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Original

Identification of Carnitine Transporter CT1 Binding Protein Lin-7 in Nervous System

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ABSTRACT

L-Carnitine is an essential component of mitochondrial fatty acid b-oxidation in the muscle and may control the acetyl moiety levels in the brain for acetylcholine synthesis. Carnitine transporter 1 (CT1) is the high affinity L-carnitine transporter whose localization was observed in the kidney, testis, liver, skeletal muscle and brain. To clarify the molecular mechanism of carnitine transport, we sought to find the interacting protein that may be related to the transport function of CT1. Using the intracellular C-terminal region of rat CT1 containing PDZ (PSD95/DLG/ZO-1) motif as bait, we performed the yeast two-hybrid screening against rat brain cDNA library. Thirty two positive clones were obtained from the 2.7×10^7 clones screened. One of them was PDZ domain-containing protein Lin-7. We found that Lin-7 interacts specifically with C-termini of CT1 : deletion and mutation of the CT1 C-terminal PDZ-motif abolished the interaction with Lin-7 in the yeast two-hybrid assay. In addition, a PDZ domain within Lin-7 associates with the CT1 C-terminal. The association of CT1 with Lin-7 enhanced L-carnitine transport activities in HEK293 cells although there is no statistical significance. Coexpression of Lin-7 and CT1 is identified in motor neurons of the spinal cord ventral horn together with Lin-2, a binding partner of Lin-7 known to assemble proteins involved in synaptic vesicle exocytosis and synaptic junctions. Therefore, Lin-7 interacts with CT1 and may regulate their subcellular distribution or function in central nervous system.

Keywords : carnitine, transporter, PDZ, yeast two-hybrid assay, SLC22

INTRODUCTION

L-Carnitine (4-*N*-trimethylammonium-3-hydroxybutyric acid) is a highly polar, water-soluble quaternary amine that exists as a zwitterion under physiological conditions[1)]. In eukaryotic cells, carnitine is biologically important for mitochondrial β-oxidation of long-chain fatty acids and to generate ATP in peripheral tissues such as muscle, heart and liver[1)]. In recent years, a different physiological role of L-carnitine has been proposed : control of acetyl moiety level in neural cells for acetylcholine synthesis[2)]. Acetyl-L-carnitine, endogenous metabolite of L-carnitine, is a source of acetyl groups available for acetylcholine synthesis and is selectively taken up by the primate brain[2)].

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Table 1 PCR primers used in this study

Construct	Sense primer	Antisense primer
CT1-CT-wt	5'-CGAATTCCCCGACACCATTGACCA-3'	5'-CCCTCGAGTTAGAAGGCTGTGCTC-3'
CT1-CT-Δ3	5'-CGAATTCCCCGACACCATTGACCA-3'	5'-CTCTCGAGTTAGCTCTTTAGGACCGTTG-3'
CT1-F557A	5'-CGAATTCCCCGACACCATTGACCA-3'	5'-CCCTCGAGTTAGGCGGCTGTGCTCTTTAGGAC-3'
CT1-T555A	5'-CGAATTCCCCGACACCATTGACCA-3'	5'-CTCTCGAGTTAGAAGGCTGCGCTC-3'
Lin7-L27 domain	5'-CGAATTCATGGCTGCGCTGGTGGAG-3'	5'-CTCTCGAGTCAATGGGCATGGCCCTCAC -3'
Lin7-PDZ domain	5'-CGAATTCCCCAGGGTCGTGGAACTA-3'	5'-CTCGTCGACTCACTCCTCCAGCACCCGAG-3'

These functions may lead to the beneficial effcts of acetyl-$_L$-carnitine against ischemic change, degenerating disease (Alzheimer disease and dementia) and age-related changes in brain[3].

