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Muskelin Interacts with Multi-PDZ Domain Protein 1 (MUPP1) through the PDZ Domain

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Protein-protein interactions have a critical role in the regulation of many cellular functions. Postsynaptic density-95/disks large/zonula occludens-1 (PDZ) domain is one of domains that mediate protein-protein interactions. PDZ domains typically bind to the specific motif at the carboxyl (C)-terminal end of partner proteins. Multi-PDZ domain protein 1 (MUPP1), which has 13 PDZ domains, serves a scaffolding function for structure proteins and signaling proteins, but the cellular function of MUPP1 has not been fully elucidated. We used the yeast two-hybrid system to identify proteins that interact with PDZ domains of MUPP1. We found an interaction between MUPP1 and muskelin. Muskelin was recently identified as a $GABA_A$ receptor ($GABA_AR$) α1 subunit binding protein and known to have a role in receptor endocytosis and degradation. Muskelin bound to the 3rd PDZ domain, but not to other PDZ domains of MUPP1. The C-terminal end of muskelin was essential for the interaction with MUPP1 in the yeast two-hybrid assay. When co-expressed in HEK-293T cells, muskelin but not the C-terminal deleted muskelin was co-immunoprecipitated with MUPP1. In addition, MUPP1 co-localized with muskelin at the same subcellular region in cells. These findings collectively suggest that MUPP1 or its interacting proteins could modulate $GABA_AR$ trafficking and turnover through the interaction with muskelin.

Key words : Adaptor protein, MUPP1, muskelin, PDZ domain, protein-protein interaction

Introduction

Protein-protein interactions can determine the localization of proteins at specific subcellular sites and the incorporation of receptors into signaling complexes [2]. Cell junctions and neuronal synapse are emerging as multi-molecular composites whose structure and regulation are governed in part by their associated proteins [12]. Protein-protein interactions mediated by a variety of domains, functionally independent unit structures of protein, are critical for the formation of functional protein networks that regulate cellular mechanisms. Postsynaptic density-95/disks large/zonula occludens-1 (PDZ) domain is one of those domains that mediate protein-protein interactions [10, 20, 25]. PDZ domain-containing proteins are generally soluble cytoplasmic proteins that act as adaptors by linking the cell membrane receptors via PDZ domains or other protein modules to cytoskeletal proteins or signaling proteins such as regulators of membrane trafficking, protein kinases and regulators of small GTPases [8-10, 12, 20, 25]. PDZ domains are built of 80~100 amino-acid residues and specialized for binding of the carboxyl (C)-terminal PDZ-association motif of partner proteins, including transmembrane receptors, channel proteins, and other adaptor proteins [7, 20, 26]. Such interactions localize membrane proteins to specific subcellular domains, thus enabling assembly of large multi-molecular complexes [25].

Multi-PDZ domain protein 1 (MUPP1), which possesses an L27 domain and 13 PDZ domains, was first identified as a protein that interacts with the C-terminus of the serotonin receptor type 2C ($5\text{-}HT_{2C}$) in brain [27]. MUPP1 is found in tight junctions, post-synaptic density (PSD), and Schwann cell incisures and has been reported to interact with a variety of integral membrane proteins, including a synaptic adhe-

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sion molecule Cadm1, junctional adhesion molecule-A, sodium channel Nav1.4, melatonin receptor MT_1, Claudin-1, and γ-aminobutyric acid receptor 2 [1, 3, 4, 6, 13, 17]. MUPP1 acts as a scaffold for attaching different proteins to the proper location in the membrane [11]. MUPP1 also interacts with synaptic Ras GTPase-activating protein SynGAP, and Ca^{2+}/calmodulin-dependent kinase (CaMKII) to regulate neuronal signaling and dendritic spine morphology [3, 11, 18, 19].

