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Translationally Controlled Tumor Protein Interacts with the Third Cytoplasmic Domain of Na,K-ATPase α Subunit and Inhibits the Pump Activity in HeLa Cells*

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Translationally controlled tumor protein (TCTP) is a growth-related protein under transcriptional as well as translational control. We screened a rat skeletal muscle cDNA library using yeast two-hybrid system and found that TCTP interacts with the third large cytoplasmic domain of α1 as well as α2 isoforms of Na,K-ATPase, believed involved in the regulation of Na,K-ATPase activity. Interaction between TCTP and Na,K-ATPase was confirmed by coimmunoprecipitation in yeast and mammalian cells. We also showed, using $^{86}Rb^{+}$ uptake assay, that overexpression of TCTP inhibited Na,K-ATPase activity in HeLa cells. Northern and Western blotting studies of HeLa cells transiently transfected with GFP-tagged TCTP showed that overexpression of TCTP did not change mRNA and protein levels of Na,K-ATPase. Recombinant TCTP protein purified from an *Escherichia coli* expression system inhibited purified HeLa cell plasma membrane Na,K-ATPase in a dose-dependent manner. Using deletion analysis, we also found that the C-terminal 102–172-amino-acid region of rat TCTP that contains the TCTP homology region 2 is essential for its association with, and inhibition of, Na,K-ATPase.

Na,K-ATPase, a multimembrane-spanning enzyme, is essential for maintaining transmembrane gradients of Na^{+} and K^{+} ions and thus for cell homeostasis (1). These ionic gradients serve to control essential cellular processes such as cell volume, membrane potential, and nutrient transport (2). In addition, Na,K-ATPase is involved in cell proliferation and differentiation, heart and vascular muscle contraction, and neurotransmitter and hormone secretion (3). Thus dysfunction of this enzyme can profoundly affect cell function.

Na,K-ATPase is composed of a catalytic 110-kDa α subunit and a glycosylated 40–60-kDa β subunit. The α subunit contains binding sites for cations, ATP, and cardiac glycosides. It has been suggested that there might exist a diffusible cytoplasmic regulator of Na,K-ATPase activity, possibly modulated by protein kinases and hormones (4, 5). The third large cytoplasmic domain (CD3)[1] of Na,K-ATPase was proposed to be one of the domains involved in the regulation of its activity by insulin, thereby playing an important role in the catalytic function and regulation of this enzyme (6). Interactions between the N-terminal region of the Na,K-ATPase α subunit with phosphoinositide-3 kinase (7), cytoplasmic domain 2 (CD2) and CD3 with ankyrin (8, 9), CD3 with cofilin (10), and purified Na,K-ATPase with actin (11) and adducin (12) have also been demonstrated.

We looked for other cytoplasmic agents that might interact with the CD3 of Na,K-ATPase α subunit and regulate its activity and found that translationally controlled tumor protein (TCTP) acts as a cytoplasmic repressor of Na,K-ATPase. TCTP is a growth-related protein, under tight transcriptional as well as translational control (13, 14). It occurs as a 23-kDa protein in humans and has a 21-kDa homologue in mice but shows no significant homology with any other family of proteins. Based on structural studies of TCTP from *Schizosaccharomyces pombe*, it has been recently proposed that TCTP belongs to the MSS4/DSS4 superfamily of proteins, which bind to the GDP/GTP-free form of Rab proteins, described as guanine nucleotide-free chaperones (15). TCTP has no known primary function, but the high degree of homology of TCTP from various sources, including plants and humans, and its expression in many tissues, suggest that it may have a basic housekeeping function (16, 17).

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen Assay—The cDNA library was constructed as described previously (10). cDNAs of rat Na,K-ATPase α1 and α2 subunits were obtained from Dr. Jerry Lingrel (University of Cincinnati College of Medicine). The CD3 region (Lys-352–Val-l756) of the α2 subunit of Na,K-ATPase was fused into the LexA DNA-binding domain and used as a bait to screen the cDNA library from rat skeletal muscle. Potential positive clones activating the reporter genes were analyzed by restriction mapping and sequencing. One of these clones had DNA sequences that correspond to the gene for rat TCTP, based on a nucleotide BLAST search in NCBI homepage.

The sequences coding for the CD3 region (Lys-354–Val-l759) of the α1 subunit were cloned into the pEG202 vector of the LexA fusion plasmid. The PCR fragments of the following were amplified and cloned into the pEG202 vector: the N-terminal portions of α1 (Met-1–Cys-93) and α2 (Met-1–Cys-91); α1CD2 (Glu-151–Ile-292) and α2CD2 (Glu-149–Ile-290) encoding the H2–H3 loop; α1CD4 (Glu-825–Arg-848) and α2CD4 (Glu-822–Arg-845) encoding the H6–H7 loop; the C-terminal portions of α1 (Ile-935–Tyr–1023) and α2 (Ile-932–Tyr-1020); and the N-terminal portion of β subunit (Ala-1–Lys-33).

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[1] The abbreviations used are: CD, cytoplasmic domain; GFP, green fluorescence protein; GST, glutathione *S*-transferase; HA, hemagglutinin; TCTP, translationally controlled tumor protein; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Yeast cells, EGY48/pSH18–34, containing *LexAop-LEU2* and *LexAop-LacZ* reporters were transformed simultaneously with both one of the resulting LexA fusions and a rat TCTP clone using the high efficiency transformation method. Protein-protein interaction results in trans-activation of the *LexAop-LEU2* and *LexAop-LacZ* reporter genes (10). Therefore, individual transformants of the yeast cells were tested on glucose $Ura^-His^-Trp^-Leu^-$, galactose $Ura^-His^-Trp^-Leu^-$, glucose $Ura^-His^-Trp^-$ X-Gal and galactose $Ura^-His^-Trp^-$ X-Gal plates. β-galactosidase activity was determined as reported previously (18) using the formula: β-galactosidase unit = $A_{420} \times 1000/t$ (min) × volume of extract (ml) × protein (μg/ml).

Co-immunoprecipitations—Co-immunoprecipitations were performed as described previously (10). Briefly, yeast cells cultured in glucose $Ura^-His^-Trp^-$ and galactose $Ura^-His^-Trp^-$ media were collected at 3,000 × *g* for 5 min and resuspended by vortexing with yeast lysis buffer (YLB) (50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 150 mM NaCl, 50 mM NaF, 2 mM $ZnCl_2$, and protease inhibitor mixture) and glass beads. After adding radioimmune precipitation buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS), the mixtures were centrifuged at 10,000 × *g* for 30 min. Affinity-purified anti-hemagglutinin (HA) 12CA5 antibody was added to the supernatants, and the mixtures were incubated for 3 h at 4 °C. Fifty percent protein A-agarose solution (Roche Applied Science) was then added, and incubation continued overnight at 4 °C. After a 5-s centrifugation at 12,000 × *g*, the pellets were washed twice with the radioimmune precipitation buffer and then with a wash buffer (1 M NaCl, 10 mM Tris-HCl, pH 8.0, and 0.1% Nonidet P-40). The pellets were then resuspended in 2× SDS sample buffer and subjected to SDS-PAGE. The blots obtained were incubated with the anti-LexA antibody.

