Mol. Cells *30*, 143-148, August 31, 2010
DOI/10.1007/s10059-010-0099-7

Molecules and Cells

Ankyrin Repeat-Rich Membrane Spanning/ Kidins220 Protein Interacts with Mammalian Septin 5

Han Jeong Park, Hwan-Woo Park, Shin-Jae Lee[1], Juan Carlos Arevalo[2], Young-Seok Park, Seung-Pyo Lee, Ki-Suk Paik, Moses V. Chao[3], and Mi-Sook Chang*

Neurotrophin receptors utilize specific adaptor proteins to activate signaling pathways involved in various neuronal functions, such as neurite outgrowth and cytoskeletal remodeling. The Ankyrin-Repeat Rich Membrane Spanning (ARMS)/kinase D-interacting substrate-220 kDa (Kidins220) serves as a unique downstream adaptor protein of Trk receptor tyrosine kinases. To gain insight into the role of ARMS/Kidins220, a yeast two-hybrid screen of a rat dorsal root ganglion library was performed using the C-terminal region of ARMS/Kidins220 as bait. The screen identified a mammalian septin, Septin 5 (Sept5), as an interacting protein. Co-immunoprecipitation using lysates from transiently transfected HEK-293 cells revealed the specific interaction between ARMS/Kidins220 and Sept5. Endogenous ARMS/Kidins220 and Sept5 proteins were co-localized in primary hippocampal neurons and were also predominantly expressed at the plasma membrane and in the tips of growing neurites in nerve growth factor-treated PC12 cells. Mapping of Sept5 domains important for ARMS/Kidins220 binding revealed a highly conserved N-terminal region of Sept5. The direct interaction between ARMS/Kidins220 and Sept5 suggests a possible role of ARMS/Kidins220 as a functional link between neurotrophin receptors and septins to mediate neurotrophin-induced intracellular signaling events, such as neurite outgrowth and cytoskeletal remodeling.

INTRODUCTION

Neurotrophins play prominent roles in regulating various neuronal functions, including cell survival, differentiation, axon guidance and neurotransmitter release in the developing and adult nervous system (Chao, 2003; Huang and Reichardt, 2003). They activate intracellular signal transduction inside the cell through two different classes of receptors: Trk as receptor tyrosine kinases and p75 receptor as a member of the tumor necrosis factor receptor superfamily. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4 all recognize the p75 receptor, while NGF binds preferentially to TrkA, BDNF and NT-4 to TrkB, and NT-3 to TrkC receptors (Chao, 2003; Huang and Reichardt, 2003). Signal transduction mediated by neurotrophins involves receptor dimerization and autophosphorylation of Trk receptors which results in recruitment of downstream adaptor proteins (Huang and Reichardt, 2003; Reichardt, 2006).

The Ankyrin-Repeat Rich Membrane Spanning (ARMS) protein (Kong et al., 2001), which is also known as a kinase D-interacting substrate of 220 kDa (Kidins220) (Iglesias et al., 2000), has been identified as a prominent downstream substrate for Trk and Eph receptors. It consists of eleven ankyrin repeats, four transmembrane domains, a proline-rich region, a sterile α motif domain and a PDZ-binding motif. ARMS/Kidins220 is highly expressed in the hippocampus, olfactory bulb, motor neurons in spinal cord and at the neuromuscular junction (Iglesias et al., 2000; Kong et al., 2001; Luo et al., 2005). ARMS/Kidins220 is tyrosine phosphorylated by neurotrophins and ephrin B through their receptor tyrosine kinases (Cabrera-Poch et al., 2004; Kong et al., 2001). It also directly interacts with Trk and p75 neurotrophin receptors to form a ternary complex inside the cell (Chang et al., 2004). One of the prominent roles of ARMS/Kidins220 is as a major platform for sustained activation of the mitogen-activated protein kinase (MAPK) pathway in neurons through the recruitment of CrkL (Arevalo et al., 2004; 2006). ARMS/Kidins220 also regulates neuronal differentiation through interaction with kinesin light chain I (Bracale et al., 2007). In addition, ARMS/Kidins220 is involved in synaptic development (Cortes et al., 2007; Wu et al., 2009), NF-kB activation (Sniderhan et al., 2008) and excitotoxicity (Lopez-Menendez et al., 2009). A decrease in ARMS/Kidins 220 levels in primary cortical neurons is associated with a reduction in the number of BDNF-induced primary neurites (Wu et al., 2009). However, the molecules involved in ARMS/Kidins 220 regulation of neurite outgrowth have not been identified.

