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Research paper

Novel translationally controlled tumor protein homologue in the buccal gland secretion of *Lampetra japonica*

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Abstract

We have cloned a homologue of the translationally controlled tumor protein (TCTP) from the buccal gland of *Lampetra japonica* according to information from a cDNA library and primary analysis of expressed sequence tags. Sequence analysis of *L. japonica* TCTP showed that it had two signature regions of high sequence homology termed TCTP-1 and TCTP-2, respectively. TCTP is highly conserved in evolution. It showed more than 40% identification similarities with parasite TCTPs that had effect on immune responses of host. Phylogeny of 31 TCTP sequences showed that lamprey was closer to jawed vertebrates than to Amphioxus and was a sister group of gnathostomes. TCTP gene from *L. japonica* was expressed in a pET23b vector and purified by using His Bind affinity chromatography. Polyclonal antibody to recombinant protein was generated in New Zealand Rabbit. Immunoblot analysis to localize the recombinant protein in buccal gland secretion proves that recombinant TCTP is a secretion protein, which may be secreted through a non-classical secretion pathway. A characterization study shows that recombinant TCTP has histamine-releasing function *in vitro*. It mediated histamine release from rat basophilic leukemia (RBL-2H3) cells. TCTP links both the innate and the adaptive immune responses by modulating the secretion of cytokines from mast cells, basophils, eosinophils, and T and B lymphocytes. These may indicate a potential role of TCTP in the inflammatory process and immune regulation between *L. japonica* and host.

Keywords: *Lampetra japonica*; Buccal gland secretion; TCTP; HRF; Immune regulation

1. Introduction

Translationally controlled tumor protein (TCTP), also known as IgE-dependent histamine-releasing factor (HRF) [1], is a hydrophilic protein. It was originally identified as a growth-related tumor protein in mouse ascites and erythroleukemic cells [2], it is regulated under transcriptional as well as translational control [3]. TCTP is highly conserved and widely expressed in all eukaryotic organisms [4] which suggests that it may play a crucial role in cell functions. TCTPs do not share significant sequence similarity with any other class of proteins, but the TCTP family has two signature regions, termed TCTP-1 and TCTP-2. TCTP is structurally similar to the Mss4/Dss4 (mammalian suppressor of Sec4) family of proteins and forms a structural superfamily with it [5]. Recently, an increasing number of researchers are focusing their attention on the cellular and extracellular activities of TCTP. Synthesis of TCTP is regulated by calcium [3], eIF4E [6] and dsRNA-dependent protein kinase (PKR) [7]. TCTP has various biological characteristics such as calcium binding [8], tubulin binding [9], and is anti-apoptotic in nature [11]. TCTP also interacts with Na, K-ATPase α subunit [10], inhibits translation, and promotes the release of histamine and interleukin [1,12,13]. It can act as growth factor for B cells [14]. In addition, it was identified as a target of

Abbreviations: BCA, bicinchoninc acid; DAB, 3,3′-diaminobenzidine; EMEM, Eagle's minimum essential medium; ELISA, enzyme-linked immunosorbent assay; EST, expressed sequence tags; HRF, histamine-releasing factor; RBL-2H3, rat basophilic leukemia cell line; rLj-TCTP, recombinant *Lampetra japonica* TCTP; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCTP, translationally controlled tumor protein.

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tumor reversion [15], a direct regulator of *Rheb* and a potential therapeutic target for tuberous sclerosis disease [16]. Phylogeny indicates that the TCTP ortholog originated in eukaryotes 1 billion years ago [17]. Although TCTP participates in various biological functions, the primary physiological roles of this protein are still unknown.

Lampreys are considered to be the most ancient living vertebrates which are scientifically accessible among the remaining jawless vertebrates; and, along with hagfishes, they represent the only existing members of the class Agnatha, whose lineage dates back over 530 million years [18]. Lampreys are parasitic to the host fishes, attaching to them with a suction-cup mouth and drilling into the fish with its tongue and teeth, its buccal gland secretion is known to act as an anticoagulant.

In the present study we describe cloning and characterization of a TCTP homologue from a cDNA library, according to the primary analysis of EST of *Lampetra japonica* buccal gland [19]. An appropriate expression system including pET23b and *Escherichia coli* BL21 (DE3) was selected. The recombinant *L. japonica* TCTP is a secretion protein, and has histamine-releasing function. Furthermore, we discuss the secretion pathway of rLj-TCTP, the plausible mechanism of the rLj-TCTP in regulating anticoagulation, and its probable role in regulating immune responses. We speculate that the recombinant protein may have some potential effects on anticoagulation therapy; however, the functional role of the rLj-TCTP in modulating immunity between *L. japonica* and host has not yet been elucidated.

2. Materials and methods

2.1. Materials

L. japonica was obtained from the Tongjiang Valley of Songhua River, Heilongjiang Province, China in December. Our lab had previously constructed the cDNA library and EST of buccal gland.