To help define the scaffolding function of MUPP1, it is necessary to identify the interacting proteins of MUPP1. We screened for proteins that interact with the PDZ domains of MUPP1 through the yeast two-hybrid assay and identified muskelin, a multi-domain scaffolding protein, known to affect cytoskeletal dynamics and microtubule-dependent $GABA_A$ receptor ($GABA_AR$) trafficking [14]. The MUPP1 and muskelin interaction suggests that MUPP1 may contribute as an adaptor protein/scaffolding protein in regulation of $GABA_AR$ trafficking through the interaction with muskelin.

Materials and Methods

Plasmid constructs

Full-length rat MUPP1 cDNA in the pCMV vector (a gift from Dr. H. Lubbert, Ruhr-Universitat, Denmark) was tagged with a FLAG-epitope at the amino (N)-terminus. Truncations of MUPP1 corresponding to different PDZ domains were prepared by PCR amplification using the appropriate primers. The amplified fragments were subcloned into T-vector. The fragments were then *EcoR*I-restricted and subcloned into the *EcoR*I site of pLexA. The correct orientation and in-frame cloning of cDNA inserts were verified by restriction enzyme analysis and DNA sequencing. EGFP-fused muskelin was constructed and used to visualize the intracellular localization in mammalian cells. General recombinant DNA techniques were performed according to standard protocol [22].

Screening of MUPP1-binding proteins by yeast two-hybrid assay

The Matchmaker LexA two-hybrid system was used for screening according to the manufacturer's manual (Clontech, Palo Alto, CA, USA). In brief, a part of the rat MUPP1 cDNA (amino acids 101-507) was fused to the DNA-BD region of the pLexA vector using the PCR and the plasmid DNA was transformed into yeast strain EGY48 carrying the p8op-lacZ gene. Transformed EGY48 yeast cells containing the MUPP1 bait plasmid were transformed with the mouse brain cDNA library and 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. Plasmids from positive clones were analyzed by restriction digestion. Unique inserts were sequenced and protein sequence analysis was performed with the BLAST algorithm at the National Center for Biotechnology Information (NCBI). Sequence-verified clones were tested again for interaction with the bait in yeast by retransformation.

Cell culture and Transfection

HEK-293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, l-glutamine, and antibiotics. Transient transfections were done with the $CaPO_4$ precipitation method.

Co-immunoprecipitation

Twenty-four hours after transfection with FLAG-MUPP1 and HA-muskelin constructs, HEK-293T cells were rinsed with ice-cold PBS twice and lysed with ice-cold lysis buffer [PBS containing 0.5% NP-40 and 1x protease inhibitor cocktail set V (Calbiochem)] by gentle rotation for 30 min. Lysates were centrifuged at 16,000 × *g* for 10 min at 4°C. The supernatant was incubated with anti-FLAG M2 agarose beads (Sigma-Aldrich) for 2 hr at 4°C with constant shaking. The beads were collected by centrifugation at 2,000 × *g* for 30 sec and washed 5 times with ice-cold lysis buffer. The immunoprecipitated proteins were analyzed by Western blotting.

Immunocytochemistry

HEK-293T cells grown on poly-D-lysine-coated coverslips were transfected with EGFP-muskelin and MUPP1 constructs. Twenty-four hours after transfection, cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS for 5 min, and permeabilized with 0.2% Triton X-100 in PBS for 10 min. After blocking with 5% normal goat serum in PBS for 30 min, cells were incubated with anti-MUPP1 antibody (BD science, San Jose, CA, USA) diluted 1:500 in PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween-20 overnight at 4°C. After washing with PBS 3 times, cells were incubated with Dylight

594-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch Labs, West Grove, PA, USA) diluted 1:800 for 40 min. After washing with PBS 3 times, the cells were mounted with Fluoromount (DAKO). Fluorescence images were acquired on Zeiss LSM510 META confocal laser scanning microscope (Carl Zeiss, Oberkochem, Germany).

