Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 369 (2008) 314–319
BBRC
www.elsevier.com/locate/ybbrc

The C-terminal end of P proteins mediates ribosome inactivation by trichosanthin but does not affect the pokeweed antiviral protein activity

Maximiliano Juri Ayub [a,*], Cristian R. Smulski [c], Kit-Wan Ma [b], Mariano J. Levin [c,d], Pang-Chui Shaw [b], Kam-Bo Wong [b]

[a] *Fundación Instituto Leloir, IIBBA-CONICET and University of Buenos Aires, Patricias Argentinas 435, 1405 Buenos Aires, Argentina*
[b] *Centre for Protein Science and Crystallography, Department of Biochemistry, The Chinese University of Hong Kong, Hong Kong, China*
[c] *Laboratorio de Biología Molecular de la Enfermedad de Chagas (LaBMECh), Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI-CONICET), Buenos Aires, Argentina*
[d] *Department of Maladies Infectieuses (U.INSERM 567)-Institut Cochin, Paris, France*

Received 29 January 2008
Available online 20 February 2008

Abstract

Ribosome inactivating proteins (RIPs) inhibit protein synthesis depurinating a conserved residue in the sarcin/ricin loop of ribosomes. Some RIPs are only active against eukaryotic ribosomes, but other RIPs inactivate with similar efficiency prokaryotic and eukaryotic ribosomes, suggesting that different RIPs would interact with different proteins. The SRL in *Trypanosoma cruzi* ribosomes is located on a 178b RNA molecule named 28Sδ. In addition, *T. cruzi* ribosomes are remarkably resistant to TCS. In spite of these peculiarities, we show that TCS specifically depurinate the predicted A^{51} residue on 28Sδ. We also demonstrated that the C-terminal end of ribosomal P proteins is needed for full activity of the toxin. In contrast to TCS, PAP inactivated efficiently *T. cruzi* ribosomes, and most importantly, does not require from the C-terminal end of P proteins. These results could explain, at least partially, the different selectivity of these toxins against prokaryotic and eukaryotic ribosomes.

Keywords: Trypanosoma; Protein synthesis; Trichosanthin; Pokeweed antiviral protein; Ribosome inactivating protein (RIP)

Ribosome inactivating proteins (RIPs) modify ribosomes through its RNA *N*-glycosidase activity that depurinates an adenine residue, A^{4324} (numbering according to the rat sequence), in the conserved α-sarcin/ricin loop (SRL) of 28S rRNA in eukaryotic ribosomes [1–5]. Such modification prevents binding of elongation factors to the SRL, and leads to the arrest of protein synthesis [6,7]. Although RIPs are able to cleave both prokaryotic and eukaryotic naked rRNA, its k_{cat} is 10^5-fold lower than that for rRNA within an intact ribosome [3]. Some RIPs (i.e. ricin and trichosanthin) are only active against eukaryotic ribosomes. In contrast, other RIPs (i.e. pokeweed antiviral protein; PAP) inactivate with similar efficiency prokaryotic and eukaryotic ribosomes. These findings strongly suggest that ribosomal proteins are involved in rendering the rRNA susceptible to inactivation by RIPs, and that different RIPs would interact with different proteins. This hypothesis is supported by the relatively low sequence identity among different RIPs. Unfortunately, reports on the interaction between RIPs and ribosomal proteins are still scarce, and different experimental approaches have been used in each case, making difficult to establish comparisons. Ricin A-chain was cross-linked to ribosomal proteins L9 and P0 of mammalian ribosome [8]. PAP has been found to interact with yeast ribosomal protein L3 [4,5]. However, those mutants with impaired binding to L3 [5] have also limited ability to depurinate *Escherichia coli* naked rRNA, where L3 protein is absent [9]. This observation suggests that these mutations would have a more general effect on the toxin molecule, and the role of these amino acids should be probably revised.

