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γ-Aminobutyric Acid Transporter 2 Binds to the PDZ Domain of Mammalian Lin-7

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Neurotransmitter transporters, which remove neurotransmittesr from the synaptic cleft, are regulated by second messenger such as protein kinases and binding proteins. Neuronal γ-aminobutyric acid transporters (GATs) are responsible for removing the inhibitory neurotransmitter γ-aminobutyric acid (GABA) from the synaptic cleft. γ-aminobutyric acid transporters 2 (GAT2/BGT1) is involved in regulating neurotransmitter recycling, but the mechanism how they are stabilized and regulated by the specific binding protein has not yet been elucidated. Here, we used the yeast two-hybrid system to identify the specific binding protein(s) that interacts with the C-terminal region of GAT2 and found a specific interaction with the mammalian LIN-7b (MALS-2). MALS-2 protein bound to the tail region of GAT2 but not to other GAT members in the yeast two-hybrid assay. The "T-X-L" motif at the C-terminal end of GAT2 is essential for interaction with MALS-2. In addition, this protein showed specific interactions in the glutathione *S*-transferase (GST) pull-down assay. An antibody to GAT2 specifically co-immunoprecipitated MALS associated with GAT2 from mouse brain extracts. These results suggest that MALS may stabilize GAT2 in brain.

Key words : γ-Aminobutyric acid, γ-aminobutyric acid transporter, PDZ Domain, protein-protein interaction, mammalian LIN-7b

Introduction

Neurons mediate fast signaling between peripheral sensory systems and the central nerve system. One of the hallmarks of normal brain function is the exquisite balance between excitatory and inhibitory activities [23]. Tightly regulated neurotransmitter release by the dynamics of synaptic vesicle and termination of synaptic neurotransmission is critical for normal brain function [24]. Plasma membrane neurotransmitter transporters participate in the reuptake of the neurotransmitters from the synaptic cleft. These transporters are located in presynaptic terminal regions and glial cells [37].

GABA is the major inhibitory neurotransmitter and a trophic factor, promoting neurite outgrowth in the mammalian nervous system [20,26,35]. The GABA system is drug targets for various psychiatric disorders [12,19] and diseases such as epilepsy, anxiety disorders, schizophrenia and pain states are related to the GABA system [21,45,47]. GATs belong to the family of Na^+/Cl^- dependent transporters that also includes transporters for the neurotransmitters such as dopamine, serotonin, norepinephrine and glycine [15,31]. The topologies of GATs consist of 12 hydrophobic transmembrane domains and a large external loop between the third and forth transmembrane domain, which possesses potential N-linked glycosylation sites [2,13,28].

Recently, four different plasma membrane GAT subtypes (GAT1-GAT4) have been identified in mammalian tissues and their pharmacological characteristics were determined through on expression in *Xenopus* oocytes [6,15,27,30]. According to northern blot analysis, GAT1 and GAT4 are localized in the brain only [18,27]. GAT3 are localized in brain and peripheral tissues [8,27]. GAT2/BGT1 is expressed in both the brain and pheriphery and believed to be involved in osmoregulation but has recently also been suggested to play a role in the control of epilepsy [5,7,40,46]. GAT2 cDNA microinjected into cultured hippomcampal neurons is sorted to extrasynaptic loci as indicated by the absence of co-localization with synapsin [1]. Therefore, it is likely that GAT1 and GAT2 would have different expression pattern in neuronal cells.

Protein-protein interactions identify a link between the machinery involved in transmitter release and uptake. Several studies have reported that GAT1 activity can be modulated by protein-protein interactions. Protein interaction between the N-terminal cytoplasmic tail of GAT1 and syntaxin 1A causes a 4-fold decrease in substrate

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transporter rates [9]. The interaction between the C-terminus of GAT1 and Pals1 contributes to the stability of GAT1, thus promoting the expression level of the transporter protein [29]. Most studies to date have focused on the role of the GAT1 in regulating GABA levels. However, the mechanism how GAT2 regulate and bind to the specific proteins has not yet been elucidated.

