

## Identification of the Self-Interaction of Rat TCTP/IgE-Dependent Histamine-Releasing Factor Using Yeast Two-Hybrid System

Taesook Yoon,<sup>\*,1</sup> Jaehoon Jung,<sup>†,1</sup> Minjeong Kim,<sup>\*</sup> Kang Man Lee,<sup>\*</sup> Eung Chil Choi,<sup>†</sup> and Kyunglim Lee<sup>\*,2</sup>

<sup>\*</sup>College of Pharmacy, Center for Cell Signaling Research and Division of Molecular Life Sciences, Ewha Woman's University, Seoul 120-750, Korea; and <sup>†</sup>College of Pharmacy, Seoul National University, Seoul 151-742, Korea

Received June 29, 2000, and in revised form August 30, 2000

**To further understand the biological function of translationally controlled tumor protein (TCTP), also known as IgE-dependent histamine-releasing factor (HRF), the yeast two-hybrid system was used to screen interacting molecules. We isolated cDNA clones coding for TCTP/HRF, suggesting that it may have a self-interacting property. Domain mapping of the interaction revealed that the C-terminal region of residue 126–172 is involved in self-interaction. The self-interacting property of TCTP/HRF was further supported by FPLC gel-filtration chromatography and coimmunoprecipitation analysis from transfected COS-7 cells. Our data suggests that TCTP/HRF may have a potential to self-interact through the C-terminal region, and the self-interaction property may be related to its biological function.** © 2000 Academic Press

**Key Words:** coimmunoprecipitation; translationally controlled tumor protein; self-interaction; yeast two-hybrid; FPLC.

Translationally controlled tumor protein (TCTP)/IgE-dependent histamine-releasing factor (HRF) was first identified as a tumor protein in an ascitic tumor of a mouse and in mouse erythroleukemia (1, 2). Subsequently it has been found in all normal cell types examined so far except kidney cells (3). TCTP/HRF homologues among the species are highly conserved (4–7), suggesting that the protein might have an essential function in the cell.

It was reported that TCTP/HRF caused a histamine degranulation from IgE<sup>+</sup> basophils (8) and was capable of various functions including calcium binding (5), metal homeostasis (9), intracellular signaling (10), and reacts with an antimalarial drug (11). However, the molecular mechanism for these activities has not yet been demonstrated.

In the present study, we employed the yeast two-hybrid system to understand the molecular mechanism and the physiological roles of TCTP/HRF. In yeast two-hybrid screens with full-length TCTP/HRF as a bait, we obtained N-terminus truncated TCTP/HRF cDNA from rat skeletal muscle cDNA library, suggesting that TCTP/HRFs are their own binding partners. We demonstrated that the C-terminus region of residue 126–172 is involved in the self-interaction. The self-interacting property of TCTP/HRF was further supported by FPLC gel-filtration chromatography and coimmunoprecipitation analysis from transfected COS-7 cells.

### MATERIALS AND METHODS

**Yeast two-hybrid screening.** The full-length cDNA of rat TCTP/HRF was cloned into a LexA-fusion plasmid, pEG202 vector, at the *EcoRI*–*XhoI* site to create pEG202-TCTP/HRF. The yeast strain EGY48/pSH18-34/pEG202-TCTP/HRF was transformed with the rat skeletal muscle cDNA library. The transformed yeast cells were recovered from the selective medium and analyzed by the  $\beta$ -galactosidase assay (12). Potential positive clones activating the reporter genes were analyzed by restriction mapping and sequencing.

**Deletion analysis of rat TCTP/HRF.** The various derivatives of clone 734 encoding for rat TCTP/HRF were fused in-frame to the pJG4-5 library vector to generate deletion constructs (Fig. 1B); clone 734A containing TCTP homology region 1 (TCTP1) and PKC phosphorylation site, clone 734B containing TCTP homology region 2 (TCTP2) and PKC phosphorylation site, clone 734C containing only PKC phosphorylation site, and clone 734D containing only TCTP homology region 2 (TCTP2). The deletion constructs were used to transform yeast EGY48/pSH18-34/pEG202-TCTP/HRF and tested

<sup>1</sup> These authors contributed equally to this work.