* Corresponding author. Fax: +54 11 5238 7501.
E-mail address: mayub@leloir.org.ar (M.J. Ayub).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.01.170

Trichosanthin (TCS) has been shown to interact with L1 (L10a) [10] and the conserved C-terminal end of the mammalian acidic ribosomal proteins P0, P1, and P2 [11,12]. These observations are puzzling, because L1 and P proteins are located at opposite protuberances on the 60S subunit [13]. Studies with mutant variants of TCS showed that *in vitro* binding to L1 correlates with their ribosome inactivating activity [10]. On the other hand, the P2-binding residues on TCS were identified as K173, R174, and K177. Triple-alanine substitutions at these positions, which abolished the interaction with P2 and with ribosomes in pull-down assays, resulted in a TCS variant with 18-fold less inhibiting activity against a rabbit reticulocyte protein synthesis system. Based on these results, interaction of TCS with the C-terminal end of P-proteins has been proposed as necessary for full activity of the toxin against mammalian ribosomes [12]. We have previously shown that *Trypanosoma cruzi* ribosomes are >1000 times more resistant to TCS than the mammalian ones [14]. However, inhibition takes place at high concentrations, suggesting that TCS depurinates the corresponding A residue on *T. cruzi* ribosomes.

In the present work, we first confirmed that TCS specifically depurinates its predicted target on the 28Sδ *T. cruzi* rRNA. By using a single chain fragment variable antibody (scFvC5) against the C-terminal end of *T. cruzi* ribosomal P proteins, we demonstrated the importance of interaction of TCS with these epitopes for the toxin activity. We also tested the ability of other RIP (PAP) to inactivate the *T. cruzi* ribosomes. In contrast to TCS, PAP was similarly active against *T. cruzi* and mammalian ribosomes, suggesting that both toxins interact with different ribosomal proteins. Sequence and 3D structure comparison showed that TCS residues corresponding to the putative P protein interaction site (K173, R174, and K177) are poorly conserved in other RIPs. This suggests that the interaction with the C-terminal end of P proteins would not be a general feature of RIPs. This hypothesis was demonstrated by the inability of scFvC5 to inhibit the activity of PAP. To our knowledge, these results show, by the first time, differential ability to interact with ribosomal proteins between two different RIPs. Moreover, these differences were demonstrated under identical experimental conditions using functional ribosomes, minimizing the possibility of artefacts. Our results could explain, at least partially, the different selectivity of these toxins against prokaryotic and eukaryotic ribosomes.

We propose that comparison of the relative activities from different RIPs toward ribosomes from different sources (e.g. mammalian and trypanosomatids) could be a valuable approach to characterize differences among ribosomes, as well as for dissecting the molecular mechanism differences among RIPs.

Materials and methods

Purification of proteins. Both wt and triple-alanine variants of TCS were expressed in *E. coli* and purified as recently described [12]. The scFvC5 was expressed and purified as described [15]. Native PAP was kindly provided by Dr Katalin Hudak.

In vitro translation assays. Purification of *T. cruzi* and rat ribosomes and *in vitro* protein synthesis experiments were performed as described [14]. Briefly, the reaction mixtures were prepared on ice and contained: 19 amino acids 50 μM each (excepting Met); 2 mM dithiothreitol; 100 mM potassium acetate; 3.5 mM magnesium acetate; 75 μg/ml wheat germ tRNA; 18 mM Hepes/KOH, pH 7.5; 1 mM ATP; 0.5 mM GTP; 7.5 mM creatine phosphate; 37.5 μg/ml creatine phosphokinase; rat liver S_{150} fraction (24 μg of protein); 0.3 A_{260} units of *T. cruzi* or rat ribosomes and 2 μCi of [^{35}S]methionine in a final volume of 30 μl. Reactions were performed at 30 °C during 60 min and stopped by adding 150 μl of 1.5 M NaOH; 1 mM Met, 170 μg/ml BSA. After incubation for 30 min at 37 °C, proteins were precipitated with 1 ml of cold TCA 25%. After 60 min on ice, the samples were filtered and washed with TCA 10% and ethanol using glass fiber filters. Radioactivity retained in the filters was measured by liquid scintillation counting.