Immunoprecipitations from extracts of HeLa cells were performed according to Jung *et al.* (19). The deletion mutants, TCTP-(1–101) and TCTP-(102–172), were constructed with Exsite PCR-based site-directed mutagenesis kit (Stratagene). The deletion cDNA constructs were PCR-amplified from GFP-TCTP using the following 5′-phosphorylated primers: for TCTP-(1–101), 5′-GCCCTTGAGTGATTTCATGTAGTCTTT-3′ (antisense) and phospho-5′-CAGTCGACGGTACCGCGGGCCCGG-GAT-3′ (sense), and for TCTP-(102–171), phospho-5′-CATGGTGACG-GAATTCGAAGCTTGAG-3′ (antisense) and 5′-AAACTTGAAGAACA-GAAACCAGAAAGG-3′ (sense).

After transient transfections with HA-tagged TCTP, GFP, GFP-TCTP, and GFP-TCTP-(1–101) and GFP-TCTP-(102–172) constructs in the LipofectAMINE PLUS™ reagent (Invitrogen), HeLa cells were incubated with ice-cold lysis buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM NaF, 2 mM Na_3VO_4, and Complete™ protease inhibitor mixture tablets (Roche Applied Science) for 30 min on ice and homogenized with a Pyrex glass homogenizer. The cell lysates were centrifuged at 10,000 × *g* for 10 min at 4 °C, and the supernatants (1 mg/ml) were preabsorbed with protein G-agarose or protein A-agarose (Roche Applied Science) for 3 h at 4 °C. Anti-HA antibody or anti-GFP antibody (Santa Cruz Biotechnology) was added to the reaction mixture and incubated at 4 °C for 3 h and incubated with protein G-agarose or protein A-agarose overnight on a rocking platform at 4 °C. The bound immune complexes so obtained were pelleted and washed three times with ice-cold phosphate-buffered saline, eluted with 2× SDS sample buffer, and separated on SDS-PAGE. The blots obtained were incubated with anti-Na,K-ATPase α1 monoclonal antibody (Upstate Biotechnology) and anti-GFP antibody.

Measurement of $^{86}Rb^+$ Uptake—HeLa cells were transiently transfected with GFP, GFP-TCTP, and GFP-TCTP-(1–101) and GFP-TCTP-(102–172) constructs and plated in 24-well dishes at 4×10^4 cells/well. After 24 h, the cells were washed with serum-free Dulbecco's modified Eagle's medium and incubated for 2 h at 37 °C in serum-free Dulbecco's modified Eagle's medium. The cells were subsequently washed three times with Krebs-Ringer buffer (KRP, 140 mM NaCl, 5 mM KCl, 10 mM Na_2HPO_4, 1 mM $MgSO_4$, 1.4 mM $CaCl_2$, and 2.5 mM glucose, pH 7.4) and incubated with or without 1 mM ouabain and/or 0.1 mM furosemide in the Krebs-Ringer buffer for 15 min at 37 °C. $^{86}Rb^+$ was added to a final concentration of 2 μCi/ml, and the reaction was allowed to proceed for 15 min at 37 °C. $^{86}Rb^+$ uptake was terminated by washing thrice with ice-chilled Krebs-Ringer buffer. The cells were then lysed with lysis buffer (50 mM NaOH, 1% Triton X-100) and incubated for 5 min at room temperature. The radioactivity of the cell lysates was determined after adding scintillation mixture solution. Protein concentration per well was determined according to Peterson's modification of the Lowry assay (20) and averaged. For this, cells contained in three wells were taken through the procedure without the addition of label.

Northern Blotting—Total RNA was isolated from TCTP-transfected HeLa cells using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. The RNA (20 μg/lane) was separated on a 1.2% agarose gel containing formaldehyde, transferred onto Hybond N^+ nylon membrane (Amersham Biosciences), and ultraviolet cross-linked. Hybridization was performed using ExpressHyb™ hybridization solution (Clontech). To determine Na,K-ATPase α1 transcription, a 641-bp fragment corresponding to nucleotides 271–911 of human Na,K-ATPase α1 cDNA was cloned after reverse transcription-PCR, using the RNA obtained as above as template, and labeled with [^{32}P]dCTP (PerkinElmer Life Sciences). After hybridization and washing, the membranes were analyzed by Imaging plate autoradiography system (Fuji) and normalized with β-actin mRNA.

Measurement of Na,K-ATPase Activity in Vitro—The plasma membrane fraction was obtained as described in a previous report (21). 0.2 mg/ml of plasma membrane fraction at 30% sucrose was incubated with various concentrations of ouabain (10^{-9}–10^{-3} M), recombinant TCTP (0.1–100 μg/ml), or recombinant TCTP alone in assay buffer containing 18 mM histidine, 18 mM imidazole, 80 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, and 0.1 mM EGTA, pH 7.4, for 20 min at 37 °C. The assay was then started with the addition of ATP to a final concentration of 5 mM in 100 μl/well of a 96-well plate. Following incubation for 40 min at 37 °C, the release of inorganic phosphate from ATP was quantified using the colorimetric method of Fiske and Subbarow (22). The reactions were read on the Bio-tek FL-600 plate reader at 630 nm. The absorbance values obtained were converted to activity values by linear regression using a standard curve of sodium monobasic phosphate that was included in the assay procedure. Total Na,K-ATPase activity was determined by subtracting the amount of inorganic phosphate resulting from incubation with TCTP alone from that with Na,K-ATPase plus TCTP. Specific activity of Na,K-ATPase was 27.9 μmol of P_i/mg of protein/h.

Deletion Analysis of TCTP—N-terminal deletion constructs of TCTP, namely 23–172 (ΔA), 64–172 (ΔB), 102–172 (ΔC), 126–172 (ΔD), and 102–125 (ΔE), were made by removing the relevant sequences from the N terminus of full strength rat TCTP (amino acid residues 1–172). Constructs ΔA and ΔB corresponded to clones 734 and 734B described previously (23). Construct ΔC was clone 292, isolated by yeast two-hybrid screening conducted in the present study. Two derivatives, ΔD and ΔE, were constructed by PCR and subcloned into the pJG4–5 library vector to generate the deletion constructs of TCTP. The N-terminal regions of different constructs were confirmed by DNA sequencing, and the expression of fusion proteins was confirmed by immunoblotting with the 12CA5 antibody against the HA epitope. The binding activity of the deletion mutants was determined by β-galactosidase assay.

