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Wdpcp, a Protein that Regulates Planar Cell Polarity, Interacts with Multi PDZ Domain Protein 1 (MUPP1) through a PDZ Interaction

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Protein-protein interactions regulate the subcellular localization and function of receptors, enzymes, and cytoskeletal proteins. Proteins containing the postsynaptic density-95/disks large/zonula occludens-1 (PDZ) domain have potential to act as scaffolding proteins and play a pivotal role in various processes, such as synaptic plasticity, neural guidance, and development, as well as in the pathophysiology of many diseases. Multi-PDZ domain protein 1 (MUPP1), which has 13 PDZ domains, has a scaffolding function in the clustering of surface receptors, organization of signaling complexes, and coordination of cytoskeletal dynamics. However, the cellular function of MUPP1 has not been fully elucidated. In the present study, a yeast two-hybrid system was used to identify proteins that interacted with the N-terminal PDZ domain of MUPP1. The results revealed an interaction between MUPP1 and Wdpcp (formerly known as Fritz). Wdpcp was identified as a planar cell polarity (PCP) effector, which is known to have a role in collective cell migration and cilia formation. Wdpcp bound to the PDZ1 domain but not to other PDZ domains of MUPP1. The C-terminal end of Wdpcp was essential for the interaction with MUPP1 in the yeast two-hybrid assay. This interaction was further confirmed in a glutathione *S*-transferase (GST) pull-down assay. When coexpressed in HEK-293T cells, Wdpcp was coimmunoprecipitated with MUPP1. In addition, MUPP1 colocalized with Wdpcp at the same subcellular region in cells. Collectively, these results suggest that the MUPP1-Wdpcp interaction could modulate actin cytoskeleton dynamics and polarized cell migration.

Key words : MUPP1, PDZ domain, Planar cell polarity (PCP), scaffolding protein, Wdpcp

Introduction

Many biological processes are regulated by protein-protein interactions. Protein-protein interactions mediated by a variety of domains, functionally independent unit structures of protein, are critical for the formation of functional protein networks [10]. Postsynaptic density-95/disks large/zonula occludens-1 (PDZ) domain is one of those domains that mediate protein-protein interactions [10, 24, 26]. PDZ domain-containing proteins are generally soluble cytoplasmic proteins that act as scaffolds by linking the cell membrane receptors via PDZ domains or other protein modules to actin cytoskeleton and actin-binding proteins or signaling proteins such as regulators of membrane trafficking, cell polarity and stabilization of cell surface proteins [8-10, 12, 24, 26]. PDZ domain-mediated interactions can play an important role in the assembly of large multi-molecular networks [26]. The importance of the PDZ-mediated multi-molecular networks is demonstrated by the fact that PDZ interactions are disrupted in pathological situations such as infectious diseases or cancers [17, 22, 30].

PDZ domains are built of 80~100 amino-acid residues and bound to the carboxyl (C)-termini of multiple binding partners, including cell surface receptors, channel proteins, and other adaptor proteins [7, 24, 28]. PDZ domain-binding partners have one of two classes of consensus PDZ-binding motifs at their C-termini (class I: S/T-X-Φ, class II: Φ-X-Φ, where Φ is a hydrophobic residue) [14]. PDZ domain-containing proteins have single or multiple copies of PDZ domain [26,

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29]. Multi-PDZ domain protein 1 (MUPP1), which possesses 13 PDZ domains, was first isolated as a protein that interacts with the C-terminus of the serotonin receptor type 2C ($5\text{-}HT_{2C}$) [29]. MUPP1 is expressed in the brain, enriched in synapse, especially in post-synaptic density (PSD) and tight junctions, and has been reported to interact with a variety of integral membrane proteins, including a synaptic adhesion molecule Cadm1 using its PDZ1-5 domain, junctional adhesion molecule-A (JAM-A) using PDZ9, neurexin 1 using PDZ2, and sodium channel Nav1.4, melatonin receptor (MT_1), Claudin-1, and γ-aminobutyric acid receptor 2 (GABAR2) using PDZ13 [1, 2, 3, 6, 13, 16, 18]. MUPP1 may act as a scaffold for the proper assembling and localization of its interacting proteins [11]. It also interacts with synaptic Ras GTPase-activating protein SynGAP, muskelin, and Ca^{2+}/calmodulin-dependent kinase (CaMKII) to regulate neuronal signaling and dendritic spine morphology [2, 11, 19, 23].

