ISSN (Print) 1225-9918
ISSN (Online) 2287-3406
Journal of Life Science **2014** Vol. 24. No. 8. 820~826 DOI : http://dx.doi.org/10.5352/JLS.2014.24.8.820

Parkin Interacts with the PDZ Domain of Multi-PDZ Domain Protein MUPP1

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Received June 23, 2014 /Revised August 14, 2014 /Accepted August 14, 2014

The localization to specific subcellular sites and the regulation of cell surface receptors and channels are crucial for proper functioning. Postsynaptic density-95/Disks large/Zonula occludens-1 (PDZ)-domain is involved in recognition of and interaction between various proteins, by which the localization and the regulation are mediated. Multi-PDZ domain protein 1 (MUPP1) contains 13 PDZ domains. MUPP1 serves a scaffolding function for structure proteins and signaling proteins, but the mechanism how MUPP1 is stabilized and signalized has not yet been 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 Parkin. Parkin is an E3 ubiquitin ligase. Loss-of-function mutations of *Parkin* gene are known to cause an autosomal recessive juvenile parkinsonism. Parkin bound to the 12th PDZ domain, but not to other PDZ domains of MUPP1. The C-terminal end of Parkin has a type II PDZ-association motif, which was essential for the interaction with MUPP1 in the yeast two-hybrid assay. When co-expressed in HEK-293T cells, Parkin co-localized with MUPP1. When co-expressed with ubiquitin in HEK-293T cells, MUPP1 has been strongly ubiquitinated by Parkin. These findings collectively suggest that MUPP1 is a novel substrate of Parkin and its function or stability could be modulated by Parkin-mediated ubiquitination.

Key words : MUPP1, Parkin, PDZ domain, protein-protein interaction, ubiquitination

Introduction

The localization of membrane receptors at specific subcellular site can be crucial for proper function. The cell-cell interactions, such as cell junctions and neuronal synapse are emerging as multimolecular composites whose structure and regulation are governed in part by their associated proteins [13]. PSD-95/Dlg/Zo-1 (PDZ)-domain containing proteins are modular proteins that act as adaptors, by linking the cell membrane receptors via PDZ domains or other protein modules to cytoskeletal proteins or signaling proteins that include regulators of membrane trafficking, protein kinase and regulators of small GTPase, such as guanine exchange factors (GEFs) and GTPase activating proteins (GAPs) [9-11, 13, 22, 24].

PDZ domains are built of 80~100 amino-acid residues and specialized for binding of the carboxyl (C)-terminus in partner proteins, which are most often transmembrane receptors and channel proteins [8, 22, 27]. Such interactions localize membrane proteins to specific subcellular domains, thus enabling assembly of large molecular complexes [24]. The role of PDZ domains in clustering and localization of proteins at the plasma membrane has important biological implications, e.g., in signaling, mediating the adhesive properties of particular cells, ion transport, and formation of the paracellular barriers also known as tight junctions [1, 10, 11, 13, 22].

Multi-PDZ domain protein 1 (MUPP1) was identified as a protein that interacts with the C-terminus of the serotonin 5-hydroxytryptamine type 2C (5-HT$_{2C}$) receptor [29]. It is highly expressed in the central nervous system, with highest levels in all cerebral cortical layers, the hippocampus, the granular layer of the dentate gyrus, and the choroid

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plexus, and enriched in synaptosomes, specifically in post-synaptic density (PSD) [4, 26]. MUPP1, which possesses an L27 domain and 13 PDZ domains has been reported to interact with a variety of integral membrane proteins, including a synaptic adhesion molecule Cadm1, junctional adhesion molecule-A, a sodium channel Nav1.4, a melatonin receptor MT_1, Claudin-1, and γ-aminobutyric acid receptor 2 using different PDZ domains [1, 3, 4, 12, 14, 18]. MUPP1 might play a multifaceted role in assembling and localizing of the integral membrane proteins.

To help define the 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 the E3 ubiquitin ligase Parkin, the causal protein responsible for hereditary recessive early-onset parkinsonism [5, 19, 21, 25]. The MUPP1 and Parkin interaction suggests that MUPP1 may be a direct substrate for Parkin-mediated ubiquitination.