Detection of A^{51} depurination on T. cruzi rRNA by RIPs. Mixtures under different conditions were performed as described above, but using non-radioactive methionine. After incubation at 30 °C for 1 h in the presence or absence of RIP, reactions were stopped by adding 100 μl H_2O and 400 μl of Trizol LS Reagent (Invitrogen). The RNA was purified and resuspended in 20 μl of H_2O. After that, 10 μl of RNA was added to 50 μl of aniline:acetic acid:H_2O (1:1.6:7.4) and incubated at 60 °C for 3 min. RNA was precipitated with 0.5 volume of ammonium acetate 7.5 M and 3 volumes of ethanol and resuspended in water. The samples were analyzed by 6–8% PAGE with urea 7 M and stained with ethidium bromide.

Inhibition of A^{51} depurination by scFvC5. *Trypanosoma cruzi* ribosomes (120 nM) were incubated under the same conditions that for the RNA glycosidase assays, but in the presence of scFvC5 500 nM and at 0 °C by 30 min. Controls were performed in the absence of scFvC5. After that, TCSwt, $TCS_{K173A/R174A/K177A}$ or PAP were added at a concentration 10 times higher than their respective IC_{50} values obtained in *in vitro* translation assays. Mixtures were incubated by 60 min at 30 °C. After that, samples were processed as for RNA glycosidase assays.

Sequence alignments. The sequence corresponding to *T. cruzi* 28Sδ rRNA (Access code **Tc00.1047053422723.20** at www.genedb.org) was used as probe to identify the homologs regions on *E. coli* 23S rRNA (AJ278710), *Rattus rattus* 28S rRNA (V01270), and *Saccharomyces cerevisiae* 25S rRNA (J01355). The sequences were aligned using the CLUSTAL W program. The amino acid sequences of TCS (1J4G_A), Ricin A chain (1J1M_A); Abrin A chain (CAA38655); saporin (CAO02571), and PAP (1922356A) were also aligned using the CLUSTAL W program.

Comparison of 3D structures. Crystal structures from TCS (1MRJ) and PAP (1PAG) where obtained from the protein data bank (www.pdb.org) and visualized with PyMOL (DeLano, W.L. The PyMOL Molecular Graphics System, 2002, available on http://www.pymol.org).

Quantification of depurination of A^{51} from T. cruzi 28Sδ rRNA. The extent of depurination of A^{51} on 28Sδ rRNA was quantified by densitometric analysis using the Scion Image program. The intensity ratios between 178b (28Sδ rRNA) and 172b (5.8S rRNA) bands in aniline treated samples was used for estimating the depurination extent. The percent of depurination of A^{51} on rRNA 28Sδ (% Depurin) was calculated as follows:

$$\%\text{Depurin} = (1 - I_{178}/I_{172}) * 100$$

being I_{178} and I_{172} the intensity of bands of 178b (28Sδ rRNA) and 172b (5.8S rRNA), respectively.

Statistical analysis. The significance of the depurination of rRNA by RIPs was analyzed with a Student's *t* test. Values of $p < 0.01$ were considered significant (**). Dose–response curves and IC_{50} values were obtained by fitting the data to a four-parameter logistic equation using non-linear regression. In all the cases, the R^2 values were >0.97.

Results and discussion

TCS depurinates the conserved A^{51} residue on the SRL of T. cruzi 28Sδ rRNA

Because of the particular processing mechanism of the trypanosomatid rRNA [16], the SRL in *T. cruzi* ribosomes