2.2. EST analysis

According to the information from the cDNA library and the primary analysis of expressed sequence tags [19], we found significant homology of the translationally controlled tumor protein (TCTP) by using NCBI's sequence similarity search tool.

2.3. Protein sequence analysis

Other homologous protein sequences of TCTP were obtained from the UniProt database. Nearly 31 different representative species were aligned with ClustalX software (version 1.81). We converted the result of multiple alignments into mega format and imported them to MEGA (version 3.1) to construct a phylogenetic tree (Neighbor-Joining tree).

2.4. RT-PCR

Total RNA of the buccal gland was extracted by Trizol (GIBCO BRL). RT-PCR amplification was carried out with an RT-PCR kit (TaKaRa) in order to obtain the open reading frame encoding the full length TCTP gene, with two primers as follows: P1: 5′-XXcatatgatcatctacaaggacatcctc-3′, P2: 5′-XXaagcttgcatttctcaatttccaggcc-3′. TaKaRa sequenced the nucleotide sequence of the target gene.

2.5. Expression vector construction

The amplified fragments, comprising an NdeI restriction site and a HindIII restriction site flanking the cDNA encoding the full length TCTP gene, were cut with NdeI and HindIII, and cloned into the corresponding sites of the pET23b vector to obtain recombinant plasmids using a DNA Ligation kit (TaKaRa). pET23b which is a bacterial expression vector with a 6 histidine tag is shown to express the fusion protein.

2.6. Expression and purification of recombinant protein

The recombinant plasmids were transformed into *E. coli* BL21 (DE3) [20] and were grown overnight in LB medium. When the OD_{600} of the medium reached 0.6 A, 0.5 mM isopropyl-1-thio-β-D-galactopyranoside was added to the cultures to induce the expression of the fusion protein. Subsequently, cells were centrifuged at 7000 rpm for 15 min at 4 °C, and the pellet was re-suspended in 5 mM imidazole, 500 mM NaCl, 20 mM Tris–HCl (pH 7.9), and then sonicated for 15 min in ice. It was further centrifuged again at 14,000 rpm for 20 min at 4 °C. The supernatants were collected and the histidine-tagged fusion protein was purified by His Bind affinity chromatography (Novagen) according to the procedure provided by the manufacturer. The recombinant protein was identified in 10% SDS-PAGE gels. The concentration of the recombinant protein was estimated using a BCA Protein Assay kit (BEYOTIME).

2.7. Production of polyclonal antibody to recombinant protein

Polyclonal antibody against purified recombinant *L. japonica* TCTP was generated in New Zealand White Rabbits. The purified recombinant protein was used as antigen. In the first immunization, equal volume of complete Freunds adjuvant was mixed with antigen solution to enhance the antigenicity. For 3 subsequent strengthening immunizations at 2-week intervals, equal volume of incomplete Freunds adjuvant was mixed with antigen solution. Then the serum of the rabbits was isolated, and stored at −20 °C. Titer of the serum was determined by ELISA.

2.8. Western blot analysis

Both the buccal gland secretion (1:10 and 1:20 dilution with PBS) and the recombinant protein were resolved on 10%

SDS-PAGE, then transferred onto nitrocellulose membrane and incubated with rabbit anti-*L. japonica* TCTP (1:500 dilution) overnight at 4 °C. After washing the membrane three times with TBST, HRP, conjugated goat anti-rabbit IgG, was added at 1:5000 dilution and color was developed using DAB substrate.

2.9. Histamine release assay

Histamine-releasing factor activity of recombinant protein was evaluated using a rat basophilic leukemia cell line, RBL-2H3 [23], which was purchased from Cell Bank, Chinese Academy of Sciences. Initially, RBL-2H3 was cultured in EMEM medium (GIBCO BRL) and incubated in a 96-well plate. When the cells became confluent, recombinant protein at a concentration of 0.3, 4.8 and 10 μg/mL was added to the wells. Following incubation at 37 °C for 30 min, supernatants were collected and stored at −20 °C until use. The well without recombinant protein was used as a negative control. Concentration of histamine in culture supernatants was determined using an enzyme immunoassay kit purchased from r-biopharm.

2.10. Statistical analysis

Means and standard deviations were analyzed with the *T* test. Differences were considered significant if *P* values were <0.01.