RESULTS

TCTP Interacts with the Third Cytoplasmic Domain of Na,K-ATPase α Subunit—Using the LexA DNA-binding domain CD3 of the Na,K-ATPase α2 subunit fusion in a yeast two-hybrid screening, several cDNA clones were isolated from the rat skeletal muscle library. One of these clones encoded a polypeptide of 172 amino acids with a molecular mass of 21 kDa, originally identified as TCTP.

The cytoplasmic domains of Na,K-ATPase were amplified by PCR and cloned into the LexA fusion plasmid, pEG202. To identify the cytoplasmic regions of Na,K-ATPase that interact with the expressed TCTP, individual $Ura^+His^+Trp^+$ transformants were streaked onto glucose $Ura^-His^-Trp^-Leu^-$ and galactose $Ura^-His^-Trp^-Leu^-$ plates and assayed for β-galactosidase activity (Table I). Transformants of pEG202/α1CD3 and pEG202/α2CD3 with pJG4–5/TCTP activated the transcription of the respective reporter genes (Table I). It is not clear whether the N-terminal portion of Na,K-ATPase interacts with TCTP because the yeast cells transformed with pEG202/α1NT or pEG202/α2NT activated reporter genes in the presence of glucose or galactose. When yeast cells were transformed with pEG202/α1CD2, pEG202/α2CD2, pEG202/α1CD4, pEG202/α2CD4, pEGα202/α1CT, pEGα202/α2CT, and pEG202/β, there was no activation of the LacZ reporter gene in a galactose-dependent colony on $Ura^-His^-Trp^-Leu^-$ plates (Table I), suggesting that CD2, CD4, and the C-terminal por-

TABLE I

Interaction of TCTP with the CD3 of Na,K-ATPase α1 and α2 subunits in yeast two-hybrid system

The CDs of the α1 and α2 subunit of Na,K-ATPase were fused to the pEG202 vector containing the LexA DNA-binding domain. Rat TCTP was fused to the pJG4–5 vector, which contained the B42 transcription activation domain. Transformants were selected for the transcription of the LexAop-LUE2 reporter on glucose $Ura^-His^-Trp^-Leu^-$ and galactose $Ura^-His^-Trp^-Leu^-$. Values given are the average of three independent determinations. $U^-H^-T^-L^-$, without Uracil, Histidine, Tyrosine, Leucine, NT: N-Terminal portion, CT: C-Terminal portion.

Na,K-ATPase subunit	Construct		Selective media plate		β-galactosidase activity (unit)[a]	
	pEG202 (DNA-binding)	pJG4–5 (Transcription activation)	Glc $U^-H^-T^-L^-$	Gal $U^-H^-T^-L^-$	Glc $U^-H^-T^-$	Gal $U^-H^-T^-$
Control	Positive		−	+++	0	328
	Negative		−	−	0	1
α1	NT (Met-1–Cys-93)	TCTP	+	+	2	12
	CD2 (Glu-151-Ile-292)		−	−	0	1
	CD3 (Lys-354–Val-759)		−	+++	0	113
	CD4 (Glu-825–Arg-848)		−	−	0	1
	CT (Ile-935–Tyr-1023)		−	−	0	1
α2	NT (Met-1–Cys-91)	TCTP	+	+	5	14
	CD2 (Glu-149–Ile-290)		−	−	0	1
	CD3 (Lys-352–Val-756)		−	+++	0	143
	CD4 (Glu-822–Arg-845)		−	−	0	1
	CT (Ile-932–Tyr-1020)		−	−	0	1
β	CD (Ala-1–Lys-33)	TCTP	−	−	0	1

[a] The β-galactosidase unit = $A_{420} \times 1{,}000/t$ (min) × volume of extract (ml) × protein (μg/ml).

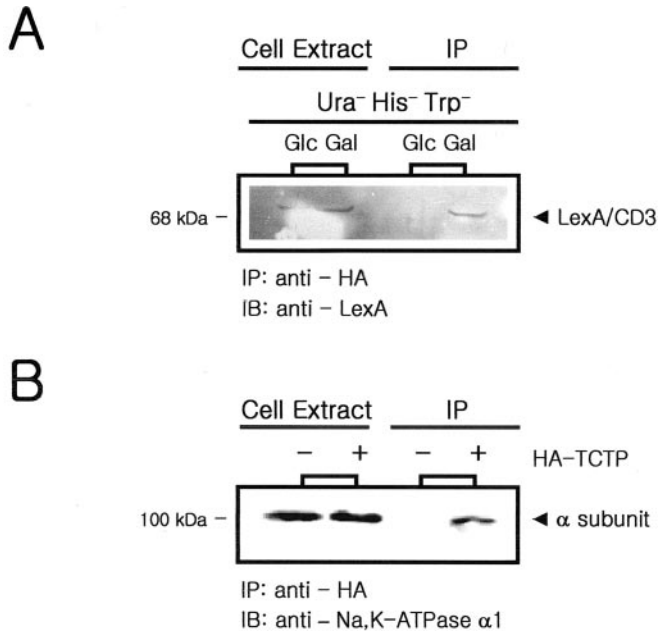

FIG. 1. **Interaction of TCTP with Na,K-ATPase α subunit in yeast and mammalian cells.** *A*, yeast cell extracts transformed with pEG202/α2CD3 and pJG4–5/TCTP were prepared in Glc $Ura^-His^-Trp^-$ and Gal $Ura^-His^-Trp^-$ liquid media, respectively. 12CA5 was used to immunoprecipitate HA-tagged proteins. LexA-fused CD3 of Na,K-ATPase was detected with anti-LexA antibody. *IP*, immunoprecipitation; *IB*, immunoblots. *B*, HeLa cells were transiently transfected with mammalian expression vector fused to HA-tagged TCTP. Immunoprecipitates were prepared from cell extracts (1 mg/ml) employing 12CA5 antibody. Total cell extract (50 μg) and immune complexes were blotted with anti-Na,K-ATPase α subunit.

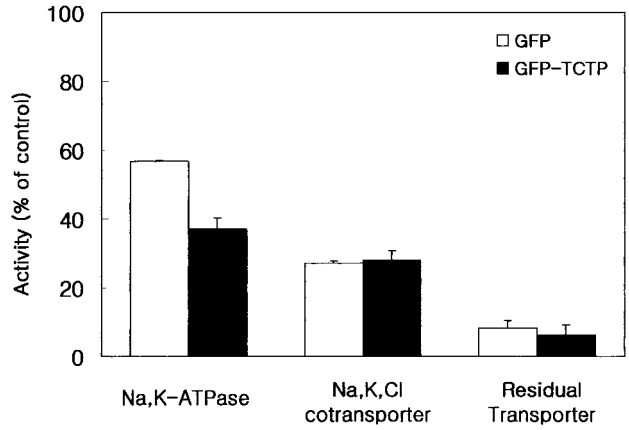

FIG. 2. **Na,K-ATPase activity in HeLa cells is inhibited by overexpression of TCTP.** HeLa cells were transiently transfected with GFP and GFP-TCTP and were plated in a 24-well plate at 0.4×10^5 cells/well. Following transfection, the cells were incubated with 1 mM ouabain and/or 0.1 mM furosemide for 15 min at room temperature. 2 μCi/ml $^{86}Rb^+$ was added, and $^{86}Rb^+$ uptake was counted in triplicate. Na,K-ATPase-dependent $^{86}Rb^+$ uptake is defined as the amount of $^{86}Rb^+$ uptake in the presence of furosemide that was inhibited by the addition of ouabain. $Na^+,K^+,2Cl^-$ cotransporter-dependent $^{86}Rb^+$ uptake is defined as the amount of control $^{86}Rb^+$ uptake in the absence of inhibitors that was inhibited by the addition of furosemide. Residual $^{86}Rb^+$ uptake is defined as $^{86}Rb^+$ uptake, which was not inhibited by either ouabain or furosemide. The bar graph shows the results of three independent experiments ± S.E.