Results

Identification of MUPP1 interacting proteins by yeast two-hybrid screening

To identify MUPP1-binding proteins, we screened a mouse brain cDNA library through the yeast two-hybrid assays using the amino (N)-terminal region of MUPP1 containing 1st-3rd PDZ domains as bait (Fig. 1B). From 6×10^6 colonies screened, we obtained one positive clone. The clone possessed a cDNA fragment of muskelin (Fig. 1A). Muskelin is a cytoplasmic multi-domain protein comprised of discoidin-like domain, LisH motif, CTLH motif, and six repeated kelch motifs [5]. To identify the domain of muskelin required for interaction with MUPP1, various fragments of muskelin were constructed and tested for interaction with MUPP1 using yeast two-hybrid system (Fig. 1A). Fig. 1A shows that the short C-terminal region of muskelin was critically required for interaction with MUPP1. To determine the domain of MUPP1 that is required for the interaction with muskelin, we constructed various fragments of MUPP1. Yeast two-hybrid assays with muskelin showed that the minimal domain required for binding was the 3rd PDZ domain of MUPP1 (Fig. 1B). Muskelin contains a putative class

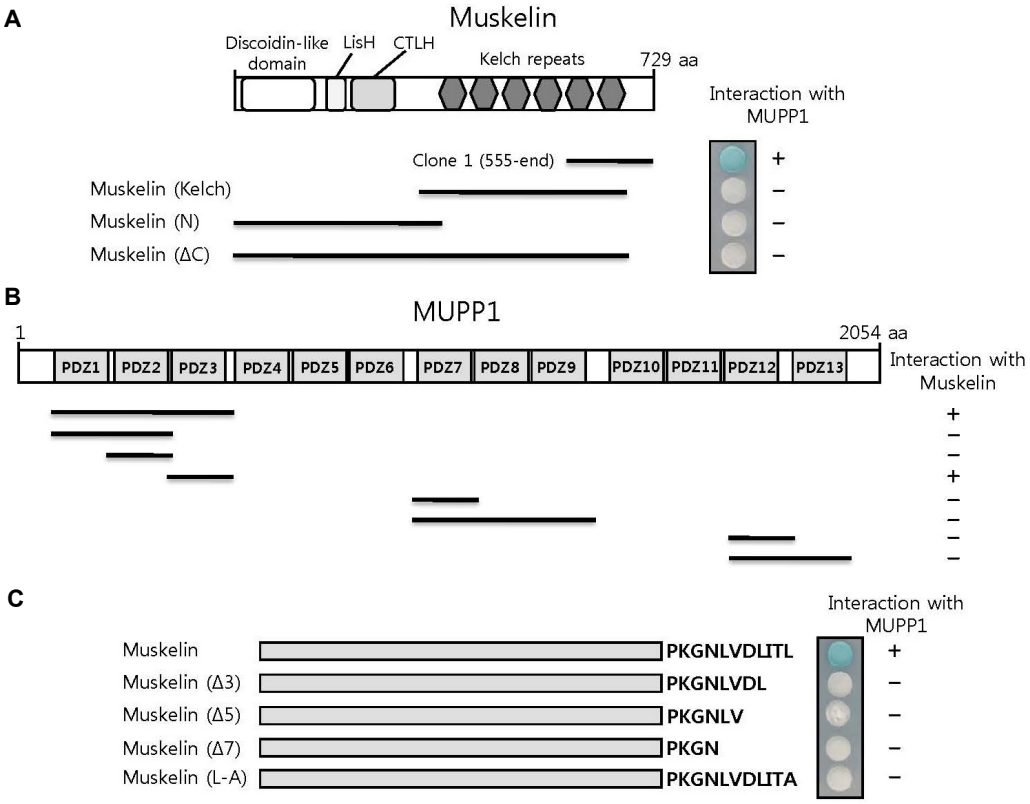

Fig. 1. Identification of the protein interacting with MUPP1 by yeast two-hybrid screening. (A) Schematic diagram of muskelin. Muskelin contains the discoidin-like domain, LisH motif, CTLH motif, and six repeated kelch motifs. Clone 1 was isolated from the yeast two-hybrid screen and different truncations of muskelin were constructed by PCR. Several truncated forms of muskelin were tested in the yeast two-hybrid assay for interaction with MUPP1. +, interaction with MUPP1; -, no interaction with MUPP1. aa, the amino acid residue number. (B) Minimal muskelin binding region in MUPP1. Different truncations of MUPP1 were constructed by PCR. Several truncated forms of MUPP1 were tested in the yeast two-hybrid assay for interaction with muskelin. +, interaction with muskelin; -, no interaction with muskelin. aa, the amino acid residue number. (C) Specific interaction of MUPP1 with the C-terminus of muskelin. Several deletion and substitution mutants of muskelin were tested in the yeast two-hybrid assay for interaction with MUPP1. +, interaction with MUPP1; -, no interaction with MUPP1.