3. Results

3.1. Sequence analysis

Our lab previously prepared the cDNA library and EST from the buccal gland of *L. japonica*. Sequence analysis of TCTP homologue using a BLAST network service at NCBI shows that TCTP homologue cDNA is 519 bp in length which ends at the termination codon TAA. It is composed of 172 amino acids with a predicted molecular mass of 22.4 kDa. In total, there are five potential phosphorylation sites in *L. japonica* TCTP, four for casein kinase II and one for protein kinase C. It also has three *N*-myristoylation and one *N*-glycosylation sites (data not shown). Sequence alignment of TCTPs with 30 other different species reveals a high degree of conservation over a long period of evolution. Significantly, twelve of the approximately 190 residues are conserved (Fig. 1A). The TCTP family has two signature regions of high sequence homology termed TCTP-1 ((IFAE)-(GA)-(GAS)-N-(PAK)-S-(GTA)-E-(GDEV)-(PAGEQV)-(DEQGAV)) and TCTP-2 ((FLIV)-x(4)-(FLVH)-(FY)-(MIVC T)-G-E-x(4,7)-(DENP)-(GAST)-x-(LIVM)-(GAVI)-x(3)-(FYWQ)). Consistent with the characteristic feature of TCTPs, *L. japonica* TCTP also has clearly defined TCTP-1 and TCTP-2 signatures, which correspond to the amino acid position from 48 to 58 and 129 to 151, respectively (Fig. 1A).

The sequence from 79 to 123 is a basic amino acid rich domain. This domain's sequence alignment shows similarity to part of the tubulin-binding domain of the microtubule-associated protein MAP-1B (Fig. 1B). The amino acid residues 81−112 which are 56% identical to rat TCTP Ca^{2+} binding domain (Fig. 1B), suggest that *L. japonica* TCTP has both microtubule binding (MTB) and calcium-binding (CaB) sites as well.

Sequence analysis of *L. japonica* TCTP to other proteins in the database reveals close matches with highly conserved amino acid residues commonly shared by other known TCTPs (Table 1). The *L. japonica*-TCTP amino acid sequence shows 69% identification with Rohu and more than 50% identification with Zebra-fish, Human, Bovine, Chicken, Rabbit, Mouse, Rat, Western clawed frog, Amphioxus, Silk moth African malaria mosquito and Yellow fever mosquito. It is about 45%, 43%, 42%, and 38% identical to the sequence of parasites like Deer tick, *Clonorchis sinensis*, and Blood fluke, *Brugia malayi*, respectively. And is about 33% identical to *Plasmodium falciparum* (data not shown).

TCTP is highly conserved in evolution. Phylogeny of 31 TCTP sequences show that clustering of TCTPs is identical with eukaryotic classification (Fig. 2). *L. japonica* TCTP clusters with teleost fish, and both of them cluster with amphibian, mammal and bird. TCTP of lamprey is closer to jawed vertebrates than to Amphioxus and is a sister group of gnathostomes. The phylogenetic tree supports that the recombinant protein cloned from the buccal gland of *L. japonica* is TCTP in another aspect.

3.2. Identification of L. japonica-TCTP gene and expression of recombinant protein

Total RNA was extracted from buccal glands of *L. japonica* and used RT-PCR to generate a 519 bp cDNA. The amplified fragments were then restricted with NdeI and HindIII and cloned into the corresponding sites of the pET23b vector, and transformed into *E. coli* DH5α. We then extracted the recombinant plasmids, screened the positive clones and identified further with NdeI and HindIII double digestion.

L. japonica-TCTP gene cloned in pET23b was expressed as histidine-tagged fusion protein. The recombinant protein has a molecular mass of approximately 23.3 kDa with the histidine tag and was purified using His Bind affinity chromatography (Fig. 3A). Protein concentration was determined using a BCA Protein Assay kit.

3.3. ELISA and Western blot analysis

The polyclonal antibody was obtained by antigen immunization of animals. ELISA showed that the titer of the antibody against recombinant protein was about 1:6400. The specificity of the antibody was determined by Western blotting. We observed that the polyclonal antibody could recognize the recombinant protein and natural protein in buccal gland secretion (Fig. 3B). This indicates that the TCTP from the buccal gland of *L. japonica* is a secretion protein.

