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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