tion of the α subunit and the cytoplasmic region of the β subunit do not interact with TCTP. These results suggest that TCTP binds to the CD3 region of the α1 and α2 subunit of Na,K-ATPase, and the binding is not α isoform-specific.

TCTP Interacts with Na,K-ATPase from Both Yeast and Mammalian Cells—Using the 12CA5 antibody against HA epitope-tagged TCTP, we next examined whether TCTP associates with the α2 subunit and co-precipitates the α2CD3 region of Na,K-ATPase in yeast. Fig. 1*A* shows SDS-PAGE results obtained by examining immunoprecipitates with anti-LexA antibody to the LexA-fused CD3 region of Na,K-ATPase. The 12CA5 antibody precipitated HA-TCTP, which co-precipitated the LexA-α2CD3 hybrid in Gal media but not in Glc media. The mobilities of the precipitated proteins were identical to those of immunoreactive bands in the yeast extracts that contained LexA-α2CD3 fusion proteins, which were expressed constitutively by alcohol dehydrogenase promoter. These results indicate that TCTP associates with a 68-kDa protein, consistent with the predicted size of α2CD3.

To determine whether TCTP and Na,K-ATPase interact *in vivo*, we transiently transfected HeLa cells with HA epitope-tagged TCTP (Fig. 1*B*). Western blotting using anti-HA antibody confirmed that the cells expressed 22.3-kDa HA epitope-tagged TCTP (data not shown). Immunoprecipitates were prepared from HeLa cell extracts with anti-HA antibody (12CA5) and blotted with anti-Na,K-ATPase α subunit antibody. HA-tagged TCTP co-precipitated the 100-kDa α subunit of Na,K-ATPase from HA-TCTP-transfected cells but not from

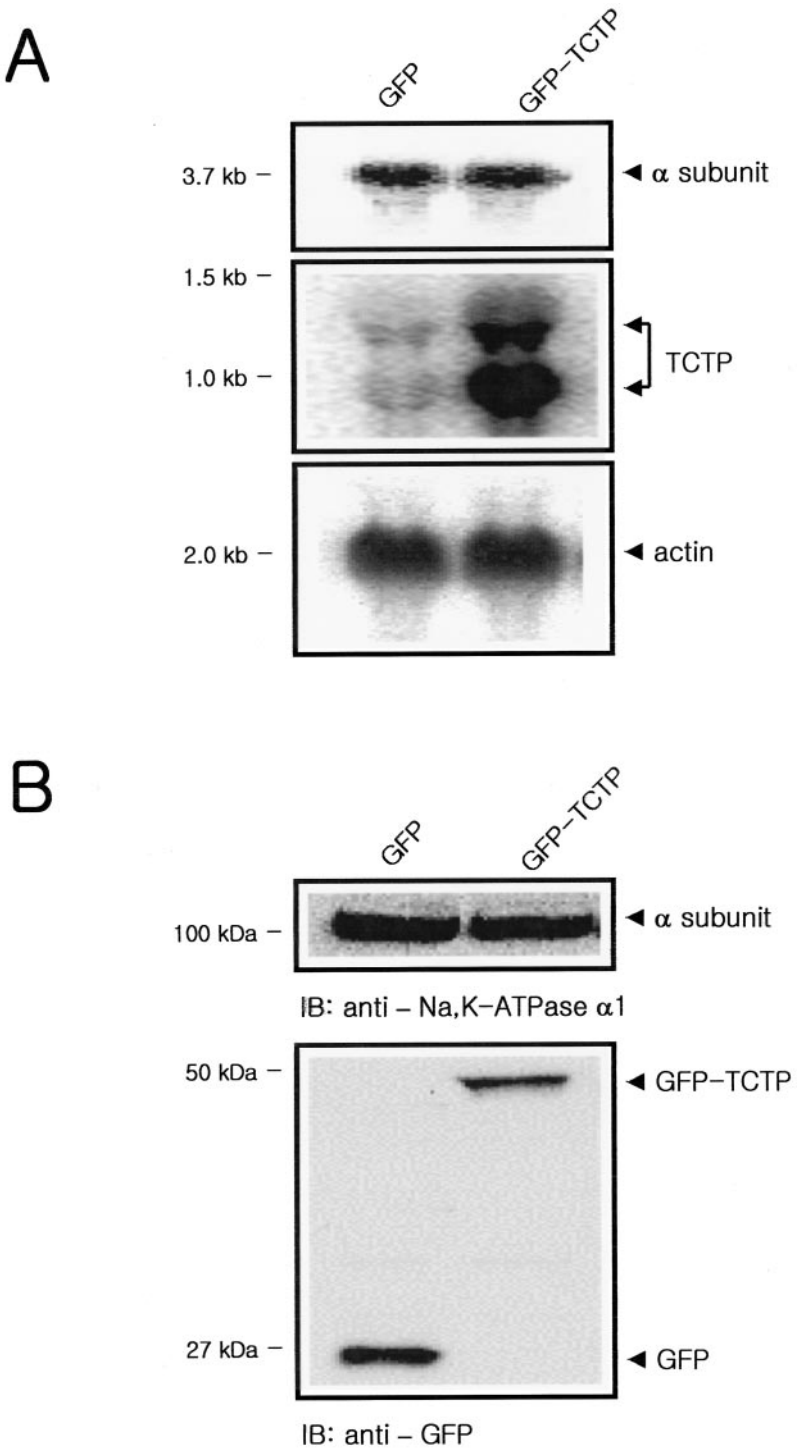

FIG. 3. **Effect of TCTP on Na,K-ATPase mRNA and protein synthesis.** *A*, total RNA was isolated from HeLa cells transiently transfected with GFP and GFP-,TCTP and the levels of target transcripts were determined by Northern blotting analysis using Na,K-ATPase α1- and TCTP-specific primers. β-actin transcript was used as an internal standard. *B*, following transient transfection of HeLa cells with GFP and GFP-TCTP, the total cell extract (50 μg) was blotted sequentially with anti-Na,K-ATPase α subunit and anti-GFP antibody. *IB*, immunoblot.

mock-transfected cells (Fig. 1*B*). This confirms that the interaction between the α subunit of Na,K-ATPase and TCTP occurs *in vivo* also.