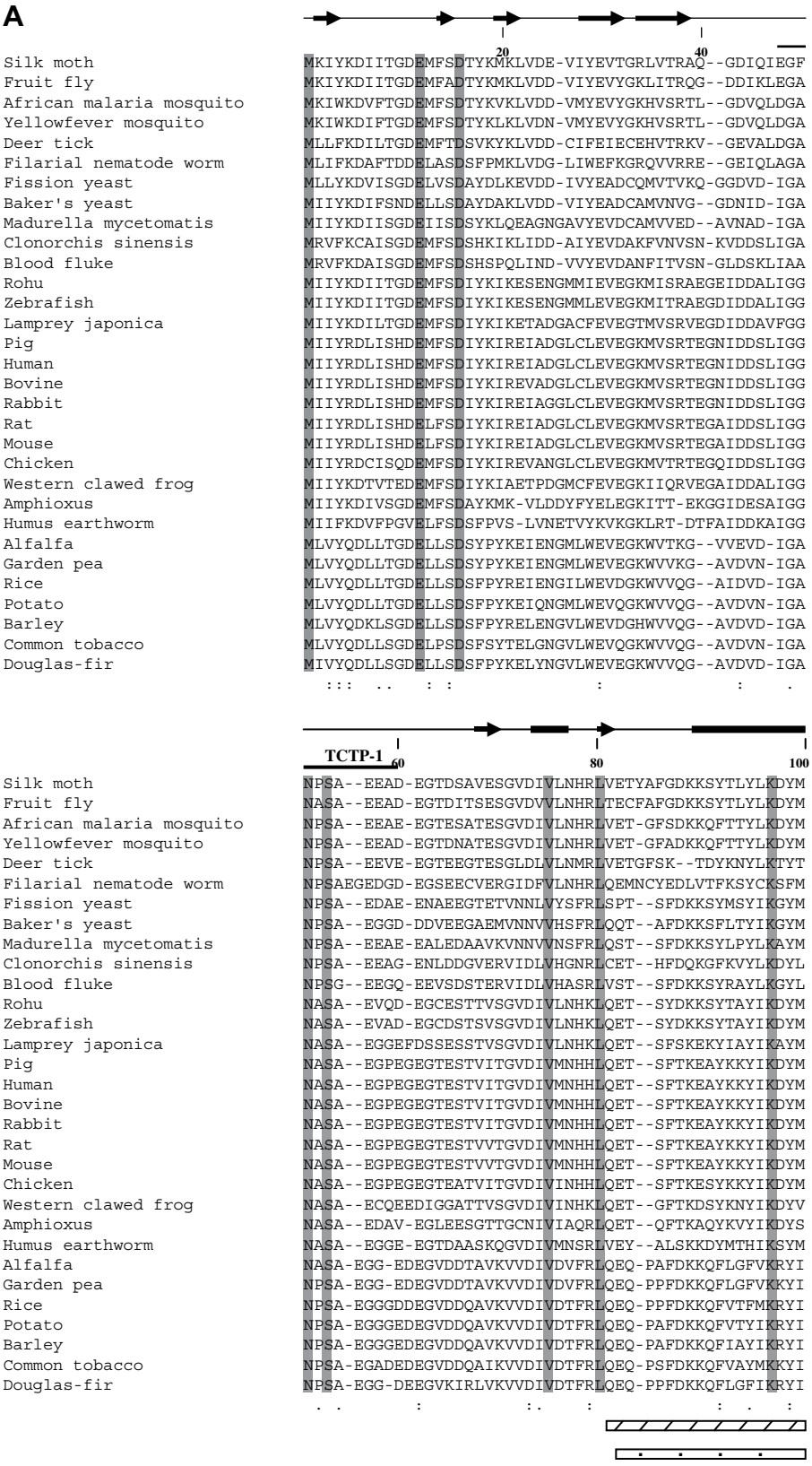

Fig. 1. Sequence analysis of the *L. japonica* TCTP. A. Sequence alignment of *L. japonica* TCTP with other TCTP families using ClustalX. Invariant residues are covered with vertical bars. Dots and colons indicate conservatively mutated positions, and there is a higher level of conservation for the latter. The TCTP-1 and TCTP-2 signature regions are marked on the solid bars. Rectangles and arrows represent secondary structure elements of *S. pombe* such as β-sheets and α-helix, respectively. Microtubule binding (MTB) and calcium-binding (CaB) regions are indicated by a bar filled with biases and dots, respectively. B. Alignment of microtubule binding (MTB) and calcium-binding (CaB) region. The histogram represents the distribution of basic and acidic amino acids of *L. japonica* TCTP. Asterisks indicate identical residues. MAP-1B is microtubule-associated protein.