In order to understand GAT2-mediated regulation of GABAergic neurotransmission, we screened for proteins that interact with the C-terminus of GAT2 through the yeast two-hybrid system and identified MALS-2 a small protein, which, in addition to the calmodulin-associated serine/threonine kinase (CASK) binding N-terminus, contains a postsynaptic density-95 (PSD-95)/discs large/zona occludens (PDZ) domain. The GAT2/MALS interaction suggests that MALS may contribute to the stability of GAT2 in the presynaptic membrane.

Materials and Methods

Plasmid constructs

The cording region of MALS was cloned by RT-PCR from mouse brain, into T-vector (Invitrogen, Carlsbad, CA, USA). After *EcoR*I digestion, the MALS fragment was inserted into the *EcoR*I site of pB42AD (Clontech, Palo Alto, CA, USA). A previously described mouse GAT2 [28] was utilized as a template to amplify the region coding for amino acids 570-628 using the appropriate primers. The amplified fragment was subcloned into T-vector. The fragment was then *EcoR*I-restricted and subcloned into the *EcoR*I site of pLexA. The correct orientation and in-frame cloning of cDNA inserts was verified by restriction enzyme analysis, and DNA sequencing. General recombinant DNA techniques were performed according to standard protocol [38].

Screening of GAT2-binding proteins by yeast two-hybrid assay

The Matchmaker LexA two-hybrid system was used for screening according to the manufacturer's manual (Clontech). In brief, a part of the GAT2 gene (amino acids [aa] 570-628) was fused to the DNA-BD region of the pLexA vector using the PCR method and the plasmid DNA was transformed into yeast strain EGY48 carrying the p8op-lacZ gene. Transformed EGY48 yeast strains containing the GAT2 bait plasmid were transformed with the mouse brain cDNA library [43] and the cells were grown on synthetic dextrose (SD) plates supplemented with glucose but with no histidine, tryptophan, or uracil (SD/-His/-Trp/-Ura). The selection of positive clones was performed on an SD/-His/-Trp/-Ura/-Leu plate containing galactose, raffinose, X-gal, and BU salts. Library plasmids from positive colonies were isolated and rescued using *Escherichia coli (E. coli)* strain KC8 strain on ampicillin-resistant plates. Library inserts were then amplified by PCR and analyzed by restriction digestion. Unique inserts were sequenced and DNA and protein sequence analysis were performed with the BLAST algorithm at the National Center for Biotechnology Information (NCBI). After isolation of the plasmids encoding the library clones, these plasmids were tested for interactions of the reporter gene in yeast by the retransformation. Activation of the reporter genes in the positive colonies was confirmed in the same experiments.

β-Galactosidase activity in liquid cultures of yeast

The strength of the interactions between MALS and GAT2 constructs was assessed by measuring the β-galactosidase activity in liquid cultures or using the two-hybrid system. Yeast was co-transformed with the expression plasmids of the positive clones and the plasmids expressing GAT2 (described above) or other GATs. The β-galactosidase activity in liquid cultures of yeast was assayed as described previously [42]. In brief, mid-log phase transformed yeast cells were collected and permeabilized with 0.1% sodium dodecyl sulphate (SDS) and chloroform. The chromogenic substrate o-nitrophenyl-β-D-galactoside was added in excess to this lysate, the mixture was incubated at 30°C, and the reaction was stopped by increasing the pH to 11 by the addition of 1 M Na_2CO_3. The formation of the reaction product, o-nitrophenol, was determined by measuring absorbance at 420 nm on a spectrophotometer and normalizing for the reaction time and the cell density.