<sup>2</sup> To whom correspondence and reprint requests should be addressed. Fax: 82-2-3277-2851. E-mail: klyoon@mm.ewha.ac.kr.

on the selective media plate. The same bait strain plus the library vector was used as a negative control. The cofilin-transformed EGY48/pSH18-34/pEG202-CD3 harboring the cytoplasmic domain of (Na,K)ATPase as a bait was used as a positive control (our unpublished results). The protein extracts from the transformed yeast cells were prepared and used for immunoblotting as described previously (13).

**FPLC gel-filtration chromatography and nondenaturing/denaturing gel electrophoresis.** The full-length TCTP/HRF was inserted at *Bam*HI-*Xho*I site of the bacterial expression vector pRSET (Invitrogen, San Diego, CA) that contains a T7 promoter and metal binding domain (His-tag) at the N-terminus of restriction site. *Escherichia coli* BL21(DE3)pLysS cells transformed with pRSET-TCTP were grown until *A*<sub>600</sub> was 0.6, induced with 1 mM IPTG (isopropylthio-β-D-galactoside) and then purified with affinity chromatography. The purified recombinant TCTP/HRF was then applied to an FPLC superdex 200 HR 10/30 column (Pharmacia Biotech) equilibrated with the 0.05 M Na-phosphate buffer, pH 7.0, containing 0.15 M NaCl. Fractions were collected, concentrated, and examined by nondenaturing and denaturing gel electrophoresis. Western blotting was also performed using TCTP/HRF rabbit polyclonal antibody.

**Coimmunoprecipitations.** COS-7 cells were transiently transfected with both the N-terminus hemagglutinin (HA)-tagged TCTP/HRF construct in pcDNAneo (Invitrogen, San Diego) and the C-terminus GFP-tagged TCTP/HRF construct (Clontech, Palo Alto) using the LIPOFECTAMINE PLUS Reagent (GIBCOBRL, Gaithersburg). After approximately 24–48 h, cells were lysed with ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protein inhibitor cocktail. After centrifugation for 10 min at 12,000*g* and 4°C, 12CA5 monoclonal antibody recognizing HA epitope was added to the supernatants. After incubation overnight at 4°C on a rocking platform, protein A agarose was then added and the suspensions were incubated for 2 h at 4°C. After washing the immune complexes three times with the ice-cold PBS, the pellets were resuspended in Laemmli sample loading buffer and subjected to a SDS-PAGE. In order to detect the target protein that should have come with the precipitated protein, Living colors peptide antibody that recognizes the GFP protein was used.

RESULTS AND DISCUSSION

Screening for Rat TCTP/HRF Binding Proteins in Yeast

To identify proteins that might interact with rat TCTP/HRF, we screened a rat skeletal muscle cDNA library using the yeast two-hybrid system. The characterization of bait protein prior to library screening demonstrated that it was correctly expressed as a 48-kDa protein, as determined by Western blotting with a LexA polyclonal antibody (data not shown). One (clone 734) of several positive clones was analyzed by DNA sequencing and had DNA sequences that correspond to the gene for TCTP/HRF protein according to a computer search of the database using BLAST (14). The insert began at residue 22 of rat TCTP/HRF and continued throughout the entire open reading frame until residue 172 (Fig. 1A). The one lettered amino acid sequences were analyzed by the computer analysis software, ANTHEPROT, and its secondary structure by GOR II method (Fig. 1).

