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Identification of the Calcium Binding Sites in Translationally Controlled Tumor Protein

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Translationally controlled tumor protein (TCTP), also known as IgE-dependent histamine-releasing factor, is a growth-related tumor protein. Although the primary sequence of rat TCTP does not reveal any recognizable Ca^{2+}-binding motif, previous studies have demonstrated that rat TCTP consisting of 172 amino acids is a Ca^{2+}-binding protein. However, the region of TCTP required for Ca^{2+} interaction has not been mapped to the molecule. Here, we reported that the Ca^{2+} binding region of TCTP, which was mapped by using a combination of deletion constructs of rat TCTP and $^{45}Ca^{2+}$-overlay assay, was confined to amino acid residues 81-112. This binding domain did not show any peculiar loop of calcium-binding motif such as CaLB domain and EF hand motif and it seems to be constituted of random coil regions neighboring the a helix. Thus, our data confirm that TCTP is a novel family of Ca^{2+}-binding protein.

Key words: TCTP, Calcium-binding sites, ^{45}Calcium-overlay assay

INTRODUCTION

The TCTP was first identified as a growth-related protein in mouse ascites and erythroleukemic cells (Yenofsky *et al.*, 1983). TCTP, also known as IgE-dependent histamine-releasing factor (HRF) is a 23 KDa protein (P23) in human and it has a 21 KDa mouse homologue (P21) (Chitpatima *et al.*, 1988; Gross *et al.*, 1989). It was thought that the synthesis of TCTP was increased at the stage of tumor proliferation. However, since TCTP is a highly conserved protein that is found not only in normal cells including mammals but also in nematodes (Bini *et al.*, 1997), trypanosomes (Haghighat *et al.*, 1992), higher plants (Pay *et al.*, 1992), and Saccharomyces (Sanchez *et al.*, 1997), it is regarded as a product of house keeping gene. Although the exact function of TCTP is not understood, human TCTP P23 has been shown to induce the release of histamine from human basophils having special group of IgE, called IgE^+ (MacDonald *et al.*, 1995). Furthermore, TCTP has been described as a protein which binds calcium with a high affinity (Sanchez *et al.*, 1997), but does not belong to any known family of calcium-binding proteins.

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Calcium ions play a key role in the regulation of cellular metabolism and gene expression. The concentration of Ca^{2+} in the cytosol of eukaryotic cells (typically 10-100 nM) is maintained in homeostatic balance with millimolar Ca^{2+} levels in both the extracellular environment and the lumen of the endoplasmic reticulum. Disruption of Ca^{2+} homeostasis impairs the normal functioning of the cell. Recently, Aimin *et al.* (1999) reported that TCTP expression is regulated by calcium at both the transcriptional and post-transcriptional level.

In the present study, we attempted to identify the calcium-binding sites of TCTP by constructing several deletion forms of TCTP. The calcium binding sites of TCTP was found to be the amino acid residues 81-112 by the $^{45}Ca^{2+}$-overlay assay.

MATERIALS AND METHODS

Plasmid construction

The DNA fragment corresponding to rat TCTP (amino acid residues 1-172) and the deletion form of rat TCTP (amino acid residues 1-80) were amplified by using PCR with the following two pairs of primers: residues 1-172; 5'-CGGGATCC (BamHI) GCCTCTGGTGTGGCTGTCTCT (sense) and 5'-CCGCTCGAG (XhoI) TGTCCTAAGTCCTGGT GT (antisense), residues 1-80; 5'-GGAATTC (EcoRI) CAT GATCATCTACCGG (sense) and 5'-CCGCTCGAG (XhoI)

TTCTTGTAAGTGATGGTT (antisense). The amplified TCTP residues 1-172 and 1-80 fragment was inserted into each enzyme sites of pRSET-A type plasmid and pET 22b (+) plasmid. The deletion constructs containing TCTP amino acid residues 1-52 and 1-112 were made by Exsite PCR-based site-directed mutagenesis kit (Strategene, CA, USA.). The deleted cDNAs containing residues 1-52 and 1-112 were PCR amplified from pRSET/TCTP using the following 5'-phosphorylated primers: for residues 1-52, TAACAAATTGGATCTATCGCCCGCCAC (sense) and AGC ATTTCCACCAATGAGTGAATCATC (antisense); for residues 1-112, TAACAAATTGGATCTATCGCCCGCCAC (sense) and CTTTACCCTTTCTGGTTTCTGTTCTTC (antisense). Upon expression of the foreign gene product in an appropriate host, these plasmids, pRSET and pET22b (+) encodes a His tag at the N-terminus and C-terminus of the recombinant protein (Novagen, WI, USA.).

