Toxicology and Applied Pharmacology 310 (2016) 120–128

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Toxicity of ricin A chain is reduced in mammalian cells by inhibiting its interaction with the ribosome

Amanda E. Jetzt [a], Xiao-Ping Li [b], Nilgun E. Tumer [b], Wendie S. Cohick [a,*]

[a] Department of Animal Sciences, Rutgers, The State University of New Jersey, New Brunswick, NJ 08901-8520, United States
[b] Department of Plant Biology and Pathology, Rutgers, The State University of New Jersey, New Brunswick, NJ 08901-8520, United States

ARTICLE INFO

Article history:
Received 23 March 2016
Revised 8 September 2016
Accepted 10 September 2016
Available online 15 September 2016

Keywords:
Ricin A chain
Ribosome binding
Mammalian cells
Cytotoxicity
Depurination
Therapeutic target

ABSTRACT

Ricin is a potent ribotoxin that is considered a bioterror threat due to its ease of isolation and possibility of aerosolization. In yeast, mutation of arginine residues away from the active site results in a ricin toxin A chain (RTA) variant that is unable to bind the ribosome and exhibits reduced cytotoxicity. The goal of the present work was to determine if these residues contribute to ribosome binding and cytotoxicity of RTA in mammalian cells. The RTA mutant R193A/R235A did not interact with mammalian ribosomes, while a G212E variant with a point mutation near its active site bound ribosomes similarly to wild-type (WT) RTA. R193A/R235A retained full catalytic activity on naked RNA but had reduced activity on mammalian ribosomes. To determine the effect of this mutant in intact cells, pre R193A/R235A containing a signal sequence directing it to the endoplasmic reticulum and mature R193A/R235A that directly targeted cytosolic ribosomes were each expressed. Depurination and protein synthesis inhibition were reduced by both pre- and mature R193A/R235A relative to WT. Protein synthesis inhibition was reduced to a greater extent by R193A/R235A than by G212E. Pre R193A/R235A caused a greater reduction in caspase activation and loss of mitochondrial membrane potential than G212E relative to WT RTA. These findings indicate that an RTA variant with reduced ribosome binding is less toxic than a variant with less catalytic activity but normal ribosome binding activity. The toxin-ribosome interaction represents a novel target for the development of therapeutics to prevent or treat ricin intoxication.

1. Introduction

Ricin is a type-2 ribosome inactivating protein (RIP) produced by the castor bean plant (*Ricinus communis*) that is highly toxic to mammalian cells. It consists of two subunits; a catalytic ricin toxin A chain (RTA) and a galactose-binding B chain (RTB) covalently linked by a single disulfide bond (Robertus and Monzingo, 2004). Following endocytosis, a small amount of ricin holotoxin enters the endoplasmic reticulum (ER) by retrograde translocation from the trans-Golgi network. Cleavage of the disulfide bond between RTA and RTB is required for RTA to enter the cytosol where it depurinates a universally conserved adenine residue within the sarcin/ricin loop (SRL) of the 28S rRNA to inhibit protein synthesis (Spooner and Lord, 2012). Due to its high toxicity, ricin has been classified as a category B bioterrorism agent (Audi et al., 2005). While two RTA-based vaccines have been tested in Phase I clinical trials (Smallshaw and Vitetta, 2012; Vitetta et al., 2012; Pittman et al., 2015), no post-exposure therapeutics for ricin intoxication are currently available.

During protein synthesis, elongation factors and other translational (t)GTPases interact with ribosomes at the GTPase-associated center, which includes the SRL and a lateral protuberance referred to as the ribosomal stalk (Uchiumi and Kominami, 1992; Wahl and Moller, 2002; Clementi and Polacek, 2010; Voorhees et al., 2010; Shi et al., 2012). The latter consists of ribosomal proteins that are phosphorylated and hence referred to as ribosomal P- proteins (Tchorzewski, 2002). The eukaryotic stalk is composed of two heterodimers of P1 and P2 proteins which bind to the base of the stalk (P0), forming a pentameric structure (Ballesta and Remacha, 1996; Guarinos et al., 2003; Krokowski et al., 2006; Grela et al., 2010). Early studies showed that the rate of depurination of native ribosomes by ricin was dramatically greater than that of naked rRNA (Endo and Tsurugi, 1988). Subsequent work indicated that RTA (Chiou et al., 2008; McCluskey et al., 2008; May et al., 2012), the catalytic subunit of the type 2 RIP Shiga toxin 1 (Stx1) (McCluskey et al., 2008, 2012), and the type-I RIP trichosanthin (TCS) (Chan et al., 2001, 2007; Too et al., 2009) bind to ribosomal P- proteins. Deletion of P1 or P2 protein decreases enzymatic activity and toxicity of RTA, Stx1 or Shiga toxin 2 (Stx2) in yeast (Chiou et al., 2008, 2011) and decreases enzymatic activity and toxicity of RTA in mammalian cells