II PDZ-association motif (ϕXϕ), where ϕ is a hydrophobic residue, at its C-terminus [7, 25, 26]. Next we investigated whether the C-terminal motif of muskelin mediates the interaction with MUPP1. For this purpose, the C-terminal deletion and substitution mutants of muskelin were constructed (Fig. 1C), and co-transfected into yeast cells with pLexA-MUPP1. As shown in Fig. 1C, the MUPP1 and muskelin interaction was impaired by the C-terminal deletion and the substitution of the last C-terminal residue of muskelin. These results indicate that MUPP1 and muskelin interact each other through their PDZ domain and PDZ-association motif, respectively, similar to the previously described class II PDZ interaction [16, 26].

MUPP1 is associated with muskelin in cells

To assess the interaction between MUPP1 and muskelin in mammalian cells, HEK-293T cells were co-transfected with constructs expressing HA-muskelin and FLAG-MUPP1. Cell lysates were immunoprecipitated with a monoclonal antibody against the FLAG epitope, followed by western blot analysis with anti-HA antibody. Fig. 2A shows that muskelin was co-precipitated with MUPP1. In contrast, HA-muskelin (Δ3) lacking the putative PDZ-association motif of muskelin failed to be co-precipitated with MUPP1 (Fig. 2B). These results further confirmed our yeast two-hybrid results, indicating that MUPP1 specifically interacts with muskelin and the C-terminal motif of muskelin is essential for the interaction.

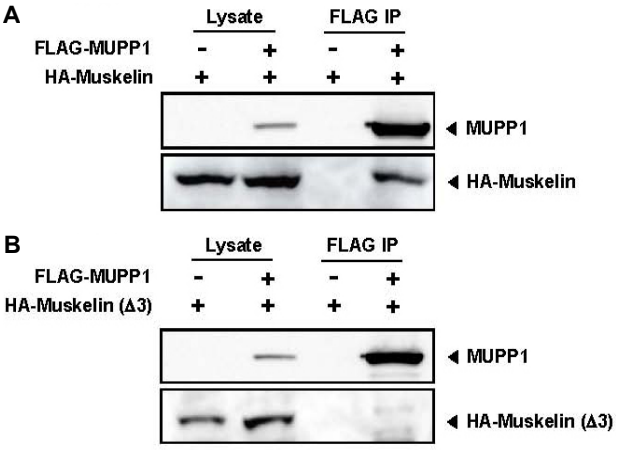

Fig. 2. MUPP1 and muskelin were co-immunoprecipitated from mammalian cells. (A) HEK-293T cells were transiently transfected with HA-muskelin plasmid (A) or HA-muskelin (Δ3) plasmid (B) and either control vector or FLAG-MUPP1 plasmid as indicated. Cell lysates were incubated with monoclonal anti-FLAG M2 agarose beads to immunoprecipitate MUPP1. Western blots were subsequently probed with anti-HA and anti-MUPP1 antibodies. Muskelin was specifically co-immunoprecipitated with MUPP1.