Subcellular fractionation and co-immunoprecipitation

Subcellular fractionation was performed as previously described [41,43]. Mouse brains were homogenized in ice-cold homogenization buffer (0.32 M sucrose, 4 mM HEPES, pH 7.3) supplemented with protease inhibitors. The homogenate was clarified by centrifugation at 900× *g* for 10 min followed by centrifugation at 1,000× *g* for 10 min, producing a pellet (P1) and supernatant (S1). The S1 supernatant was centrifuged again at 12,000× *g* for 15 min, and the resulting supernatant (S2) was saved. For im-

munoprecipitation of the S2 fraction, the samples were diluted in the same volume of 2× binding buffer (50 mM HEPES, 240 mM KCl, 2 mg/ml BSA, 0.2% Triton X-100, pH 7.4) and incubated with anti-GAT2 antibody (Biocompare, South San Francisco, CA, USA) or with control IgG overnight at 4°C, followed by precipitation with protein-A Sepharose (Amersham Pharmacia, Piscataway, NJ, USA). The beads were collected by brief centrifugation and washed three times with TBS-T (20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% tween 20). The pellets were resuspended with Laemmli's loading buffer, the proteins were eluted and denatured by boiling for 2 min and then separated by SDS-PAGE. The gel was transferred to a nitrocellulose membrane and incubated with antibodies against the specific MALS antibodies (Biocompare).

Glutathione S-transferase (GST) pull-down assays

Pull-down assays using GST fusion proteins were performed as follows. cDNAs encoding the C-terminal cytoplasmic region of GAT2 was cloned in pET 41, and the recombinant GST-GAT2 fusion proteins were expressed in bacterial strain BL21 GOLD (Stratagene, La Jolla CA, USA) after induction with 1 mM isopropyl thio-β-D-galactopyranoside (Fisher Biotech, South Australia, Australia). The fusion proteins were purified using glutathione-agarose beads (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. GST alone or GST fusion proteins were dialyzed for 2 hr in PBS using Slide-A-Lyzer (Pierce, Rockford, IL, USA). Ten μg of each of the GST fusion proteins was then coupled to 50 μl of glutathione-agarose beads for each reaction by incubating at room temperature for 1 h, followed by rinsing several times with PBS. The mouse brain S2 fraction was incubated overnight at 4°C with the GST fusion protein-coupled glutathione beads. The beads were pelleted by centrifugation, washed three times with the extraction buffer (1% Triton X-100 in PBS containing 10 μg/ml each aprotinin, leupeptin, and pepstatin and 1 μM phenylmethanesulfonyl fluoride), and once with PBS. The bound proteins were eluted from the glutathione beads with 100 μl of SDS sample buffer. The samples were boiled for 5 min and then processed for SDS-PAGE and Western blotting with antibodies to MALS.