To identify proteins interacting with ARMS/Kidins220, a yeast two-hybrid screen of a rat dorsal root ganglion (DRG) library was presently performed using the C-terminal region of ARMS/Kidins220 as bait. Here, we report that ARMS/Kidins220 spe-

Department of Oral Anatomy, School of Dentistry and Dental Research Institute, Seoul National University, Seoul 110-749, Korea, [1]Department of Orthodontics, School of Dentistry and Dental Research Institute, Seoul National University, Seoul 110-749, Korea, [2]Institute of Neurosciences Castilla y Leon, University of Salamanca, 37007 Salamanca, Spain, [3]Molecular Neurobiology Program, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, USA
*Correspondence: mschang@snu.ac.kr

Received April 7, 2010; revised May 6, 2010; accepted May 7, 2010; published online July 23, 2010

Keywords: adaptor protein, neurotrophin, receptor, septin, signal transduction

cifically interacts with Septin 5 (Sept5), also known as CDCrel-1, a member of a family of highly conserved GTP-binding proteins called septins (Macara et al., 2002). Septins in the budding yeast are very important to regulate cytokinesis required for the completion of budding (Hartwell, 1971; Weirich et al., 2008). Although the role of mammalian septins is not yet fully elucidated, it has been reported that Sept5 inhibits exocytosis by binding to syntaxin 1A (Beites et al., 1999; 2005). Sept5 is also accumulated in large amounts at the plasma membrane and in the tips of neurites in NGF-treated PC12 cells (Beites et al., 1999). Thus, it is possible that Sept5 may regulate neurite outgrowth by regulating vesicle trafficking in these areas.

The present study was designed to address our hypothesis that ARMS/Kidins220 may act as a functional link between neurotrophin receptor and Sept5 to regulate neurite outgrowth and cytoskeletal remodeling in the cell after neurotrophin receptor activation. To test this hypothesis, the Sept5 domains important for ARMS/Kidins220 interaction were mapped using deletion constructs of Sept5 in glutathione *S*-transferase (GST)-pull down and co-immunoprecipitation assays. The expression of Sept5 deletion constructs in PC12 cells also indicated that Sept5 displayed discrete effects upon NGF-mediated neurite outgrowth and cytoskeletal remodeling.

MATERIALS AND METHODS

Cell culture

HEK293 human embryonic kidney cells were grown in Dulbecco's modified Eagle's medium Dulbecco's modified Eagle's medium (DMEM; Hyclone) containing 10% fetal bovine serum (FBS; Gibco). PC12 cells were cultured in DMEM containing 10% FBS and 5% heat-inactivated horse serum. Hippocampal neurons were obtained from E17 Sprague-Dawley rats and cultured in Neurobasal-A medium (Gibco) supplemented with B-27 and 1 mM L-glutamine on poly-D-lysine/laminin-coated coverslips. All cells were cultured at 37°C with 5% CO_2.

Construction of the two-hybrid DRG library and yeast two-hybrid screen

The construction of a cDNA library has been previously described (Kong et al., 2001). Briefly, polyadenylated RNA, which had been purified from adult mouse and postnatal day 1 (P1) rat DRG by the use of Trizol (Invitrogen) and the PolyA Tract System (Promega), was used as the template for reverse transcription (Invitrogen) with an oligo-dT-*Not*I primer. Subsequent ligation into a *Bst*XI/*Not*I-digested pJG4-5 vector and electroporation into DH5α yielded a library of ~10^6 cfu and an average insert size of 1.5-2.0 kb. A two-hybrid interaction screen, based on the LexA system (Gyuris et al., 1993), was performed in EGY191. The bait consisted of the C-terminus of rat ARMS/Kidins220 (amino acids 1603-1715) as an in-frame fusion with the LexA DNA-binding domain. Library cDNAs were expressed as in-frame fusions with the Gal4 transcriptional activation domain. Approximately 100 million yeast transformants were screened for their ability to survive in the absence of leucine. Sequence analysis identified a cDNA clone of ~2.2 kb that includes the total 369 amino acids of Sept5.

Plasmids

The full-length cDNA of ARMS/Kidins220 cloned in pEGFPC3 vector to generate GFP-Sept5 has been previously described (Arevalo et al., 2006). pCDNA Flag-tagged full-length Sept5 cDNA was kindly provided by Dr. William S. Trimble, University of Toronto, Canada. The Myc-tagged expression vectors for wild-type and mutant Sept5 constructs were generated by PCR using pCDNA Flag-tagged Sept5 cDNA as a template, cut with *Bam*HI and *Eco*RI and ligated into a *Bam*HI/*Eco*RI-digested pCMV-tag3B vector. All constructs were verified by DNA sequencing.

Antibodies

A polyclonal antibody (892) against the C terminal of ARMS/Kidins220 has been previously described (Kong et al., 2001). Anti-Sept5 Sp20 monoclonal antibody was generously provided by Dr. William S. Trimble. The following antibodies were used: polyclonal anti-GFP (Abcam), monoclonal anti-GFP (Roche Applied Science), monoclonal anti-c-Myc (Santa Cruz).