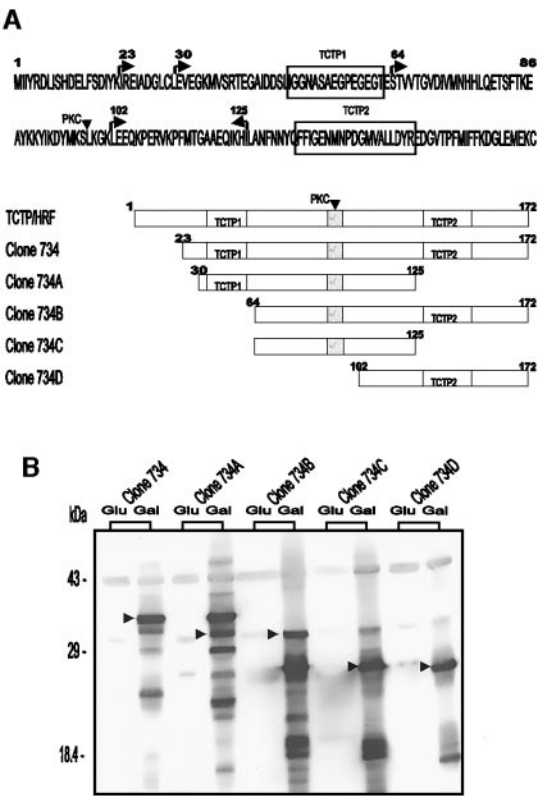


**FIG. 1.** Schematic representation of clone 734 and its deleted constructs and the expression of the constructs. (A) The clone 734 was divided into clone 734A containing TCTP homology region 1 (TCTP1) and PKC binding site, clone 734B containing TCTP homology region 2 (TCTP2) and PKC binding site, clone 734C containing only PKC binding site, and clone 734D containing only TCTP homology region 2 (TCTP2). (B) Expression of clone 734 and its deleted constructs were confirmed with 12CA5 monoclonal antibody on 12% SDS-PAGE.

Deletion Analysis of Clone 734 of Rat TCTP/HRF

The results obtained from the yeast screening suggest that the TCTP/HRF may have a potential to self-interact in cells to play a biological function. To define the binding region of the potential self-interaction, we constructed the deletion derivatives of clone 734. Clone 734 and its deletion constructs 734A, 734B, 734C, and 734D encode residue 22–172, 30–125, 64–172, 64–125, and 102–172 of the open reading frame, respectively (Fig. 1A). The protein expression of these deletion constructs was confirmed by immunoblots of yeast transformed with each construct. The 12CA5 monoclonal antibody detected 41.5-, 30.5-, 32-, 26.8-, and 27.8-kDa polypeptides in clone 734, 734A, 734B, 734C, and 734D expressing cells in galactose media, respectively, but not in glucose media (Fig. 1B). Since the expression of the pJG4-5 vector was controlled by the Gal1 promoter, the detection of protein with the 12CA5 antibody was galactose-dependent as shown in Fig. 1B. The 12CA5 antibody also detected other galactose-dependently ex-

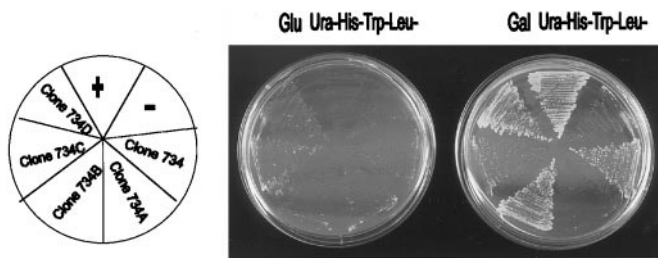


**FIG. 2.** Interaction of the deleted polypeptides with the bait pEG202-TCTP/HRF. Individual colonies of EGY48/pSH18-34 yeast cells, which were transformed with pEG202-TCTP/HRF and pJG4-5/clone 734 derivatives, were streaked onto a glucose Ura<sup>-</sup>His<sup>-</sup>Trp<sup>-</sup>Leu<sup>-</sup> or galactose Ura<sup>-</sup>His<sup>-</sup>Trp<sup>-</sup>Leu<sup>-</sup> plate. Strong growth with clone 734, 734B, and 734D, no growth with clone 734A and 734C were observed.

pressed proteins in the yeast extract (Fig. 1B). We do not know the exact origin of them but this might be due to the protein degradation.

