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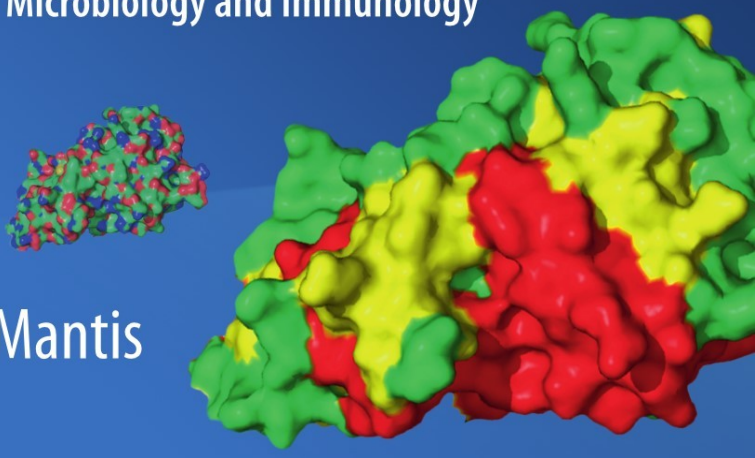

Nicholas J. Mantis
Editor

Ricin and Shiga Toxins

Pathogenesis, Immunity, Vaccines and Therapeutics

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Preface

At first glance, a volume dedicated to two seemingly very different toxins, ricin and Shiga, may seem puzzling. Ricin toxin is a by-product of castor beans, which are cultivated and processed on an industrial scale throughout the world for their oils. Shiga toxins (Stx), on the other hand, are produced by enteric bacterial pathogens, namely *Shigella dysenteriae* serotype 1 and certain strains of *Escherichia coli*, which collectively are responsible for causing illness in thousands of individuals each year. Ricin is a heterodimer, consisting of a single enzymatic subunit and a single receptor-binding subunit, while Stx is comprised of a single enzymatic subunit and five receptor-binding subunits. The two toxins show very limited primary amino acid identity and have little to no demonstrable immune cross-reactivity.

Upon closer examination, however, the toxins are remarkably similar. Ricin and Stx are both ribosome-inactivating proteins (RIPs), by virtue of their ability to depurinate a highly conserved adenosine residue within the sarcin-ricin loop (SRL) of 28S ribosomal RNA. Structural analysis by X-ray crystallography, in conjunction with mutagenesis studies, has revealed that the two toxins have identical catalytic centers and enzymatic mechanisms. Although ricin and Stx use different receptors to adhere to cell surfaces, the two toxins follow the same intracellular retrograde trafficking pathways from the cell surface to the endoplasmic reticulum and beyond. Both toxins induce apoptosis of mammalian cells via complicated signaling cascades involving the ribotoxic stress response (RSR), as well as stress activated protein kinases (SAPK) pathways. Thus, ricin and Stx are more similar than they are different.

The intent of this volume of Current Topics in Microbiology and Immunology was to bring together a collection of in-depth and cutting edge reviews that highlight our current understanding of the biology of ricin and Stx, with the long term goal of advancing the development of countermeasures against these toxic agents. In May of this year, Western Europe experienced a severe outbreak of Stx-producing *E. coli* (STEC) that culminated in more than 3,299 cases and over 40 deaths. While Stx is not the only virulence factor associated with STEC, it is certainly the primary determinant associated with the onset of hemolytic uremic

syndrome (HUS). At the present time, there are no clinically approved measures to neutralize Stx in individuals suffering from STEC infection. Nor are there any preventatives or therapeutics for ricin toxin. Although incidents of ricin exposure are largely unheard of, federal agencies and public health officials consider it a significant threat. It is well documented that domestic and international terrorist groups have stockpiled, and in some cases weaponized ricin with the intent of releasing it into the public sphere and causing panic, illness and/or death on a local, regional, or possibly national scale.

As the title of this volume indicates, the chapters, written by leading experts in the field, are organized so as to cover all aspects of ricin and Stx, including pathogenesis, immunity, vaccines and therapeutics. I would like to express my sincere appreciation to all my colleagues who took great care and effort in putting their chapters together and making this volume a success. I am confident that this outstanding collection of reviews will serve as an important and readily accessible resource for the research community in the coming years.

Albany, NY, November 2011 Nicholas J. Mantis

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Interaction of Ricin and Shiga Toxins with Ribosomes

Nilgun E. Tumer and Xiao-Ping Li

Abstract Ricin and Shiga toxins designated as ribosome inactivating proteins (RIPs) are RNA *N*-glycosidases that depurinate a specific adenine (A_{4324} in rat 28S rRNA) in the conserved α-sarcin/ricin loop of the large rRNA, inhibiting protein synthesis. Evidence obtained from a number of studies suggests that interaction with ribosomal proteins plays an important role in the catalytic activity and ribosome specificity of RIPs. This review summarizes the recent developments in identification of the ribosomal proteins that interact with ricin and Shiga toxins and the principles governing these interactions.