Transfection of mammalian cells, immunoprecipitation and immunoblotting

The mammalian expression plasmids were transiently transfected into HEK293 cells (2×10^6 cells/10-cm dish) using the calcium phosphate method. Immunoprecipitation was performed as previously described (Chang et al., 2004). Briefly, cells were harvested and lysed 40 h after transfection by incubation for 30 min on ice in 1 ml of 10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 0.5% NP-40 (TNE buffer) containing protease inhibitors. After centrifugation and removal of the insoluble fraction, the supernatants of equivalent protein content were incubated with either aniti-Flag or anti-GFP antibody. The immune complexes were immobilized on protein A-Sepharose beads (PIERCE), washed seven times with ice-cold TNE buffer, boiled in SDS-sample buffer, separated by SDS-PAGE and western blotting was performed as previously described (Chang et al., 2004).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde, permeabilized and blocked with 0.1% Triton X-100 and 5% normal goat serum in phosphate-buffered saline (PBS). Cells were incubated overnight at 4°C with the following primary antibodies: polyclonal anti-ARMS/Kidins220, 1:1000, monoclonal anti-Sept5 antibody, 1:20. Cells were then incubated with the following secondary antibodies: FITC-conjugated goat anti-rabbit IgG and Cy3- or Cy5-conjugated goat anti-mouse IgG (Jackson Laboratories). F-actin was visualized by rhodamin-phalloidin (Invitrogen). The z-stack images were captured using a laser-scanning confocal microscope (Olympus).

Preparation of GST fusion proteins and *in vitro* binding assays

The wild-type and mutant Sept5 constructs were amplified by PCR and subcloned in pGEXKG vector. Expression of GST fusion proteins in BL21 bacteria was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 6 h at 30°C. The bacteria were collected, resuspended in lysis buffer (25 mM HEPES ; pH 7.8, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.05% Tween-20, 0.5% Triton X-100, 1 mM PMSF, 1 μg/ml leupeptin and pepstatin, 2 mM benzamidine) and sonicated. The glutathione-agarose beads (Sigma) were then incubated with lysates of ARMS/Kidins220-transfected HEK293 cells for 4 h at 4°C, washed and analyzed by SDS-PAGE and Western blot.

Stably transfected PC12 cells

PC12 cells were transfected with pCMV Myc-tagged wild-type and mutant Sept5 constructs with Lipofectamine 2000 (Invitrogen). Cells were treated with 500 μg/ml G418 for 2 weeks. Resistant clones were subjected to Western blot analysis using Myc antibodies (9E10, Santa Cruz) to verify protein expression. Positive clones were maintained in 200 μg/ml G418 thereafter.

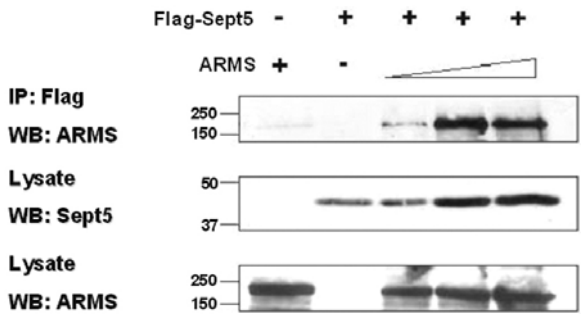

Fig. 1. Co-immunoprecipitation of Sept5 with ARMS/Kidins220. HEK293 cells were transiently co-transfected with cDNAs encoding full-length Flag-Sept5 and full-length ARMS/Kidins220. Cell lysates were immunoprecipitated with anti-Flag antibody for Sept5 and immunoblotted either with anti-ARMS/Kidins220 892 antibody or anti-Flag antibody.

In vitro neurite analysis

The stably transfected PC12 cells were plated on polyethyleneimine (PEI)-coated coverslips. After 24 h, cells were starved overnight and treated with NGF (Harlan, 100 ng/ml) for 2 weeks before immunostaining with anti-ARMS/Kidins220 antibody. Neurites were visualized under the z-stack laser-scanning confocal microscope and digitally captured. Neurites of imaged PC12 cells were counted and the length of each neurite was measured by Olympus Fluoview 300 software.

Statistical analysis

Statistical analyses were carried out using SAS 9.1 (SAS Institute). After checking the normality and equality of variance of the data, analysis of covariance (ANCOVA) was used to compare the effects of expressing wild type and deletion constructs of Sept5 mutants on NGF-mediated neurite outgrowth. Probability (P) values < 0.05 were considered statistically significant. Data were expressed as the mean ± standard error of mean (SEM).

RESULTS

ARMS/Kidins220 interacts with a mammalian septin, Sept5

To identify proteins interacting with ARMS/Kidins220, a yeast two-hybrid screen of a rat DRG library was undertaken using the C-terminal region of rat ARMS/Kidins220 (amino acids 1603-1715) as bait. A positive cDNA clone was identified that encoded the total 369 amino acids of Sept5, a member of mammalian septins. This report describes Sept5 as an interacting protein with ARMS/Kidins220.

To determine whether an association exists between Sept5 and ARMS/Kidins220, HEK293 cells were transiently co-transfected with cDNAs encoding full-length Flag-tagged rat Sept5 and an increasing amount of full-length rat ARMS/Kidins 220. Lysates were then prepared and subjected to co-immunoprecipitation with anti-Flag antibody to confirm the interaction between the two proteins. The interaction between Sept5 and ARMS/Kidins220 is clearly detected (Fig. 1).