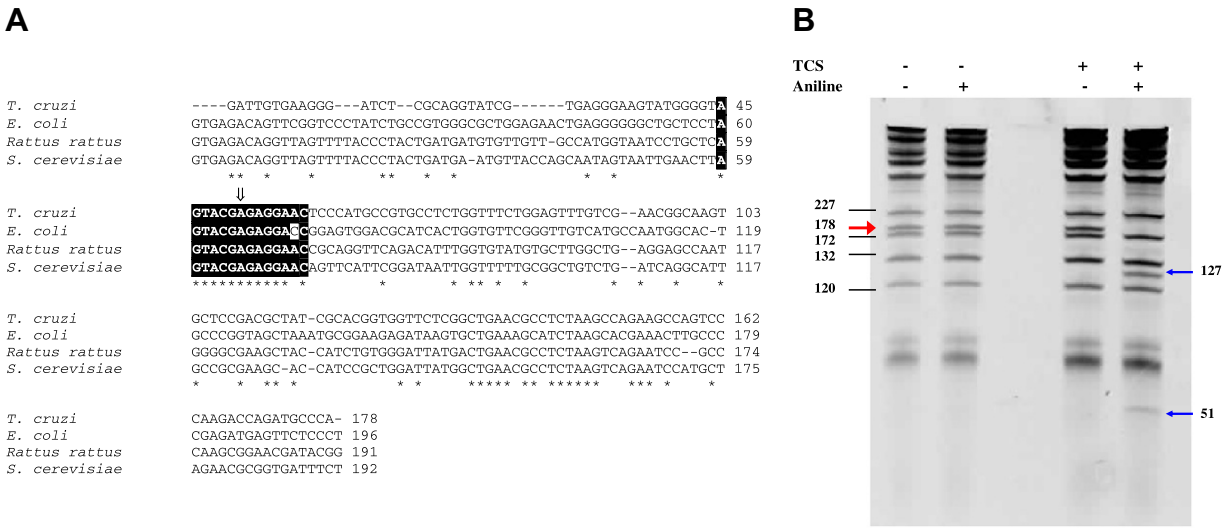

Fig. 1. (A) Sequence alignment of the *T. cruzi* rRNA 28Sδ with the corresponding regions from *E. coli* 23S rRNA, *S. cerevisiae* 25S rRNA and rat 28S rRNA. The highly conserved SRL sequence is shown in black background. The arrow indicates the conserved adenine that is the target of RIPs. (B) *T. cruzi* ribosomes were treated with TCS 10 μM and then the RNA was purified and treated with aniline as described in Materials and methods. The 178b 28Sδ molecule is cleaved rendering two fragments of 127b and 51b.

is located on a 178b RNA molecule named 28Sδ (Fig. 1A). We have previously reported that *T. cruzi* ribosomes are >1000 times more resistant to TCS than the mammalian ones [14]. However, at high concentration, the toxin is able to inhibit protein synthesis. To confirm that the effect at that high doses is still specific, we treated *T. cruzi* ribosomes with TCS and then analyzed the effect on the rRNA. Fig. 1B shows that TCS specifically depurinates the conserved A^{51} residue on the SRL, as detected by treatment of RNA with acid aniline, generating two RNA fragments of 127b and 51b. No alteration in the pattern of other bands was observed. This suggests that resistance of trypanosomatids ribosomes is not due to inability of TCS to depurinate rRNA, in contrast to the resistance of *E. coli* ribosomes to ricin.

scFvC5 specifically blocks interaction between TCS K173, R174, and K177 residues and P proteins protecting ribosomes from inactivation

TCS has been shown to interact *in vitro* with ribosomal L1 and P proteins [10,12]. The relevance of these interactions for the depurinating activity of the toxin remains to be demonstrated, since L1 and P proteins form two opposite protuberances on the large ribosomal subunit [13]. Studies with mutants of TCS showed a strong correlation between the ability to interact with P proteins and their activity against mammalian ribosomes [12]. However, the role of this interaction on the action of TCS has not been demonstrated. Replacement by alanine of positively charged P2-interacting residues K173, R174, and K177, which are in an exposed loop of the protein structure, could have additional effects (i.e. decreased conformational stability or catalytic efficiency) on the toxin molecule leading to decreased activity. Therefore, we used a different approach to evaluate the effect of the interaction of TCS with the C-terminal peptides of *T. cruzi* P proteins. By using the scFvC5 we specifically blocked these peptides on active ribosomes. When *T. cruzi* ribosomes (around 120 nM) were preincubated with the scFvC5 500 nM before adding TCS, it was clear that ribosomes were protected from the toxin action (Fig. 2). This observation is consistent with a significant role of the C-terminal end of P proteins for the full activity of TCS. The inhibition is not a direct effect of scFvC5 on TCS, since the antibody had no effect on the action of TCS on rat ribosomes, consistently with the inability of this antibody to bind mammalian P proteins (not shown). To rule out the possibility that the effect of the antibody could be due to steric effects rather than a specific blocking of its epitope, we assayed the effect of scFvC5 on inactivation of *T. cruzi* ribosomes by $TCS_{K173A/R174A/K177A}$. As can be seen in Fig. 2, scFvC5 has no effect on the activity of this toxin, definitely demonstrating that its effect is mediated by specific blockage of the C-terminal end of P proteins, and that the decreased activity of $TCS_{K173A/R174A/K177A}$ is due to its inability to interact with these peptides. Altogether, these results demonstrate that interaction of TCS with the ribosomal P proteins is necessary for full activity of the toxin, and that this interaction is mediated by residues K173, R174, and K177, confirming the hypothesis presented by Chan et al. [12]. Our results also make extensive that model to ribosomes from *T. cruzi*, an ancient eukaryotic organism. This strongly suggests that this mechanism is a general rule for the inactivation of eukaryotic ribosomes by TCS.