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 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 were verified by restriction enzyme analysis and DNA sequencing. EGFP-fused Parkin were constructed and used to visualize the intracellular localization in mammalian cells. General recombinant DNA techniques were performed according to standard protocol [23].

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 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.

β-Galactosidase activity in liquid cultures of yeast

The β-galactosidase activity of yeast was assayed as described previously [28]. Mid-log phase yeast cells were collected and permeabilized with 0.1% sodium dodecyl sulphate (SDS) and chloroform. An excess amount of o-nitrophenyl-β-D-galactoside (ONPG) was added to yeast lysate, and the mixture was incubated at 30℃, and then the reaction was stopped by increasing 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. The units of enzyme activity were calculated by the following equation: units $=1000\times[(OD_{420}-1.75\times OD_{550})]$ / (reaction time (min) × culture volume (ml) × OD_{600}) [2]. All experiments were independently performed at least three times.

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.

Immunocytochemistry

Cells grown on poly-D-lysine-coated coverslips were transfected with EGFP-Parkin 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℃. 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).

Ubiquitination Assay

Twenty-four hours after transfection of myc-Parkin, HA-Ub, and MUPP1 constructs, cells were rinsed with PBS twice and lysed with SDS lysis buffer [PBS containing 1% SDS and 1X protease inhibitor cocktail set V (Calbiochem, Darmstadt, Germany)]. Lysates were subjected to sonication and then boiled for 10 min. Lysates were centrifuged at 16,000× *g* for 10 min at room temperature. The supernatant was diluted 1:10 with PBS containing 0.5% NP-40 and incubated with anti-FLAG M2 agarose beads (Sigma-Aldrich, St. Louis, MO, USA) for 4 h at room temperature with constant rocking. The beads were collected by centrifugation at 2,000× *g* for 30 sec and washed 5 times with PBS containing 0.5% NP-40. The immunoprecipitated proteins were analyzed by Western blotting.

Results

Identification of MUPP1 interacting proteins by yeast two-hybrid screening

To identify MUPP1-interacting proteins, we screened a mouse brain cDNA library through the yeast two-hybrid assays using the C-terminal region of MUPP1 containing 12th and 13th PDZ domains as bait (Fig. 1A). From 8×10^{6} colonies screened, we obtained one positive clone. This clone possessed a cDNA fragment of Parkin (Fig. 1B). MUPP1 contains 13 homologous protein binding PDZ domains (Fig. 1A) [29]. To determine the binding domain of MUPP1 that is required for the interaction with Parkin, we constructed various fragments of MUPP1. Yeast two-hybrid assays with Parkin showed that the minimal domain required for binding was critically dependent on the 12th PDZ domain of MUPP1 (Fig. 1A). Parkin contains a type II PDZ-association motif (ϕXϕ), where ϕ is a hydrophobic residue, at its C-terminus [8, 24]. Next we investigated whether the C-terminal PDZ-association motif of Parkin mediates protein-protein interaction. For this purpose, a series of C-terminal deletion mutants of Parkin were constructed (Fig. 1B), and co-transfected into yeast cells with pLexA-MUPP1. As shown in Fig. 1B, the C-terminal deletion mutants of Parkin did not interact with MUPP1. These result indicated that the inter-