Co-localization of ARMS/Kidins220 with Sept5 and F-actin

We carried out immunocytochemistry to examine the endogenous expression of ARMS/Kidins220 and Sept5 in primary rat hippocampal neurons. Using a polyclonal antibody against ARMS/Kidins220 (Fig. 2A) and a monoclonal antibody against Sept5 (Fig. 2B), we were able to detect co-localized ARMS/Kidins220 and Sept5 in the cell body of neurons and neurites

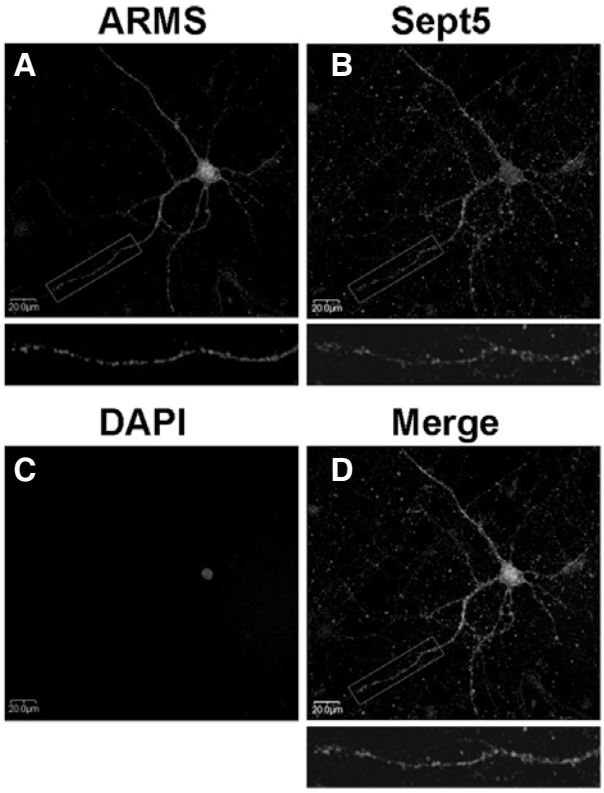

Fig. 2. Co-localization of ARMS/Kidins220 and Sept5 in rat hippocampal neurons. Hippocampal neurons isolated from E17 rat embryos were incubated with anti-ARMS/Kidins220 antibody/FITC-conjugated goat anti-rabbit antibody (A) and anti-Sept5 antibody/Cy3-conjugated goat anti-mouse antibody (B). Endogenously expressed ARMS/Kidins220 and Sept5 were co-localized in hippocampal neurons from the cell body to the axons and dendrites. The boxed area shows an enlargement of the co-localization signals. Scale bars: 20 μm.

(Fig. 2D). To examine expression of ARMS/Kidins220 and Sept5 at the growing neurites in more detail, immunocytochemistry was performed in NGF-treated PC12 cells. Both ARMS/Kidins220 and Sept5 were highly concentrated at the plasma membrane and in the tips of growing neurites (Fig. 3).

Mapping of interaction between ARMS/Kidins220 and Sept5

To determine the domains of Sept5 involved in interacting with ARMS/Kidins220, a series of mutant Sept5 constructs were examined (Fig. 4A). Cell lysates from ARMS/Kidins220-overexpressing HEK293 cells were prepared and subjected to GST pull-down assays with GST-tagged wild-type Sept5 (GST-Sept5-WT), Sept5 with a point mutation in the GTP-binding domain (S58N, GST-Sept5-MT), N-terminal region of Sept5 (GST-Sept5-NT, amino acids 1-213), C-terminal region of Sept5 (GST-Sept5-CT, amino acids 235-368), and the coiled-coil domain of Sept5 (GST-Sept5-CO, amino acids 337-368). The full-length and the N-terminal region of Sept5 pulled down ARMS/Kidins220, while the C-terminal region of Sept5 did not (Fig. 4A). This result suggested that the N-terminal region of Sept5 (amino acids 1-213) was necessary for binding to ARMS/Kidins220. On the other hand, the point mutation of GTP-binding domain did not disrupt the interaction with ARMS/Kid-