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1 Introduction to Ribosome Inactivating Proteins

Ricin and abrin, from the seeds of *Ricinus communis* and *Abrus precatorius* respectively, have been recognized as toxins and used in Greek and Egyptian medicine (Olsnes 2004; Stirpe and Battelli 2006). In 1891, Paul Ehrlich showed that both ricin and abrin are proteins and was the first to raise antibodies against them (Olsnes 2004). However, the structures and modes of action of these proteins were paid little attention for several decades until Sjur Olsnes demonstrated the subunit composition and enzymatic activity of ricin (Olsnes and Pihl 1972a, b). Ricin and related proteins maintain one common feature, the ability to inhibit translation in vitro by damaging the ribosome. As a result, these proteins were named "ribosome inactivating proteins" (RIPs). Ricin, abrin and Shiga toxin (from *Shigella dysenteriae*) are classified as type II RIPs because they consist of an enzymatically active A-chain disulfide-linked to a B-chain. Pokeweed antiviral protein (PAP), saporin (from *Saponaria officinalis*), gelonin (from *Gelonium multiflorum*), β-luffin (from *Luffa cylindrica*) and trichosanthin (from *Trichosanthes kirilowii*) are classified as type I RIPs because they consist of only an enzymatically active chain. Type II RIPs are considerably more toxic than type I RIPs, and consequently have received recent attention as agents of bioterrorism. A third class of RIPs, termed type III, includes only a few members, with the maize RIP being the most prominent. They are found as a single chain and become active after the removal of a short internal peptide (Barbieri et al. 1993; Walsh et al. 1991). Type I RIPs, type III RIPs and the A-chains of type II RIPs are each approximately 30 kDa in size. The B-chain of ricin (RTB) is a galactose specific lectin that is responsible for binding ricin to glycoproteins or glycolipids on the surface of cells to promote endocytosis of ricin (Endo et al. 1987; Endo and Tsurugi 1987). Receptor-dependent internalization of ricin involves retrograde transport to the endoplasmic reticulum (ER), where protein disulfide isomerase activates the toxin by reducing the disulfide bond connecting the A and B subunits (Spooner et al. 2006). The ricin holotoxin is enzymatically inactive and is activated after reduction of the disulfide bond, which allows release of the catalytically active A chain into the cytoplasm (Sandvig and van Deurs 1996).

Shiga toxin family is a group of structurally and functionally related toxins including Shiga toxin (Stx) produced by *S. dysenteriae* and Shiga-like toxin 1 (Stx1) and Shiga-like toxin 2 (Stx2) produced by enterohemorrhagic *Escherichia coli* (EHEC). The Japanese microbiologist Kiyoshi Shiga first identified the bacterium causing dysentery, and named it as *S. dysenteriae* in 1897. In 1977, a group of *E. coli* isolates were found to produce a toxin that was able to kill Vero cells in culture. The toxin was termed verotoxin and the bacteria producing the toxin was termed verotoxin-producing *E. coli* (VTEC) (Konowalchuk et al. 1977). Soon after, O'Brien et al. (1984) identified Shiga-like toxin-producing *E. coli* (ETEC). Shiga and Shiga-like toxins were shown to be highly related and these terms have been used interchangeably since then.

Stx, Stx1 and Stx2 are the key virulence factors leading to either hemorrhagic colitis (HC) or hemolytic uremic syndrome (HUS) that can result in severe morbidity and mortality (Paton and Paton 1998; Pickering et al. 1994). There are no antidotes or therapeutics effective against Stx-mediated HUS. A recent outbreak of HUS in Germany represented one of the largest outbreaks of HUS worldwide and was the deadliest on record (Bielaszewska et al. 2011). Shiga toxins are AB_5 toxins consisting of an enzymatically active A subunit associated with a pentamer of receptor binding B subunits. Shiga toxins can bind to eukaryotic cells through interaction between the B pentamer and host receptors on the cell surface that are concentrated in lipid rafts. While Stx1 is highly conserved and differs by only one amino acid from Stx produced by *S.dysenteriae*, greater sequence variation exists within Stx2 family members (O'Brien et al. 1992). Most known Stx variants have high binding affinity for the neutral glycolipid globotriaosylceramide (Gb3), although other receptors exist (Jacewicz et al. 1986; Lingwood et al. 1987; Waddell et al. 1988). After binding, the holotoxin is internalized by clathrin-mediated endocytosis and is transported in a retrograde manner from the early endosomes to the Golgi, and eventually to the cytosol (Sandvig et al. 1992; Sandvig and van Deurs 2002). The A subunit of the Shiga toxins can be proteolytically cleaved into an enzymatically active A_1 chain and an A_2 chain, which remain associated through a disulfide bond (Garred et al. 1995). The disulfide bond is ultimately reduced in the ER lumen, releasing the A_1 chain from the A_2–B_5 complex (Sandvig and van Deurs 2005).