Residues interacting with ribosomal proteins are not conserved among different RIPs

The fact that TCS requires from P proteins for full activity, arose the question if this is also true for other RIPs.

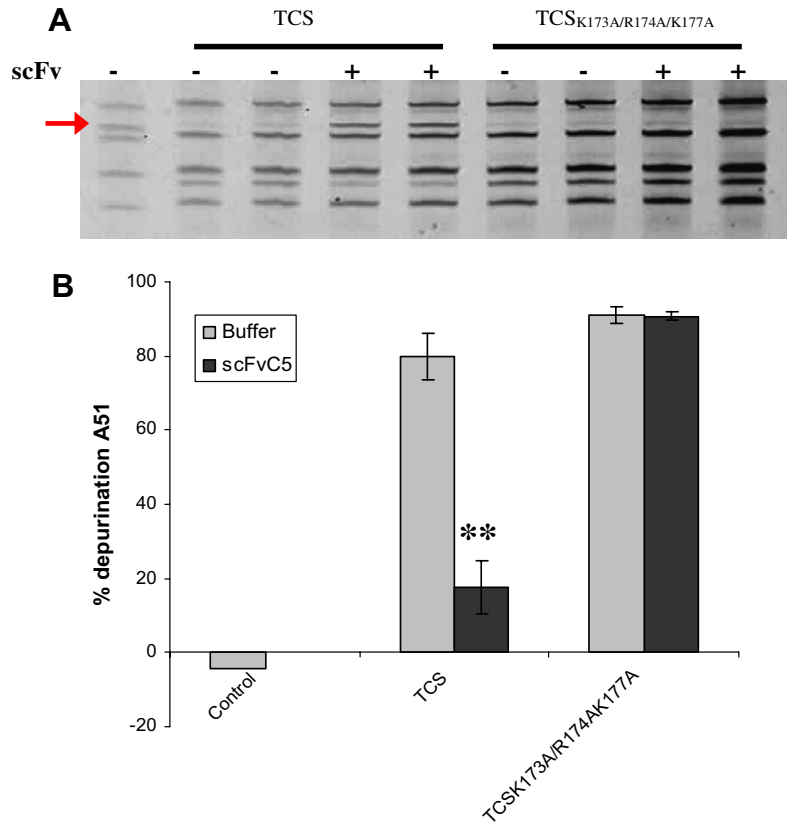

Fig. 2. (A) *Trypanosoma cruzi* ribosomes were preincubated in the presence or in the absence of scFvC5 before treatment with wt TCS or $TCS_{K173A/R174A/K177A}$. RNA was purified and treated with aniline. Samples were analyzed by PAGE as described in Materials and methods. (B) Quantification of the depurination of the TCS target by densitometric analysis. Data are presented as media ± SD and (**) indicates significant differences with control ($p < 0.01$).

Supplementary Fig. 1 shows a sequence alignment of TCS and other RIPs. As expected, amino acids forming the active site [9] are strictly conserved. In contrast, residues involved in interactions with ribosomal proteins, seem to be not conserved at all. For instance, the PAP residues that have been involved in interaction with ribosomal L3 protein [5] are poorly conserved among RIPs. A similar situation is observed with TCS K173, R174, and K177 residues interacting with P proteins. Altogether, these observations support the view that different RIPs would access to the SRL by interacting with different ribosomal proteins.