To clearly define the scaffolding function of MUPP1, further identification of the interacting proteins of MUPP1 is required. We screened for proteins that interact with the amino (N)-terminal PDZ domain of MUPP1 through the yeast two-hybrid assay and identified Wdpcp, known to contain two WD40 domains and regulate cell polarity and polarized cell migration by modulation of the actin cytoskeleton through the interaction with septin 2 (Sept2) [4, 5, 8]. The MUPP1 and Wdpcp interaction suggests that MUPP1 may serve a critical scaffolding function for regulation of planar cell polarity and polarized cell migration.

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 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 *Eco*RI-restricted and subcloned into the *Eco*RI site of pLexA. The correct orientation and in-frame cloning of cDNA inserts were verified by restriction enzyme analysis and DNA sequencing. EGFP-fused Wdpcp was constructed and used to visualize the intracellular localization in HEK-293T cells. General recombinant DNA techniques were performed according to standard protocol [25].

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, the rat MUPP1 cDNA fragment corresponding to PDZ1 domain (amino acids 102-251) was fused to the DNA-BD region of the pLexA vector using PCR and the resulting plasmid 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 [16]. 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, 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 = $1{,}000\times [OD_{420} - 1.75x\ OD_{550}]$ / [reaction time (min) x culture volume (ml) x OD_{600}]. All experiments were independently performed at least three times.

Glutathione S-transferase (GST) pull-down assays

cDNA encoding the full length Wdpcp was cloned into pET41a. The recombinant GST-Wdpcp fusion protein was expressed in bacterial strain BL21 GOLD (Stratagene, La Jolla CA, USA) after induction with 0.5 mM isopropyl thio-β-D-galactopyranoside (IPTG) for 3 hr. The fusion proteins

were purified by attachment to glutathione-agarose beads (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. 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 Laemmli's loading buffer. The pulled-down proteins were analyzed by immunoblotting with anti-MUPP1 antibody (BD SCIENCE, San Jose, CA, USA).

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 myc-Wdpcp 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 PBS containing 0.5% NP-40. The immunoprecipitated proteins were analyzed by immunoblotting.

Immunocytochemistry

HEK-293T cells grown on poly-D-lysine-coated coverslips were transfected with EGFP-Wdpcp and MUPP1 constructs. Twenty-four hours after transfection, cells were washed with 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 diluted 1:500 in PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween-20 overnight at 4°C. After washing 3 times with PBS, 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 3 times with PBS, 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 the binding proteins of MUPP1, we screened a mouse brain cDNA library through the yeast two-hybrid assays using the N-terminal region of MUPP1 containing PDZ1 domain as bait (Fig. 1B). From $9×10^6$ colonies screened, we obtained two positive clones. The clones possessed cDNA fragments of Wdpcp (Fig. 1A). To determine whether the other PDZ domains of MUPP1 also interact with Wdpcp, we constructed various PDZ domain-containing fragments of MUPP1. Yeast two-hybrid assays with Wdpcp showed that, among the PDZ domains tested, only the PDZ1 domain of MUPP1 is required for binding (Fig. 1B). Wdpcp is a multi-domain protein comprised of one coiled-coil domain and two WD40 repeated domains [4]. To identify the domain of Wdpcp required for interaction with MUPP1, various fragments of Wdpcp were constructed and tested for interaction with MUPP1. As shown in Fig. 1C, the short C-terminal region of Wdpcp was required for interaction with MUPP1. The C-terminus of Wdpcp contains a putative class II PDZ-binding motif [4, 14]. Therefore, we investigated whether the putative C-terminal motif of Wdpcp mediates the interaction with MUPP1. For this purpose, the C-terminal deletion and substitution mutants of Wdpcp were constructed and co-transfected into yeast cells with pLexA-MUPP1. As shown in Fig. 1D, the MUPP1 and Wdpcp interaction was impaired by the C-terminal deletion or the substitution of the last C-terminal residue of Wdpcp. These results indicate that MUPP1 and Wdpcp interact with each other through its PDZ domain and PDZ-binding motif, respectively, consistent with the previously described class II PDZ interaction [26]. Next, we compared the binding of Wdpcp to MUPP1 with those of two known MUPP1 interacting proteins, muskelin and neurexin 1 [15, 16]. A yeast two-hybrid assay (Fig. 2A) and a quantitative β-galactosidase assay (Fig. 2B) showed that Wdpcp has a binding affinity comparable to muskelin and neurexin 1.