Expression of the recombinant proteins of TCTP and TCTP deletion constructs

For expression of TCTP and TCTP deletion constructs, each of pRSET plasmids harboring TCTP and TCTP deletion constructs (residues 1-52 and 1-112), pET 22b (+) plasmid harboring TCTP deletion construct (residues 1-80) were then transformed into *E. coli* BL21 (DE3) pLysS host cells. Transformed cells were grown in LB media containing ampicillin and chloramphenicol for selection at 37°C until reaching at OD_{600} of 0.6. Culture of the transformed *E. coli* was used to inoculate the same media to a 1:1000 (v/v) dilution and then incubated at 37°C with the continuous shaking until reaching at OD_{600} of 0.6. Maximal TCTP expression was obtained by inducing transformed cultures with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for pET 22b (+) plasmid and 0.4 mM IPTG for pRSET plasmid, followed by the continued incubation of the cultures for 2~3 h at 37°C. Bacterial cells were then pelleted by centrifugation at 5,000 × g for 5 min at 4°C and stored at -70°C before purification.

Purification of the recombinant proteins of TCTP and TCTP deletion constructs

Cell pellets were resuspended in the ice-cold binding buffer (40 mM imidazole, 4 M NaCl, and 160 mM Tris-HCl, pH 7.9) and sonicated on ice until the lysate was no longer viscous. The lysate was centrifuged at 30,000 × g for 30 min. After the nickel-affinity columns (Novagen) were charged orderly with the sterile deionized water, charge buffer (400 mM NiSO4), and binding buffer, the supernatant containing TCTP and TCTP deletion constructs was then passed through the charged column. The columns were then washed with the binding buffer, wash buffer (480 mM imidazole, 4 M NaCl, and 160 mM Tris-HCl, pH 7.9) for reducing non-specific ionic interactions of cellular proteins. TCTP and TCTP deletion constructs were eluted with the elution buffer (4 M imidazole, 2 M NaCl, and 160 mM Tris-HCl, pH 7.9). The eluted proteins were then dialyzed against double distilled water. The protein concentration was determined by Bradford method using bovine serum albumin as the standard. The TCTP and TCTP deletion proteins containing several protease inhibitors (0.5 mM PMSF, 5 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM benzamidin) was stored at -70°C until using.

Gel electrophoresis and western blot analysis

SDS-PAGE (15%) was performed as described by Laemmli *et al.* (1970). Proteins were visualized by Coomassie Blue staining. Western blot analysis was performed with the purified TCTP and TCTP deletion proteins to determine the authenticity of the protein. Each 1 μg of the affinity purified His tag fusion proteins was separated in 15% SDS-PAGE and blotted onto a nitrocellulose membrane using transfer buffer (39 mM glycine, 48 mM Tris base, 20% methanol). The non-specific binding was blocked by soaking the membrane in blocking solution containing 3% gelatin in Tris-buffered saline (TBS) and incubated under continuous shaking for 1 h at room temperature. After washing with TBS/Tween-20 (TTBS, 100 mM Tris-HCl, 0.9% NaCl, 0.5% Tween-20, pH 7.5), the membrane was incubated with the 1/500 diluted anti-rabbit TCTP polyclonal antibody for 1-2 h. Excessive antibodies were removed by washing with TTBS. Target antibodies were detected with anti-rabbit IgG antibody (Serotec, Oxford, UK.) diluted 1:2000 in blocking solution. Antibody labelings of protein bands were detected with ECL reagents according to the supplier's protocol (Amersham, Buckinghamshire, UK.). After electrophoresis, proteins in the membrane were visualized by auto-radiography, phosphor-imaging (Las 2000, Fujifilm, Japan). Protein spots were detected and analyzed using Imagegauge software (Fujifilm, Japan)

$^{45}Ca^{2+}$-overlay assay

The $^{45}Ca^{2+}$ -overlay assay was performed according to Garrigos et *al.* (1991). For the preparation of all solutions, double-distilled water was used and all incubation steps were carried out at room temperature with gentle movement. The purified proteins were resolved by 15% SDS-PAGE and the proteins were transferred to nitrocellulose membrane. The membrane was rinsed briefly with blotting buffer to remove residual acrylamide, washed three times in buffer (60 mM KCl/ 5 mM $MgCl_2$/ 10 mM imidazole-HCl, pH 6.8) for 20 min each. Subsequently, the membrane was incubated in the same buffer containing 2 μCi/ml $^{45}CaCl_2$ for 20 min. The radioactive solution was removed and the membrane was washed in 50% ethanol three times for 20 min each. The washed membrane was dried completely overnight. Radioactivity asso-

ciated with selected proteins was quantified using a phosphorimager (BAS-2500, Fujifilm, Japan).