We introduced clone 734 and its deleted derivatives into yeast cells harboring reporter genes. Figure 2 shows that colonies of clone 734A and 734C on the galactose Ura<sup>-</sup>His<sup>-</sup>Trp<sup>-</sup>Leu<sup>-</sup> plate were greatly diminished in number in contrast to those of clone 734, 734B, and 734D. We also measured the activity of the LacZ-reporter gene in the cells grown in glucose or galactose media by liquid culture  $\beta$ -galactosidase assay. Clone 734A and 734C resulted in nearly complete loss of activity in the  $\beta$ -galactosidase assay and clone 734B and 734D showed about half of an activity of clone 734 (Table I). Therefore, it appears that the C-terminal domain of residue 126–172 plays a prominent role in self-interaction, although we do not know whether the interaction was head to tail or tail to tail.

#### Characterization of Recombinant of TCTP/HRF Protein

In order to characterize the recombinant TCTP/HRF, gel-filtration FPLC, and nondenaturing gel

**TABLE I**

| Construct                  | Selective media                                               |                                                                 |
|----------------------------|---------------------------------------------------------------|-----------------------------------------------------------------|
|                            | Glucose<br>Ura <sup>-</sup> His <sup>-</sup> Trp <sup>-</sup> | Galactose<br>Ura <sup>-</sup> His <sup>-</sup> Trp <sup>-</sup> |
| Positive control           | 2                                                             | 3665                                                            |
| Negative control           | 1                                                             | 2                                                               |
| pEG202-TCTP/HRF/clone 734  | 2                                                             | 691                                                             |
| pEG202-TCTP/HRF/clone 734A | 4                                                             | 75                                                              |
| pEG202-TCTP/HRF/clone 734B | 4                                                             | 398                                                             |
| pEG202-TCTP/HRF/clone 734C | 6                                                             | 83                                                              |
| pEG202-TCTP/HRF/clone 734D | 4                                                             | 456                                                             |

*Note.*  $\beta$ -gal unit = O.D.<sub>420</sub>  $\times$  1000/*t* (min)  $\times$  vol. of extract (ml)  $\times$  protein ( $\mu$ g/ml).

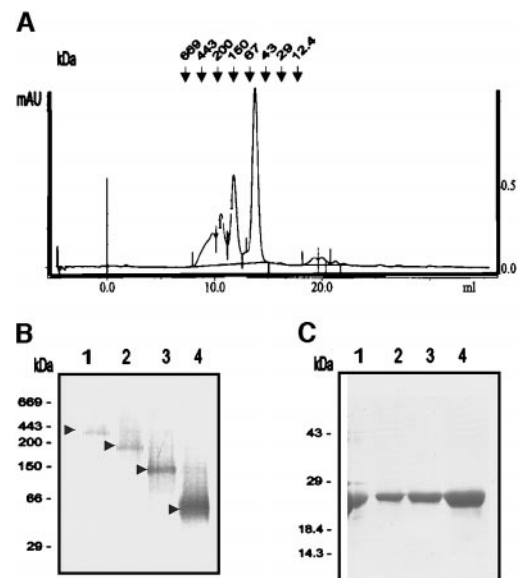


**FIG. 3.** Characterization of recombinant rat TCTP/HRF protein. (A) Gel-filtration FPLC of TCTP/HRF. X axis is retentive volume and Y axis is OD value at 280 nm. (B) The eluates of TCTP/HRF from FPLC column were confirmed by nondenaturing gel electrophoresis. Lane 1 is a eluate peak of which retentive volume is 9.8 after FPLC column passage, lane 2 is a eluate peak of 10.61, lane 3 is a eluate peak of 11.8, lane 4 is a eluate peak of 13.8. (C) The eluates of TCTP/HRF were also confirmed by denaturing gel electrophoresis as described above.