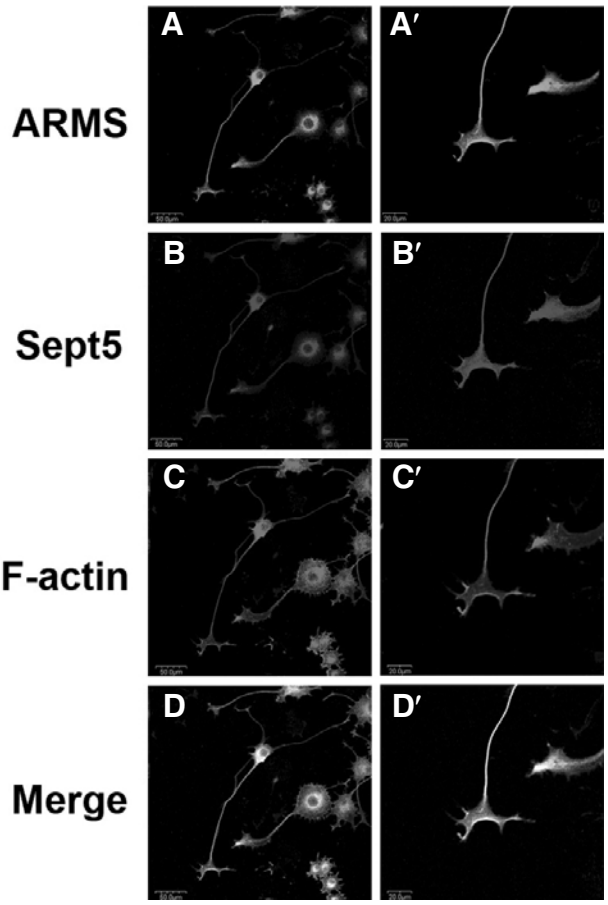

Fig. 3. Localization ARMS/Kidins220, Sept5 and F-actin in NGF-treated PC12 cells. PC12 cells were treated with NGF (50 ng/ml) for 2 weeks. Cells were fixed, permeabilized and subjected to immunocytochemistry with anti-ARMS/Kidins220 antibody/FITC-conjugated goat anti-rabbit antibody (A, A′) or with anti-Sept5 antibody/Cy5-conjugated goat anti-mouse antibody (B, B′). F-actin was visualized by rhodamin-phalloidin (C, C′). ARMS/Kidins220 and Sept5 were co-localized with F-actin in the cell body and at lamelipodia, but they were not co-localized with F-actin at filopodia of growth cones in differentiated PC12 cells. Scale bars: 50 μm (A, B, C, and D), 20 μm (A′, B′, C′, and D′).

ins220 (Fig. 4A). To more precisely delineate the region of Sept5 involved in the interaction with ARMS/Kidins 220, further N-terminal deletions of Sept5 (GST-Sept5-G3, amino acids 1-124) were generated using PCR. Clearly, GST-Sept5-G3 failed to pull down ARMS/Kidins220 (Fig. 4A). As expected, the coiled-coil domain of Sept5 (GST-Sept5-CO) failed to pull down with ARMS/Kidins220 (Fig. 4A). Taken together, these results demonstrated that amino acids 125-213 of Sept5 were crucial for binding to ARMS/Kidins220.

To further confirm the region of Sept5 required for interacting with ARMS/Kidins220, HEK293 cells were transiently co-transfected with GFP-tagged full-length ARMS/Kidins220 and Myc-tagged full-length Sept5 (Sept5-WT), N-terminal region of Sept5 (Sept5-NT), C-terminal region of Sept5 (Sept5-CT) and the further deleted N-terminal region of Sept5 (Sept5-G3) (Fig. 4B). Cell lysates were prepared and subjected to immunoprecipitation with anti-GFP antibody for ARMS/Kidins220, followed by Western blot with either anti-Myc antibody or anti-ARMS/Kidins220 antibody. Consistent with results from the GST pull-down assays, Myc-tagged full-length Sept5 and the N-terminal region of Sept5 were co-immunoprecipitated with ARMS/Kidins 220 (Fig. 4B). These results were consistent with the suggestion that a specific region of the N-terminus of Sept5 (amino acids 125-213) is required for ARMS/Kidins220 binding (Fig. 4C). Interestingly, the N-terminal region of Sept5 that interacts with ARMS/Kidins220 is highly conserved among different species (Fig. 4D).

Effects of Sept5 deletion constructs on neurite outgrowth of PC12 cells

Next, we sought to determine whether different Sept5 deletion constructs affected neurite outgrowth of NGF-treated PC12 cells. Changes of neurite outgrowth in differentiated PC12 cells stably expressing Sept5 deletion constructs were evident (Table 1). Image analyses indicated that there were no differences in the percentage of neurite-bearing cells. However, the total number of neurites per cell was increased in PC12 cells expressing Sept5-CT or Sept5-G3 compared to PC12 cells expressing Sept5-NT ($P < 0.05$) (Table 1). Similarly, the total neurite length per cell was also increased in PC12 cells expressing Sept5-CT or Sept5-G3 compared to PC12 cells expressing Sept5-NT, but the values failed to reach statistical significance (Table 1).

DISCUSSION

Although neurotrophins play important roles in neurite outgrowth and cytoskeletal remodeling, a direct interaction between neurotrophin receptors and relevant effectors has not been fully elucidated. ARMS/Kidins220 acts as a platform to recruit downstream effectors to the neurotrophin receptors (Arevalo et al., 2004). To identify interacting molecules with ARMS/Kidins220, a yeast two hybrid screening was performed, which identified Sept5 as one of the interacting molecules. Septins were originally identified as filamentous proteins essential for cytokinesis in yeast (Hartwell, 1971). Although the precise functions of mammalian septins remain unclear, they may be involved in mechanisms other than cytokinesis since some of them, such as Sept5 and Septin 7 (Sept7), are predominantly expressed in the postmitotic neurons in the brain (Honer et al., 1993; Kinoshita et al., 2000). In addition, mammalian septins can associ- ate with membranes, microtubules and F-actin to mediate changes in cytoskeletal dynamics and membrane remodeling by acting as structural scaffolds (Weirich et al., 2008). For example, Sept7 specifically localizes to the dendritic branch points and overexpression of Sept7 increases dendrite branching, whereas a decreased expression of Sept7 reduces dendritic arborization (Tada et al., 2007). This suggests that Sept7 may play important roles in regulating growth and morphology of dendritic spine (Tada et al., 2007; Xie et al., 2007).