Since P proteins are not present in prokaryotes, it would be expected that those RIPs that inactivate with similar efficiency prokaryotic and eukaryotic ribosomes does not bind ribosomes via P proteins. One of these broad specificity RIPs is PAP. Sequence of PAP (Supplementary Fig. 1) reveals that residues corresponding to TCS K173 and R174 are replaced in PAP by T and N, respectively. In contrast, K177 is replaced by another positively charged residue (R).

Structural studies based on crystal data support the sequence analysis. Comparison of TCS and PAP showed a structurally conserved active site composed by residues Y72, Y123, E176, R179, and W208 on PAP and the corresponding Y70, Y111, E160, R163, W192 residues on TCS (Fig. 3A). In contrast, TCS residues K173, R174, and K177 are exposed and forming a strongly positively charged region that is not conserved in PAP (Fig. 3B).

scFvC5 does not protects ribosomes from inactivation by PAP

As it was mentioned before, PAP inactivates both eukaryotic and prokaryotic ribosomes, suggesting that this toxin should interact with highly conserved proteins. Consistently with this hypothesis, and in contrast to TCS [14], PAP inactivated *T. cruzi* and rat ribosomes with similar efficiency, supporting the hypothesis that PAP and TCS interact with different ribosomal proteins (Supplementary Fig. 2). Since P proteins are not present in prokaryotes, we hypothesized that PAP should bind ribosomes independently of these proteins. When *T. cruzi* ribosomes were preincubated with scFvC5, no effect was observed on the depurinating activity of PAP, demonstrating that binding of this toxin to ribosomes is not mediated by the C-terminal end of P proteins (Fig. 4). To our knowledge, this is the first report showing a differential interaction of two RIPs with ribosomal proteins. The binding of TCS to P proteins could explain, at least partially, the specificity of this toxin by eukaryotic ribosomes.