Further, we examined the MUPP1 and Wdpcp interaction

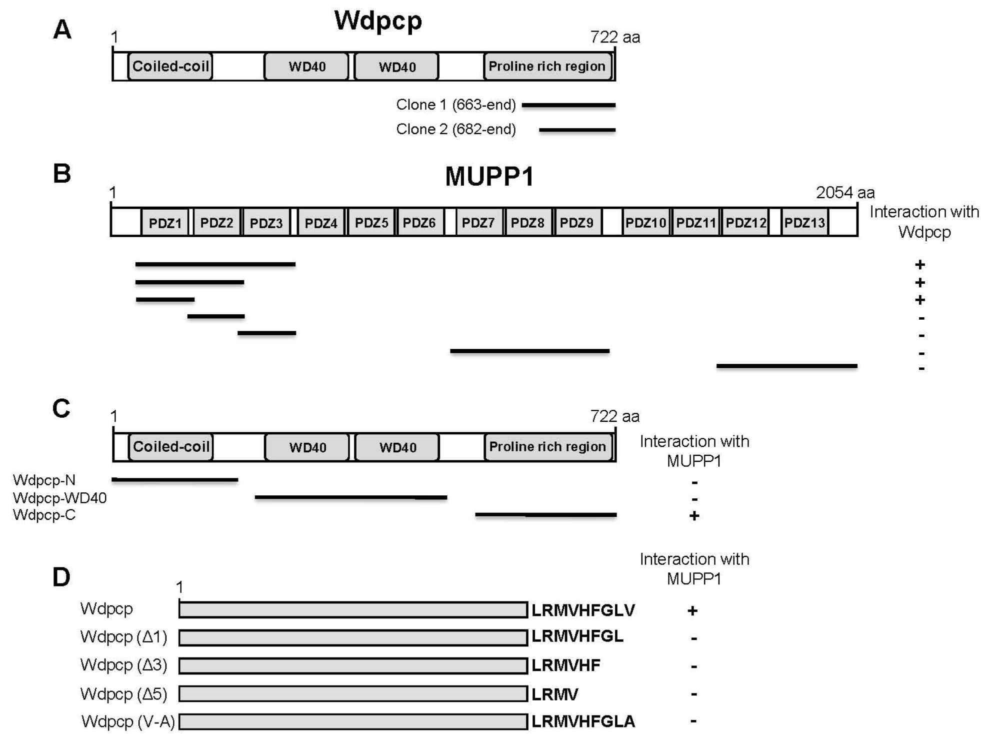

Fig. 1. Identification of the protein interacting with MUPP1 by yeast two-hybrid screening. (A) Schematic diagram of Wdpcp. Wdpcp contains the coiled-coil domain, WD40 domains, and proline-rich region. Clone 1 and clone 2 were isolated from the yeast two-hybrid screen. (B) Minimal Wdpcp binding region in MUPP1. Different truncations of MUPP1 were constructed by PCR and tested in the yeast two-hybrid assay for interaction with Wdpcp. +, interaction with Wdpcp; -, no interaction with Wdpcp. aa, the amino acid residue number. (C, D) Specific interaction of MUPP1 with the C-terminal region of Wdpcp. Several different truncated forms and deletion and substitution mutants of Wdpcp were constructed by PCR and tested in the yeast two-hybrid assay for interaction with MUPP1. +, interaction with MUPP1; -, no interaction with MUPP1.