Sept5 inhibits exocytosis by blocking vesicle fusion through its binding to syntaxin and, thus, inhibits soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) interactions (Beites et al., 1999). Interestingly, the Sept5 domains important for syntaxin 1A binding (amino acids 1-213, Sept5-NT in this study) (Beites et al., 2005) includes a specific region of the N-terminus of Sept5 (amino acids 125-213) required for ARMS/Kidins220 binding. Both ARMS/Kidins220 and Sept5 were co-localized with F-actin at the plasma membrane and in the tips of growing neurites in NGF-treated PC12 cells. Sept5 is co-localized with syntaxin 1A at the plasma membrane and in neurites of NGF-treated PC12 cells (Beites et al., 1999).

The previous and present results suggest that Sept5 may interact with ARMS/Kidins220 and syntaxin 1A simultaneously. It will be very interesting to examine whether Sept5 and ARMS/Kidins220 may be functional links between neurotrophin receptors and SNARE proteins. They may mediate extrinsic signals from the plasma membrane to the SNARE proteins in the vesicle to affect vesicle dynamics in neurons in response to neurotrophin receptor activation.

In response to a 2-week NGF treatment, there was a significant decrease in average number of neurites per cell in PC12 cells expressing Sept5-NT compared to PC12 cells expressing either Sept5-CT or Sept5-G3 ($P < 0.05$). There were also some increases in average length of neurites in cells expressing either Sept5-CT or Sept5-G3 compared to cells expressing Sept5-NT, although these increases failed to reach statistical significance. Since neither Sept5-CT nor Sept5-G3 bound to ARMS/Kidins220, and Sept5-NT displayed stronger binding to syntaxin 1A than Sept5-WT (Beites et al., 2005), Sept5-NT may act as a dominant negative mutant to prevent endogenous Sept5 from interacting with ARMS/Kidins220. Alternatively, Sept-NT may act as a dominant negative mutant to prevent endogenous Sept5 from interacting with syntaxin 1A to affect the function of syntaxin 1A. Overexpression of syntaxin 1A inhibits NGF-induced neurite extension in PC12 cells (Zhou et al., 2000). It is also possible that Sept5-NT may bind to ARMS/Kidins220 and syntaxin 1A simultaneously to affect the functions of two proteins at the same time. Thus, further studies are needed to examine the interactions among these proteins at

Table 1. Effects of Sept5 deletion constructs on neurite outgrowth of NGF-treated PC12 cells

	Total neurite number/cell	Total neurite length/cell (μm)	Neurite-bearing cells (%)
Vector	3.40 ± 0.30	389.03 ± 41.90	84.46 ± 7.25
Sept5-WT	3.27 ± 0.20	387.70 ± 47.44	92.64 ± 3.18
Sept5-NT	3.04 ± 0.21	365.45 ± 42.32	94.49 ± 1.92
Sept5-CT	3.36 ± 0.23*	412.91 ± 15.40	92.95 ± 2.17
Sept5-G3	3.29 ± 0.17*	405.95 ± 31.95	90.33 ± 3.10

*, $p < 0.05$ compared to Sept5-NT. At least 50 cells were analyzed in randomly chosen fields. The values are expressed as the mean ± SEM of five independent experiments.