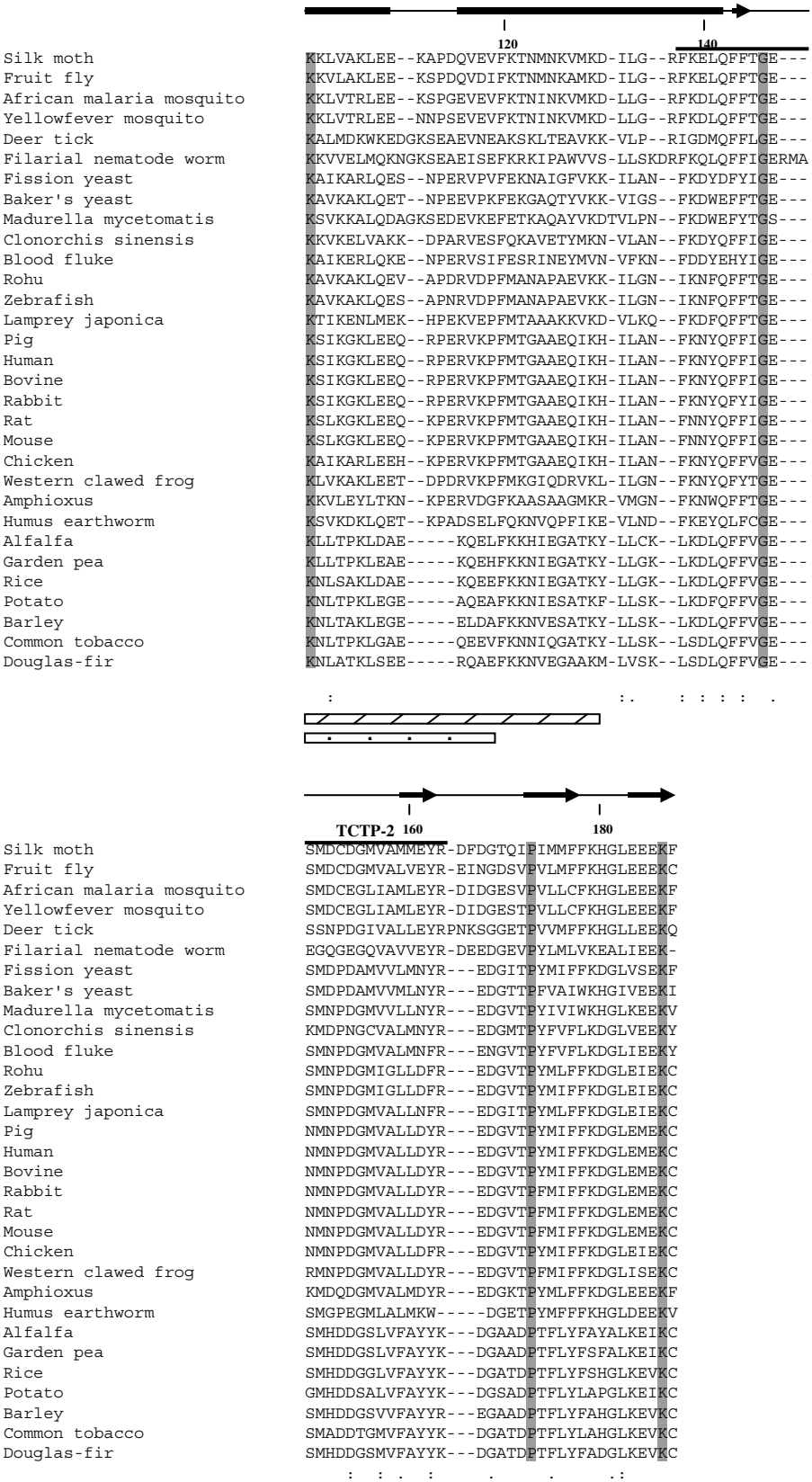

Fig. 1. (*continued*)

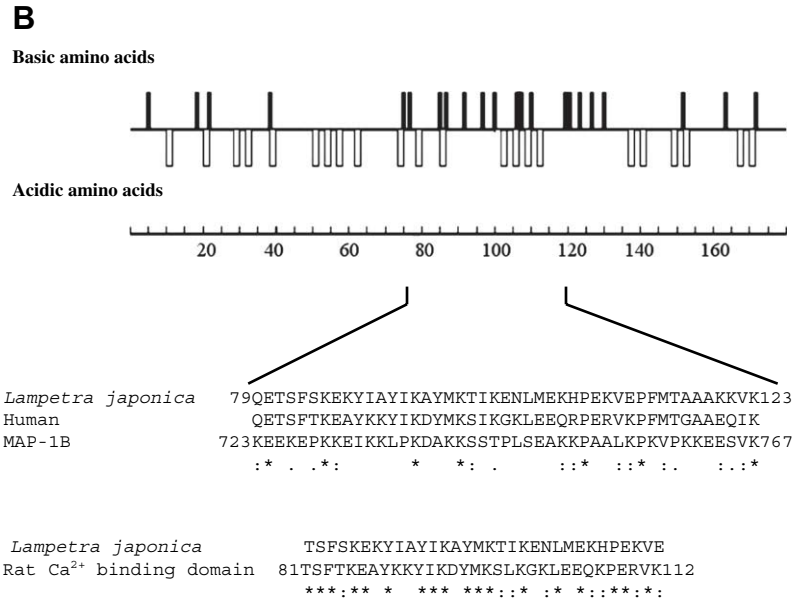

Fig. 1. (*continued*)

Table 1
The TCTP sequence identity of *Lampetra japonica* compared with other 30 species