Carnitine transporter CT1 (*Slc22a5*), also termed as Octn2, was isolated from rat intestine and mediates a high-affinity sodium-dependent transport of $_L$-carnitine[4,5]. Systemic carnitine deficiency (SCD, OMIM 212140) in human and in juvenile visceral steatosis (jvs) mouse is caused by the mutations of *SLC22A5*/*Slc22a5*[5]. CT1-mediated $_L$-carnitine uptake was strongly inhibited by acetyl-$_L$-carnitine, suggesting that acetyl-$_L$-carnitine is putative transport substrate of CT1. CT1 mRNA was strongly expressed in the testis, colon, kidney and liver, and weakly in the skeletal muscle and brain[4]. Since the brain depends on carnitine supplied from the plasma, control of the transport function of CT1 seems to be essential for the neuronal cell metabolism. Therefore, it is suggested that the transport of both $_L$-carnitine and acetyl-$_L$-carnitine via CT1 is important to maintain the homeostasis in the central nervous system (CNS).

A number of recent studies have shown that association of neuronal ion channels and receptors with adapter and cytoskeletal proteins such as PDZ protein influences their localization and regulation[6]. We have performed the yeast two-hybrid screening to identify the interacting proteins for CT1 and to clarify the regulation mechanism of carnitine transport in CNS. Here we report that the PDZ protein Lin-7 interacts with the C terminus of CT1 and that Lin-7 and CT1 are coexpressed in rat brain such as neurons in the spinal cord ventral horn.

METHODS

Materials

$_L$-[^{3}H]carnitine hydrochloride (84 Ci/mmol) was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). All other chemicals and reagents used were of analytical grade and obtained from commercial sources. A rat brain cDNA library for the yeast two-hybrid screening was purchased from OriGene Technologies (Rockville, MD).

Plasmid construction

The C-terminal fragments of wild-type rat CT1 cDNA and three mutants (designated Δ3, F557A, and T555A) were generated by PCR using specific primers (Table 1) and cloned into the *Eco* RI and *Xho* I sites of bait vectors (pEG202) to construct CT1-C-terminus (CT) wt, CT1-CTΔ3, CT1-F557A, and CT1-T555A. The pcDNA3.1 vector containing the full-length rat Lin-7 (rLin-7) and preys (pJG4-5) containing L27 and PDZ domains of rLin-7 were prepared as described previously[7].

Yeast two-hybrid assay

A CT1 C-terminal bait corresponding to the last 39 amino acids of CT1 was used to screen 4.5×10^6 clones of the rat brain cDNA library with the LexA-based *GFP* two-hybrid system (Grow'n' Glow system ; MoBiTec) as described previously[7]. Several positive clones were obtained and confirmed in a second round of screening using the yeast system. The expression of all the bait constructs in yeast was confirmed by western blot analysis of yeast protein extracts using an anti-LexA antibody (Santa Cruz Biotechnology) (data not shown).

Preparation of Antibodies

Corresponding to the 14 amino acids of the NH_2-terminus of rat CT1, rabbit anti-CT1 polyclonal antibody raised against the keyhole limpet hemocyanin-conjugated synthesized peptides, MTSTFNPREC

(amino acids 1-10 of the rat CT1 amino acid sequence) was generated.

Immunohistochemistry

Eight-week-old male Wistar rats weighing 250-300 g (SLC, Shizuoka, Japan) were anesthetized by intraperitoneal injection of pentobarbital (5 mg/100 g body weight ; Abbott, North Chicago, IL, USA), and perfused transcardially with ice-cold 25 mM phosphate-buffered saline (pH7.4), followed by a fixative containing 3% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains and spinal cords were dissected out, cryoprotected with 0.1 M PB (pH 7.4) containing 20% glycerol for 16 h at 4℃ and frozen with dry-ice. Frozen sections 30 μm thick were prepared using a sliding microtome. The sections were then subjected to the immunohistochemical staining for CT1.