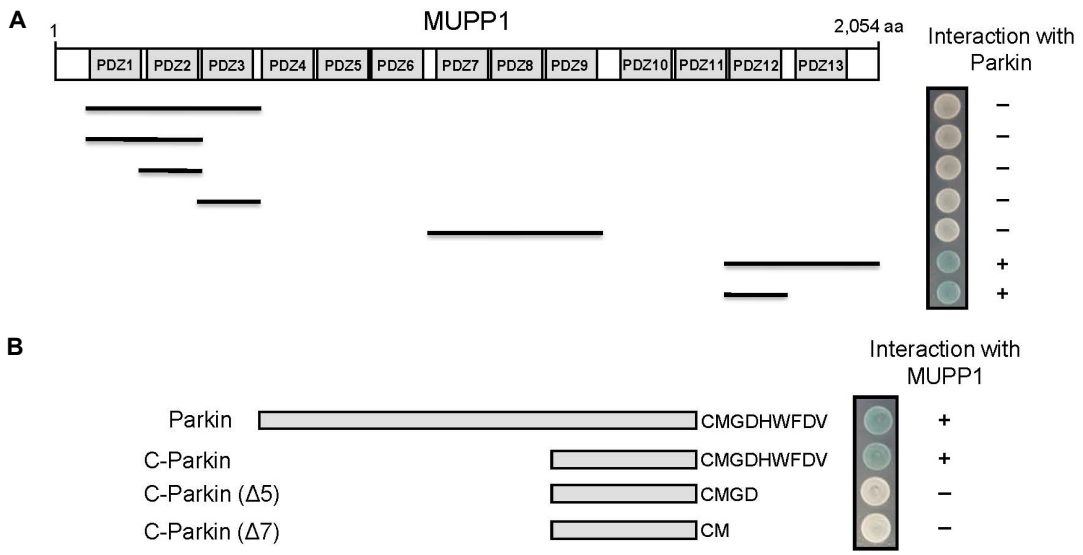

Fig. 1. Identification of the proteins interacting with MUPP1 by yeast two-hybrid screening. (A) Minimal Parkin binding region in MUPP1. Different truncations of MUPP1 were constructed by PCR. Several truncated forms of MUPP1 were tested in the yeast two-hybrid assay for interaction with Parkin. +, interaction with Parkin; -, no interaction with Parkin. PDZ domains are indicated in gray. aa, the amino acid residue number. (B) Specific interaction of MUPP1 with the C-terminus of Parkin. Several deletion mutants of Parkin were tested in the yeast two-hybrid assay for interaction with MUPP1. +, interaction with MUPP1; -, no interaction with MUPP1.

action between Parkin and MUPP1 is mediated through a PDZ-mediated interaction similar to the previously described type II PDZ interaction [8, 24].

To determine whether other PDZ domain containing proteins interact with Parkin, we tested four different PDZ domain containing proteins using yeast two-hybrid assay. Fig. 2A shows that Parkin did not interact with other PDZ domain containing proteins, glutamate receptor-interacting protein 1 (GRIP1), X11L/Mint-2, PSD-95, and Veli-2. A quantitative β-galactosidase assay showed that the C-terminus of Parkin is essential in binding MUPP1 and its deletion impairs the binding with MUPP1 (Fig. 2B). These data indicated a specific interaction between MUPP1 and Parkin through the 12th PDZ of MUPP1 and the PDZ-association motif of Parkin.

MUPP1 colocalizes with Parkin in cells and is a substrate for Parkin-mediated ubiquitination

Conjugation of ubiquitin to protein requires the conserted activity of three enzymes, an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme, and an E3 ubiquitin ligase. A previous report has shown that Parkin is an E3 ubiquitin ligase [30]. To determine whether Parkin ubiquitinates MUPP1, we carried out an ubiquitination assay by co-transfection with FLAG-MUPP1, HA-ubiquitin, and myc-Parkin plasmids in HEK-293T cells. High molecular weight, HA-ubiquitinated proteins were observed in the presence of FLAG-MUPP1, HA-ubiquitin, and myc-Parkin but not in control cultures lacking either myc-Parkin or HA-ubiquitin (Fig. 3A). This result indicates that MUPP1 is a direct substrate for Parkin-mediated ubiquitination.

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

Discussion

In this study, we have shown by yeast two-hybrid assay that Parkin associates with MUPP1 through the C-terminal region. We have further shown that Parkin and MUPP1 co-localizes in HEK-293T cells, and that MUPP1 is a direct substrate for Parkin-mediated ubiquitination.