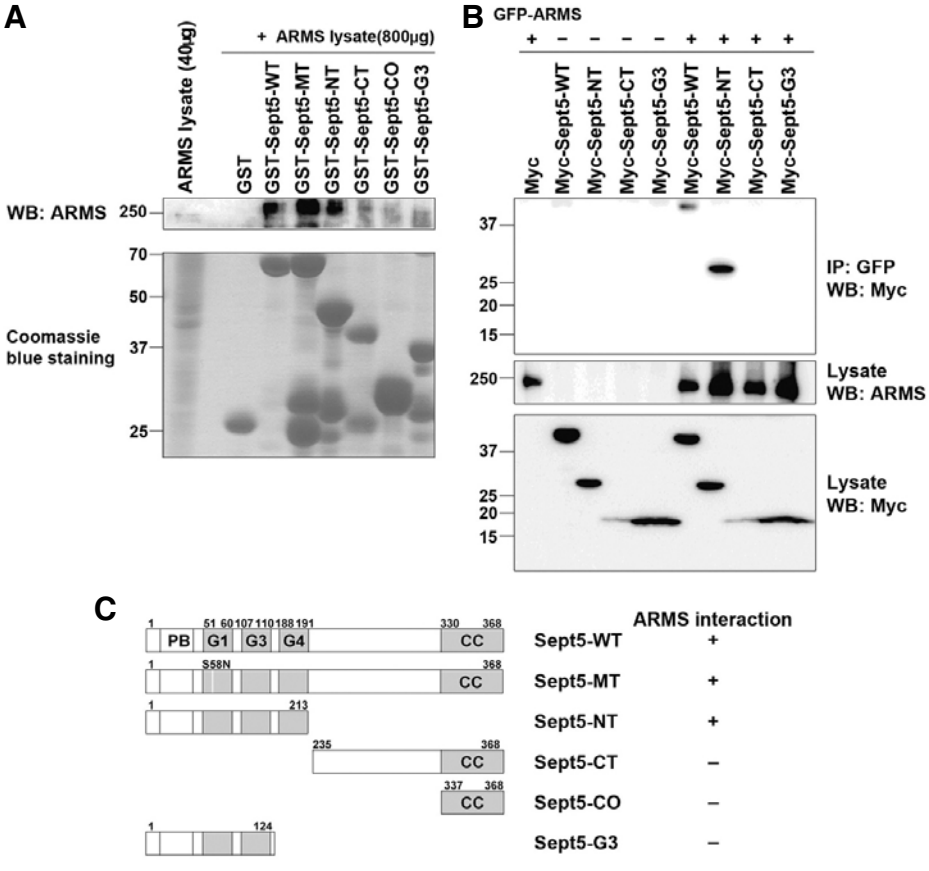

Fig. 4. Mapping the interaction between ARMS/Kidins220 and Sept5. (A) GST pull-down assays using GST-tagged full-length and deletion constructs of Sept5. GST protein alone (GST), GST-tagged full-length (GST-Sept5-WT), Sept5 with a point mutation in the GTP-binding domain (GST-Sept5-MT), N-terminal domain of Sept5 (GST-Sept5-NT), C-terminal domain of Sept5 (GST-Sept5-CT), coiled-coil domain of Sept5 (GST-Sept5-CO) and further deleted N-terminal domains of Sept5 (GST-Sept5-G3) were immobilized on glutathione-agarose beads and incubated with ARMS/Kidins220-overexpressing HEK293 cell lysates. Bound proteins were electrophoretically resolved and immunoblotted with anti-ARMS/Kidins220 antibody. (B) Co-immunoprecipitation of ARMS/Kidins220 with Sept5 deletion constructs. HEK293 cells were transiently co-transfected with GFP-tagged full-length ARMS/Kidins220, Myc-tagged full-length and deletion constructs of Sept5. Cell lysates were subjected to immunoprecipitation with anti-GFP antibody for ARMS/Kidins220, followed by Western blot with either anti-Myc antibody or anti-ARMS/Kidins220 antibody. Both full-length and the N-terminal regions of Sept5 were co-immunoprecipitated with ARMS/Kidins220. All experiments were repeated five times with the same results. (C) Amino acids 125-213 of Sept5 are required for binding to ARMS/Kidins220. PB; polybasic region, G1, G3 and G4; GTP binding domains, CC; coiled-coil domain. (D) Alignment of Sept5 N-terminal sequences involved in ARMS/Kidins220 interaction. Asterisk; fully conserved residues, two dots; conserved amino acids.

the growing tips of neurites and to examine the effects of down-regulation or deletion of Sept5 on NGF-induced neurite outgrowth of PC12 cells. Since homozygotic Sept5 null mice do not have any developmental abnormalities or function due to compensatory changes in the expression of other septins in the brain (Peng et al., 2002), it is possible that there may be functional redundancy among septins to replace Sept5 in interacting with ARMS/Kidins220.

In summary, we demonstrate that ARMS/Kidins220 specifically interacts with Sept5 through a highly conserved N-terminal region of Sept5. The direct interaction between ARMS/Kidins 220 and Sept5 suggests that septins act in mammalian cells to mediate neurotrophin-induced intracellular signaling events, such as neurite outgrowth and cytoskeletal remodeling.

ACKNOWLEDGMENTS

We thank Dr. William S. Trimble, University of Toronto, Canada, for providing us anti-Sept5 Sp20 monoclonal antibody and plasmids (pCDNA Flag-tagged full-length Sept5 cDNA, GST-Sept5-WT, GST-Sept5-MT, GST-Sept5-NT, GST-Sept5-CT and GST-Sept5-CO). This research was supported by a grant from the NIH (MVC, NS21072) and a grant from the National Research Foundation of Korea (M-SC, 2006-KRF-531-C00043) funded by the Ministry of Education, Science and Technology, Republic of Korea.

REFERENCES

Arevalo, J.C., Yano, H., Teng, K.K., and Chao, M.V. (2004). A unique pathway for sustained neurotrophin signaling through an ankyrin-rich membrane-spanning protein. EMBO J. *23*, 2358-2368.