Species	Common name	Uniport no	Sequence identity (%)
Labeo rohita	Rohu	Q98SJ7	69
Danio rerio	Zebra-fish	Q9DGK4	68
Homo sapiens	Human	P13693	67
Bos taurus	Bovine	Q5E984	67
Suc scrofa	Pig	P61288	67
Gallus gallus	Chicken	P43347	66
Oryctolagus cuniculus	Rabbit	P43348	65
Mus musculus	Mouse	P63028	65
Rattus norvegicus	Rat	P63029	65
Xenopus tropicalis	Western clawed frog	Q66JC5	64
Branchiostoma belcheri	Amphioxus	Q95VY2	53
Bombyx mori	Silk moth	Q75VN3	52
Anopheles agmbiae	African malaria mosquito	Q7QCK2	52
Aedes aegypti	Yellowfever mosquito	Q1HR79	50
Madurella mycetomatics		Q2VET3	49
Schizosaccharomyces pombe	Fission yeast	Q10344	48
Drosophila melanogaster	Fruit fly	Q9VGS2	48
Saccharomyces cerevisiae	Baker's yeast	P35691	46
Ixodes scapularis	Deer tick	Q4PLZ3	45
Lumbricus rubellus	Humus earthworm	O18477	44
Clonorchis sinensis		A1KZ95	43
Schistosoma japonicum	Blood fluke	P91800	42
Hordeum vulgare	Barley	Q9M5G3	39
Brugia malayi	Filarial nematode worm	P90697	38
Medicago sativa	Alfalfa	P28014	38
Pisum sativum	Garden pea	P50906	38
Oryza sativa subsp. japonica	Rice	P35681	37
Solanum tuberosum	Potato	P43349	37
Pseudotsuga menziesii	Douglas-fir	Q9ZRX0	36
Nicotiana tabacum	Common tobacco	Q9XHL7	35

3.4. Recombinant protein-induced histamine release from RBL-2H3 cell line

HRF belongs to a class of proteins called translationally controlled tumor protein homologies. We determined whether the recombinant protein had any direct histamine-releasing effect on rat basophilic leukemia (RBL-2H3) cells. In the present study, the recombinant protein, in various concentrations, was incubated with RBL-2H3 and histamine in the culture supernatant was determined using enzyme immunoassay kit. Although the recombinant protein didn't act in dose dependent manner, the result shows that it mediates histamine release from RBL-2H3 cells compared with the control and performs in trace. This release is significantly ($P < 0.01$) higher than spontaneous release (Fig. 4).

4. Discussion

TCTP is highly conserved and widely expressed in all eukaryotic organisms. There are by far two molecular functions of the TCTPs, microtubulebinding (MTB) and calcium-binding (CaB) domains, respectively [8,9]. Furthermore, the MTB domain is longer than the CaB domain. It has been demonstrated that human translationally controlled tumor protein, which is called P23, is a tubulin-binding protein, where the amino acids region from 79 to 123 of P23 is able to bind the acidic protein-tubulin and is a basic region [9]. Based on sequence analysis, we find that the corresponding area of *L. japonica* TCTP is also a basic amino acid rich domain. Sequence alignment shows similarity to part of the tubulin-binding domain of the microtubule-associated protein MAP-1B (Fig. 1B). So, we speculate that *L. japonica* TCTP may have properties of a tubulin-binding protein that participates in the regulation and control of the cell cycle. In addition,