Proteins containing PDZ domains usually form multimeric complexes [11, 22, 24]. PDZ domains contain a conserved peptide-binding groove that associates with the extreme C-terminus of ligands [8]. Interestingly, MUPP1 has

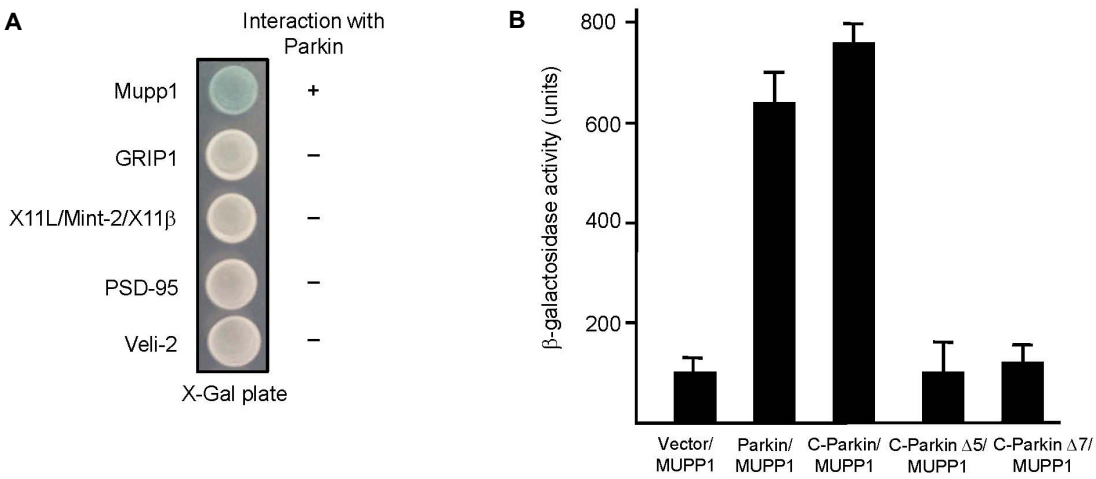

Fig. 2. Interaction between MUPP1 and Parkin. PDZ domain containing proteins were fused to the pLexA DNA binding domain. (A) Parkin specifically interacted with MUPP1 but not with GRIP1, X11/Mint-2/X11β, PSD-95, or Veli-2. +, interaction with Parkin; -, no interaction with Parkin. (B) The strength of interactions between several deletion mutants of Parkin and MUPP1 were examined quantitatively using β-galactosidase activity in yeast two-hybrid reporter assay.

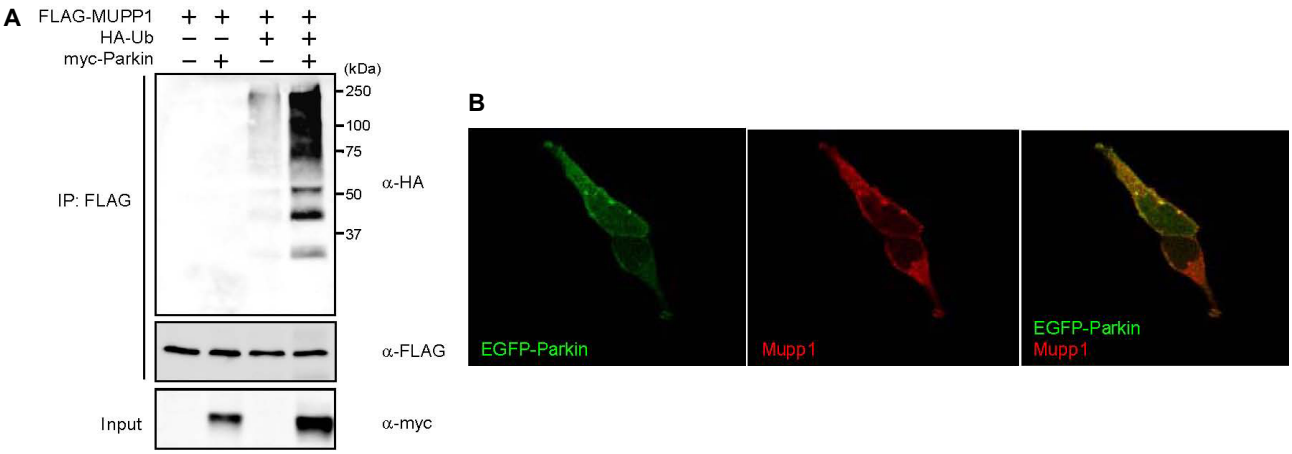

Fig. 3. Parkin-mediated ubiquitination of MUPP1 and co-localization of MUPP1 and Parkin in mammalian cells. (A) HEK-293T cells were transiently transfected with MUPP1 and HA-Ub plasmids and either control vector or Parkin plasmid as indicated. Cell lysates prepared with SDS lysis buffer were incubated with anti-FLAG M2 agarose beads to immunoprecipitate MUPP1. Western blots were subsequently probed with anti-HA antibody. MUPP1 was modified by ubiquitin. (B) Twenty-four hours after transfection with MUPP1 and EGFP-Parkin plasmids, HEK-293T cells were immunostained using anti-MUPP1 antibody. MUPP1 and EGFP-Parkin are seen at the same subcellular region in cells.

