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Ribosomal protein L10a, a bridge between trichosanthin and the ribosome

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Abstract

Trichosanthin is a type I ribosome-inactivating protein (RIP) with many pharmacological activities. The trichosanthin-coupled Sepharose affinity purification revealed a protein, which was identified by mass spectrometry as the ribosomal protein L10a. The interaction between trichosanthin and recombinant L10a was further confirmed by in vitro binding assay. Kinetic analysis by surface plasmon resonance technology revealed that L10a had a high affinity to trichosanthin with a K_D of 7.78 nM. The study with mutated forms of trichosanthin demonstrated that this specific association correlates with the ribosome-inactivating activity of trichosanthin. This finding might provide insight into the mechanisms by which trichosanthin inactivates ribosome and that underlies its pharmacological effect.

Keywords: Trichosanthin; L10a; Interact; Mass spectrometry; Binding assays; Surface plasmon resonance

Trichosanthin (TCS) is type I single chain ribosome-inactivating protein (RIP). It was isolated from *Trichosanthes kirilowii* and has been used to terminate early and midtrimester pregnancies and to treat ectopic pregnancies, hydatidiform moles, and trophoblastic tumors [1]. Pharmacological studies reported that TCS was able to inactivate eukaryotic ribosome [2] and to suppress the immune responses [3]. In the early 1990s, TCS was administered in the treatment of patients with AIDS or AIDS-related complex in phase I and II studies [4].

Ribosomal protein L10a is highly conserved in eukaryotes and its function is not clear [5]. L10a was downregulated in many cell types during development, indicating its role in embryogenesis and organogenesis [6,7]. Interestingly, L10a was downregulated as well in vivo by chronic treatments with cyclosporin-A (Csa), which was a potent, highly specific immunosuppressive drug [6].

In this study, we purified a 30 kDa protein by affinity chromatography, which was identified as the ribosomal protein L10a by mass spectrometry. It was then cloned and expressed in *Escherichia coli* to further characterize its interaction with TCS. This finding might provide new insights into the mechanisms of TCS inactivating ribosome and inducing abortion and suppressing the immune responses.

Materials and methods

Preparation of TCS- and BSA–Sepharose beads. TCS was purified from freshly harvested root tubes of *T. kirilowii* as previously described [8]. TCS or BSA was conjugated to CNBr-activated Sepharose 4B beads (Amersham Pharmacia Biotech) according to the instructions of the manufacturer. The quality of TCS-coupled Sepharose beads was demonstrated by its capability to purify anti-trichosanthin polyclonal antibody raised from Chinese big ear rabbit using purified TCS as the antigen (data not shown).

Affinity purification of TCS-binding protein from detergent-solubilized total membrane fraction of rat liver. Crude membrane fraction from rat liver was prepared as previously described method [9]. In brief, young rats (100–150 g), fasted overnight, were killed by decapitation. The livers were rapidly excised, perfused with ice-cold 150 mM NaCl, cut into pieces, and weighed. Homogenization buffer (10 mM Hepes, pH 7.4, 0.25 M sucrose, 1 mM EDTA, and 0.1 mM PMSF) was added in a 5:1 ratio (w/w) and homogenized on ice with 10–20 strokes. The homogenate was filtered through two layers of cotton gauze and centrifuged at 600*g* for 10 min at 4 °C to get rid of the unbroken cells and nuclei. The supernatant was centrifuged again at 8000*g* for 10 min at 4 °C to pellet the mitochondria.

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Finally, the supernatant was spun at 100,000*g* for 20 min at 4 °C. The pellet was suspended in homogenization buffer homogenized, and spun again. The pellet was thoroughly rehomogenized and incubated with solubilization buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, and 1% Triton X-100) for 1 h at 4 °C. The soluble fraction was recovered by centrifugation at 200,000*g* for 30 min at 4 °C.

The soluble fraction was loaded on TCS–Sepharose column after the BSA–Sepharose column. After extensive washing with homogenization buffer and elution buffer (10 mM Hepes, pH 7.4, 1 mM EDTA, and 0.5 M NaCl), the binding fraction was eluted with 0.1 M glycine, pH 2.2. The eluant was dialyzed against 20 mM PBS, pH 7.4, and concentrated by Amicon Ultra-4 (Millipore).