The crystal structures of ricin, ricin A chain (RTA) (Katzin et al. 1991; Rutenber et al. 1991) *Shigella* Stx and Stx2 (Fraser et al. 1994, 2004) have been solved and amino acids involved in catalysis have been identified (Kim et al. 1992; Ready et al. 1991; Di et al. 2011). Although the amino acid sequences share less than 50% identity among the RIPs, the crystal structures of RIPs are similar and the amino acids involved in catalysis are highly conserved. RIPs consist of N-terminal and C-terminal domains with the active site in a cleft between these two domains. Structural differences that might result in differences in the mechanisms of action have been identified in Stx and Stx2 (Fraser et al. 2004, 2006). The active site of Stx is blocked by the A_2 chain (Fraser et al. 2006). In contrast, the active site of Stx2 is accessible to the adenine substrate and Stx2 cleaves the adenine when it is crystallized in the presence of adenosine (Fraser et al. 2006). Stx1 and Stx2 display indistinguishable enzymatic activity in cell free systems (Head et al. 1991; Brigotti et al. 1997; Tesh et al. 1993). However, epidemiological data indicate that Stx2 is more important than Stx1 in the development of HUS (Nataro and Kaper 1998; Siegler et al. 2003). The German outbreak isolates, which were the deadliest on record, contained only Stx2 and possessed the typical characteristics of enteroaggregative *E. coli* [2]. Among variants with identical B subunits, sequence differences within the A subunit of Stx2 can lead to differences in lethality for mice (Tesh et al. 1993). Differences have also been observed in the affinity of Stx1 and Stx2 holotoxins for the glycolipid receptor, globotriaosylceramide (Gb3) (Nakajima et al. 2001; Head et al. 1991; Tesh et al. 1993). The B pentamers of Stx1 and Stx2 show differential stability (Conrady et al. 2010). However, these

differences do not entirely account for the increased potency of Stx2 and further work is needed to address this important question.

2 Regulation of Enzymatic Activity

In 1988, Endo and Tsurugi demonstrated that RTA is an RNA *N*-glycosidase that hydrolyzes the *N*-glycosidic bond between a specific adenine (A_{4324} in the rat 28S rRNA and A_{2660} in the *E. coli* 23S rRNA) and the sugar, releasing adenine from a highly conserved stem-loop, the α-sarcin/ricin loop (SRL) of the rRNA (Endo and Tsurugi 1988). The SRL is also targeted by α-sarcin, a site-specific endoribonuclease that cleaves the phosphodiester bond between G_{4325} and A_{4326} of the 28S rRNA and is therefore designated as a ribotoxin (Endo et al. 1987; Endo and Tsurugi 1987). The SRL contains the longest known universally conserved ribosomal sequence (A2654–A2665 in the *E. coli* 23S rRNA and A4318–A4329 in the rat 28S rRNA) (Szewczak and Moore 1995). The SRL is important for binding and GTPase activation of the translational GTPases, which include the elongation factor 1 (EF1) and the elongation factor 2 (EF2), by the ribosome (Voorhees et al. 2010). The crystal structure of EF-Tu and aminoacyl-tRNA bound to the bacterial ribosome with a GTP analog revealed that codon recognition leads to a series of conformational changes needed for GTP hydrolysis that position a conserved histidine residue in EF-Tu by A_{2662} of the bacterial SRL (Voorhees et al. 2010). Structural analysis suggested that this positioning of the catalytic His by the SRL is a universal mechanism for triggering GTP hydrolysis by the translational GTPases on the ribosome (Voorhees et al. 2010). The irreversible depurination of the SRL by RTA prevents the binding of EF2 to ribosomes and affects both the EF1- and EF2-dependent GTPase activities with subsequent arrest of protein synthesis at the translocation step (Montanaro et al. 1975; Nygard and Nilsson 1989; Fernandez-Puentes and Vazquez 1977). In contrast, cleavage of the SRL by α-sarcin inhibits both EF1-dependent binding of aminoacyl-tRNA and the GTP-dependent binding of EF2, inhibiting GTP hydrolysis and translocation step of protein synthesis (Brigotti et al. 1989). Recent results indicated that cleavage of the SRL of 23S rRNA by α-sarcin differentially affected elongation factor G (EF-G) and elongation factor Tu (EF-Tu) binding and was more critical for the activity of EF-G (Garcia-Ortega et al. 2010).

Manipulation of specific functional groups on the ribosome demonstrated that A2660, the target of RIPs on bacterial ribosomes, is a key determinant triggering GTP hydrolysis on EF-G (Clementi et al. 2010). Synthetic oligonucleotides containing the SRL sequence reproduce EF-G binding (Munishkin and Wool 1997) and can be cleaved by the toxins (Wool et al. 1992; Gluck et al. 1992). The crystal structure of the SRL is a distorted hairpin, and the loop portion folds into a GAGA tetraloop and a G-bulged cross-strand A-stack (Correll et al. 1998; Szewczak and Moore 1995). Structural studies indicated that both motifs contribute to ribotoxin recognition (Yang et al. 2001). These motifs are also recognized by the elongation

