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Solution structure and mapping of a very weak calcium-binding site of human translationally controlled tumor protein by NMR

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Abstract

Human translationally controlled tumor protein (TCTP) is a growth-related, calcium-binding protein. We determined the solution structure and backbone dynamics of human TCTP, and identified the calcium-binding site of human TCTP using multi-dimensional NMR spectroscopy. The overall structure of human TCTP has a rather rigid well-folded core and a very flexible long loop connected by a short two-strand β-sheet, which shows a conserved fold in the TCTP family. The C-terminal portions of loop $L_{\alpha 3\beta 8}$ and strand β9 and the N-terminal region of strand β8 may form a calcium-binding site in the human TCTP structure, which is largely conserved in the sequence alignment of TCTPs. The K_d value for the calcium binding is 0.022–0.025 M indicating a very weak calcium-binding site.

Keywords: TCTP; HRF; Calcium-binding; Solution structure; NMR

The translationally controlled tumor protein (TCTP)[3] is a highly conserved protein in most eukaryotes from yeast to human with various biological functions and medical importance, which has been studied for more than 20 years [1]. TCTP is a 20–25 kDa protein abundantly and ubiquitously expressed in cell. In yeast of exponential growth phase, TCTP is one of the top 20 most abundant proteins detected by two-dimensional gel analysis [2]. The mRNA of human TCTP (hTCTP) is one of the top 50 most abundant transcripts in 84,103 unique genes of 19 normal and diseased tissue types [3]. hTCTP gene is one of the top 10 most ubiquitously expressed genes in human by examining 1753 libraries from kinds of tissues [4]. However, TCTP expression levels vary widely with the cell/tissue types and the developmental stages, and are highly regulated in response to a wide range of extracellular signals and cellular conditions [1,5]. TCTP expression is regulated not only at the translational level as indicated by its name, but also at the transcriptional level, which may provide the rapid adaptation of TCTP levels in various conditions [1,6].

The abundance and ubiquity indicate that TCTP may have important primary functions, which are still not understood. However, a large number of cellular and biochemical functions have been found since 1980s. Most of these functions can be classified into two groups. One is growth-related biological functions, including microtube-binding, anti-apoptosis, tumor-related, proliferation, etc [7–15]. The other is immunity-related, medical importantly functions, in which TCTP was also named as histamine release factor (HRF) [16–21]. TCTP was identified as a

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[3] *Abbreviations used:* TCTP, translationally controlled tumor protein; HRF, histamine release factor; hTCTP, human TCTP; sTCTP, *Schizosaccharomyces pombe* TCTP; pTCTP, *Plasmodium knowlesi* TCTP; GEF, guanine nucleotide exchange factor; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycoltetraacetic acid; HSQC, heteronuclear single-quantum coherence; RMSD, root mean square deviation; PDB, Protein Data Bank; NOE, nuclear Overhauser effect.

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calcium-binding protein first in parasite *Trypanosoma brucei* in 1992 [22], following in human, rat and several kinds of parasites [23–28]. Functional relationship between TCTP and calcium was reported as TCTP is regulated by calcium at both the transcriptional and post-transcriptional levels [24]. More recently, TCTP was suggested to be involved in the calcium handling of eukaryotic cells [29]. The calcium-binding site was preliminarily identified by constructing several fragments of rat TCTP, which confirmed that TCTP is a novel family of calcium-binding proteins [25].

The first structure of TCTP solved by NMR spectroscopy in 2001 was from *Schizosaccharomyces pombe*, which revealed significant structural similarities to the human protein Mss4, a guanine nucleotide-free chaperone of Rab GTPases [30]. This finding suggested that TCTP may interact with GTPase as a guanine nucleotide exchange factor (GEF) or guanine nucleotide-free chaperone. Later research found that TCTP is involved in the elongation step of translation by interacting with the large GTPase eEF1A [12]. More recently, Hsu et al. reported that TCTP displays GEF activities *in vivo* and *in vitro* and directly associates with Rheb, a small GTPase, and is an essential new component of the tuberous-sclerosis-Rheb pathway [15].

Although the structures of *S. pombe* TCTP (45.9% identity and 2.3% gap with hTCTP) and *Plasmodium knowlesi* TCTP (35.4% identity and 4.0% gap with hTCTP) have been reported [30,31], most functional studies of TCTP were based on mammalian TCTPs for which no structure determination was reported. In this research, the solution structure of hTCTP was determined by heteronuclear NMR method. The structure has a rigid well-folded core and a flexible long loop connected by a short two-strand β-sheet. The backbone dynamics of hTCTP indicates that the three parts of hTCTP have different dynamic characteristics in agreement with the structure. The calcium titration experiments and chemical shift mapping analysis determined a very weak calcium-binding site on hTCTP which is conserved in the TCTP family.