13 PDZ domains that mediate interaction with the other proteins [29]. In this study, we demonstrated through domain analysis that the 12th PDZ domain of MUPP1 specifically mediates the interaction with the C-terminal region of Parkin.

What would the association between MUPP1 and Parkin mean? A first possibility is the turn-over of MUPP1 by Parkin. Parkin functions as an E3 ubiquitin ligase [17, 25, 30]. The process of ubiquitination occurs through the transfer of an ubiquitin molecule from an activated E1 enzyme to the conjugating E2 enzyme [6, 15]. The E3 enzyme confers substrate specificity and acts as a scaffold for the covalent attachment of ubiquitin. Our data showed that MUPP1 is a direct substrate for Parkin, indicating that Parkin may regulates MUPP1 by ubiquitination. Further study on this possibility may help to understand the pathogenesis of Parkinson's disease.

A second possibility is subcellular targeting of Parkin to appropriate subcellular localization by MUPP1. Several PDZ proteins, such as GRIP1 and syntenin serve to act as targeting/scaffolding proteins that have potential to bring their interacting proteins to appropriate subcellular localization [16, 20]. Therefore, the association of Parkin with MUPP1 could target Parkin to specific subcellular location for appropriate functions. A thorough understanding of the factors that regulate Parkin may help to develop therapeutics for the treatment of Parkinson's disease. Our findings provide insight into the possible regulation of Parkin by MUPP1 through PDZ-mediated interaction, and could provide a novel therapeutic target [7].

Acknowledgments

The research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) by the Ministry of Science, ICT and Future Planning (NRF-2012R1A1A2020689).

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초록 : Parkin과 Multi-PDZ Domain Protein (MUPP1) 단백질 간의 PDZ 결합

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세포표면 수용체와 통로가 적절히 기능하려면 특정 세포 내 위치로 배치되고 조절되어야 한다. PSD95/Dlg/Zo-1 (PDZ) 도메인은 이러한 배치와 조절을 매개하는 다양한 단백질들을 인식하고 이 단백질들이 서로 결합하는데 관여한다. MUPP1은 13개의 PDZ domain을 가지는 단백질로서 여러 구조 단백질 및 신호전달 단백질과 상호결합하지만, MUPP1이 어떻게 안정화되며, 어떻게 신호전달과정에 관여하는지에 대해 아직 명확히 밝혀지지 않았다. 본 연구에서 MUPP1의 PDZ 도메인과 상호 작용하는 단백질을 규명하기 위하여 효모 two-hybrid 방법을 이용하였고, Parkin이 MUPP1과 결합하는 것을 확인하였다. Parkin은 E3 ubiquitin ligase로서, Parkin 유전자의 기능상실 돌연변이는 autosomal recessive juvenile parkinsonism을 일으키는 것으로 알려져 있다. Parkin은 MUPP1의 12번째 PDZ domain과 결합하지만, 다른 PDZ 도메인과는 결합하지 않았다. Parkin의 C-말단부위는 II 형 PDZ-결합모티프를 가지고 있는데, 이 모티프가 MUPP1과의 결합에 필수적임을 확인하였다. HEK-293T 세포에 MUPP1과 Parkin을 동시에 발현하여 발현위치를 확인한 결과 세포내의 같은 위치에서 발현하였다. 또한 Parkin은 MUPP1을 강하게 유비퀴틴화 하였다. 이러한 결과들은 MUPP1이 Parkin의 기질이며, Parkin에 의한 유비퀴틴화에 의해 MUPP1의 기능 혹은 안정성이 조절될 수 있음을 시사한다.