Mass spectrometric analysis. The concentrated samples were subjected to SDS–PAGE analysis and the gel was stained with Coomassie brilliant blue. Protein band of interest was cut out and digested in-gel with trypsin. Peptide mass maps were generated by Applied Biosystem Voyager System 6192. The mass spectra were fitted to databases by the program MS-Fit (University of California, San Francisco).

Expression and purification of recombinant L10a. Full length coding sequence of L10a was amplified by PCR with primers (5′-gccatatgagcagcaaagtttcacg-3′ and 5′-tcggatcctaatacagacgctggggcttg-3′) from the plasmid pL10-5 (a gift from Dr. Ira. G. Wool, the University of Chicago), digested by *Nde*I and *Bam*HI, and ligated to pET-28a(+) (Novagen). The inserts were confirmed by DNA sequencing. Recombinant proteins were expressed in *E. coli* BL21 (DE3). After induction for 3 h with 1 mM IPTG at 37 °C, the bacteria were harvested, lysed by sonication, and centrifuged at 12,000*g* for 20 min at 4 °C. The soluble fraction was loaded onto NTA–Ni^{+} Sepharose (Qiagen) according to the instruction of the manufacturer. His-tagged L10a was eluted with 500 mM imidazol and dialyzed against PBS, pH 7.4.

In vitro binding assays. The pull-down assay was performed as previously described [10]. In brief, recombinant L10a was incubated with TCS–Sepharose or BSA–Sepharose on ice for 4 h. The beads were slightly washed with PBS and the binding products were loaded onto SDS–PAGE, followed by either Coomassie blue staining or Western blot.

The immunoprecipitation experiment was performed as previously reported [10]. TCS was incubated with anti-TCS antibody and protein A–Sepharose (Calbiochem) for 2 h on ice. The recombinant L10a was then added and incubated for another 2 h. After washing with PBS, the immunoprecipitated complexes absorbed onto protein A–Sepharose were loaded onto 10% SDS–PAGE, followed by Western blot analysis. TCS present in the samples was detected using anti-TCS antibody, and the presence of L10a on the same blot was detected using anti-His monoclonal antibody (Novagen) after stripping the antibodies off by incubating in 62.5 mM Tris–HCl, pH 6.7, 100 mM of 2-ME, and 2% SDS at 70 °C for 30 min.

The cross-linking was performed by using disuccinimidyl suberate (DSS; Pierce) following the manufacturer's instructions. In brief, the recombinant L10a (10 μg) was incubated with or without DSS (100 μM) at room temperature for 30 min in the presence or absence of TCS (10 μg) in a total volume of 200 μl. The reaction was terminated by adding cold Tris–HCl (1 M pH 7.4) to a final concentration of 10 mM and incubated for additional 15 min. The sample was analyzed by Western blotting, and the cross-linked complex of TCS and L10a was detected by rabbit anti-TCS antibodies.

Surface plasmon resonance (SP) analysis. The binding of TCS to L10a was studied by SPR analysis on a BIAcore 1000 instrument (Pharmacia LKB Biotechnology). Briefly, BIAcore sensor chip CM5 (Pharmacia LKB Biotechnology) was activated according to the instruction of the BIAcore 1000 amine coupling kit by injecting volumes ranging from 50 to 100 μl of 100 mM NHS/400 mM EDC (1:1, v:v). Purified TCS was then covalently coupled to the sensor chip through reaction with primary amines by injecting 40–100 μl TCS across the activated surface. Excess reactive groups were deactivated by reacting with a large excess (100 μl) of 1 M ethanolamine adjusted to pH 8.5. Satisfactory results were obtained when around 0.01–0.05 pmol/mm^2 of TCS was immobilized. The purified L10a prepared as indicated concentrations was injected at 25 °C into the flow cells at a flow rate of 10 μl/min using HBS as running buffer. The resonance signal (absolute response in RU) was recorded continuously during passage of a sample and the difference between the optical signals measured before and after sample injection (relative response in RU) was related to the amount of bound L10a. The resulting binding curves were analyzed with BIAevaluation 2.1 software (Pharmacia Biosensor), to obtain values for the apparent dissociation rate constants.

TCS mutations. The constructs of two TCS mutations and the determination of their ribosome inactivating activity were performed according to previous reports [8,11]. TCS_{119} was completely deprived of ribosome inactivating activity, in which position 120–123 was deleted. TCS_{KDEL} exhibited almost the same ribosome inactivating activity as natural TCS, by adding an ER retrieval signal (C-terminal KDEL sequence) onto the C-terminal of TCS, to facilitate the transport of the protein to the ER.