RESULTS AND DISCUSSION

Although the primary sequences of TCTP do not reveal any recognizable Ca^{2+}-binding motif, previous studies have demonstrated a Ca^{2+}-binding property for a recombinant form of human TCTP expressed in *Escherichia coli* and in a similar homologue of TCTP from *Trypanosoma brucei* (Sanchez *et al.*, 1997) and *Plasmodium falciparum* (Bhisutthibhan *et al.*, 1998). TCTP of Cos-7 cells shared this capacity as well (Aimin *et al.*, 1999). Since calcium-binding region of the protein was not known, we tried to map the calcium-binding region of the rat TCTP. Calcium-binding proteins, such as calmodulin, troponin c, myosin, and paralbumin, commonly have the EF-hand motif. Another calcium-binding-related domain, the CaLB domain is a 43-amino acid sequence motif found in a number of functionally diverse signaling proteins including Ras-specific GTPase activating proteins (GAPs) and Ca^{2+}-dependent phospholipid-binding protein annexin VI. CaLB domain is a convergence point in Ca^{2+} and Ras signaling pathways.

TCTP seems to contain three helix-loop-helix motifs that do not belong to the CaLB domain nor to the EF-hand motif by computer search (Fig. 1A). We had applied the fluorescence method using quin2 to detect the calcium-binding sites of TCTP, but failed to detect TCTP as a fluorescent band. This supports the work of Tatsumi group that the fluorescence method using quin2 was demonstrated only on EF hand-conformation of calcium-binding proteins (Tatsumi *et al.*, 1997). To identify the calcium-binding sites of TCTP consisting of 172 amino acids, we constructed three deletion plasmids of TCTP (the amino acid residues 1-52, 1-80, and 1-112) (Fig. 1B). To confirm that the TCTP and TCTP deletion proteins were correctly expressed, we carried out 15% SDS-PAGE for Coomassie Blue staining (Fig. 2A) and Western blot analysis with anti-rabbit TCTP polyclonal antibodies (Fig. 2B). As shown in Fig. 2A, Coomassie staining visualized the expected molecular sizes of TCTP and TCTP deletion proteins (TCTP full length 1-172 in lane 1; 27KDa, TCTP 1-112 in lane 2; 21KDa, TCTP 1-80 in lane 3; 15KDa, TCTP 1-52 in lane 4; 10KDa). Western blotting also confirmed that all of the expressed proteins are indeed TCTP. Despite the same volume (1 mg) of each protein loaded, the signal in TCTP in lane 4(1-52) was a little stronger and

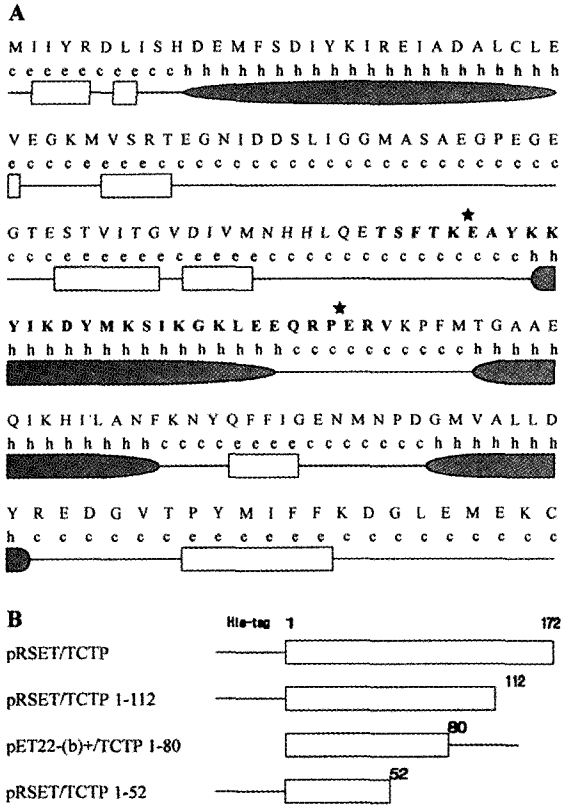

Fig. 1. The secondary structure of TCTP predicted by Network Protein Sequence analysis (A) and the deletion constructs of TCTP (B). Deletion constructs were amplified by PCR using oligonucleotide primer and inserted to expression vectors, pET 22b (+) and pRSET. Random coil, (–) Extended strand, (▭) Alpha helix (⬬).