Overexpression of TCTP in HeLa Cells Inhibits Na,K-ATPase Activity—To understand the role of TCTP in the regulation of the Na,K-ATPase activity, we transiently overexpressed exogenous TCTP in HeLa cells and measured Na,K-ATPase activity using $^{86}Rb^+$ uptake assay. Because 90% of $^{86}Rb^+$ uptake in HeLa cells is accounted for by Na,K-ATPase (~60%) and the $Na^+,K^+,2Cl^-$ cotransport system (~30%) (24), we employed 1 mM ouabain (a specific inhibitor of the Na,K-ATPase) and 0.1 mM furosemide (an inhibitor of the $Na^+,K^+,2Cl^-$ cotransporter). As can be seen in Fig. 2, the activity of Na,K-ATPase of the GFP-TCTP-transfected cells was 22.3 ± 3.3% lower than that of GFP-transfected cells. However, cells overexpressing either GFP or GFP-TCTP showed a similar $Na^+,K^+,2Cl^-$ cotransporter and residual transporter activity. These results suggest that the increased level of TCTP in HeLa cells inhibits $^{86}Rb^+$ uptake and that this inhibition is due to its effect on the Na,K-ATPase but not on the $Na^+,K^+,2Cl^-$ cotransport system.

Overexpression of TCTP in HeLa Cells Does Not Affect Na,K-ATPase at Either mRNA or Protein Levels—Northern blotting using human Na,K-ATPase α1-specific probe demonstrated that the mRNA level of Na,K-ATPase α1 subunit was the same in both GFP and GFP-TCTP-transfected cells, whereas that of TCTP remarkably increased in GFP-TCTP-

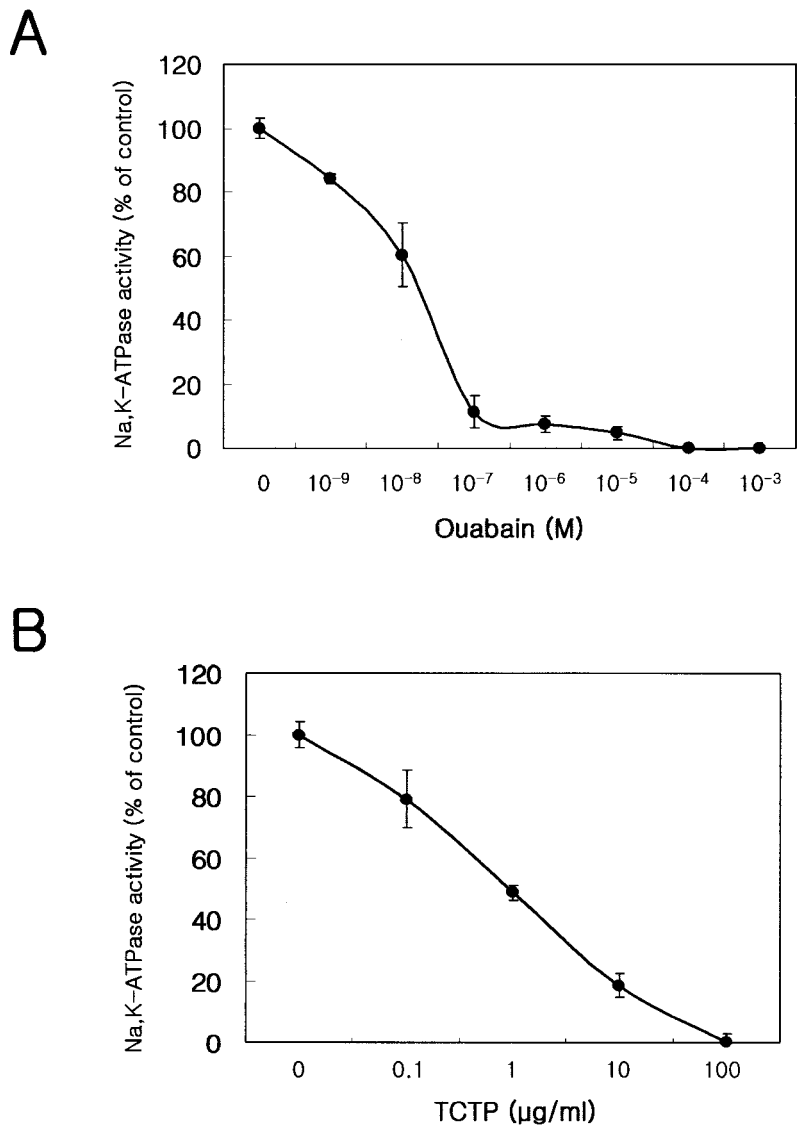

FIG. 4. **Inhibition of the purified HeLa cell plasma membrane Na,K-ATPase activity by recombinant TCTP.** *A*, 0.2 mg/ml of HeLa cell plasma membrane fraction was incubated with various concentration of ouabain in assay buffer for 20 min at 37 °C. The assay was started with the addition of ATP (final concentration 5 mM) in 100 μl/well of a 96-well plate. Following incubation for 40 min at 37 °C, the release of inorganic phosphate from ATP was read on the Biotek FL-600 plate reader at 630 nm. *B*, recombinant TCTP (0.1–100 μg/ml) were incubated with 0.2 mg/ml of HeLa cell plasma membrane fraction or with TCTP alone in assay buffer for 20 min at 37 °C. The release of inorganic phosphate was then measured as described above. Total Na,K-ATPase activity was determined by subtracting the amount of inorganic phosphate in TCTP alone incubation from that in Na,K-ATPase plus TCTP. Data are the average of three independent experiments ± S.E.

transfected cells (Fig. 3*A*). We also determined the level of expression of Na,K-ATPase α1 subunit using α1-specific monoclonal antibodies. The expression level of α1 subunits was the same in GFP as well as GFP-TCTP-transfected cells (Fig. 3*B*), suggesting that an increase in TCTP protein does not affect the expression of Na,K-ATPase α1 subunits.

TCTP Reduces in Vitro Na,K-ATP Activity in HeLa Cell Plasma Membrane in a Dose-dependent Manner—The effect of TCTP on membrane-bound Na,K-ATPase was studied using HeLa cell plasma membrane enriched with ouabain-sensitive Na,K-ATPase by removal of extraneous protein. The purified HeLa cell plasma membrane Na,K-ATPase activity decreased with increased concentration of ouabain (IC_{50} = 2.20 ± 0.37 × 10^{-8} M) (Fig. 4*A*). The addition of recombinant GST-TCTP to the purified plasma membrane resulted in a significant decrease in Na,K-ATPase activity in a dose-dependent manner (IC_{50} = 48 nM) (Fig. 4*B*), whereas the addition of GST and bovine serum albumin did not (data not shown). It appears that recombinant TCTP protein specifically inhibits HeLa cell plasma membrane Na,K-ATPase activity.