ELISA. TCS was labeled with biotin (biotin-TCS) following the instruction of the manufacturer (Pierce). A 96-well flat-bottomed ELISA plate (NUNC, Roskilde Denmark) was coated overnight with recombinant L10a in PBS (100 μl/well) at 4 °C. The rest of the experiment was carried out at room temperature. The wells were blocked with 2% BSA in PBS for 1 h. After washing with PBS/0.05% Tween 20, the plate was incubated for 2 h with biotin-TCS in 100 μl PBS/2% BSA in the presence or absence of TCS or TCS mutations. The wells were washed four times with PBS/Tween and incubated for 2 h with a 1:1000 dilution of HRP-conjugated streptavidin (Vector Laboratories) in PBS/2% BSA. Wells were washed four times with PBS/Tween and detected by adding 100 μl/well working substrate solution of *O*-phenylenediamine (0.8 mg/ml OPD in 0.2 M citrate-phosphate buffer, pH 5.0, containing 0.04% H_2O_2). Reactions were stopped after 10 min with 50 μl/well 2 M sulfuric acid and the OD_{490} was measured using a Microplater reader (Bio-Rad).

Results and discussion

TCS is a potent ribosomal inactivating protein, presumably due to its specifically depurinating activity for a single adenine base at 4324 of 28S rRNA [12]. It has been postulated that the depurination of 28S rRNA by RIP alters the conformation of the rRNAs and inhibits the binding of elongation factors, which in turn inhibits protein synthesis. There are increasing evidences that ribosomal proteins play an important role in making rRNA highly susceptible to the attack by RIPs [13–15].

In this study, we isolated a TCS-binding protein by affinity chromatography (Fig. 1, lane 3). It was then identified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Fig. 2). The spectrum shows eight peptides that matched the theoretical *m*/*z* values for residues in the sequence from 60S ribosome protein L10a (Table 1). The eight matching peptides covered 53% of the sequence.

The full-length coding sequence of L10a was cloned into pET-28a vector and expressed in *E. coli* BL21 (DE3). The recombinant protein was purified by NTA–Ni^{+} Sepharose (Fig. 3, lane 4). From a 100 ml culture of *E. coli* cells, 100 μg of soluble protein was obtained.

The interaction between TCS and L10a was investigated using several approaches. As shown in Figs. 4A and B, L10a was pulled down by the TCS–Sepharose (AB, lane 3) but not by BSA–Sepharose (AB, lane 2). Coimmunoprecipitation of L10a with TCS was also detected (Fig. 4D, lane 3). In cross-linking experiments, incubation of TCS and L10a with DSS resulted in an upshift of the TCS band to a position of about 50 kDa, approximately the sum of

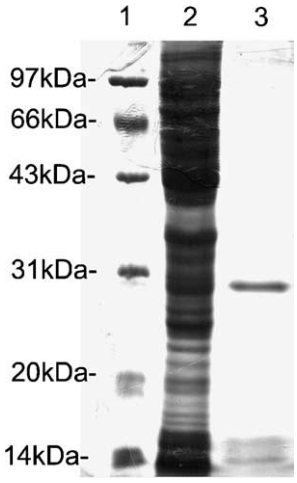

Fig. 1. Affinity purification of TCS-binding protein. SDS–polyacrylamide gel electrophoresis analysis of affinity purification of TCS-binding protein from the crude membrane fraction of rat liver with the TCS–Sepharose column. The proteins were separated on polyacrylamide gel (12%) and stained by Coomassie. Lane 1, molecular mass marker; lane 2, the crude membrane fraction from rat liver; lane 3, purified TCS-binding protein.

TCS (27 kDa) and L10a (30 kDa) (Fig. 4E, lane 3). These results strongly indicated the specific association of TCS with L10 in vitro.

Table 1
Mass fragments fitted for ribosomal protein L10a by MS-Fit search

m/z	Start	End	Peptide sequence
816.4	197	202	NWQNVR
904.5	208	215	STMGKPQR
952.5	16	23	EVLHGNQR
966.5	8	15	DTLYEAVR
1308.6	5	15	VSRDTLYEAVR
1484.8	106	118	KYDAFLASESLIK
1484.8	197	207	NWQNVRALYIK
1600.8	134	147	FPSLLTHNENMVAK

Moreover, we employed Biacore 1000 as an alternative approach to perform the kinetic analysis of the interaction of TCS and L10a (Fig. 5). During the assay, TCS was immobilized on the CM5 sensor chip as ligand according to the standard amine-coupling wizard. By injecting various concentrations of L10a under kinetic conditions, we obtained the dissociation constants: 7.78×10^{-9} M, which quantitatively revealed the highly binding affinity of L10a against TCS.