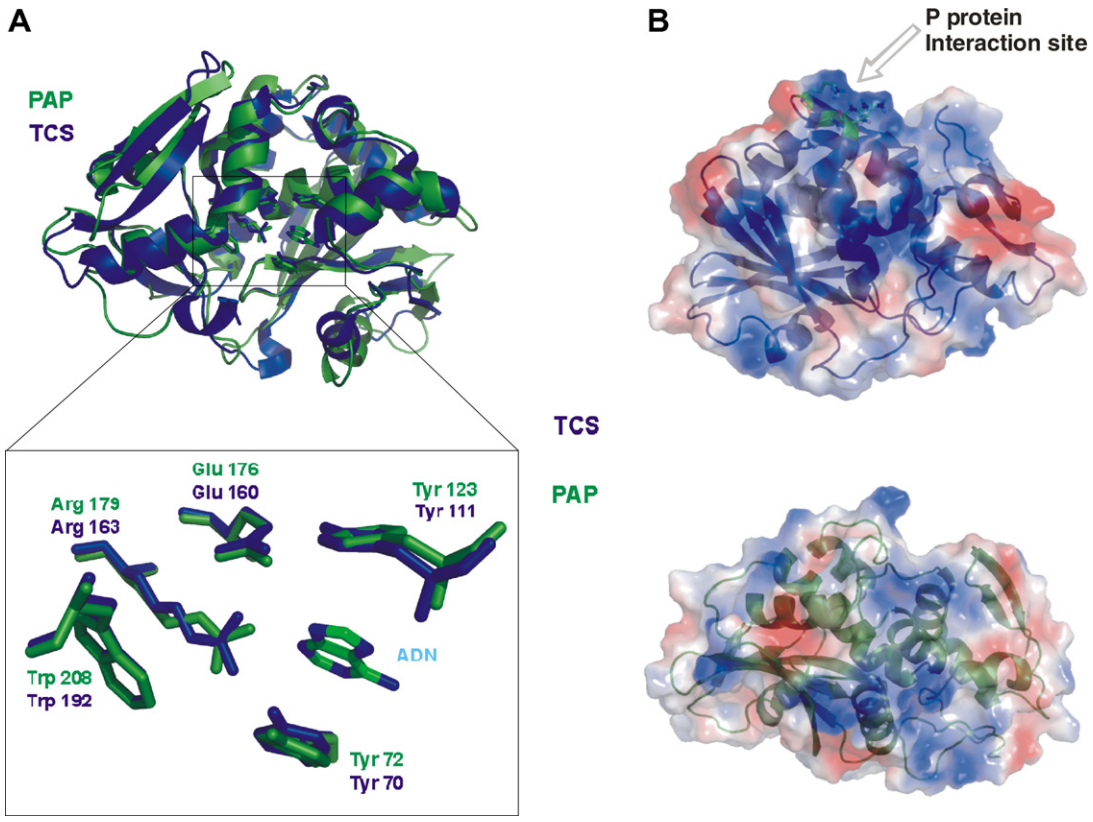

Fig. 3. 3D structure comparison between TCS and PAP. (A) Overlay of the 3D structures of PAP and TCS. Inset shows an amplified view of amino acids forming the active site. (B) Comparison of surface electrostatic potential of TCS and PAP. The location of the positively charged region of TCS interacting with the C-terminal end of P proteins is shown.

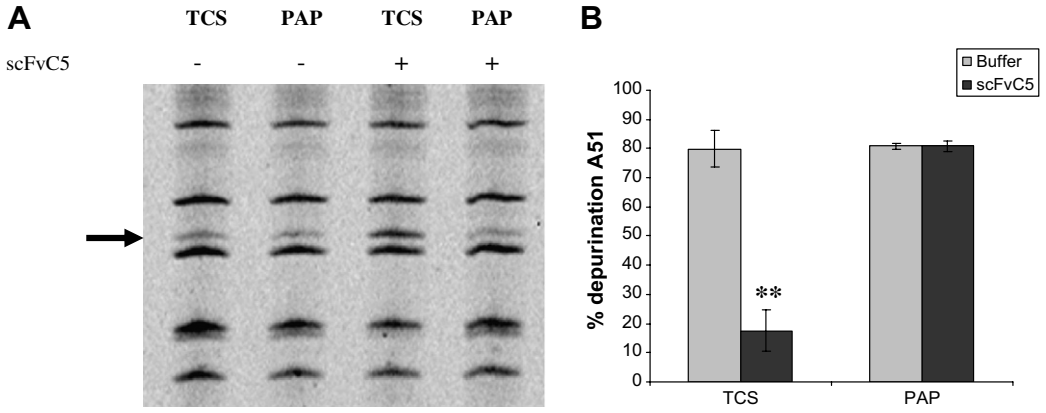

Fig. 4. (A) *Trypanosoma cruzi* ribosomes were preincubated in the presence or in the absence of scFvC5 before treatment with TCS or PAP. RNA was purified and treated with aniline. The arrow indicates the 28Sδ rRNA band. (B) Quantification of the depurination of the RIP target by densitometric analysis. Data are presented as media ± SD and (**) indicates significant differences with control ($p < 0.01$).

In summary, in the present work we show that TCS is able to depurinates its specific target within the *T. cruzi* 28Sδ rRNA. Taking advantage of the availability of scFvC5 and $TCS_{K173A/R174A/K177A}$, we showed, under the same conditions used for *in vitro* protein synthesis assays, that interaction of TCS with the C-terminal epitope of P proteins is needed for full activity of the toxin, in line with previous data on mammalian P proteins and ribosomes. Therefore, resistance of *T. cruzi* ribosomes to TCS seems to be due to differences in yet unknown interactions between this RIP and ribosomal proteins. Finally, we demonstrated that interaction with P proteins is not a general feature of RIPs, since PAP activity is not modified by preincubation with scFvC5. This result shows that different RIPs can access to the same target via interaction with different ribosomal proteins.

Acknowledgments

This work was partially supported by grants from CONICET and University of Buenos Aires. We are grateful to Dr Katalin Hudak for kindly providing PAP. We are indebted to Dr. Israel D. Algranati for continuous support, helpful discussions and careful reading of the manuscript. We also thank to A. Soler-Bistué, E. Trejo and C. Czadikian for valuable advices and technical assistance. M.J.A. and M.J.L. are members of the career of scientific investigator of CONCIET. C.R.S. is a Doctoral fellowship of CONICET. M.J.L. is a Marie Curie Fellow-EU project SMAAD 40402.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.01.170.

References

[1] Y. Endo, K. Tsurugi, RNA *N*-glycosidase activity of ricin A-chain. Mechanism of action of the toxic lectin ricin on eukaryotic ribosomes, J. Biol. Chem. 262 (1987) 8128–8130.