For the potential interaction between MUPP1 and muskelin to be physiologically relevant, two proteins must co-localize at the same subcellular region in cells. To determine whether MUPP1 and muskelin co-localize, we generated the N-terminal EGFP-fused muskelin construct. MUPP1 was co-transfected with EGFP-muskelin into HEK-293T cells. Confocal microscopic images of EGFP-muskelin (green channel) and MUPP1 (red channel) showed that MUPP1 and muskelin co-localized at the same subcellular region in cells (Fig. 3A). Both proteins formed puncta along cytoplasmic membrane and extensively overlapped at the same subcellular region in cells (Fig. 3B). These findings indicate that MUPP1 and muskelin interact with each other in cells.

Discussion

In this study, we have shown that the scaffold protein MUPP1 associates with muskelin. Using the N-terminal PDZ domains of MUPP1 as bait, we identified muskelin in a yeast two-hybrid screen of a mouse brain cDNA library. When MUPP1 and muskelin were expressed in HEK-293 T cells, they co-immunoprecipitated and co-localized in cells

Specific protein-protein interactions are important for intracellular protein transport and biological signal transduction. The PDZ domain is one of the most abundant protein interaction modules. Proteins containing PDZ domains usually form large multimeric protein complexes [10, 20, 25]. PDZ domains contain a conserved peptide-binding groove that associates with the extreme C-terminus of ligands [7, 26]. Interestingly, MUPP1 contains multiple PDZ domains and plays an important role as a multivalent scaffold protein that recruits various proteins [27]. In this study, we demonstrated through domain analysis that the 3rd PDZ domain of MUPP1 specifically mediates the interaction with the C-terminal region of muskelin.

The N-terminal region of muskelin containing the discoidin domain and LisH motif binds to the $GABA_AR$ and this interaction regulates the endocytosis and degradation of $GABA_AR$ [14]. In recent report, the LisH motif acts as a dimerization element of muskelin and the LisH-dependent dimerization is required to assemble a muskelin tetramer by intermolecular head-to-tail interaction [5]. Interestingly, the loss of the LisH-dependent dimerization leads to relocali-

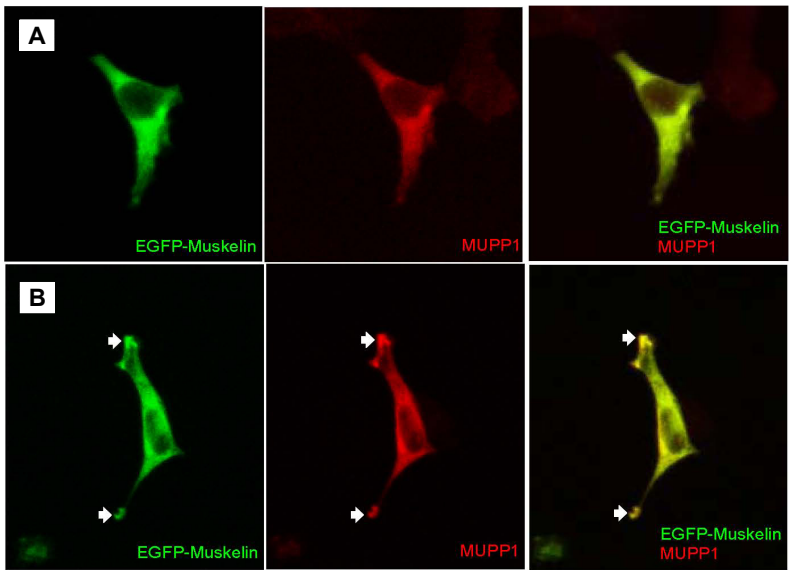

Fig. 3. Co-localization of MUPP1 and muskelin at subcellular region. Twenty-four hours after transfection, cells were immunostained using anti-MUPP1 antibody. (A) EGFP-muskelin and MUPP1 co-localize largely in cells. (B) EGFP-muskelin and MUPP1 are seen at the same subcellular region in cells (arrow).

zation of muskelin from the cytoplasm to the nucleus and impairs the $GABA_AR$ transport [5].