factors. However, the primary determinant of recognition does not seem to be the nucleotide structure, but rather the conformation of the SRL (Munishkin and Wool 1997; Correll et al. 1999; Correll et al. 2003). Co-crystal structures of a ribotoxin, restrictocin, bound to several different substrate analogs suggested that ribotoxins may use a base flipping mechanism to enable cleavage at the correct site of the SRL substrates (Yang et al. 2001). Two distant regions of the ribotoxin participate in the specific interaction with the SRL. Three lysine residues (K110, K111 and K113) located adjacent to the active site (Plantinga et al. 2011) and the active site residues, His49 and Tyr47 (Plantinga et al. 2011). Maximum catalytic activity occurs when both motifs of the SRL are present with the major contribution involving the bulged-G motif. The interactions of RIPs and ribotoxins with the SRL alone do not explain the exquisite specificity and the unusually fast rate of interaction of the toxins with the ribosome (Korennykh et al. 2006, 2007; Li et al. 2009). These results suggest that the ribosomal context enhances the specificity and the reaction rate of RIPs and ribotoxins with ribosomes.

3 Interaction with the Ribosomal Proteins

Although the SRL is the universal substrate for all RIPs, ribosomal proteins play an important role in ribosome depurination. RTA has a similar K_m measured with rat ribosomes or the naked 28S rRNA. However, the rat ribosome is depurinated by RTA with a k_{cat} nearly 10^5-fold greater than that measured using the naked 28S rRNA (Endo and Tsurugi 1988). RTA depurinates the naked 23S rRNA from *E. coli* at the corresponding position, but not the ribosomes from *E. coli* (Endo et al. 1988). Binding affinity of PAP for the naked rRNA is tenfold weaker than for the ribosome (Rajamohan et al. 2001a). These results suggest that ribosomal proteins play an important role in increasing the susceptibility of the rRNA to depurination by the RIPs. The efficiency of ribosome inactivation among fungal, protozoan, plant, insect and prokaryotic ribosomes varies between the RIPs (Stirpe and Battelli 2006). For example, $Stx1A_1$ has about the same IC_{50} against both bacterial and eukaryotic ribosomes (Suh et al. 1998), while RTA is more active on rat liver ribosomes than on plant or yeast ribosomes, and is not active on bacterial ribosomes (Endo et al. 1988; Taylor et al. 1994; Harley and Beevers 1982). In contrast, PAP is equally active on ribosomes from all five kingdoms. These observations suggest that the differential sensitivity of ribosomes to RIPs that have identical rRNA substrate specificities may be due to differences in their interactions with the ribosomal proteins (Chan et al. 2007; Hudak et al. 1999; McCluskey et al. 2008).

PAP, a type I RIP, did not inhibit growth of yeast harboring the *mak8-1* allele of ribosomal protein L3 (*RPL3)*, which contains two point mutations, W255C and P257S in L3 (Hudak et al. 1999). Unlike ribosomes in wild-type cells, ribosomes from *mak8-1* cells were not depurinated when PAP expression was induced (Hudak et al. 1999). Both wild-type PAP and an inactive variant PAP_{E176V} were associated with ribosomes in wild-type cells but not in *mak8-1* cells.

PAP co-immunoprecipitated with L3 from ribosomes of wild-type cells, but not from *mak8-1* cells, demonstrating that *RPL3* is essential for binding of PAP to ribosomes and subsequent depurination of the SRL. Since L3 is in close proximity of the SRL in yeast 25S rRNA, PAP might dock onto L3 to access the SRL and to orient its active site toward the SRL. Furthermore, L3 is highly conserved between prokaryotic and eukaryotic ribosomes, which may explain why PAP is able to depurinate both types of ribosomes (Chiou et al. 2008). Expression of a truncated form of yeast L3 protected transgenic plants from the toxicity of PAP, suggesting that PAP interacts with L3 in plants as in yeast (Di and Tumer 2005). For PAP-ribosome interaction, N69, F90, N91 and D92 in the active site cleft of PAP were shown to be important for binding to L3 (Rajamohan et al. 2001b).