Two mutated forms of TCS, TCS_{KDEL} and TCS_{119}, showed different ribosome inactivating activity. TCS_{119} was completely deprived of ribosome inactivating activity and TCS_{KDEL} showed almost the same ribosome inactivating activity as wild type TCS [8,11]. Fig. 6A showed that

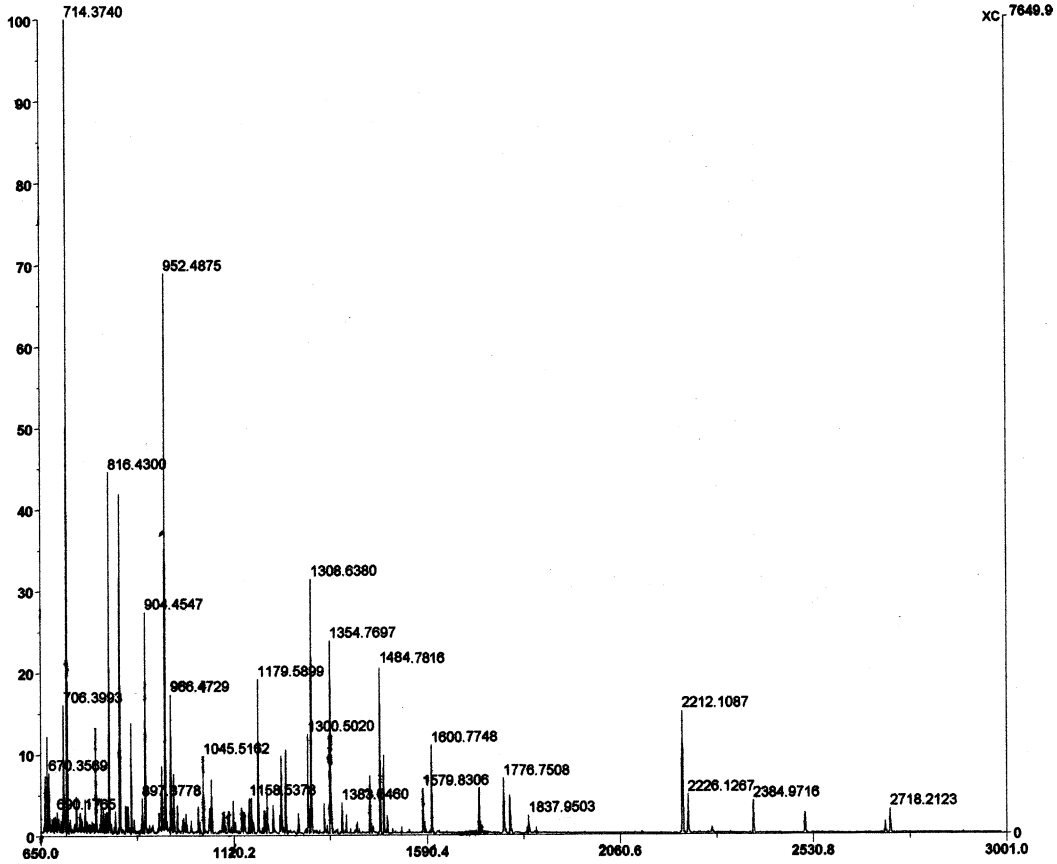

Fig. 2. Mass spectrum of the tryptic digest of TCS-binding protein in the *m/z* range 650–3000 Da. The TCS-binding molecule indicated in Fig. 1 (lane 3) was in-gel digested with trypsin and subjected to MALDI-TOF MS spectrometry.