What would the association between MUPP1 and muskelin mean? First, the interaction may have a role in regulation of the cell surface expression level of $GABA_AR$. The association with MUPP1 and muskelin possibly affects internalization of $GABA_AR$ from membrane surface. Protein-protein interactions not only determine the specific membrane surface expression of receptor proteins, but can also affect the membrane surface expression level by altering endocytic rates [15]. Direct interacting proteins of the receptor could serve as a tag that identifies receptor proteins to be internalized [15, 21]. This might occur because the tag is indicative of a receptor protein in an appropriate conformational state for internalization. Thus, like PSD-95 and GRIP, the interaction between MUPP1 and muskelin may indicate conformational state that determines $GABA_AR$ internalization from membrane surface [15]. Second, MUPP1-muskelin complex may mediate subcellular targeting of $GABA_AR$ to appropriate subcellular localization. Several PDZ domain-containing proteins, such as mLin-10 and GRIP1 act as targeting/scaffolding proteins that have potential to bring their interacting proteins to appropriate subcellular localization [23, 24]. Therefore, the association of muskelin with MUPP1 could target $GABA_AR$ to specific subcellular location for appropriate functions. Our findings provide insight into the possible regulation of $GABA_AR$ by MUPP1-muskelin complex through PDZ domain-mediated interaction. Further functional studies on the possibilities mentioned above and identification of other MUPP1 interacting proteins may help to shed light on regulation of $GABA_AR$.

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초록 : Muskelin과 multi-PDZ domain protein 1 (MUPP1) 단백질의 PDZ 도메인을 통한 결합

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단백질-단백질 결합은 다양한 세포내 반응 조절에서 중요한 역할을 한다. Postsynaptic density-95/disks large/zonula occludens-1 (PDZ) 도메인은 널리 알려진 단백질-단백질 결합 매개 도메인 중 하나이다. PDZ 도메인은 결합 단백질의 카르복실(C)-말단의 특정 motif와 결합한다. Multi-PDZ domain protein 1 (MUPP1)은 13개 PDZ 도메인을 가지는 단백질로서 다양한 구조단백질 및 신호단백질에 대한 scaffold로 작용한다고 알려져 있지만 MUPP1의 세포 내 기능은 아직 명확히 밝혀지지 않았다. 본 연구에서 MUPP1의 PDZ 도메인과 결합하는 단백질을 규명하기 위하여 효모 two-hybrid 방법을 이용하였고 muskelin이 MUPP1과 결합하는 것을 확인하였다. Muskelin은 $GABA_A$ 수용체($GABA_AR$)의 α1 subunit와 결합하며 수용체의 endocytosis와 분해에 관여하는 것으로 알려져 있다. Muskelin은 MUPP1의 3번째 PDZ 도메인과 결합하지만, 다른 PDZ 도메인과는 결합하지 않았다. 또한 MUPP1과의 결합에 muskelin의 C-말단부위가 필수적임을 효모 two-hybrid 방법으로 확인하였다. HEK-293T 세포에 MUPP1과 muskelin을 동시에 발현하여 면역 침강한 결과 두 단백질은 같이 면역 침강하였다. 반면에 C-말단 결손 muskelin은 MUPP1과 같이 면역 침강하지 않았다. 또한 muskelin과 MUPP1은 세포내의 같은 위치에서 발현하였다. 이러한 결과들은, muskelin과의 결합을 통해, MUPP1 혹은 MUPP1과 결합하는 단백질이 $GABA_AR$의 세포내 이동과 회전(turnover)을 조절할 가능성을 시사한다.