Briefly, free-floating sections were rinsed with TBS, incubated for 1 h in a blocking solution containing 5% normal goat serum (NGS), 0.5% Triton X-100 in Tris-buffered saline (TBS, pH 7.4), and then in the blocking solution containing anti-CT1 (1 : 200) for 16 h at 4℃. After rinsing with TBS, the sections were incubated with biotinylated anti-rabbit IgG (1 : 200, BA-1000, Vector, Burlingame, CA, USA) in TBS, rinsed with TBS and processed using Elite ABC Standard Kit (PK-6100, Vector). After rinsing with 0.1 M PB, localization of HRP was visualized with 0.02% DAB and 0.003% H_2O_2 in 50 mM Tris-Cl solution, pH 7.4 for 5-10 min. The sections were then mounted, dehydrated and coverslipped with DPX (Fluka, Buchs, Switzerland). As a control, the primary antibody was replaced with the same concentration of rabbit pre-immune IgG. For absorption experiments, primary antibody was absorbed with 100 μg/ml of synthetic peptide for 16 h at 4℃ prior to immunohistochemistry.

Images were photographed using a microscope (BX-60, Olympus) equipped with a digital CCD camera (DP50, Olympus).

Double immunostaining method using two antibodies from rabbits

Since our antibodies for CT1, Lin-7 and Lin-2 were made from rabbits, we employed ECSS, a new method for chromogen-based immunohistochemical method for elucidation of the co-expression of two antigens using antibodies from the same species[8].

In brief, sections (30 um) were rinsed several times in TBS and incubated in a blocking solution containing 5% Normal goat serum (NGS) and 0.5% Triton in TBS for 1 h. The sections were then incubated for 16 h at 4℃ in the primary antibody solution After rinsing in TBS, the sections were incubated with HRP-Labeled anti-rabbit secondary antibody solution (K4002, DakoCytomation, Kyoto, Japan) for 1h followed by development with AEC (K3464, Dakocytomation, Japan). The sections were rinsed in 0.1M PB (pH 7.4), mounted temporarily on slides and photographed using the microscope system mentioned above. After removing coverslips, sections were microwaved in 10 mM Citrate buffer (pH 6.0) for ten minutes to inactivate the antigenecity of IgG and the HRP enzyme activity. Subsequently, the sections were incubated in the second primary antibody solution, rinsed with TBS and incubated with HRP-Labeled anti-rabbit secondary antibody solution (K4002, DakoCytomation, Kyoto, Japan). After rinsing in TBS, the sections were stained with 50 mM Tris-Cl (pH 7.4) containing 0.02% DAB and 0.001% H_2O_2. After rinsing with 0.1 M PB, the sections were dehydrated and coverslipped with DPX. In this step, AEC staining on the sections were dissolved in the organic solvents so that the sections did not show any signal for the first primar antibody. The same areas were photographed again to show the coexpression of the signals. Since no labeling was observed following the omission of the primary antibody in the second staining, there was virtually no cross-reactivity between the first staining and the second staining.

The primary antibodies used in this study were listed below : polyclonal rabbit anti-CT1 (1 : 100, original), polyclonal rabbit anti-Lin-7 (1 : 100, SIGMA), polyclonal rabbit anti-Lin-2 (1 : 100, SIGMA)

Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen) at 37°C and 5% CO_2. Transient transfection with Lipofectamine

2000 (Invitrogen, Gaithersburg, MD) was performed according to the manufacturer's instructions. After transfection, the cells were grown 36–48 h before the experiments.

Carnitine transport activity assay

HEK293 cells were plated on 24-well culture plates at a density of 2×10^5 cells/well 24 h prior to transfection, and they were transfected as described above. After 36 h, the culture medium was removed, and the cells were washed three times and incubated in serum-free Hank's solution (containing in mM : 125 NaCl, 5.6 glucose, 4.8 KCl, 1.2 $MgSO_4\cdot7H_2O$, 1.2 KH_2PO_4, 1.3 $CaCl_2\cdot2H_2O$, 25 HEPES (pH 6.0)) for 10 min. The uptake study was started by adding 500 μl of solution containing 40 nM [^{3}H]carnitine to the plate. After 2 min, the cells were washed twice in an ice-cold solution, and lysed in 0.1 N NaOH for 20 min for scintillation counting.

Statistical analysis

Uptake experiments were conducted three times, and each uptake experiment was performed in triplicate. Values are presented as the mean ± standard error. Statistical significance was determined by Student's t test. Differences were considered significant at $P<0.05$.