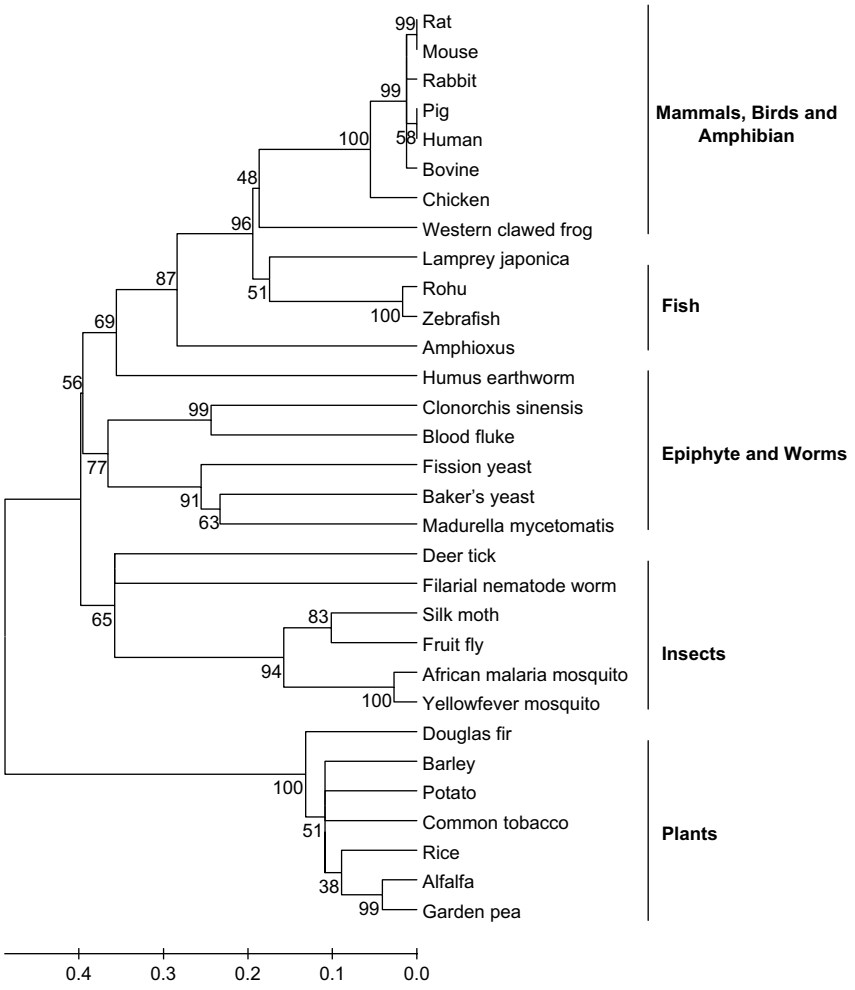

Fig. 2. Phylogenetic tree of TCTPs. Clusters of TCTP sequence are identical with eukaryotic classification. *L. japonica* TCTP clusters with teleost fish, and both of them cluster with amphibian, mammal and bird.

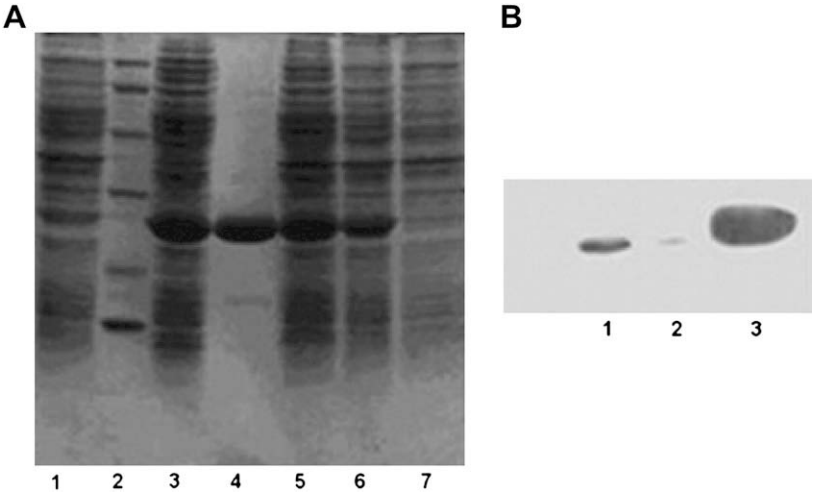

Fig. 3. Expression of recombinant protein and Western blot analysis. A. SDS-PAGE analysis of soluble recombinant protein expression in *E. coli* BL21. 1, induced expression of BL21/pET23b; 2, low molecular weight protein marker. From the top down, molecular weight of each band is 97.2, 66.4, 44.3, 29.0, 20.1 and 14.3 kDa, respectively; 3, 5 and 6, induced expression of BL21/pET23b-TCTP; 4, the purified fusion protein; 7, non-induced expression of BL21/pET23b-TCTP. B. Western blot with serum. 1, the hybridization between buccal gland secretion (1:10 dilution) and polyclonal antibody; 2, the hybridization between buccal gland secretion (1:20 dilution) and polyclonal antibody; 3, the hybridization between recombinant TCTP and polyclonal antibody.

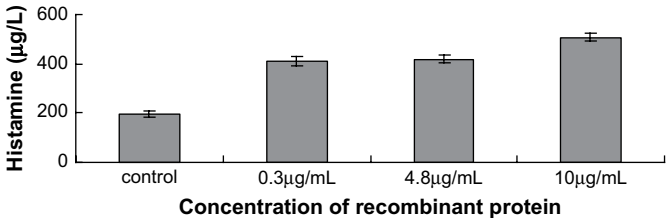

Fig. 4. Recombinant protein-induced histamine release from RBL-2H3. 1×10^5 RBL-2H3 cells were incubated with 0.3, 4.8 and 10 μg/mL rLj-TCTP for 30 min at 37 °C. Concentrations of histamine in culture supernatants were determined using an enzyme immunoassay. The well without recombinant protein was used as a negative control. Values shown are the mean (±SD) of three experiments. All three concentrations of recombinant protein released significantly higher amounts of histamine than negative controls ($P < 0.01$).

intriguingly, the translationally controlled tumor protein is a calcium-binding protein, but it does not present any known calcium-binding motif. Research of rat TCTP shows that TCTP's Ca^{2+} binding domain is confined to amino acid residues 81−112 which is 56% identical to the corresponding area of *L. japonica* TCTP (Fig. 1B). It is likely that *L. japonica* TCTP has calcium-binding activity.