Trichosanthin (TCS), another type 1 RIP, has been shown to interact with the acidic ribosomal stalk proteins, P0 and P1 by yeast two-hybrid analysis and by in vitro pull-down assay (Chan et al. 2001, 2007). Deletion mutagenesis indicated that TCS interacted with the conserved C-terminal tail of ribosomal stalk protein P2 (Chan et al. 2001). The ribosomal stalk is a lateral protuberance of the large ribosomal subunit that recruits translation factors to the ribosome (Gonzalo and Reboud 2003; Diaconu et al. 2005) and is involved in GTPase activation by EF-Tu and EF-G (Mohr et al. 2002). A unique feature of the stalk is that its activity depends mainly on ribosomal proteins (Gonzalo and Reboud 2003). The prokaryotic stalk is composed of L12 protein dimers anchored to the ribosome through the L10 protein, which together with the L11 protein form the stalk base (Diaconu et al. 2005). The eukaryotic stalk is composed of phosphorylated P proteins, P0, P1 and P2, which form a pentameric complex, P0-$(P1\text{-}P2)_2$ where the P1 and P2 proteins are in the form of heterodimers (P1/P2) that attach to P0 (Guarinos et al. 2001, 2003; Gonzalo et al. 2001; Tchorzewski et al. 2003). P1 and P2 are the eukaryotic orthologs of the prokaryotic L12 protein, and P0 is equivalent to the prokaryotic L10. The eukaryotic P proteins have little sequence similarity to their prokaryotic counterparts, but have similar organization and function (Gonzalo and Reboud 2003; Wahl and Moller 2002). The N-termini of the P1 proteins directly interact with P0, which docks on the ribosome and interacts with the rRNA through its N-terminal domain. The P protein stalk together with the SRL forms the main interaction site for binding of elongation factors to the ribosome (Gonzalo and Reboud 2003). A notable characteristic of the stalk is that the ribosome-bound P1 and P2 proteins are exchangeable with free P1 and P2 proteins present in a cytoplasmic pool, resulting in ribosome subpopulations containing different amounts of P1/P2 proteins (Ballesta and Remacha 1996; Remacha et al. 1995). The stalk represents the last structure on the ribosome with unknown molecular architecture. A structural model for the prokaryotic stalk has been presented (Diaconu et al. 2005). More recently, the crystal structure of the archaeal ribosomal stalk core structure (Naganuma et al. 2010) and a model based on small-angle X-ray scattering (SAXS) and nuclear magnetic resonance (NMR) for the prokaryotic L12 have been published (Bernado et al. 2010). In addition, an NMR structure of a mammalian protein P2 homodimer has been reported (Lee et al. 2010), but structures of the biologically important P1/P2 heterodimer and

the co-crystal structures between the RIPs and ribosomes have not yet been resolved.

Triple alanine substitutions in three basic residues in TCS at positions K173, R174 and K177 abolished the interaction of TCS with P2 and resulted in a TCS variant with less activity in an in vitro translation system (Chan et al. 2001). These results suggested that the basic residues in TCS form charge–charge interactions with the conserved acidic residues in the C-terminal domain of P proteins of the ribosomal stalk. Crystal structure of TCS complexed with a conserved 11mer motif (SDDDMGFGLFD) corresponding to the C-terminus of P2 protein indicated that the N-terminal region of this peptide interacts with K173, R174 and K177, while the C-terminal region is inserted into a hydrophobic pocket in TCS (Too et al. 2009). Therefore, charge–charge interactions are likely involved at the N-terminal region of the P peptide and hydrophobic interactions at the C-terminal region (Too et al. 2009). Docking of the P2 C-terminal peptide to RTA, saporin and Stx1 showed that these RIPs may interact with P proteins in a similar manner to TCS (Too et al. 2009). In contrast, docking the C-11 peptide to PAP indicated that there was no clear molecular interaction surface on PAP. A monoclonal antibody that recognizes the C-terminal peptide of P proteins protected ribosomes from TCS, but not PAP, suggesting that the C-terminal region of the P protein was not needed for PAP to depurinate ribosomes (Ayub et al. 2008). The anti-parallel beta-sheets that correspond to K173, R174 and K177 in TCS are replaced by a short alpha helix in maize RIP, and no positively charged residues are found, suggesting that the ribosome interaction site on maize RIP might be different from that of TCS (Yang et al. 2010). These results provided further evidence that not all RIPs interact with the stalk proteins.

Stx1 was shown to bind P0, P1 and P2 proteins of the ribosomal stalk by in vitro pull-down experiments (McCluskey et al. 2008). Removal of the last 17 amino acids of either P1 or P2 abolished the interaction with the A_1 chain of Stx_1, while P0 lacking the common C-terminus bound to the A_1 chain. In vitro pull-down experiments using fusion proteins tagged with C-terminal peptides corresponding to the conserved 7, 11 and 17 C-terminal residues of P1 and P2 confirmed that the A chain of Stx1 and ricin bind to this C-terminal peptide motif. A synthetic peptide corresponding to the 17 amino acid C-terminus of P1 and P2 was shown to inhibit the ribosome inactivating function of the A_1 chain of Stx1 in an in vitro translation assay. These results suggested that the ribosomal stalk may enable $Stx1A_1$ to localize its catalytic domain near the depurination site of the 28S rRNA in vivo (McCluskey et al. 2008). However, these studies provided only a fragmentary view of the ribosome interactions because they were carried out with individual P proteins, which are not found in the cytoplasm by themselves. The only stalk components that are found free in the cytoplasm are the P1/P2 heterodimers (Nusspaumer et al. 2000).

RTA has been chemically cross-linked to ribosomal proteins P0 and L9 in human lung carcinoma cells (Vater et al. 1995). The interaction between labeled RTA and the ribosomes was inhibited by excess unlabeled RTA, but not by excess unlabeled gelonin, a type I RIP having an identical mechanism of action

(Vater et al. 1995). These experiments suggested that neither L9 nor P0 serve as binding sites for gelonin, providing evidence that alternative binding partners are possible for different RIPs (Vater et al. 1995). It was proposed that RTA is inactive against *E. coli* ribosomes, because of lack of homology of P0 and L9 to the bacterial homologs, L10 and L6, respectively (Vater et al. 1995).