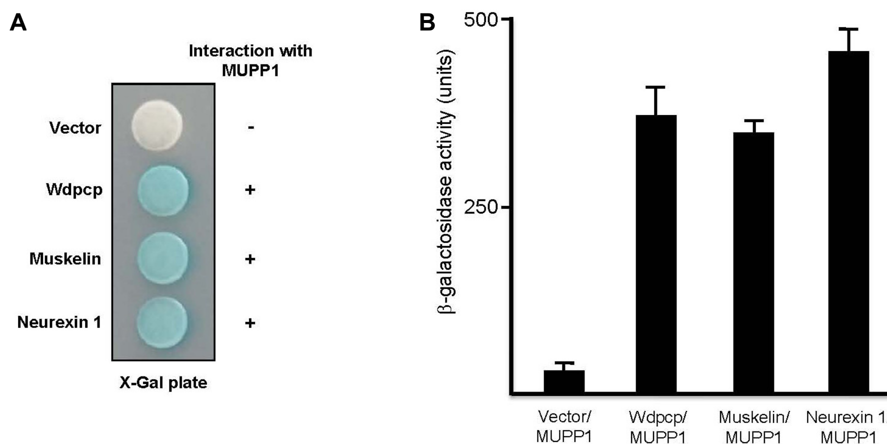

Fig. 2. Interaction between MUPP1 and Wdpcp. (A) Wdpcp and proteins containing class II PDZ-binding motif (muskelin and neurexin 1) were fused to the pLexA DNA binding domain. Wdpcp interacted with MUPP1. +, interaction with MUPP1; -, no interaction with MUPP1. (B) The strength of interaction between MUPP1 and Wdpcp were compared with those of muskelin and neurexin 1 quantitatively using β-galactosidase activity in yeast two-hybrid reporter assay.

at the protein level using GST pull-down experiments. Recombinant GST-Wdpcp fusion protein was expressed in *E. coli*. The purified GST fusion protein was allowed to interact with mouse brain lysates. Immunoblot analysis revealed that MUPP1 interacted with GST-Wdpcp, but not with GST, and the C-terminal deletion of Wdpcp impaired the inter-