Materials and methods

Expression and purification of the human TCTP

For expression of hTCTP, the protein was fused with a His-tag at the C-terminus using the vector pET22b. Uniformly ^{15}N-labeled and ^{15}N/^{13}C-double labeled hTCTP were expressed in *Escherichia coli* BL21(DE3) with M9 minimal medium at 37 °C. When the optical density at 600 nm of the cell culture reached about 0.8, the protein expression was induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside for 6 h at 30 °C. The cells were harvested by centrifuging and resuspended in 50 mM Tris–HCl, pH 8.0, 250 mM NaCl. The resuspended cell pellet was thawed and lysed by sonication. After centrifugation, the supernatant of the lysate was loaded onto a Chelating Sepharose Fast Flow column (Pharmacia). The hTCTP fraction was eluted from the column with 250 mM imidazole. The eluate containing hTCTP was concentrated to 5 ml and further purified using a Sephadex G50 (Pharmacia) gel filtration column pre-equilibrated with 100 mM NH_4HCO_3. The purified hTCTP was lyophilized and stored at −20 °C.

NMR spectroscopy

The NMR sample contained 1.0–2.0 mM ^{15}N- or ^{15}N/^{13}C-labeled hTCTP, 200 mM NaCl, 0.01% NaN_3, 0.01% 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), and 10% (v/v) D_2O in 50 mM phosphate buffer (pH 7.8). All NMR experiments were performed at 308 K on a Bruker DMX 600 MHz spectrometer equipped with a cryo-probe. The 2D ^{1}H–^{15}N HSQC and 3D HNCA, HNCACB, CBCA(CO)NH, HNCO, HN(CA)CO, HBHA(CBCA)NH, HBHA(CBCA)(CO)NH, H(C)CH-COSY, (H)CCH-COSY, H(C)CH-TOCSY, and (H)CCH-TOCSY experiments were adopted for the resonance assignments. NOE distance constraints were derived from 3D ^{1}H–^{15}N NOESY-HSQC experiment, and two 3D ^{1}H–^{13}C NOESY-HSQC experiments for the aliphatic region and for the aromatic region. The mixing times for ^{13}C–^{13}C TOCSY and ^{1}H–^{1}H NOESY experiments were 12 ms and 80 ms, respectively. All NMR data were processed and analyzed using software FELIX 98.0 (Accelrys Inc.). ^{1}H chemical shifts were referenced to internal DSS. ^{15}N and ^{13}C chemical shifts were referenced to DSS indirectly [32].

Structure calculations

NOEs were manually assigned using software FELIX. A homology-modeling structure generated by MODELLER [33] and yeast TCTP structure (PDB ID: 1H6Q) [30] was also used to assistant the NOEs assignments for the initial structure calculations. According to the peak volumes, all assigned NOEs were grouped into four classes of distance restraints: 1.8–2.5 Å, 1.8–3.5 Å, 1.8–4.5 Å, and 1.8–5.5 Å. Dihedral angle restraints were obtained using the program TALOS [34]. Hydrogen bond restraints were added according to the regular secondary structure patterns. The structures were calculated using the program CNS version 1.1 [35]. A family of 100 structures was generated, and the 20 structures with lowest energies were selected. Structural analysis and statistics were obtained using programs MOLMOL [36] and PROCHECK-NMR [37]. The molecular figures were generated with MOLMOL. The atomic coordinates of hTCTP and all restraints have been deposited in the Protein Data Bank under accession ID 2HR9.

Measurement and analysis of ^{15}N relaxation parameters

Heteronuclear steady-state ^{1}H–^{15}N NOE, ^{15}N-transverse relaxation rate (R_2), and ^{15}N-longitudinal relaxation rate (R_1) were measured using standard pulse sequences. The steady-state ^{1}H–^{15}N NOE spectra were acquired with and without ^{1}H saturation in an interleaved manner. The relaxation delay times for R_2 measurements were set to 17.0, 33.9, 55.9, 67.8, 84.8, 101.8, 118.7, 135.7, 169.6, and 203.5 ms. The relaxation delay times for R_1 measurements were set to 0.060, 0.140, 0.240, 0.360, 0.530, 0.760, 1.150, 1.500 and 2.000 s. The NOE values were calculated from the ratios of the peak intensities with and without proton saturation. R_1 and R_2 relaxation rates were determined using the CurveFit software (http://cpmcnet.columbia.edu/dept/gsas/biochem/labs/palmer/software/curvefit.html).

Calcium titration

The samples containing 0.4 mM ^{15}N-labeled hTCTP were dialyzed extensively for removing the residual calcium in the sample solution. The dialysis was made firstly against the sample buffer (20 mM Tris–HCl, 200 mM NaCl, pH 7.8) containing 5 mM EDTA and 5 mM EGTA, and then against the sample buffer without EDTA/EGTA. Small volumes of calcium chloride stock solution were added into the sample to an appropriate ratio. The dissociation constant K_d for Ca^{2+} interaction with hTCTP was obtained by fitting the observed chemical shifts to the equations:

$$Y = Y_{free} \times F_{free} + Y_{bound} \times F_{bound}; K_d = [Ca][P]/[CaP]$$

where Y is the observed chemical shift; Y_{free} and Y_{bound} are the chemical shifts for Ca^{2+}-free and Ca^{2+}-bound hTCTP; $F_{free} = [P]/([CaP] + [P])$ and $F_{bound} = [CaP]/([CaP] + [P])$ are the fraction of Ca^{2+}-free and Ca^{2+}-bound