electrophoresis methods were used. The FPLC eluates of the recombinant TCTP/HRF revealed four peaks corresponding to a mass of approximately 292.8-, 214.1-, 135.2-, and 62.4-kDa (Fig. 3A). Four bands can also be discerned as a mass of approximately 307-, 225.8-, 122.2-, and 48.6-kDa when subjected to nondenaturing gel electrophoresis (Fig. 3B). However, an equal loading of the protein on SDS gels gave a single band when visualized by Coomassie staining (Fig. 3C) and was confirmed by Western blotting with TCTP/HRF polyclonal antibody (data not shown). Assignment of the degree of oligomerization to bands is not straightforward because the mobility of a given band depends on molecular charge as well as size and shape. However, gel filtration and electrophoresis data show that TCTP/HRF is capable of self-assembly to form stable and reproducible oligomeric structures in addition to dimer. We confirmed that 7.8-kDa protein expressed from pRSET vector did not self-interact (data not shown), thus excluding the possibility that the oligomerization seen might be due to the presence of the His-tag on the TCTP/HRF protein.

To demonstrate that TCTP/HRF can self-interact *in vivo*, we cotransfected COS-7 cells with HA-tagged TCTP/HRF and GFP-tagged TCTP/HRF. We then immunoprecipitated extracts from the transfected

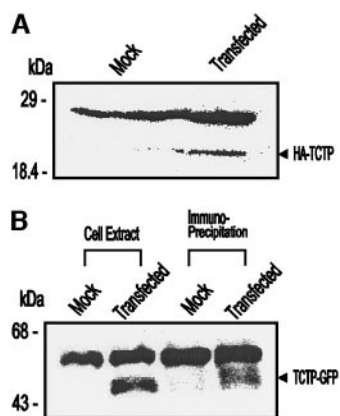


**FIG. 4.** Coimmunoprecipitation of TCTP/HRF fusion proteins. (A) HA-tagged TCTP/HRF expression (22.3 kDa) in COS-7 cells was confirmed with 12CA5 monoclonal antibody on 15% SDS-PAGE. (B) Extracts were prepared from COS-7 cells transfected with both the HA-tagged TCTP/HRF and the GFP-tagged TCTP/HRF constructs. The extracts were subjected to immunoprecipitation with 12CA5 monoclonal antibody and detected by hybridization with anti-GFP polyclonal antibody.

COS-7 cells with anti-HA monoclonal antibody and analyzed the immunoprecipitates by immunoblotting with the GFP antibody. The HA-tagged and the GFP-tagged TCTP/HRF protein were correctly expressed as a 22.3 kDa (Fig. 4A) and a 48-kDa protein (Fig. 4B, Cell Extract) as determined by Western blotting with a 12CA5 monoclonal antibody and a GFP antibody, respectively. As shown in Fig. 4B, GFP-tagged TCTP/HRF was coimmunoprecipitated with HA-tagged TCTP/HRF, suggesting that they do self-interact in cell extracts.

In many functional molecules, they exist in dimer or oligomer for the benefits of their activity and stability (15–19). These dimeric or oligomeric forms are involved in various functions such as modulation of smooth muscle myosin light chain kinase activity (18), chaperone-like activity of alpha A and alpha B crystallins (20), and the hormone-sensitive lipase function (21). Dimerization is also an important process for hormones and growth factors to bind to their receptors on the cell surface and exert their activity (22–24). In the present study, we demonstrated that TCTP/HRF form dimer/oligomers *in vivo* and *in vitro*. Although the functional significance of TCTP/HRF is currently unknown, our data suggests that TCTP/HRF has a potential to self-interact and that the self-interaction of TCTP/HRF is likely to be required to exert the biological function of the protein.