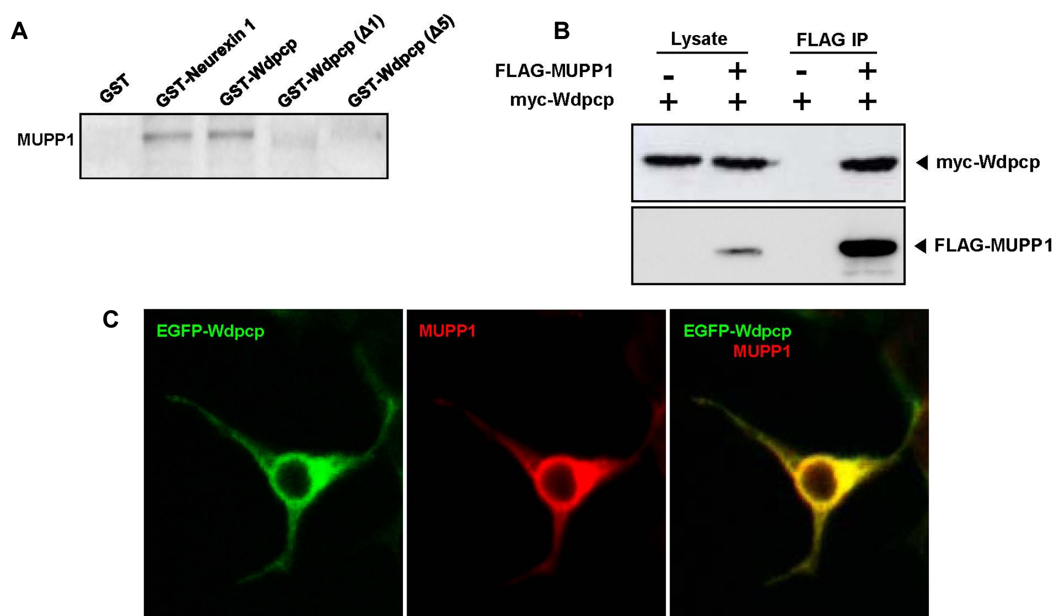

Fig. 3. MUPP1 and Wdpcp were co-immunoprecipitated and co-localized in mammalian cells. (A) Proteins in the mouse brain lysate were allowed to bind to GST alone, GST- Wdpcp, GST-Wdpcp (△1), GST-Wdpcp (△5), and GST-Neurexin 1 fusion proteins. The elution fractions were resolved by SDS-PAGE and immunoblotting was performed using anti-MUPP1 antibody. (B) HEK-293T cells were transiently transfected with FLAG-MUPP1 and myc-Wdpcp plasmids as indicated. Cell lysates were incubated with monoclonal anti-FLAG M2 agarose beads to immunoprecipitate MUPP1. Immunoblots were subsequently probed with anti-FLAG and anti-myc antibodies. Wdpcp was specifically co-immunoprecipitated with MUPP1. (C) Twenty-four hours after transfection with MUPP1 and EGFP-Wdpcp, HEK-293T cells were subjected to immunoflourescent staining using anti-MUPP1 and Dylight 594-conjugated secondary antibodies. EGFP-Wdpcp and MUPP1 co-localize largely in cells.

action (Fig. 3A).

MUPP1 is associated with Wdpcp in cells

To examine the interaction between MUPP1 and Wdpcp in mammalian cells, HEK-293T cells were co-transfected with constructs expressing FLAG-MUPP1 and myc-Wdpcp. Cell lysates were immunoprecipitated with a monoclonal antibody against the FLAG epitope, followed by immunoblot analysis with anti-myc and anti-FLAG antibodies. Fig. 3B shows that Wdpcp was co-precipitated with MUPP1 (Fig. 3B). This result indicates that MUPP1 specifically interacts with Wdpcp in cells. To determine whether MUPP1 and Wdpcp co-localize in cells, MUPP1 was co-transfected with EGFP-Wdpcp into HEK-293T cells. Confocal microscopic images of EGFP-Wdpcp and MUPP1 showed that MUPP1 and Wdpcp co-localized at the same region in cells (Fig. 3C). These findings indicate that MUPP1 interacts with Wdpcp in cells.

Discussion

In the present study, we have shown that the scaffold protein MUPP1 interacts with Wdpcp. Using the PDZ1 domain containing region of MUPP1 as bait, we identified Wdpcp in a yeast two-hybrid screen. The C-terminal end region of Wdpcp can interact with PDZ1 domain of MUPP1. Furthermore, using a combination of GST pull-down assay and co-immunoprecipitation, we confirmed that MUPP1 interacted with Wdpcp at the protein level. Moreover, when MUPP1 and Wdpcp were co-expressed in mammalian cells, they co-localize at the same region in cells.

Specific protein-protein interactions are important for the localization and clustering of cell surface receptors at the specific subcellular site [26]. The PDZ domain is one of most abundant interaction modules found in the proteins that act as scaffolds of multi-protein complexes through specific interaction of their PDZ domains to the C-terminal binding motif of interacting proteins, thereby linking the interacting proteins to cytoskeletal proteins or other protein modules [10, 24]. Therefore, PDZ domain-containing proteins usually form large multimeric protein complexes [24, 26]. Interestingly, MUPP1 contains 13 PDZ domains and plays an important role as a scaffold of protein complexes via PDZ domains and binding protein's modules [29]. Wdpcp has G-L-V

seqeunce at C-terminus, which is similar to the class II PDZ-binding motif [4]. In this study, we demonstrated through domain analysis and deletion analysis that the C-terminal three amino acids of Wdpcp act as a binding motif required for specific interaction with the PDZ1 domain of MUPP1.