[2] Y. Endo, K. Mitsui, M. Motizuki, K. Tsurugi, The mechanism of action of ricin and related toxic lectins on eukaryotic ribosomes. The site and the characteristics of the modification in 28S ribosomal RNA caused by the toxins, J. Biol. Chem. 262 (1987) 5908–5912.

[3] Y. Endo, K. Tsurugi, The RNA *N*-glycosidase activity of ricin A-chain. The characteristics of the enzymatic activity of ricin A-chain with ribosomes and with rRNA, J. Biol. Chem. 263 (1988) 8735–8739.

[4] K.A. Hudak, J.D. Dinman, N.E. Tumer, Pokeweed antiviral protein accesses ribosomes by binding to L3, J. Biol. Chem. 274 (1999) 3859–3864.

[5] F. Rajamohan, Z. Ozer, C. Mao, F.M. Uckun, Active center cleft residues of pokeweed antiviral protein mediate its high-affinity binding to the ribosomal protein L3, Biochemistry 40 (2001) 9104–9114.

[6] L. Nilsson, O. Nygard, The mechanism of the protein-synthesis elongation cycle in eukaryotes. Effect of ricin on the ribosomal interaction with elongation factors, Eur. J. Biochem. 161 (1986) 111–117.

[7] S. Sperti, L. Montanaro, A. Mattioli, F. Stirpe, Inhibition by ricin of protein synthesis in vitro: 60S ribosomal subunit as the target of the toxin, Biochem. J. 136 (1973) 813–815.

[8] C.A. Vater, L.M. Bartle, J.D. Leszyk, J.M. Lambert, V.S. Goldmacher, Ricin A chain can be chemically cross-linked to the mammalian ribosomal proteins L9 and L10e, J. Biol. Chem. 270 (1995) 12933–12940.

[9] F. Rajamohan, M.J. Pugmire, I.V. Kurinov, F.M. Uckun, Modeling and alanine scanning mutagenesis studies of recombinant pokeweed antiviral protein, J. Biol. Chem. 275 (2000) 3382–3390.

[10] X. Xia, F. Hou, J. Li, H. Nie, Ribosomal protein L10a, a bridge between trichosanthin and the ribosome, Biochem. Biophys. Res. Commun. 336 (2005) 281–286.

[11] S.H. Chan, F.S. Hung, D.S. Chan, P.C. Shaw, Trichosanthin interacts with acidic ribosomal proteins P0 and P1 and mitotic checkpoint protein MAD2B, Eur. J. Biochem. 268 (2001) 2107–2112.

[12] D.S. Chan, L.O. Chu, K.M. Lee, P.H. Too, K.W. Ma, K.H. Sze, G. Zhu, P.C. Shaw, K.B. Wong, Interaction between trichosanthin, a ribosome-inactivating protein, and the ribosomal stalk protein P2 by chemical shift perturbation and mutagenesis analyses, Nucleic Acids Res. 35 (2007) 1660–1672.

[13] C.M. Spahn, R. Beckmann, N. Eswar, P.A. Penczek, A. Sali, G. Blobel, J. Frank, Structure of the 80S ribosome from *Saccharomyces cerevisiae*—tRNA-ribosome and subunit–subunit interactions, Cell 107 (2001) 373–386.

[14] M. Juri Ayub, K.W. Ma, P.C. Shaw, K.B. Wong, *Trypanosoma cruzi*: High ribosomal resistance to trichosanthin inactivation, Exp. Parasitol. 118 (2008) 442–447.

[15] C. Smulski, V. Labovsky, G. Levy, M. Hontebeyrie, J. Hoebeke, M.J. Levin, Structural basis of the cross-reaction between an antibody to the *Trypanosoma cruzi* ribosomal P2beta protein and the human beta1 adrenergic receptor, FASEB J. 20 (2006) 1396–1406.

[16] T.C. White, G. Rudenko, P. Borst, Three small RNAs within the 10 kb trypanosome rRNA transcription unit are analogous to domain VII of other eukaryotic 28S rRNAs, Nucleic Acids Res. 14 (1986) 9471–9489.