The *L. japonica*-TCTP amino acid sequence shows 45%, 43%, 42%, and 38% identification with the sequence of parasites like Deer tick, *C. sinensis*, and Blood fluke, *B. malayi,* respectively. And is about 33% identical to *P. falciparum*. TCTP is also called HRF. Interestingly, all parasitic versions of TCTP/HRF proteins seem to be secreted into the vertebrate host organism. Studies of tick saliva-secreted functional HRF homologue suggest that it is injected into the host during tick feeding [21]. Furthermore, the malarial TCTP, which can be secreted into the host, is a functional homologue of immune mediator. It can also differentially modulate the secretion of cytokines such as histamine and IL-8 from basophils and eosinophils, respectively [22]. Molecular characterization of TCTP from the filarial parasites *B. malayi* and *Wuchereria bancrofti* shows that when injected intra-peritoneal, both of the filarial TCTPs induce inflammatory infiltration of esinophils, suggesting that the filarial TCTPs may have an effect on allergic inflammatory responses associated with filarial infections [23]. All of these suggest that TCTP participates in a series of immune responses. So, it is possible that *L. japonica* TCTP has some undiscovered functions, such as immune regulation between *L. japonica* and host. Further studies with recombinant products of such activities are needed.

Classical secretion is mediated by an N-terminal signal peptide, which directs the protein through the ER/Golgi-dependent secretory pathway to the extracellular space. In addition to this manner, several intracellular proteins such as FGF-1 and FGA-2 can be exported without a classical N-terminal signal peptide, which is called an alternative pathway or unconventional protein secretion. TCTP is cytoplasmic. It does not have signal peptide. Then, how is it exported out of the cell? Recent studies found TCTP in preparations of small secreted vesicles called exosomes [24]. Exosomes are small membrane vesicles that are secreted by a multitude of cell types as a consequence of fusion of multivesicular late endosomes/lysosomes with the plasma membrane [25]. Over-expression of TSAP6, a p53-inducible 5-6 trans-membrane protein, can enhance exosome production, increase TCTP levels in exosome preparations and facilitate the secretion of TCTP via a non-classical pathway [24,26]. It is thus likely that the recombinant protein rLj-TCTP in this study may be secreted from the buccal gland of *L. japonica* in such a manner. In fact, more and more research shows that exosomes are secreted by some immune cells, such as cytotoxic T cells, ensuring specific targeting of cytolytic substances to target cells [25], B cell and dendritic cell responsible for the persistence of antigen presentation and T cell proliferation *in vitro* [25,27]. Although the biological functions of exosomes remain unclear, it is possible that exosomes can regulate a series of immune responses. Based on this we speculate that the secreted protein, *L. japonica* TCTP, may be concerned with immunology.

L. japonica is one of the ancient cyclostomatous representative animals. Its buccal gland secretion has been known to act in anticoagulation. The recombinant protein has the ability of HRF, and histamine might increase endothelial cell-surface expression of thrombomodulin, a tissue anticoagulant, which can activate protein C. Protein C, is a vitamin K-dependent glycoprotein and plasma serine protease precursor. Trombomodulin converts protein C into activated protein C. Activated protein C exerts anticoagulant activity by inhibiting factors Va and VIIIa [28], inhibiting the combination of Xa and platelets, promoting the lyses of fibrin. All of these pathways may modulate the anticoagulant cascade. In addition, histamine has the ability to exert vasodilator effects and may participate in the immune response of the host. Given the histamine-releasing factors of recombinant protein and functions of histamine discussed above, it is possible that the recombinant protein have some potential effects on anticoagulation therapy.

HRF is a collective term used for a heterogeneous group of factors with different modes of action. The current review is focused on IgE-dependent HRF that requires the presence of certain types of IgE (designated IgE^+) to induce histamine release [29]. Mast cells are an integral component of the innate immune system. De-regulation of mast cells by TCTP can result in the release of histamine. Histamine, involved in many allergic reactions, via specific activation of H2 receptors, may be an important regulator of human NK cell activity [30]. These may indicate a potential role of TCTP in the inflammatory process. In addition to acting as a histamine-releasing factor, TCTP can also differentially modulate the secretion of cytokines from human basophils, eosinophils, T cells [31], mast cells and murine B cells, suggesting that it may induce a complex array of responses at sites of allergic inflammation. TCTP participates in the regulation of immune cells, which suggests that it may play a crucial role in the immune response. As the recombinant protein is a new secretion protein, we speculate that it may have some important effects on completed immune regulation between *L. japonica* and host, but the exact roles are still unclear.

5. Conclusion

We have cloned a homologue of the TCTP from the buccal gland of *L. japonica* according to the information from a cDNA library, and the primary analysis of EST. It has two signature regions of high sequence homology termed TCTP-1 and TCTP-2, respectively. Phylogeny analysis shows that clustering of TCTPs was identical with eukaryotic classification. Immunoblot analysis to localize the recombinant protein in buccal gland secretion proves that rLj-TCTP is a secretion protein. It has a histamine-releasing function *in vitro*. TCTP links both the innate and the adaptive immune responses by modulating the secretion of cytokines from mast cells, basophils, eosinophils and T and B lymphocytes. These may indicate a potential role of TCTP in the inflammatory process and immune regulation between *L. japonica* and host.

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