Using P protein mutants, our group demonstrated that the ribosomal stalk is required for ribosome depurination in *Saccharomyces cerevisiae*. A markedly reduced association was observed between RTA and ribosomes from the yeast mutants that contained deletions of P1(ΔP1), P2 (ΔP2) or all four P proteins (ΔP1ΔP2) (Chiou et al. 2008). Ribosomes from the P protein mutants were depurinated less than the wild-type ribosomes when treated with RTA in vitro. Ribosome depurination was reduced when RTA was expressed in the ΔP1 and ΔP2 mutants in vivo and these mutants were resistant to the cytotoxicity of RTA. Ribosomes from the P protein mutants were also more resistant to $Stx1A_1$ and $Stx2A_1$, but not to PAP in vitro. These results demonstrated that RTA interacts with the P1 and the P2 proteins of the ribosomal stalk to localize the SRL in vivo and provided evidence that the interaction of RIPs with different ribosomal proteins might be responsible for their ribosome specificity (Chiou et al. 2008). The interaction with the stalk most likely positions the catalytic domain of RTA in close proximity to the SRL on the rRNA. Recent analysis of the structural relationships among the ribosomal stalk proteins indicated that the archaeal and the bacterial stalk proteins are not structurally related (Grela et al. 2008). These observations suggest that the differences in the primary sequence and the architecture of the ribosomal stalk between prokaryotic and eukaryotic ribosomes account for the difference in the sensitivity of the eukaryotic and prokaryotic ribosomes to ricin (Chiou et al. 2008).

To determine if Stx1 and Stx2 require the ribosomal stalk for depurination in vivo, the activity and cytotoxicity of the A subunits were examined in the yeast P protein deletion mutants. Stx1A and Stx2A were less toxic and depurinated ribosomes less in a strain lacking P1/P2 on the ribosome and in the cytosol (ΔP2) than in a strain lacking P1/P2 on the ribosome, but containing free P2 in the cytosol (ΔP1), suggesting that cytoplasmic P proteins facilitated ribosome depurination by Stx1 and Stx2 (Chiou et al. 2011). To determine if cytoplasmic P proteins facilitated ribosome depurination, Stx1A and Stx2A were expressed in the P0ΔAB mutant, in which the binding sites for P1/P2 were deleted on the ribosome, and P1/P2 accumulated in the cytosol. Stx1A was less toxic and depurinated ribosomes less in P0ΔAB, suggesting that intact binding sites for P1/P2 were critical. In contrast, Stx2A was toxic and depurinated ribosomes in P0ΔAB as in wild type, suggesting that it did not require the P1/P2 binding sites. Depurination of ΔP1, but not P0ΔAB ribosomes increased upon addition of purified $P1\alpha/P2\beta$ in vitro, and the increase was greater for $Stx1A_1$ than for $Stx2A_1$. These results indicated that cytoplasmic P proteins stimulated ribosome depurination by $Stx1A_1$ by facilitating the access of the toxin to the ribosome. In contrast, $Stx2A_1$ was less dependent on the stalk proteins for activity than $Stx1A_1$ and could depurinate ribosomes with an incomplete stalk better than $Stx1A_1$ (Chiou et al. 2011).

These studies indicated that Stx1 and Stx2 differ in their requirements for the stalk proteins, and provided the first evidence that $Stx1A_1$ and $Stx2A_1$ may interact differently with ribosomes (Chiou et al. 2011).

4 Ribosome Targeting

Ribosome cleavage by restrictocin showed strong dependence on salt concentration, unusually fast k_{cat}/K_m and multiple binding sites on the ribosomal surface (Korennykh et al. 2006). Electrostatic interactions were also shown to facilitate diffusion of the RIP, gypsophilin toward the SRL over a wide range of salt concentrations (Korennykh et al. 2007). Similar studies showed that RIPs share with ribotoxins a common mechanism of electrostatically facilitated ribosome targeting (Korennykh et al. 2007). As a consequence, these enzymes can interact with the ribosome with a speed exceeding their basal encounter frequency (Korennykh et al. 2007).

Binding to the stalk proteins may anchor RIPs near the SRL and allow them to find their substrate more efficiently. Furthermore, the ability of RIPs to interact with multiple copies of the C-terminal tails of the stalk proteins may increase the association rate between RIPs and the ribosome. Since RIPs must interact with a very large substrate, the ribosome, a double step mechanism may be envisaged for the molecular recognition, involving first interaction with ribosomal proteins and then attack on rRNA. Evidence for this was presented by surface plasmon resonance (SPR) analysis of the interaction of RTA with wild type and mutant yeast ribosomes deleted in the stalk proteins (Li et al. 2009). The interaction between RTA and wild-type ribosomes did not follow a single step binding model, but was best characterized by two distinct types of interactions. The AB1 interaction had very fast association and dissociation rates, was saturable and required an intact stalk, while the AB2 interaction had slower association and dissociation rates, and did not require the stalk. RTA interacted with the mutant ribosomes by a single type of interaction, which was similar to the AB2 interaction with the wild-type ribosomes. Both interactions were dominated by electrostatic interactions and the AB1 interaction was stronger than the AB2 interaction. Based on these results, a two-step interaction model was proposed (Li et al. 2009). This model is summarized in Fig. 1. In the first step, the slow and nonspecific electrostatic AB2 interactions concentrate the RTA molecules on the surface of the ribosome and guide RTA to the stalk. In the second step, AB2 interactions promote the faster, more specific AB1 interactions with the ribosomal stalk. In the third step, the C-terminal domain (CTD) of the stalk proteins transfer RTA to the SRL, thus restricting its diffusion and leading to rapid recruitment. In the fourth step, RTA removes a specific adenine (A_{4324}) from the SRL. The electrostatic AB1 and AB2 interactions work together allowing RTA to depurinate the SRL at a much higher rate on the intact ribosomes than on the naked 28S rRNA (Li et al. 2009).