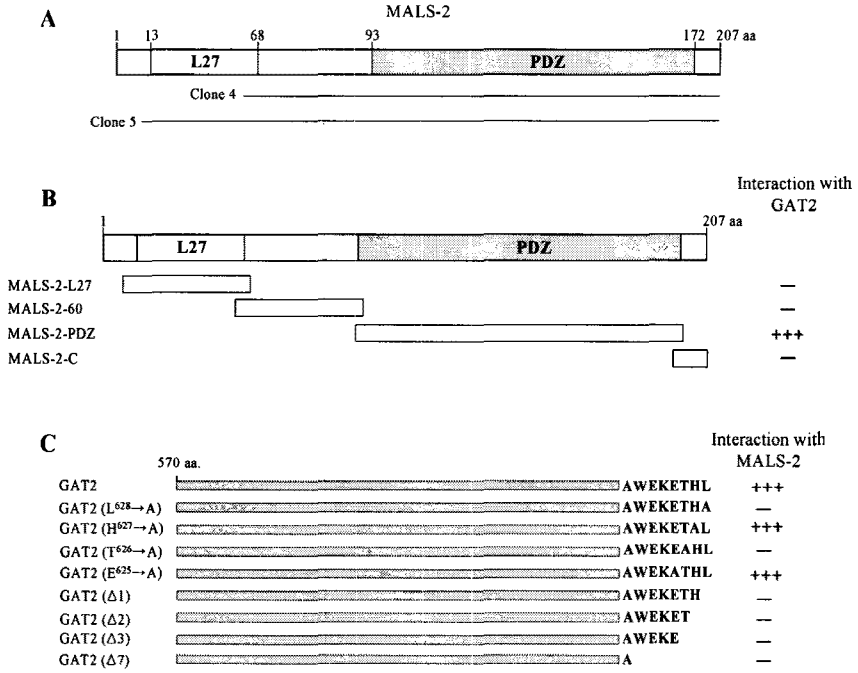

Fig. 1. Identification of the proteins interacting with GAT2 by yeast two-hybrid screening. (A) The domain structure of MALS-2 illustrating that clones 4 and 5 overlap in the C-terminal region. MALS-2 has one PDZ domain. PDZ domain is indicated in gray. aa, the amino acid residue number. (B) Minimal GAT2 binding region in MALS-2. PDZ domain is indicated in gray. Different truncations of MALS-2 were constructed by PCR. Several truncated forms of MALS-2 were tested in the yeast two-hybrid assay for interaction with GAT2. +, interaction with GAT2; -, no interaction with GAT2. aa, the amino acid residue number. (C) Specific interaction of MALS-2 with the C-terminus of GAT2. Several point mutant and deletion mutant forms of GAT2 were tested in the yeast two-hybrid assay for interaction with MALS-2. +, interaction with MALS-2; -, no interaction with MALS-2.

Results

Identification of GAT2 interacting proteins by yeast two-hybrid screening

To examine GAT2-interacting proteins, we screened a mouse brain cDNA library through yeast two-hybrid assays using a portion of GAT2 cytoplasmic region as bait (Fig. 1C). From 1×10^7 colonies screened, we obtained five positive clones. Among these, two clones (clone 4 and 5) were identified as MALS-2 and overlapped at the C-terminal region (Fig. 1A). MALS-2 is a small protein that contains a PDZ domain (Fig. 1B) [32]. To determine the binding domain of MALS-2 that is required for the interaction with GAT2, we constructed a series of deletion mutants of MALS-2. Yeast two-hybrid assays with GAT2 showed that the minimal domain required for binding was critically dependent on the PDZ containing region of the MALS-2 (Fig. 1B).

Next we investigated whether the last three amino acids of GAT2 contain a functional PDZ target motif (T-X-L; T, Thr; X, any amino acid; L, Leu) that mediates protein-protein interaction. For this purpose, a series of C-terminal point mutants and deletion mutants of GAT2 were constructed (Fig. 1C), and co-transfected into yeast cells with pLexA-MALS-2. As shown in Fig. 1C, the T-X-L motif-containing GAT2 can interact with MALS-2. In contrast, the T-X-L deletion mutants and point mutants could not interact with MALS-2. These result indicated that the interaction between GAT2 and MALS-2 is mediated through a PDZ-mediated interaction similar to the previously described interaction between PSD-95 and the N-methyl-D-aspartate (NMDA) receptor 2B subunit of NMDA receptor [11,25,39].

Next we quantified the binding affinity of GAT2 to MALS-2 by measuring β-galactosidase activity in liquid cultures of yeast transformed with the each constructs. The interaction of the GAT2 with MALS-2 yielded approximately 530 U of β-galactosidase activity (data not shown), indicating a sufficient strength to mediate molecular sorting *in vivo* [41]. When the C-terminal cytoplasmic tails of GAT1, GAT2, GAT3 and GAT4 were tested for MALS-2-binding by yeast two-hybrid assays, there was no detectable binding between MALS-2 and the tail domains of GAT1, GAT3 and GAT4 (Fig. 2). These data indicate that the interaction of MALS-2 with GATs is specific to the cytoplasmic region of GAT2 isoform.