## ACKNOWLEDGMENTS

We thank Dr. Jongsun Kim for previewing the manuscript and Dr. Man Wook Hur for providing us TCTP/HRF polyclonal antibody. This work was supported by KOSEF through CCSR at Ewha Woman's University, KISTEP research fund for Women's University, and the Korean Ministry of Education. T. Yoon was supported by the Brain Korea 21 Project fellowship.

## REFERENCES

- Yenofsky, R., Cereghini, S., Krowczynska, A., and Brawerman, G. (1983) *Mol. Cell. Biol.* **3**, 1197–1203.
- Chipatima, S. T., Makrides, S., Bandyopadhyay, R., and Brawerman, G. (1988) *Nucleic Acids Res.* **16**, 2350.
- Sanchez, J. C., Schaller, D., Ravier, F., Golaz, O., Jaccoud, S., Belet, M., Wilkins, M. R., James, R., Deshusses, J., and Hochstrasser, D. (1997) *Electrophoresis* **18**, 150–155.
- Bini, L., Heid, H., Liberatori, S., Geier, G., Pallini, V., and Zwilling, R. (1997) *Electrophoresis* **18**, 557–562.
- Haghighat, N. G., and Ruben, L. (1992) *Mol. Biochem. Parasitol.* **51**, 99–110.
- Rasmussen, S. W. (1994) *Yeast* **10** (Suppl. A), S63–S68.
- Pay, A., Heberle-Bors, E., and Hirt, H. (1992) *Plant Mol. Biol.* **19**, 501–503.
- MacDonald, S. M., Rafnar, T., Langson, J., and Lichtenstein, L. M. (1995) *Science* **269**, 688–690.
- Sturzenbaum, S. R., Kille, A., and Morgan, A. J. (1998) *Biochim. Biophys. Acta* **1398**, 294–304.
- Nielsen, H. V., Johnsen, A. H., Sanchez, J. C., Hochstrasser, D. F., and Schiøtz, P. O. (1998) *Allergy* **53**, 642–645.
- Bhisutthibhan, J., Pan, X. Q., Hossler, P. A., Walker, D. J., Yowell, C. A., Carlton, J., Dame, J. B., and Meshnick, S. R. (1998) *J. Biol. Chem.* **273**, 16192–16198.
- Gyrus, J., Golemis, E., Chertkov, H., and Brent, R. (1993) *Cell* **75**, 791–803.
- Yoon, T., and Lee, K. (1998) *Mol. Cells* **8**, 606–613.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215**, 403–410.
- Lin, W. H., Hung, C. H., Hsu, C. I., and Lin, J. Y. (1997) *J. Biol. Chem.* **272**, 20044–20048.
- Hanada, M., Aime-Sempe, C., Sato, T., and Reed, J. C. (1995) *J. Biol. Chem.* **270**, 11962–11969.
- Propper, C., Huang, X., Roth, J., Sorg, C., and Nacken, W. (1999) *J. Biol. Chem.* **274**, 183–189.
- Babiyuchuk, E. B., Babiyuchuk, V. S., and Sobieszek, A. (1995) *Biochemistry* **34**, 6366–6372.
- Nadeau, O. W., Traxler, K. W., Fee, L. R., Baldwin, B. A., and Carlson, G. M. (1999) *Biochemistry* **38**, 2551–2559.
- Kumar, L. V., and Rao, C. M. (2000) *J. Biol. Chem.* **275**, 22009–22013.
- Shen, W. J., Patel, S., Hong, R., and Kraemer, F. B. (2000) *Biochemistry* **39**, 2392–2398.
- Tsuda, T., Ikeda, Y., and Taniguchi, N. (2000) *J. Biol. Chem.* **275**, 21988–21994.
- Overton, M. C., and Blumer, K. J. (2000) *Curr. Biol.* **10**, 341–344.
- Heldin, C. H. (1995) *Cell* **80**, 213–223.