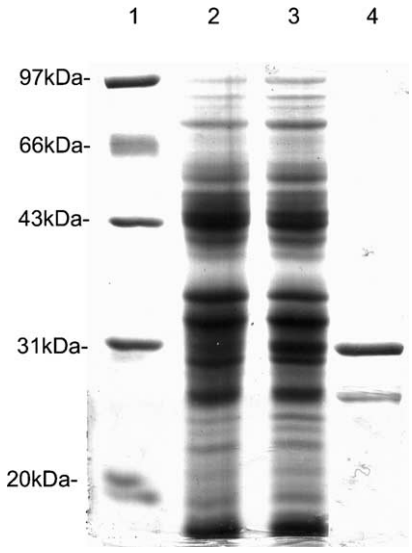

Fig. 3. Expression and purification of recombinant L10a. A fusion of L10a with a hexa-histidine epitope (His-L10a) was produced by cloning of the L10a encoding sequence into vector pET-28a(+) and by purification of the fusion protein from BL21 bacteria via routine Ni–NTA affinity chromatography. SDS–polyacrylamide gel electrophoresis analysis of protein markers (lane 1), uninduced sample (lane 2), induced sample (lane 3), and affinity purification sample (lane 4). The protein was visualized by Coomassie staining.

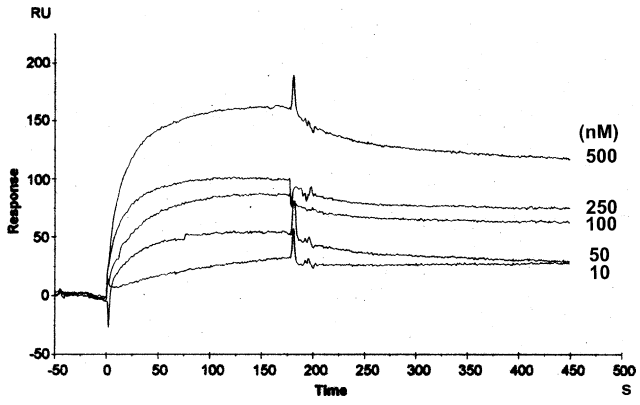

Fig. 5. Surface plasmon resonance analysis of the binding of recombinant L10a to TCS immobilized on Biacore 1000. The apparent K_D values were obtained from the BIAevaluation 2.1 software.

the interaction of TCS and L10a could also be detected with ELISA. Fig. 6B showed that TCS_{KDEL} exhibited the same inhibitory activity as wild type TCS while TCS_{119} almost lost its inhibitory activity. These results demonstrated that the specific association of TCS and L10a correlated with the ribosomal inactivating activity of TCS.

Although the mechanism underlying the catalytic activity of RIPs was understood, the difference among their activity on ribosome remains elusive. Some studies suggested that the differences in sensitivity of ribosome to RIPs might reflect differences in their interactions with ribosomal

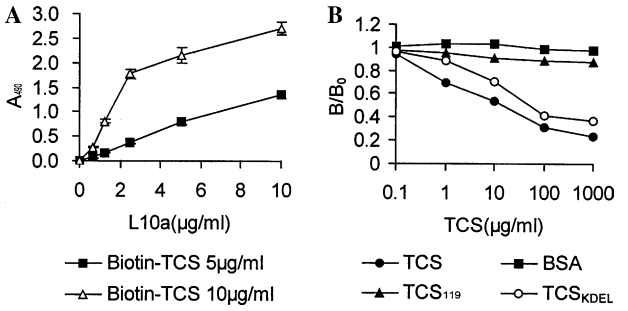

Fig. 6. Characterization of TCS binding to L10a by ELISA. TCS was labeled with biotin and detected with HRP-streptavidin. Specific binding was determined by subtraction of values obtained from binding to plates coated with 10% BSA. (A) Binding of biotin-TCS (■, 5 μg/ml; Δ, 10 μg/ml) to L10a coated on microtiter plates at different concentrations. The results shown represent means of three independent experiments. (B) Inhibition of biotin-TCS binding to L10a (10 μg/ml) on microtiter plates by BSA (■), TCS (●), TCS_{119} (▲) or TCS_{KDEL} (○). Each point is the mean of results obtained from three individual wells and the standard deviation is less than 5%.