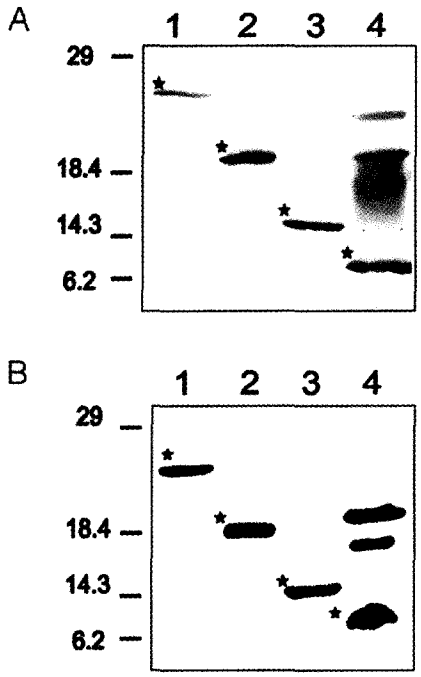

Fig. 2. Detection of the purified recombinant deletion constructs by Coomassie staining and Western blotting. TCTP and the deletion proteins of TCTP were purified by His·tag-Ni^{2+} affinity column chromatography. The purified proteins were subjected to 15% SDS-PAGE for Coomassie staining (A) and Western blot analysis with anti-rabbit TCTP polyclonal anti-body (B). Lane 1, TCTP full-length; lane 2, TCTP (1-112); lane 3, TCTP (1-80); lane 4, TCTP (1-52).

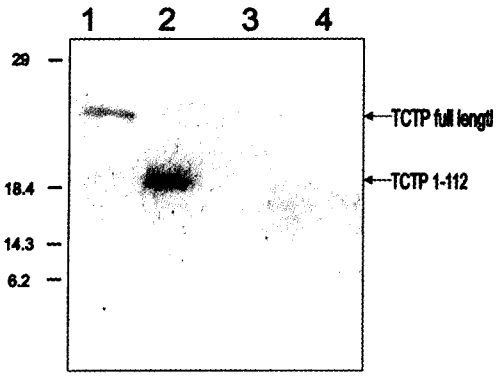

Fig. 3. Detection of the calcium-binding sites of TCTP. Each affinity purified fusion protein (8μg) obtained from the TCTP and its deletion constructs was separated in 15% SDS-PAGE and blotted onto nitrocellulose membrane and then subjected to $^{45}Ca^{2+}$-overlay assay. Lane 1, TCTP full-length as a positive control; lane 2, TCTP (1-112); lane 3, TCTP (1-80); lane 4, TCTP (1-52).

showed several upper bands. This might be due to the slight difference of epitope against anti-rabbit TCTP polyclonal antibodies and protein aggregation. By using $^{45}Ca^{2+}$-overlay assay, we were able to detect $^{45}Ca^{2+}$ which was selectively retained by TCTP full length and TCTP 1-112 (Fig. 3). Other deletion constructs of TCTP 1-80 and TCTP 1-52 did not bind to $^{45}Ca^{2+}$. This result suggests that amino acid residues 81-112 of rat TCTP are the calcium-binding sites.

Ca^{2+} can be bound in a loop between two β strands, as well as in a structurally similar loop between two a helices or between an α helix and β strand (Timothy *et al.*, 2000). Although amino acid residues 81-112 do not have any peculiar loop of calcium-binding motif, random coil regions neighboring the α helix might bind to Ca^{2+} (Fig. 1A). Interestingly, the negatively charged amino acids in random coil regions, E86 and E109 were found.

Our identification of the calcium binding sites of TCTP may contribute to the understanding of the regulatory mechanism by calcium and the biological function of calcium-binding property of TCTP. Further study such as x-ray crystallography will help to characterize the new calcium-binding motif of TCTP.

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