RESULTS

Isolation of Lin-7 in yeast two-hybrid genetic screen

In our search for putative CT1 binding partners, we used the CT1 C-terminal tail (CT1-CT) as bait in a yeast two-hybrid screen of a cDNA library of the rat adult brain. From 2.7×10^7 independent colonies screened, 32 positive clones were obtained. Of these positive clones, 1 yielded an identical sequence encoding some portions of the gene for rat Lin-7. Lin-7 is a 297-amino acid protein that contains one PDZ domain[9,10] (GenBank accession number AF012281). We could not detect interaction between the CT1-CT with any other PDZ proteins from rat brain cDNA library. For further experiments, we cloned full-length rat Lin-7 by long PCR and subcloned it into a prey vector.

The C-terminus of CT1 is necessary for the interaction with Lin-7

To identify the sites in CT1 that interact with Lin-7, we made three mutant baits. The first one (CT1-CTΔ3) is a bait that lacks the last three residues of CT1, which are known to play a crucial role in PDZ domain recognition. The second and third ones (F557A and T555A), the extreme C-terminal phenylalanine (0 position) or threonine (-2 position) of CT1, have been replaced by alanine, which was expected to abolish or strongly suppress the binding of PDZ proteins[6]. These three baits did not interact with Lin-7 (Fig. 1A). Thus, binding through the C-terminus of CT1 suggests that the PDZ motif of CT1 is the site of interaction with Lin-7.

Domain analysis of Lin-7 protein-protein interaction with CT1 C-terminus

Lin-7 possesses two kinds of protein-protein interaction modules. One is L27 (Lin2/7) domain that preferentially hetero-dimerize and another is PDZ domain that assemble target proteins by binding to a C-terminal motif with a consensus sequence such as (S/T)-X-Ø. To determine the possible interactions of the CT1 C-terminal region with the PDZ domains of Lin-7, we produced prey vectors each containing L27 domain or PDZ domain. The interaction with CT1 C-terminus was observed for PDZ domain of Lin-7 but not for L27 domain (Fig. 1B).

Effect of Lin-7 coexpression on CT1 transport activity

To determine whether CT1/Lin-7 interactions change CT1 activity, we transfected transiently HEK293 cells with the pcDNA3.1 (+) construct containing full-length CT1. At an incubation time of 1 minutes, we demonstrated that the uptake of [^{3}H]carnitine by the wild-type CT1 was from 2.8-fold higher than that by the mock (Fig. 2). When full-length CT1 coexpressed with pcDNA3.1 (+) containing Lin-7, the coexpression clearly increased carnitine transport activity by 1.2-folds (Fig. 2) although there is no statistical significance.

Coexpression of CT1 and Lin-7 in the rat brain

Colocalization of CT1, Lin-7 and Lin-2 were observed in motor neurons in the ventral horn of the lumbar enlargement (Fig. 3a, 3c, 3d). These signals

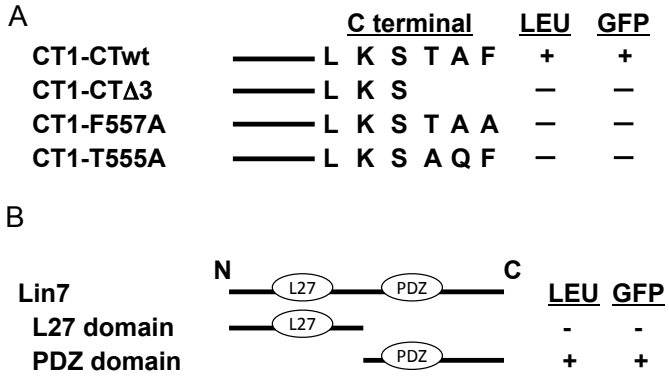

Fig. 1 Specificity of Lin-7 for interaction with C-terminus of CT1 in the yeast two-hybrid system