Abbreviations: CTD, C-terminal domain; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; qRT-PCR, quantitative reverse transcription PCR; RIP, ribosome-inactivating protein; RTA, ricin toxin A chain; RTB, ricin B-chain; RU, resonance units; SRL, sarcin-ricin loop; Stx1, Shiga toxin 1; Stx2, Shiga toxin 2; TCS, trichosanthin; WT, wild-type.

* Corresponding author at: Department of Animal Sciences, Rutgers, The State University of NJ, 59 Dudley Rd, New Brunswick, NJ 08901-8520., United States.
E-mail address: cohick@aesop.rutgers.edu (W.S. Cohick).

http://dx.doi.org/10.1016/j.taap.2016.09.004
0041-008X/

(May et al., 2012). Collectively, these studies indicate that interaction with ribosomal P-proteins is required for full enzymatic activity of these RIPs and suggest a role for the ribosomal stalk in localizing the toxins near the SRL on the ribosome.

We have previously examined the ability of different RTA mutants to affect depurination activity, protein synthesis inhibition and apoptosis in mammalian cells (Jetzt et al., 2012). These studies showed that a relatively low level of depurination by RTA is sufficient to inhibit protein synthesis and activate stress-induced cell signaling and apoptosis such that a substantial reduction in the depurination activity of RTA is required to prevent its inhibitory effect on protein synthesis. All of the mutated residues in our study were on the side of RTA where the active site is located (Jetzt et al., 2012). However, several studies have shown that arginine residues in RTA located away from the active site cleft affect its enzymatic activity *in vitro* as well as its ability to bind the ribosome (Watanabe and Funatsu, 1986; Watanabe et al., 1994; Kitaoka, 1998; Li et al., 2013). In yeast, mutation of arginine residues 193 and 235 to alanine (R193A/R235A) results in a variant that folds similarly to wild-type (WT) but is unable to bind the ribosome or the stalk pentamer (Li et al., 2013). This mutant exhibits reduced cytotoxicity even though it depurinates naked RNA at a similar catalytic rate as WT RTA (Li et al., 2013). Whether these specific arginine residues contribute to binding of RTA to mammalian ribosomes and affect the toxicity of RTA in mammalian cells *in vivo* has not been determined. Here we examine the ribosome binding mutant R193A/R235A, as well as the G212E mutant, which contains a point mutation near the active site, to determine whether ribosome binding is important for ribosome depurination, translation inhibition and apoptosis in mammalian cells. Since the 35-residue signal sequence would target precursor (pre) RTA to the ER and thus mimic retrograde trafficking while the mature form of RTA would represent catalytic activity in the absence of retrograde trafficking, both the pre- and mature forms of RTA were expressed.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L D-glucose and penicillin/streptomycin were purchased from Life Technologies (Carlsbad, CA). Phenol red-free DMEM with low glucose, gentamicin, insulin, D-(+)-glucose, fetal bovine serum (FBS), GTP, puromycin, heparin, PMSF, aprotinin, leupeptin, and trypsin inhibitor were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Recombinant RTA protein purification

Wild type (WT) RTA and RTA mutants were cloned into the *E. coli* expression vector, pET28, with an N-terminal His_{10} followed by the mature RTA (Li et al., 2013). WT RTA and His_{10}-tagged EGFP were purified using nickel-nitrilotriacetic acid (NTA)-agarose from Qiagen (Li et al., 2013). RTA mutants were purified by the Northeast Biodefense Center protein expression core facility (Wadsworth Center, Albany, NY).