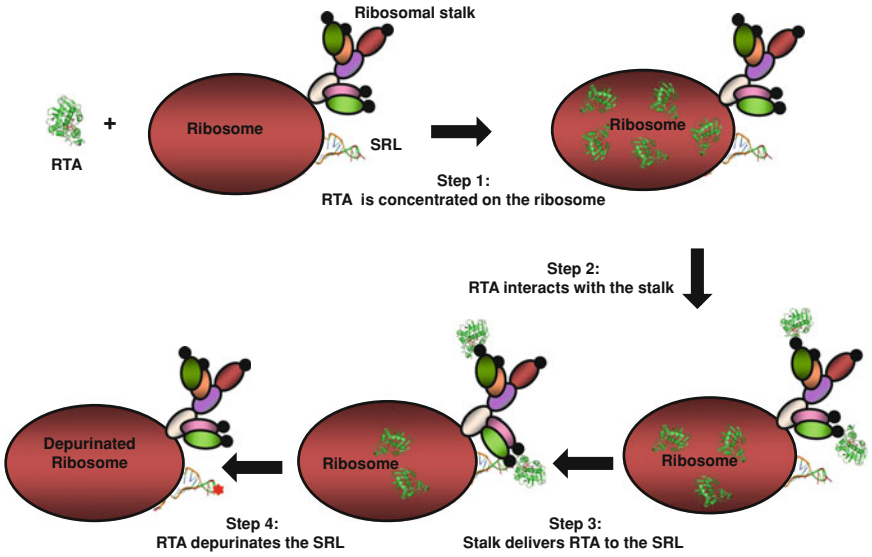

Fig. 1 A model representing the interaction of RTA with ribosomes. In the first step, the slow and nonspecific electrostatic interactions concentrate the RTA molecules on the surface of the ribosome and guide RTA to the stalk. In the second step, RTA binds to the stalk through more specific and stronger electrostatic interactions, which are saturable. Subsequently, the C-terminal domain of the stalk proteins transfers RTA to the SRL, thus restricting its diffusion and leading to rapid recruitment. Finally, RTA removes a specific adenine (A_{4324} in rat 28S rRNA) from the GAGA tetraloop of the SRL

Experimental evidence for this model was obtained using purified, in vivo synthesized yeast ribosomal stalk complexes. Purified ribosomal stalk complexes interacted with C-termini of RTA directly in a simple 1:1 interaction model confirming the proposed two-step interaction model of RTA with ribosomes (Li et al. 2010). Furthermore, the association rate of RTA with stalk complexes containing two pairs of P1/P2 dimers (stalk pentamer) was twice the association rate of stalk complexes containing only one dimer (stalk trimer). These results demonstrated that the stalk is the main landing platform for RTA on the ribosome and that pentameric organization of the stalk accelerates recruitment of RTA to the ribosome for depurination (Li et al. 2010).

The stalk binds the elongation factors and determines the specificity of ribosomes for the elongation factors. For example, replacing prokaryotic stalk complex with the eukaryotic P protein complex changes the specificity of ribosomes for the eukaryotic elongation factors (Uchiumi et al. 1999, 2002). The C-termini of P proteins contain a conserved protein sequence and monoclonal antibody against this conserved sequence can block the binding of elongation factors and the ribosome-dependent GTPase activity in protein synthesis in vitro (Uchiumi et al. 1990). It has been proposed that the flexible stalk proteins, which are protruding out of the ribosome, increase the association rate of elongation

factors by fetching them to the binding site on the ribosome (Diaconu et al. 2005). While eukaryotes exclusively have pentameric organization of the stalk, Archaea have heptameric organization and bacteria have pentameric as well as heptameric organization. Therefore, multiple copies of P1/P2 dimers might also accelerate recruitment of the translation factors as has been shown for the RIPs (Li et al. 2010).