GAT2 are associated with MALS *in vitro* and in vivo

To confirm the GAT2 - MALS-2 interaction found using the yeast two-hybrid assay, the direct interaction between GAT2 and MALS-2 was assayed using the GST pull-down assay. Recombinant GST-GAT2 and GST-GAT2 C-terminal deletion fusion proteins were expressed in *E. coli* and purified by affinity chromatography. Immunoblot analysis showed that GAT2 interacted with MALS-2, but not with GST alone, consistent with the yeast two-hybrid results (Fig. 3). GAT2, moreover, interacted with MALS-1 and MALS-3 (Fig. 3). These results were not surprising in view of the fact that MALS-1, MALS-2 and MALS-3 have same PDZ domains. Deletion mutation of the last amino acid

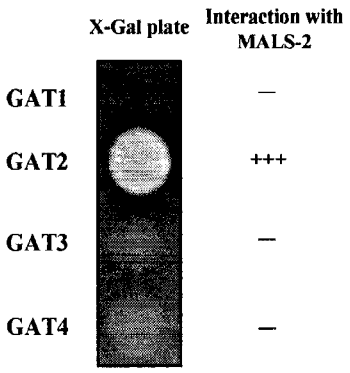

Fig. 2. Interaction between GATs and MALS-2. The indicated amino acid residues of each GATs protein were fused to the pLexA DNA binding domain. MALS-2 specifically interacted with GAT2 protein but not with GAT1, GAT3 or GAT4 (+++, interaction with MALS-2; -, no interaction with MALS-2).

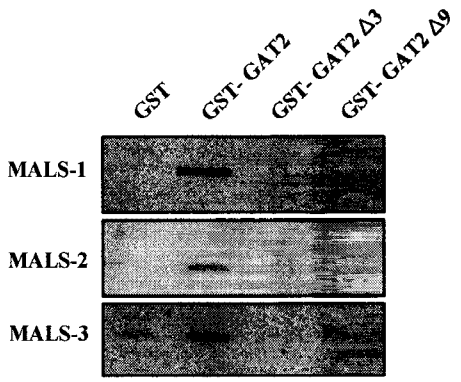

Fig. 3. Association of GAT2 with MALS-2 in the GST pull-down assay. Proteins in the mouse brain lysate were allowed to bind to GST alone or to GST- GAT2 fusion proteins containing the several truncated tail domains of GAT2. The elution fractions were resolved by SDS-PAGE, and Western blotting was performed using an antibody to each MALS.

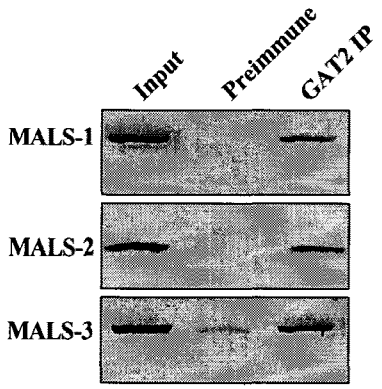

Fig. 4. Co-immunoprecipitation of KLC1 and JSAP1 from brain extracts. Mouse brain lysate was immunoprecipitated with anti-GAT2 antibody or preimmune serum, and the precipitates were immunoblotted with anti-MALS antibodies. Input: 5% of the mouse brain lysates used for each co-immunoprecipitation assay.

residue of GAT2 interrupted interaction with MALS-2 (Fig. 3), indicating that GAT2 and MALS binding is dependent on the PDZ domain of MALS and the C-terminus of GAT2. This result further indicates that the GAT2 directly interacts with MALS.

In order to determine whether the interaction between GAT2 and MALS-2 occurs *in vivo*, we performed co-immunoprecipitation experiments using mouse brain extracts. Lysates from mouse brain were incubated with a GAT2 antibody. Protein G-agarose beads selectively precipitated the immuno-complexes, which were subsequently separated by SDS-PAGE and immunoblotted with MALS antibodies (Fig. 4). As shown in Fig. 4, the three isoforms of MALS was co-immunoprecipitated with GAT2, which are consistent with the yeast two-hybrid and pull-down results. This result indicates that MALS is a specific binding partner of GAT2 *in vivo*.

Discussion

In this study we demonstrate for the first time that the GAT2 directly interacts with MALS in the brain. Their interaction was verified through yeas two-hybrid screen of a mouse brain cDNA library using the inetracellular C-terminal tail of GAT2 as bait, and their interaction in vivo was verified through affinity pull-down assays and co-immunoprecipitations. This study further showed that the three C-terminal amino acids (T-H-L) of GAT2 that encode a PDZ interaction motif are required for the association of MALS with GAT2.