A, Lin-7 specifically interacted with C-terminal wild-type CT1 but not with the C-terminal CT1 mutants Δ3 (555-557), F557A and T555A and. **B**, the CT1 C-terminal wild-type bait interacts with the prey containing PDZ domain of Lin-7, but not with the prey containing only L27 domain. The bars represent the approximate length of the baits, and the sequence of the last 10 amino acids is shown. The system used for the two-hybrid screen includes the reporter genes LEU2 and GFP, which replace the commonly used *lacZ* gene ; it allows a fast and easy detection of positive clones with long-wave length UV. The results of growth assay and GFP fluorescence monitoring are shown on the right.

were disappeared by absorbing the primary antibody with the synthetic peptide prior to immunohistochemistry (Fig. 3b). Both CT1 and Lin-7 signals were observed in the same neurons (arrows in Fig. 3e and 3i). In the control experiments, no cross reactivity was observed between the first and second stainings (arrowhead in Fig. 3f-h and 3j-l). Both CT1 and Lin-2 signals were observed in the same neurons (arrows in Fig. 3m and 3n). On the other hand, some neurons exhibited only CT1 signals (arrowheads in Fig. 3m and 3n). In the control experiments where the primary antibody was omitted in the second staining, no cross reactivity was observed between the first and second stainings (arrowhead in Fig. 3o and 3p).

DISCUSSION

CT1 is thought to be importance in regulating blood carnitine level in the body. Through a yeast two-hybrid screen of a rat brain cDNA library, we identified Lin-7 as a binding partner of CT1. Lin-7 is a PDZ domain-containing protein that was originally identified as a protein that directly interacts with Lin-2 (or CASK), another PDZ protein in the synapse[11,12]. In addition to Lin-2, Lin-7 has also been reported to interact with several membrane proteins through its PDZ domain ; these proteins include LET 23 receptor tyrosine kinase[13], acid-sensing ion channel ASIC3[14], Kir2.3 channel[15], GABA transporter

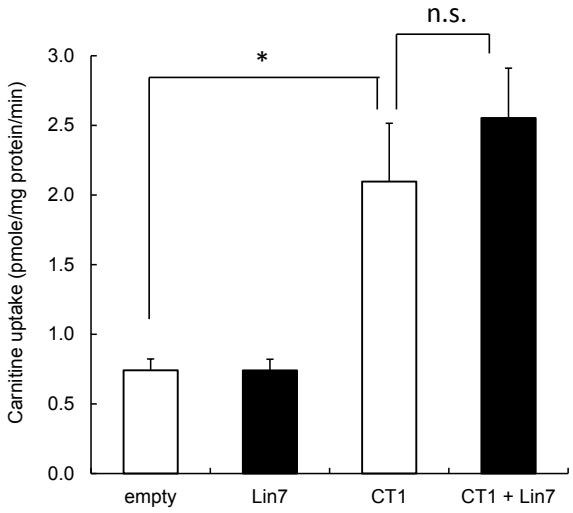

Fig. 2 Effect of Lin-7 on[^{3}H]carnitine transport activity

Coexpression of CT1 and Lin-7 increased carnitine uptake (40 nM) clearly but not significantly in cells transfected with CT1 alone. *, $p<0.05$.