2.3. Isolation of bovine ribosomes

MAC-T cells were lysed in buffer A [20 mM HEPES-KOH pH 7.6, 5 mM Mg $(CH_3COO)_2$, 50 mM KCl, 10% glycerol, 1 mg/mL heparin, 1 mM PMSF, 1 mM DTT and 10 μg/mL leupeptin, trypsin inhibitor and aprotinin]. Cells were then homogenized by 10 passages through a 23 gauge needle. After addition of Triton X-100 to a final concentration of 1%, lysates were mixed gently and centrifuged at 30,000 ×*g* for 20 min at 4 °C. The resulting supernatant was applied to 1 mL buffer C cushion [50 mM HEPES-KOH pH 7.6, 5 mM Mg $(CH_3COO)_2$, 50 mM NH_4Cl, 0.1 mM PMSF, 0.1 mM DTT and 25% glycerol] and centrifuged at 303,800 ×*g* for 2 h at 4 °C. The pellet was washed with buffer C and resuspended in buffer B [20 mM HEPES-KOH pH 7.6, 20 mM Mg $(CH_3COO)_2$, 0.5 M KCl, 10% glycerol, 1 mg/mL heparin, 1 mM PMSF, 1 mM DTT and 10 μg/mL leupeptin, trypsin inhibitor and aprotinin] by shaking on a vortex at 4 °C for 1.5 h. Puromycin and GTP, both at 1 mM final concentration, were added, and the sample was incubated at 30 °C for 30 min. After centrifugation at 14,000 rpm for 10 min at 4 °C, the supernatant was applied to 1 mL buffer B cushion [20 mM HEPES-KOH pH 7.6, 20 mM Mg $(CH_3COO)_2$, 0.5 M KCl, 25% glycerol, 1 mg/mL heparin, 1 mM PMSF, 1 mM DTT and 10 μg/mL leupeptin, trypsin inhibitor and aprotinin] and centrifuged at 303,800 ×*g* for 2 h at 4 °C. The ribosome pellet was washed and resuspended in buffer C.

2.4. Isolation of rat ribosomes

Animal care was performed in accordance with guidelines from the Rutgers Institutional Animal Care and Use Committee (IACUC) and complied with NIH policy. Rat livers were collected following decapitation, flash frozen in liquid nitrogen and stored at −80 °C. Approximately 3–4 g of tissue were cut into smaller pieces using scissors, and homogenized in Buffer A using a glass homogenizer. After addition of Triton X-100 to a final concentration of 2%, lysates were mixed gently and centrifuged at 30,000 ×*g* for 20 min at 4 °C. The resulting supernatant was applied to 4 mL buffer C cushion and centrifuged at 199,600 ×*g* for 2 h at 4 °C. The pellet was washed with buffer C and resuspended in buffer B by mixing gently. Subsequent puromycin treatment and salt washing of ribosomes was as described above for bovine ribosomes.

2.5. Interaction of wild type and mutant RTA with bovine and rat ribosomes

A Biacore T200 (GE Healthcare) was used to measure RTA-ribosome interactions as previously described (Basu et al., 2016). Briefly, an NTA chip was used to capture His_{10}-tagged RTA and its mutants. R193A R235A was captured on flow cell (Fc) 2 between 937 and 1284 resonance units (RU), WT RTA and G212E were captured either on Fc3 or Fc4 between 879 and 970 RU. His_{10}-tagged EGFP was captured on the reference channel (Fc1) between 1113 and 1183 RU as the control. The running buffer contained 10 mM Hepes pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 50 μM EDTA and 0.003% Surfactant P20. Bovine or rat ribosomes at 0.5, 1.0, 2.0, 4.0 and 8.0 nM were passed over the surface at 50 μL/min using the single kinetic injection method. Association was 2 min and dissociation was 5 min. The surface was regenerated with a 1 min injection of 0.35 M EDTA plus a 1 min injection of 0.3% SDS. The RTA was freshly captured at each cycle. The experiments were repeated 3 times. The apparent affinity of interaction was fitted by Biacore T200 Evaluation Software.

2.6. In vitro depurination assay

For RNA depurination, purified MAC-T RNA (1 μg) was diluted in 18 μL of reaction buffer containing 20 mM citrate, pH 5.0. The reaction was started by adding 2 μL RTA and then incubating at 37 °C for 30 min. RNA was precipitated with ethanol and the extent of depurination was determined by qRT-PCR as described below using 375 ng total RNA in the reverse transcription reaction. For ribosome depurination, the reaction mixture contained 30 nM bovine or rat ribosomes, 1× reaction buffer [10 mM Tris-HCl, pH 7.4, 60 mM KCl, and 10 mM $MgCl_2$], and different concentrations of RTA ranging from 0 to 205 nM in a total reaction of 100 μL. The reaction was started by adding RTA, and then incubated at 30 °C for 3 min. The reaction was stopped by adding 100 μL of 2× RNA extraction buffer [50 mM Tris-HCl, pH 8.8, 240 mM NaCl, 20 mM EDTA, and 2% SDS]. The RNA was extracted once with phenol, and again with phenol/chloroform, and then precipitated twice with ethanol. The extent of depurination was determined by qRT-PCR as described below using 375 ng rRNA in the reverse transcription reaction.