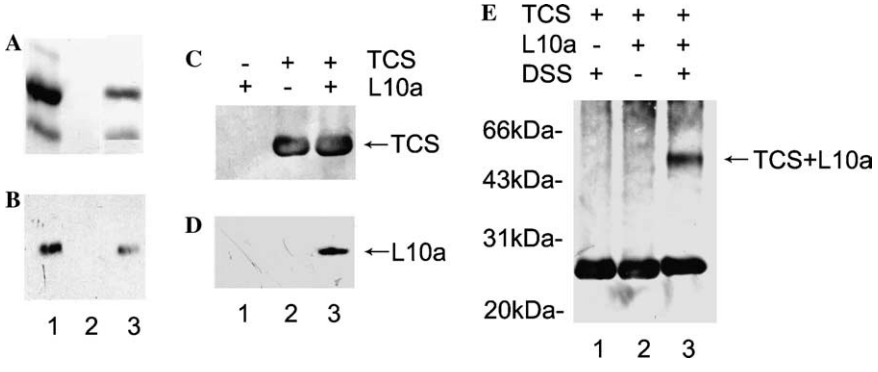

Fig. 4. Interaction of TCS and L10a in vitro. Pull-down assays were performed by incubating L10a with TCS–Sepharose or BSA–Sepharose (A,B). Samples of input (lane 1) and elution from BSA–Sepharose (lane 2) or TCS–Sepharose (lane 3) were analyzed by SDS–PAGE. The protein was visulized by Coomassie staining (A) or detected by anti-His monoclonal antibody (B). The coimmunoprecipitation experiment of TCS and L10a was performed as in Materials and methods (C,D). The presence of TCS (C) and L10a (D) in the immunocomplexes was detected with anti-TCS antibody and anti-His monoclonal antibody after Western blotting. Lane 1, L10a without TCS; lane 2, TCS without L10a; lane 3, TCS with L10a. The cross-linking experiments were performed by incubating TCS with L10a as described under Materials and methods (E). The existence of TCS cross-linking to L10a was detected by anti-TCS antibody. Lane 1, TCS + DSS; lane 2, TCS + L10a; lane 3, TCS + L10a + DSS.

proteins [13–15]. Interactions of RIPs with several ribosome proteins have been reported. For example, ricin A-chain has been shown to be able to be cross-linked to L9 and L10e of human lung carcinoma cells [13]. In addition, a ribosomal protein with a molecular mass of 35 kDa from the 60S ribosome was found to interact with saporin [14]. The yeast ribosomal protein L3 can be coimmunoprecipitated with pokeweed antiviral protein [15]. In this paper, we showed that TCS interacted with L10a. Although the precise roles of L10a in the process of eukaryotic protein synthesis are unknown, it is reasonable to postulate that the interaction of TCS and L10a might provide receptor sites for RIPs to gain access to the ribosome or interfere with the process of ribosome assembly and protein synthesis.

The expression level of L10a was highly specific in different tissues and was hardly detectable in adult placenta, kidney, and brain, which were also the targets of TCS in the selective cytotoxicity [1,16,17]. The interaction between TCS and L10a indicates that the specific cytotoxicity of TCS seemed to be relevant to the distribution of L10a.

Overexpression of L10a was able to restore the capacity of the oxidative stress-sensitive yap1 null strain of *Saccharomyces cerevisiae* to resist against H_2O_2, paraquat, menadione, and UV light due to increased carotenoid levels [18]. Zhang et al. [19] showed that reactive oxygen species were involved in trichosanthin-induced apoptosis of human choriocarcinoma cells. Thus, it would be of interest to determine if the physical association of TCS to L10a might prevent the resistance of L10a to reactive oxygen species and result in it most sensitivity of choriocarcinoma cells to TCS due to the lowest expression level of L10a in choriocarcinoma cells.

Although the role of L10a in the immunosuppression induced by cyclosporin-A (Csa) remains to be elucidated, there are common features suggesting that TCS and L10a participate in the similar events in lymphocytes. It has been reported that L10a was downregulated in the thymus by Csa and might be involved in controlling the late stage of thymocyte development [6]. Csa is a potent, highly specific immunosuppressive drug and blocks thymocyte differentiation at the immature $CD4^+CD8^+$stage, preventing them from developing into mature single positives [20]. TCS was a potent immunosuppressive protein that could affect humoral immunity and a variety of cell-mediated processes [3]. Clinical studies show that TCS treatment may help to prevent loss of $CD4^+$ cells in AIDS patients failing treatment with antiretrovial agents such as zidovudine [21] and even to increase $CD4^+$ cells in other cases [22,23]. Given our result that L10a interacted with TCS, it was plausible that L10a might be involved in the immunosuppression triggered by TCS. However, the precise mechanism remains to be revealed.

In conclusion, our study shows that type I ribosomal inactivating protein TCS interacts with ribosome protein L10a, a protein that presumably influences ribosome resembling, protein synthesis, oxidative stress response, and immunosuppressive events. These might shed new light on the potential mechanisms by which TCS inactivates ribosome, induces abortion, and immunomodulates.

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