C-terminal Homology Region 2 of TCTP (Residues 102–172) Is Essential for Its Association with, and Inhibition of, Na,K-ATPase—To identify the regions of TCTP that interact with the CD3 regions of α1 and α2 subunits of Na,K-ATPase, we introduced full-length TCTP and its deleted constructs (23–172 (ΔA), 64–172 (ΔB), 102–172 (ΔC), 126–172 (ΔD), and 102–125 (ΔE) (Fig. 5*A*) into yeast cells harboring reporter genes and examined the behavior of these strains in glucose $Ura^-His^-Trp^-Leu^-$ and galactose $Ura^-His^-Trp^-Leu^-$ media. The proteins expressed by these constructs were first confirmed by Western blotting in yeast extracts transformed with each construct (data not shown).

We measured, employing liquid culture β-galactosidase assay, the activity of the *LacZ* reporter gene in cells grown in glucose- or galactose-containing media. As shown in Fig. 5*B*, constructs of full-length TCTP, ΔA, ΔB, and ΔC showed similar β-galactosidase activities, suggesting that residues 1–101 are not involved in the interaction with CD3 of Na,K-ATPase. However, β-galactosidase activity of the construct ΔD-(126–172) was reduced 6-fold when compared with that of full-length TCTP, suggesting that residues 102–125 (ΔE) are necessary for the association between TCTP and the CD3 of Na,K-ATPase and for the full expression of the enzyme activity. When we constructed ΔE containing residues 102–125 and determined its effect on β-galactosidase activity, there was an almost complete loss of β-galactosidase activity. It appears that residues 102–172 of TCTP are required for the interaction between TCTP and the CD3 region of the α1 as well as the α2 subunit of Na,K-ATPase.

To obtain direct evidence that the C-terminal region of TCTP

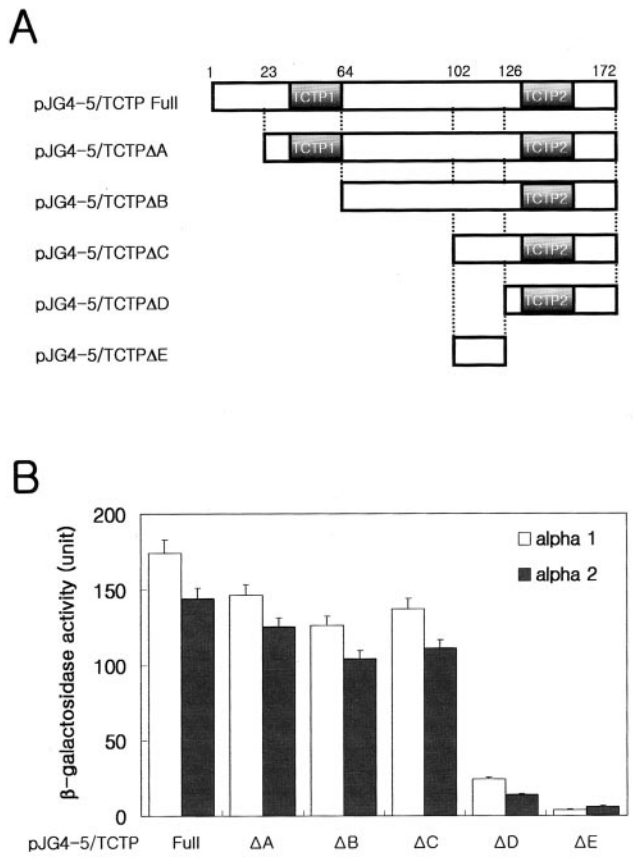

FIG. 5. **Interaction of C-terminal domain of TCTP containing TCTP homology region 2 with CD3 of Na,K-ATPase α subunit.** *A*, schematic representation of rat TCTP deletion constructs. The *numbers* refer to the amino acid sequence of rat TCTP and its deletion constructs. The TCTP homology region 1 (TCTP1) and 2 (TCTP2) are depicted by *shaded boxes*. *B*, CD3 of the α1 and α2 subunits of Na,K-ATPase were fused to the pEG202 vector, and the deletion construct of TCTP was fused to the pJG4–5 vector. The interaction was determined by assay of β-galactosidase activity. Data are the average of three independent experiments ± S.E.

interacts with Na,K-ATPase in mammalian cells, we subcloned deletion mutants that encode residues 1–101 (N-terminal region) and 102–172 (C-terminal region) and overexpressed them in HeLa cells. Immunoprecipitates with anti-GFP antibody were prepared from HeLa cell extracts expressing the deletion mutants and blotted with anti-Na,K-ATPase α subunit and anti-GFP. As shown in Fig. 6*A*, full-length TCTP and C-terminal GFP-TCTP formed complexes with the 100-kDa fragment of the α subunit of Na,K-ATPase but not the GFP and N-terminal regions. These results confirm that the C-terminal region of TCTP is essential for its interaction with α subunit of Na,K-ATPase *in vivo*.

We also compared the $^{86}Rb^+$ uptake of HeLa cells transfected with full-length TCTP with that of HeLa cells transfected with deletion constructs. The $^{86}Rb^+$ uptake in cells transfected with the full-length and C-terminal regions (102–172) was about 20.6 ± 2.6 and 29.6 ± 3.5%, respectively, lower than in GFP-transfected cells, suggesting that the C-terminal region (102–172) of TCTP is sufficient to reduce the activity of Na,K-ATPase in HeLa cells (Fig. 6*B*). On the other hand, the $Na^+,K^+,2Cl^-$ cotransporter and residual transporter activities in GFP, GFP-TCTP, GFP-TCTP-(1–101), and GFP-TCTP-(102–172) overexpressing cells were similar (Fig. 6*B*). These data also suggest that the C-terminal region (102–172) of TCTP is essential for interaction with Na,K-ATPase α subunit and is sufficient to reduce the Na,K-ATPase activity.

DISCUSSION

TCTP has been identified in the proliferative stage of breast tumor (25) and has been initially reported to be present in all normal cell types, with the exception of kidney cells (16). However, we detected endogenous TCTP in COS-7 cells and rat kidney tissue by Western blotting,[2] and it now appears that TCTP exists in all normal cell types. Also, TCTP is widely distributed and exhibits a high degree of homology among various species. This suggests that this protein must have some essential cellular function. In the present study, we identified TCTP as a Na,K-ATPase-binding protein and as a cytoplasmic repressor of Na,K-ATPase activity. One might speculate that the basic function of TCTP may be to sustain all normal cell types by regulating the Na,K-ATPase activity.