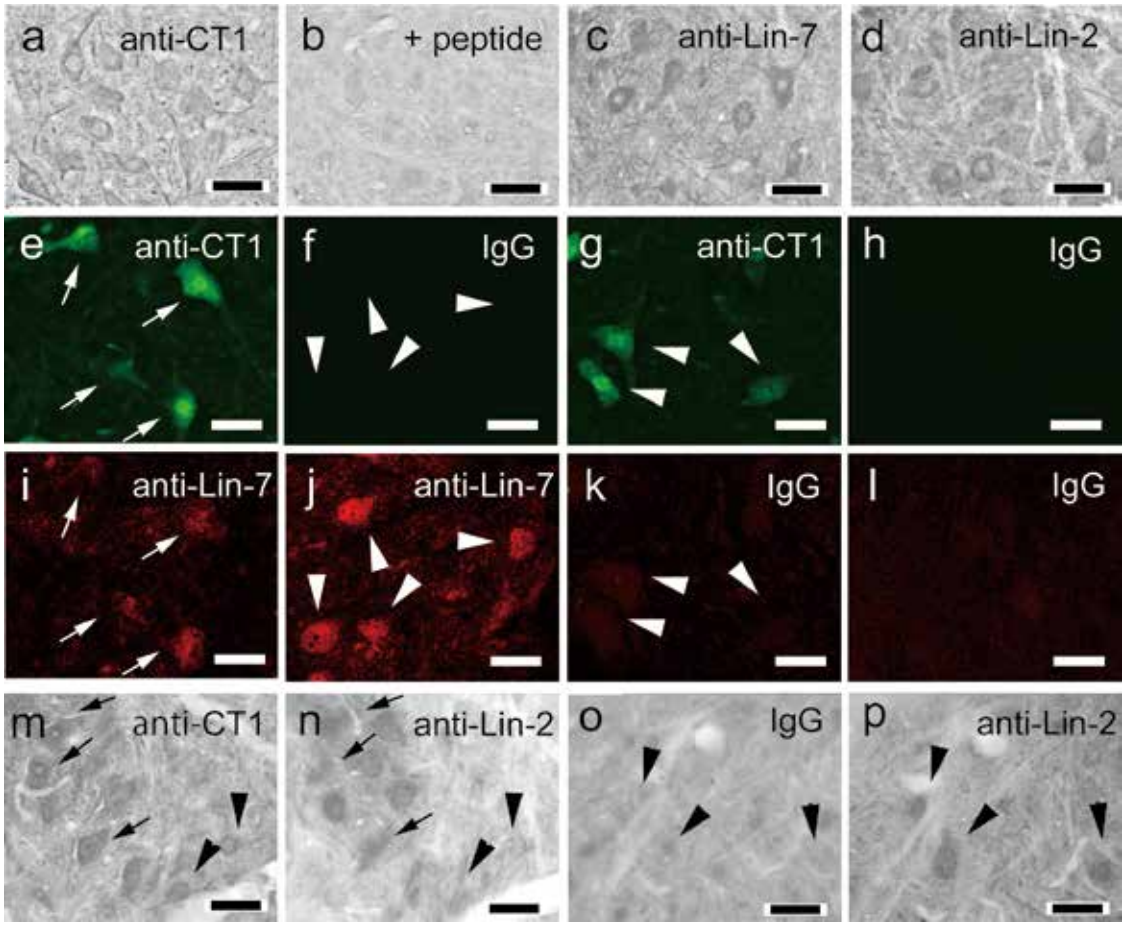

Fig. 3 Colocalization of CT1 and Lin-7 in nervous system

Localization of CT1 (**a**), Lin-7 (**c**) and Lin-2 (**d**) in the ventral horn of the lumbar enlargement. Note that the signals were observed in motor neurons. These signals were disappeared by absorbing the primary antibody with the synthetic peptide prior to immunohistochemistry (**b**). Coexpression of endogenous CT1 and Lin-7 in motor neurons of the ventral horn (e-l). Both CT1 and Lin-7 signals were observed in the same neurons (arrows in **e** and **i**). In the control experiments, no cross reactivity was observed between the first and second stainings (arrowhead in f, g, j and k). (**f** and **j**) The primary antibody was omitted in the second staining. (**g** and **k**) The primary antibody was omitted in the first staining. (**h** and **l**) The primary antibodies were omitted both in the first and second staining. Coexpression of CT1 and Lin-2 in motor neurons of the ventral horn (m-p). Both CT1 and Lin-2 signals were observed in the same neurons (arrows in **m** and **n**). On the other hand, some neurons exhibited only CT1 signals (arrowheads in m and n). In the control experiments where the primary antibody was omitted in the second staining, no cross reactivity was observed between the first and second stainings (arrowhead in **o** and **p**). Scale bars = 50 μm.