5 Conclusions

The plant toxin ricin is one of the most potent and lethal substances known. Its high toxicity makes ricin an attractive tool for bioterrorism (Audi et al. 2005). Stx1 and Stx2 produced by *E. coli* O157:H7 cause significant morbidity and mortality and are major concerns for public health (Boyce et al. 1995). Currently, no US Food and Drug Administration-approved vaccines or therapeutics exist to protect against ricin or Shiga toxins. Therefore, understanding how they kill cells and developing antidotes to protect exposed people remain priorities. In addition, ricin and Shiga toxin are important in medicine because of their anti-cancer activities. RTA has been used as a component of immunotoxins that target cancer cells (Kreitman et al. 2011). Stx1 can kill human melanoma cells (Cheung et al. 2010) and can remove contaminated tumor cells in stem cell graft (LaCasse et al. 1999). Stx1 has antiviral activity (Ferens et al. 2007, 2006; Ferens and Hovde 2007, 2000), as has been reported for PAP and TCS (Parikh and Tumer 2004). In contrast, RTA is not antiviral even though it has the same enzymatic activity as the antiviral RIPs (Parikh and Tumer 2004). Several RIPs also have anti-fungal activity in transgenic plants (Nielsen and Boston 2001; Tumer et al. 1999). These differences may be due to the ability of certain RIPs to recognize RNAs other than the rRNA as substrates (Parikh and Tumer 2004).

Evidence obtained from a number of studies suggests that interaction with the ribosome plays an important role in the activity of RIPs. Several type I and type II RIPs interact with the P proteins of the ribosomal stalk (Chan et al. 2007; Chiou et al. 2008; McCluskey et al. 2008; Li et al. 2009, 2010). The conserved C-terminal domain of P proteins may interact with basic residues on RIPs through electrostatic interactions, which enable extremely rapid and specific ribosome targeting (Too et al. 2009). Interaction with the ribosomal proteins may induce conformational changes on RIPs and increase their enzymatic activity. Alternatively, binding of RIPs to the ribosome may also change the conformation of the ribosome, making the SRL more susceptible to depurination. Studies with the EF2-sordarin-ribosome complex demonstrated that EF2 directly binds to the SRL and interacts with the P proteins (Spahn et al. 2004). Docking TCS to the SRL substrate indicated that the P protein binding site on TCS is close in proximity to the EF2 binding site (Chan et al. 2001). Since RIPs and elongation factors both interact with P proteins of the ribosomal stalk, RIPs that bind well to P proteins may hinder the association of elongation factors with the ribosome. Therefore,

interaction with P proteins may help RIPs to compete with the elongation factors and block the access of the factors to the SRL. This is supported by the early studies indicating that ricin inhibits binding of EF2 to the ribosome (Nolan et al. 1976). The EF2 can protect ribosomes from RTA (Fernandez-Puentes et al. 1976) and block binding of RTA to the ribosome (Cawley et al. 1979). More recent studies indicated that a catalytically inactive α-sarcin mutant, which could not cleave the SRL was able to inhibit protein synthesis possibly by binding to the ribosomes (Alvarez-Garcia et al. 2011). These results indicated that site-specific damage to the SRL may not account entirely for the activity of RIPs and ribotoxins. An additional activity may be associated with ribosome binding, which may block the interaction of elongation factors and the other translational GTPases with the ribosome.

Several studies indicated that ribosome depurination and translation inhibition by ricin, Shiga toxins and PAP are not sufficient for cell death (Li et al. 2007; Hudak et al. 2004; Di et al. 2011). Mutant forms of these RIPs, which depurinated ribosomes and inhibited translation were reduced in cytotoxicity (Li et al. 2007). Similarly, an α-sarcin mutant that could not inhibit protein synthesis was cytotoxic (Alford et al. 2009). These results provide evidence that RIPs and ribotoxins can promote cell death by a mechanism that is independent of depurination or cleavage of the SRL (Alford et al. 2009; Alvarez-Garcia et al. 2011; Hudak et al. 2004; Li et al. 2007; Di et al. 2011). Understanding ribosome interactions of RIPs and how they affect toxicity at the molecular level is important not only for developing antidotes, but also for understanding ribosome function. A high-resolution structure of the eukaryotic stalk complex is not available and is necessary to fully understand complex formation. The main problem is that the stalk has very high structural mobility and there are no good molecular probes for structural and functional analyzes. The recent observations, indicating that several RIPs interact with the stalk structure (Chan et al. 2007; Chiou et al. 2008; McCluskey et al. 2008; Li et al. 2010) may lead to a breakthrough in this area. RIPs may be excellent tools to probe the function of the eukaryotic stalk and to identify the functional parts of the stalk structure.

Major unsolved questions remain. If electrostatic interactions dominate the interaction of ricin and Shiga toxins with ribosomes, what is the molecular basis for the specificity of these interactions? Experimental evidence summarized here suggests that RTA, Stx1 and Stx2 interact differently with ribosomes (Chiou et al. 2011, 2008). Although they interact with the stalk, there are differences in their requirement for the stalk proteins. Stx2 is less dependent on the stalk proteins and may have additional docking sites. What determines which ribosomal proteins ricin and Shiga toxins interact with? How many ribosomal proteins are involved in these interactions? Which amino acids are involved in ribosome recognition and the specificity of the RIPs? After binding to the ribosome, how do they find their adenine target on the SRL? What is the role of ribosome binding in translation inhibition and cell death? Further structural and biochemical studies on ribosome recognition are required to answer these questions.

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