Wdpcp has a short N-terminal coiled-coil domain consisting of five heptad repeats and two WD40 domains [4]. The WD40 domain has been identified in many proteins, and forms a β-propeller structure that provides surfaces for protein-protein interaction [27]. WD40 domain-containing proteins have been shown to serve as scaffolds for assembly of multi-protein complexes. Wdpcp is localized in the actin cytoskeleton and interacts with Sept2, also found in actin filaments [21]. Septins are highly conserved GTP-binding proteins which form filamentous cytoskeleton by hetero-oligomerization [20]. In recent report, Wdpcp-deficient cells showed a disruption in the actin cytoskeleton required for planar cell polarity and polarized cell migration [5]. Wdpcp appears to modulate the actin cytoskeleton by mediating Sept2 interaction with actin filaments [5]. Therefore, it is speculated that the MUPP1-Wdpcp interaction may indicate the formation of a multi-scaffold for regulation of cell polarity and cell migration by modulating the actin cytoskeleton.

Our findings provide insight into the possible regulation of maintaining cell polarity and polarized cell migration by MUPP1-Wdpcp complex through PDZ domain-mediated interaction. Further experiments are needed to examine the effect of MUPP1-Wdpcp interaction on Sept2 interaction with actin filament. Studies on the possibilities mentioned above and identification of other MUPP1 interacting proteins may help to shed light on regulation of maintaining cell polarity and polarized cell migration.

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초록 : Planar cell polarity 조절단백질 Wdpcp와 multi-PDZ domain protein 1 (MUPP1)의 PDZ 결합

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단백질-단백질 결합은 수용체 단백질, 효소, 세포 골격 단백질의 세포내 위치 결정 및 기능 조절에 중요한 역할을 한다. Postsynaptic density-95/disks large/zonula occludens-1 (PDZ) 도메인을 가진 단백질들은 시냅스 가소성, 신경세포 성장과 분화뿐만 아니라 많은 질병의 병태생리에 중요하게 관여하는 scaffold 단백질로 작용한다. Multi-PDZ domain protein 1 (MUPP1)은 13개 PDZ 도메인을 가지는 단백질로서 세포막 수용체 군집화, 신호전달 복합체 구성, 세포 골격 조정에 대한 매개 역할을 하는 것으로 알려지고 있지만 MUPP1의 세포 내 기능은 아직 명확히 밝혀지지 않았다. 본 연구에서 MUPP1의 아미노 말단 PDZ 도메인과 결합하는 새로운 단백질을 규명하기 위하여 효모 two-hybrid 방법을 이용하였고 Wdpcp (전에 Fritz로 알려짐)이 MUPP1과 결합하는 것을 확인하였다. Wdpcp는 planar cell polarity (PCP) effector로서 세포 이동과 섬모형성에 관여하는 것으로 알려져 있다. Wdpcp는 MUPP1의 첫 번째 PDZ 도메인과 결합하지만, 다른 PDZ 도메인과는 결합하지 않았다. 또한 MUPP1와 Wdpcp의 결합에서 Wdpcp의 C-말단부위가 결합에 필수적임을 효모 two-hybrid 방법으로 확인하였다. 이러한 단백질간 결합은 glutathione *S*-transferase (GST) pull-down assay, 공동면역침강, HEK-293T 세포에서의 발현위치를 통하여 추가적으로 확인하였다. 이러한 결과들은, MUPP1과 Wdpcp 결합은 세포내 액틴 다이내믹스 (dynamics)와 세포이동 조절에 역할을 할 가능성을 시사한다.