Modulation of intracellular ion balance by Na,K-ATPase is an important mechanism by which cell growth, differentiation, and apoptosis are regulated in diverse tissues. Recently, evidence has been obtained that moderate inhibition of Na,K-ATPase provides a prosurvival signal in addition to its mitogenic effect, whereas complete inhibition of the enzyme sharply decreases cell survival (26, 27). TCTP has been shown to bind to the proapoptotic myeloid cell leukemia protein-1, a BCl-2 homologue (28). Li *et al.* (29) showed that transient overexpression of TCTP in HeLa cells prevented etoposide-induced apoptosis, suggesting that TCTP influences cell survival through its novel anti-apoptotic effect and regulation of apoptosis. Therefore, it seems that reduction of Na,K-ATPase activity resulting from the overexpression of TCTP in HeLa cells is unlikely due to decreased cell viability.

Xie and Askari (30) demonstrated that Na,K-ATPase is a signal transducer, with two distinct but coupled signal transducing pathways. One pathway involves the classical energy transducing ion pump enzymes, whose partial inhibition by ouabain initiates the increase in intracellular Ca^{2+}, whereas the second involves signal transducing enzymes that activate a host of signaling intermediates and increase intracellular reactive oxygen species in cardiac myocytes. It is possible that inhibition of Na,K-ATPase activity by TCTP causes an increase in intracellular Na^+, which in turn affects the Na^+,Ca^{2+} exchanger, leading to an increase in intracellular Ca^{2+}. Since HeLa cells do not have Na^+,Ca^{2+} exchanger, we are currently studying cardiac myocytes to determine how TCTP raises intracellular Ca^{2+}.

ATPase is subject to both short and long term regulation by a variety of phenomena. Long term regulatory mechanisms generally involve *de novo* Na,K-ATPase synthesis or degradation, whereas short term regulation involves either direct effect on the kinetic behavior of the enzyme or translocation of Na,K-ATPase to the plasma membrane. The reduction of Na,K-ATPase activity described in the present studies is due to binding of Na,K-ATPase to TCTP and not due to changes in Na,K-ATPase synthesis.

Because of the possible central role that Na,K-ATPase plays in physiological and pathological phenomena, it is important to elucidate the mechanisms that underlie the regulation of Na,K-ATPase activity by TCTP. Lingham and Sen (31) were the first to suggest that cAMP-dependent protein kinase requires an intermediate protein to mediate its effect on the Na,K-ATPase in rat brain. It has also been reported that a protein kinase C-mediated decrease in plasma membrane Na,K-ATPase activity is not associated with direct phosphorylation (32). TCTP is a Ca^{2+}-binding protein with four potential phosphorylation sites, one site for protein kinase C and three sites for casein

[2] J. Jung, M. Kim, M.-J. Kim, J. Kim, J. Moon, J.-S. Lim, M. Kim, and K. Lee, unpublished results.

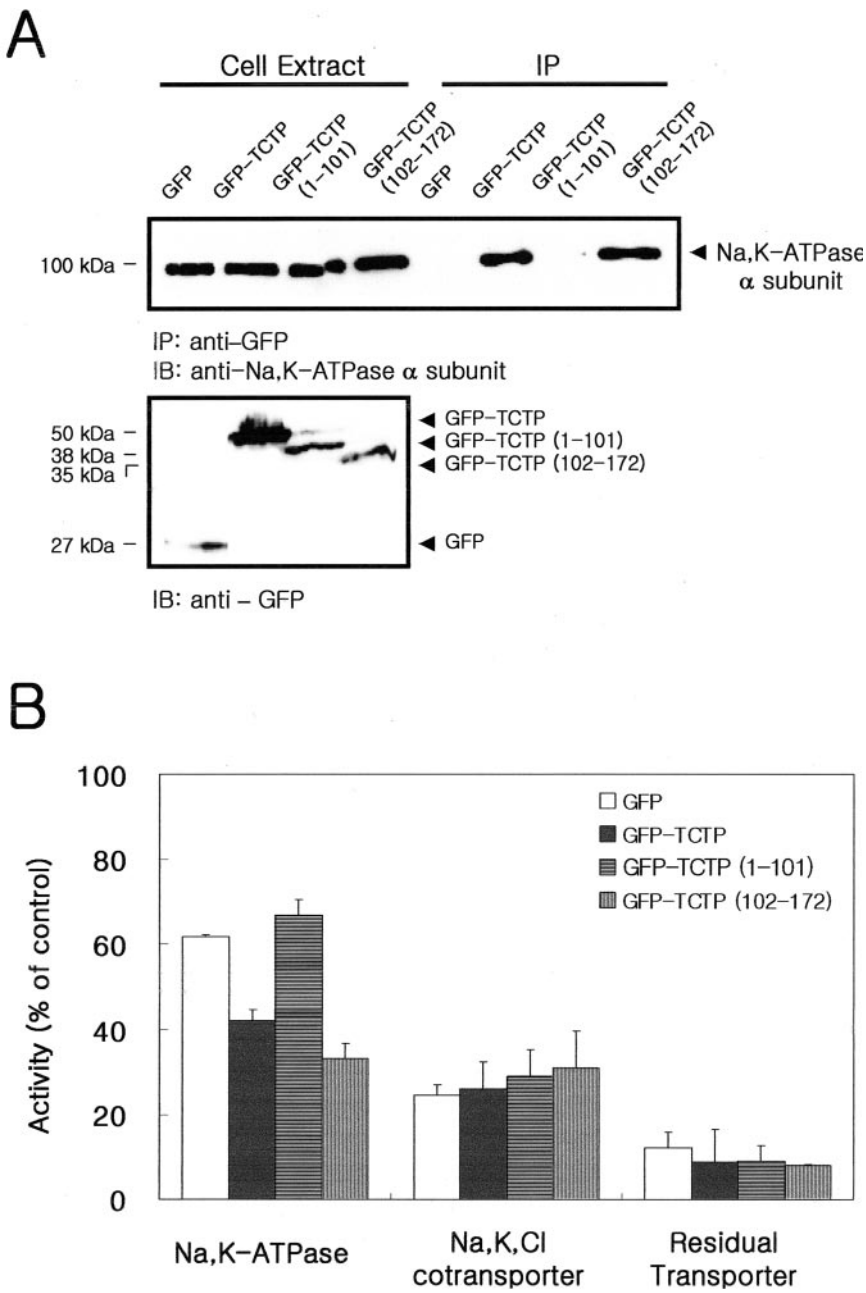

FIG. 6. **Effect of *in vivo* interaction of C-terminal region (102–172) of TCTP with Na,K-ATPase α subunit on Na,K-ATPase activity.** *A*, HeLa cells were transiently transfected with GFP, GFP-TCTP, GFP-TCTP-(1–101), and GFP-TCTP-(102–172) constructs. Immunoprecipitates were prepared from the cell extracts (1 mg/ml) with anti-GFP. Total cell extract (50 μg) and immune complexes (*IP*) were sequentially stained with anti-Na,K-ATPase α subunit and anti-GFP. *IB*, immunoblot. *B*, HeLa cells were transiently transfected, and the experimental procedures were performed as described in the legend for Fig. 2. The bar graph shows the results of three independent experiments ± S.E.

kinase II, according to a ScanProsite search. In addition, it was reported that TCTP is phosphorylated at serine residues 46 and 64 by a polo-like protein kinase that regulates spindle function (33). Thus it is possible that TCTP might be regulated by unknown kinases to modulate the Na,K-ATPase activity. McGill and Guidotti (6) reported that both α1 and α2 isoforms of Na,K-ATPase in adipocytes were stimulated by insulin and that this stimulatory effect was lost during the isolation of the membrane, suggesting the presence of a diffusible repressor of Na,K-ATPase, regulated by insulin. It will be of interest to examine whether insulin also plays a role in the regulation of TCTP binding to Na,K-ATPase in adipocytes.

