bai et al., 2009, 2010; Pang et al., 2011; Pruet et al.,

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2011; Wahome et al., 2011) or disruption of toxin uptake or trafficking (Paton et al., 2000, 2001; Saenz et al., 2007; Stechmann et al., 2010; Yermakova and Mantis, 2011; Mukhopadhyay and Linstedt, 2012; Park et al., 2012; Rasooly et al., 2012; Yermakova et al., 2012). Although some of these approaches have shown modest efficacy, a more complete understanding of the activity of ricin and other RIPs will be required to develop improved inhibitors of these toxins.

2. Path of ricin to the ribosome

Ricin is comprised of two subunits: a 32 kDa catalytic subunit (RTA) and a 34 kDa galactose/*N*-acetylgalactosamine-binding subunit (RTB) that are coupled by a single disulfide bond (Olsnes and Pihl, 1972, 1973). RTB is a lectin that facilitates uptake of the toxin into mammalian cells through interactions with glycoproteins or glycolipids at the cell surface (Lord et al., 1994). Following endocytic uptake, a limited amount of ricin proceeds to the *trans*-Golgi network and undergoes retrograde trafficking to the endoplasmic reticulum (ER) (Yoshida et al., 1991; Rapak et al., 1997; Lord and Roberts, 1998; Sandvig et al., 2010). Within the ER lumen, a resident protein disulfide isomerase facilitates reductive separation of RTA and RTB (Spooner et al., 2004). Subsequently RTA is thought to exploit cellular pathways that function to transport misfolded proteins across the ER membrane into the cytosol for degradation by proteasomes (Simpson et al., 1999; Stolz and Wolf, 2010; Spooner and Lord, 2012). RTA utilizes specific components of the endoplasmic reticulum (ER) associated degradation (ERAD) pathway for dislocation, and has unique characteristics compared with typical ERAD substrates (Spooner and Lord, 2012).

Recent work from our group examined the structural features within RTA that affect transport out of the ER into the cytoplasm, a process that indirectly affects subsequent ribosome depurination and cytotoxicity (Yan et al., 2012). Ricin is synthesized as a glycoprotein in *R. communis* (Lord, 1985). *N*-glycosylation occurs on asparagines 10 and 236 in RTA (Rutenber et al., 1991). The role of glycosylation in transport of RTA out of the ER was not known. Recombinant nonglycosylated RTA inhibits protein synthesis almost as effectively as plant-derived glycosylated RTA *in vitro* (Schlossman et al., 1989), suggesting that glycosylation does not affect catalytic activity. Since very few RTA molecules reach the ER in mammalian cells and are difficult to visualize, we used yeast as a model to study the intracellular trafficking of RTA by expressing enhanced green fluorescent protein (EGFP) tagged RTA with its native signal sequence (Yan et al., 2012). The precursor form of RTA (preRTA) was initially targeted to the ER by the native N-terminal signal peptide and was subsequently transported to the vacuole. The preRTA accumulated in the vacuole at 4 and 6 h post induction when expressed from the *GAL1* promoter in yeast (Fig. 1). The mature form of RTA without the N-terminal extension was not localized in the ER or in the vacuole and remained in the cytosol. When the two glycosylation sites in RTA were mutated, the cytosolic mature form was fully active and toxic, indicating that the mutations did not affect its catalytic activity. However, the nonglycosylated precursor form had reduced levels of depurination and toxicity, indicating that *N*-glycosylation affected transport of RTA to the cytosol (Yan et al., 2012). Mutations of *N*-glycosylation sites also delayed transport of RTA to the vacuole, indicating that *N*-glycosylation promotes transport of RTA out of the ER. The timing of depurination coincided with the timing of vacuole transport, suggesting that RTA may enter the cytosol during anterograde transport to the vacuole (Yan et al., 2012). A C-terminal hydrophobic sequence within RTA was critical for transport across the ER membrane (Yan et al., 2012). A C-terminal mutation (P250A) was also found to reduce transport of RTA from the ER to the cytosol in mammalian cells (Sokołowska et al., 2011). Collectively, these results demonstrated that *N*-glycosylation and the C-terminal hydrophobic sequence contribute to the toxicity of RTA by promoting its transport out of the ER (Yan et al., 2012).

It is thought that partial unfolding of RTA facilitates its dislocation from the ER to the cytosol. Indeed RTA exhibits conformational lability and partial unfolding *in vitro* (Argent et al., 2000). Partially unfolded RTA was compact, retained significant secondary structure but lacked stable tertiary structure. Furthermore, compared to native RTA, RTA folding intermediates exhibited increased protease sensitivity and increased exposure of hydrophobic residues, as evidenced by an increased ability to bind to the hydrophobic fluorescent probe, anilino naphthalene sulfonate (Argent et al., 2000). All these properties are consistent with a molten globular state (Ptitsyn, 1995), observed for other proteins that traverse membranes (van der Goot et al., 1992). It is thought that specific conditions within cellular compartments or extra-cellular milieu, including changes in pH or temperature, or reduction of disulfide bonds, trigger partial unfolding into molten globular states permissive of membrane translocation (van der Goot et al., 1992). Remarkably, re-folding of RTA *in vitro* was stimulated by the addition of ribosomes leading the authors to propose that ribosomes support re-folding of RTA following its dislocation from the ER to the cytosol (Argent et al., 2000). Once ricin has gained access to the cytosol it is thought that RTA evades proteasomal degradation due to a paucity of lysine residues, which can be polyubiquitinated, a process that mediates targeting to the proteasome (Hazes and Read, 1997).

3. The role of ribosomal proteins in ricin activity and specificity

It has long been established that RTA inhibits translation through its *N*-glycosidase activity and its ability to cleave a specific adenine residue of the universally conserved SRL of 28S ribosomal RNA, a process termed depurination (Endo and Tsurugi, 1987). The SRL is part of the GTPase center of the ribosome and is critical for ribosome function as it facilitates the binding and activation of translational GTPases that regulate protein synthesis (Agrawal et al., 1998; Clementi and Polacek, 2010; Voorhees et al., 2010; Shi et al., 2012). Although the SRL has been established as a common substrate for all RIPs, early work suggested that ricin might also interact with other ribosomal elements. In particular, it was observed that although the K_m of RTA for

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