Three MALS genes exist in mammals [22]. Each contains a single N-terminal L27 domain that mediates interaction with the CASK [3,16,32]. This study demonstrated through domain analysis in yeast two-hybrid assay that the interaction is dependent upon the PDZ domain of MALS and the C-terminal amino acids of GAT2. These three amino acids represent a class I PDZ domain ligand [42]. PDZ-domain proteins play important roles in establishing and maintaining an asymmetry of membrane proteins in polarized cells [25]. Proteins containing PDZ domains usually possess multiple protein-protein interaction domains, allowing them to orchestrate multimeric complex formation on specific membrane domains [14]. PDZ domains contain a conserved peptide-binding groove that associates with the extreme C-terminus of interacting protein ligands or with appropriate internal binding motifs [10,17]. Pull-down assays in this study suggest that GAT2 interacts with the PDZ domain of MALS. Similarly, dopamine transporter has been shown to link Pick1. As Pick1 is a PKC-associated protein, this interaction demonstrates the potential for a physical coupling of transporters and regulators [44]. Although we did not show whether GAT2 can bind with other PDZ domain containing proteins, our results suggest that the interaction between GAT2 and MALS-2 is mediated through a PDZ interaction.

An important property of GATs in general, is their ability to be functionally regulated by a wide variety of signal factors [9]. These factors may act directly on the transporter protein or by regulating the interaction of the transporter with other synaptic proteins [11,12,14,16]. Protein-protein interactions not only determine the subcellular localization of membrane proteins, but can also affect their expression level by stabilizing the protein, altering endocytic or exocytic rates. In neuronal culture cell, coexpression of PSD-95 and NMDA receptor NR2B results in decreased endocytosis of NR2B, while deletion of the PDZ interaction motif yields an increase in the percentage of receptor that is internalized [36]. Interestingly, the carboxyl tail of dog GAT2 is responsible for proper targeting and maintenance of GAT2 at basolateral membrane of Madin-Darby canine kidney (MDCK) cells [4,33,34].

How is the association between MALS and GAT2 regulated? One potential regulatory mechanism is the increased level of GAT2 in synaptic terminal membrane. Recent evidence for many different transporter systems suggests that direct interacting proteins of the transporter serves as a tag

that identifies transporters to be internalized [16,36]. This might occur because the tag is indicative of a transporter in an appropriate conformational state for internalization. Thus, like PSD-95, it may be that the interaction between GAT2 and MALS induces conformational state that confers a slowing of GAT2 internalization from synaptic terminal membrane. Further functional studies of this and other MALS interacting proteins may help to shed light on the role of MALS in regulating GAT2 activity.

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초록 : γ-Aminobutyric acid transporter 2와 mammalian Lin-7의 PDZ결합

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신경전달물질을 수송하는 신경전달물질 수송체는 연접전막에서 신경전달물질의 농도를 조절한다. 신경세포에 발현하는 GATs들은 연접에서 억제성 신경전달물질인 GABA의 재흡수에 관여한다. GAT2/BGT1가 어떻게 연접전막에 안정적으로 존재하는지, 어떤 결합단백질과 결합하여 조절을 받는지는 알려져 있지 않다. 본 연구에서 효모 two-hybrid system을 사용하여 GAT2의 C-말단과 특이적으로 결합하는 mammalian Lin-7 (MALS)-2을 분리하였다. GAT2의 C-말단에 존재하는 "T-X-L"아미노산 배열이 MALS-2와의 결합에 필수적으로 관여하였다. 또한 이 단백질간의 결합을 pull-down assay로 확인한 결과 MALS는 glutathione *S*-transferase (GST)와는 결합하지 않으나 GST-GAT2와는 결합하였다. 또한 생쥐의 뇌 균질액에서 GAT2는 MALS와 함께 침강함을 면역침강으로 확인하였다. 이러한 결과들은 MALS가 GAT2와 결합하여 GAT2를 연접전막에서 안정화시키는 역할을 함을 시사한다.