It has been reported that TCTP has a tubulin-binding region (residues 80–120) and that TCTP associates transiently with microtubules during the cell cycle (34). We reported previously that the C-terminal region (126–172) of TCTP is involved in interaction with itself (23). In the present study, we found that the C-terminal region of TCTP-(102–172) is essential for its interaction with Na,K-ATPase α subunit and is sufficient to inhibit Na,K-ATPase activity. Thus it seems that TCTP plays multiple roles by interacting with different kinds of proteins through diverse domains.

In summary, this is the first demonstration that TCTP is a Na,K-ATPase-binding protein serving as an intracellular inhibitor of Na,K-ATPase activity. TCTP acts on the cytoplasmic side of the cell membrane, in contrast to ouabain, which inhibits the enzyme by binding to the outer side of the cell membrane. This demonstration of negative regulation of Na,K-ATPase activity by TCTP suggests a possible new mechanism for the regulation of the machinery of the cell and for the maintenance of ion homeostasis. Further work is under way to elucidate the precise regulatory role of TCTP.

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REFERENCES

1. Therien, A. G., and Blostein, R. (2000) *Am. J. Physiol.* **279,** C541–C566
2. Ullrich, K. J. (1979) *Annu. Rev. Physiol.* **41,** 181–195
3. Bertorello, A. M., and Katz, A. I. (1993) *Am. J. Physiol.* **265,** F743–F755
4. Jorgensen, P. L., and Andersen, J. P. (1988) *J. Membr. Biol.* **103,** 95–120
5. Forbush, B., III, Kaplan, J. H., and Hoffman, J. F. (1978) *Biochemistry* **17,** 3667–3676
6. McGill, D. L., and Guidotti, G. (1991) *J. Biol. Chem.* **266,** 15824–15831
7. Yudowski, G. A., Efendiev, R., Pedemonte, C. H., Katz, A. I., Berggren, P. O., and Bertorello, A. M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 6556–6561
8. Devarajan, P., Scaramuzzino, D. A., and Morrow, J. S. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 2965–2969
9. Jordan, C., Puschel, B., Koob, R., and Drenckhahn, D. (1995) *J. Biol. Chem.* **270,** 29971–29975
10. Lee, K., Jung, J., Kim, M., and Guidotti, G. (2001) *Biochem. J.* **353,** 377–385
11. Koob, R., kraemer, D., Trippe, G., Aebi, U., and Drenckhahn, D. (1990) *J. Cell Biol.* **53,** 93–100
12. Ferrandi, M., Salardi, S., Tripodi, G., Barassi, P., Rivera, R., Manunta, P., Goldshleger, R., Ferrari, P., Bianchi, G., and Karlish, S. J. (1999) *Am. J. Physiol.* **277,** H1338–1349
13. Bommer, U. A., Lazaris-Karastzas, A., De Benedetii, A., Nurnberg, P., Benndorf, R., Bielka, H., and Sonenberg, N. (1994) *Cell. Mol. Biol. Res.* **40,** 633–641
14. Xu, A., Bellamy, R., and Taylor, J. A. (1999) *Biochem. J.* **342,** 683–689
15. Thaw, P., Baxter, N. J., Hounslow, A. M., Price, C., Waltho, J. P., and Craven, C. J. (2001) *Nat. Struct. Biol.* **8,** 701–704
16. 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
17. Chitpatima, S. T., Makrides, S., Bandyopadhyay, R., and Brawerman, G. (1988) *Nucleic Acids Res.* **16,** 2350
18. Himmelfarb, H. J., Pearlberg, J., Last, D. H., and Ptashne, M. (1990) *Cell* **63,** 1299–1309
19. Jung, J., Yoon, T., Choi, E. C., and Lee, K. (2002) *J. Biol. Chem.* **277,** 48931–48937
20. Fehlmann, M., and Freychet, P. (1981) *J. Biol. Chem.* **256,** 7449–7453
21. Coppi, M. V., and Guidotti, G. (1997) *Arch. Biochem. Biophys.* **346,** 312–321
22. Fiske, C. H., and Subbarow, Y. (1925) *J. Biol. Chem.* **66,** 375–400
23. Yoon, T., Jung, J., Kim, M., Lee, K. M., Choi, E. C., and Lee, K. (2000) *Arch. Biochem. Biophys.* **384,** 379–382
24. Kort, J. J., and Koch, G. (1990) *J. Cell. Physiol.* **145,** 253–261
25. Gross, B., Gaestel, M., Bohm, H., and Bielka, H. (1989) *Nucleic Acids Res.* **17,** 8367
26. Murata, Y., Matsuda, T., Tamada, K., Hosoi, R., Asano, S., Takuma, K., Tanaka, K., and Baba, A. (1996) *Jpn. J. Pharmacol.* **72,** 347–353
27. Kaplowitz, P. B., and Moscona, A. A. (1976) *Cell Differ.* **5,** 109–119
28. Zhang, D., Li, F., Weidner, D., Mnjoyan, Z. H., and Fujise, K. (2002) *J. Biol. Chem.* **277,** 37430–37438
29. Li, F., Zhang, D., and Fujise, K. (2001) *J. Biol. Chem.* **276,** 47542–47549
30. Xie, Z., and Askari, A. (2002) *Eur. J. Biochem.* **269,** 2434–2439
31. Lingham, R. B., and Sen, A. K. (1982) *Biochim. Biophys. Acta* **688,** 475–485
32. Nestor, N. B., Lane, L. K., and Aperia, A. (1997) *Ann.* N. Y. *Acad. Sci.* **834,** 579–581
33. Yarm, F. R. (2002) *Mol. Cell. Biol.* **22,** 6209–6221
34. Gachet, Y., Tournier, S., Lee, M., Lazaris-Karatzas, A., Poulton, T., and Bommer, U. A. (1999) *J. Cell Sci.* **112,** 1257–1271

Translationally Controlled Tumor Protein Interacts with the Third Cytoplasmic Domain of Na,K-ATPase α Subunit and Inhibits the Pump Activity in HeLa Cells

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