BGT1[16], and cholesterol transporter ABCA1[17]. Lin-7 can function as a tripartite complex of Lin-7, Lin-2 and Lin-10[18]. To form this complex, N-terminal of Lin-7 (L27 domain) interacts with the PDZ domain of Lin-2. And the CaM kinase domain of Lin-2 interacts with N-terminal of Lin-10. PDZ domain of Lin-7 interacts with the interacting protein mentioned earlier. The Lin-2/7/10 complex plays the direct role as the membrane protein targeting especially for basolateral localization of LET 23 RTK and neurotransmitter receptor on the postsynaptic neuron in the brain[9].

PDZ (PSD-95, DglA, and ZO-1) domains have been identified in various proteins and are known to be modular protein-protein recognition domains that play a role in protein targeting and protein complex assembly. These domains range from 80 to 90 amino acids in length and bind typically to proteins containing the tripeptide motif (S/T)-X-Ø (X = any amino acid and Ø = a hydrophobic residue) at their C-termini. These multidomain molecules not only target and provide

scaffolds for protein-protein interactions but also modulate the function of receptors and ion channels, by which they associate. The disruption of the association between PDZ proteins and their targets contributes to the pathogenesis of a number of human diseases, most probably because of the failure of PDZ proteins to appropriately target and modulate the actions of associated proteins[6].

Here we report the specific interaction between CT1 and Lin-7 as demonstrated by yeast two-hybrid assays and colocalization experiments. The C-terminal of CT1 (S-T-A-F) constitutes with class I (S/T-X-Ø) of the PDZ-binding motif[6]. All the proteins that interact with Lin-7 have the PDZ-binding motif belonging to class I ; LET-23 RTK, E-T-C-L[13] : ASIC3, V-T-Q-L[14] : Kir2.3, E-S-A-I[15] : BGT1, E-T-H-L[16] : and ABCA1, E-S-Y-V[17]. The results obtained using the various mutants of the CT1 C-terminal in yeast two-hybrid assays (Fig. 1) confirmed the importance of the 0 and –2 positions of the PDZ-binding motif.

The carnitine transport study revealed that the coexpression of Lin-7 with CT1 in HEK293 cells leads to an enhancement of CT1-mediated [^{14}C]carnitine transport although it was not statistical significant (Fig. 2). Previously, we demonstrated that PDZK1, another PDZ protein that has four PDZ domains expressed in several epithelial tissues, interacts and alters the transport function of renal apical transporters such as URAT1[18], OAT4[19], and PEPT2[20], so we examined the possibility that PDZ protein Lin-7 interacts CT1 and alters its transport function. Since there is no report concerning the functional alteration after the binding with Lin-7 to its interacting proteins, the association of Lin-7 with CT1 may be a new example of the role of Lin-7 in modulating the function of their associated proteins.

Rat CT1 mRNA expression was detected in brain as well as the testis, colon, kidney, liver, the skeletal muscle, placenta, and small intestine[4]. Northern blot analysis revealed a message of rat Lin-7 in heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis[10]. Based on these reports, it seemed to be not difficult to detect the coexpression of CT1 and Lin-7 but it was not so easy. We could detect the CT1 protein expression in several region of the brain : for example, in the neocortex, signals were localized in the cell bodies and dendrites of pyramidal cells in layers II, III, V, and VI : in the cerebellum, signals were localized in the cell bodies and dendrites of Purkinje cells : in the hippocampus, the dentate granule cells and the pyramidal cells in Ammon's horn showed strong signals (data not shown). Although we could detect CT1 protein expression in those regions, we could not confirm the colocalization of Lin-7. After the several efforts, we finally found the coexpression of CT1 and Lin-7 together with Lin-2 in motor neurons of the ventral horn (Fig. 3). These results confirm that the protein-protein interaction between CT1 and Lin-7 is not artificial due to the yeast two-hybrid screen but truly occurs in native tissues with physiological importance.

CONCLUSION

In summary, we have identified that the PDZ protein Lin-7 is a binding partner of CT1, which may regulate CT1-mediated carnitine transport activity. A further study of transporter-interacting proteins, such as PDZ proteins, will encourage our understanding of the important regulatory mechanisms different from other transporters.

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Conflict of Interest.

No COI for all authors.

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