Arevalo, J.C., Pereira, D.B., Yano, H., Teng, K.K., and Chao, M.V. (2006). Identification of a switch in neurotrophin signaling by selective tyrosine phosphorylation. J. Biol. Chem. *281*, 1001-1007.

Beites, C.L., Xie, H., Bowser, R., and Trimble, W.S. (1999). The septin CDCrel-1 binds syntaxin and inhibits exocytosis. Nat. Neurosci. *2*, 434-439.

Beites, C.L., Campbell, K.A., and Trimble, W.S. (2005). The septin Sept5/CDCrel-1 competes with alpha-SNAP for binding to the SNARE complex. Biochem. J. *385*, 347-353.

Bracale, A., Cesca, F., Neubrand, V.E., Newsome, T.P., Way, M., and Schiavo, G. (2007). Kidins220/ARMS is transported by a kinesin-1-based mechanism likely to be involved in neuronal differentiation. Mol. Biol. Cell *18*, 142-152.

Cabrera-Poch, N., Sanchez-Ruiloba, L., Rodriguez-Martinez, M., and Iglesias, T. (2004). Lipid raft disruption triggers protein kinase C and Src-dependent protein kinase D activation and Kidins220 phosphorylation in neuronal cells. J. Biol. Chem. *279*, 28592-28602.

Chang, M.S., Arevalo, J.C., and Chao, M.V. (2004). Ternary complex with Trk, p75, and an ankyrin-rich membrane spanning protein. J. Neurosci. Res. *78*, 186-192.

Chao, M.V. (2003). Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat. Rev. Neurosci. *4*, 299-309.

Cortes, R.Y., Arevalo, J.C., Magby, J.P., Chao, M.V., and Plummer, M.R. (2007). Developmental and activity-dependent regulation of ARMS/Kidins220 in cultured rat hippocampal neurons. Dev. Neurobiol. *67*, 1687-1698.

Gyuris, J., Golemis, E., Chertkov, H., and Brent, R. (1993). Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. Cell *75*, 791-803.

Hartwell, L.H. (1971). Genetic control of the cell division cycle in yeast : IV. Genes controlling bud emergence and cytokinesis. Exp. Cell. Res. *69*, 265-276.

Honer, W.G., Hu, L., and Davies, P. (1993). Human synaptic proteins with a heterogeneous distribution in cerebellum and visual cortex. Brain. Res. *609*, 9-20.

Huang, E.J., and Reichardt, L.F. (2003). Trk receptors: roles in neuronal signal transduction. Annu. Rev. Biochem. *72*, 609-642.

Iglesias, T., Cabrera-Poch, N., Mitchell, M.P., Naven, T.J., Rozengurt, E., and Schiavo, G. (2000). Identification and cloning of Kidins220, a novel neuronal substrate of protein kinase. D. J. Biol. Chem. *275*, 40048-40056.

Kinoshita, A., Noda, M., and Kinoshita, M. (2000). Differential localization of septins in the mouse brain. J. Comp. Neurol. *428*, 223-239.

Kong, H., Boulter, J., Weber, J.L., Lai, C., and Chao, M.V. (2001). An evolutionarily conserved transmembrane protein that is a novel downstream target of neurotrophin and ephrin receptors. J. Neurosci. *21*, 176-185.

Lopez-Menendez, C., Gascon, S., Sobrado, M., Vidaurre, O.G., Higuero, A.M., Rodriguez-Pena, A., Iglesias, T., and Diaz-Guerra, M. (2009). Kidins220/ARMS downregulation by excitotoxic activation of NMDARs reveals its involvement in neuronal survival and death pathways. J. Cell Sci. *122*, 3554-3565.

Luo, S., Chen, Y., Lai, K.O., Arevalo, J.C., Froehner, S.C., Adams, M.E., Chao, M.V., and Ip, N.Y. (2005). {alpha}-Syntrophin regulates ARMS localization at the neuromuscular junction and enhances EphA4 signaling in an ARMS-dependent manner. J. Cell Biol. *169*, 813-824.

Macara, I.G., Baldarelli, R., Field, C.M., Glotzer, M., Hayashi, Y., Hsu, S.C., Kennedy, M.B., Konoshita, M., Longtine, M., Low, C., et al. (2002). Mammalian septins nomenclature. Mol. Biol. Cell *13,* 4111-4113.

Peng, X.R., Jia, Z., Zhang, Y., Ware, J., and Trimble, W.S. (2002). The septin CDCrel-1 is dispensable for normal development and neurotransmitter release. Mol. Cell. Biol. *22*, 378-387.

Reichardt, L.F. (2006). Neurotrophin-regulated signalling pathways. Philos. Trans. R. Soc. B. *361*, 1545-1564.

Sniderhan, L.F., Stout, A., Lu, Y., Chao, M.V., and Maggirwar, S.B. (2008). Ankyrin-rich membrane spanning protein plays a critical role in nuclear factor-kappa B signaling. Mol. Cell Neurosci. *38*, 404-416.

Tada, T., Simonetta, A., Batterton, M., Kinoshita, M., Edbauer, D., and Sheng, M. (2007). Role of Septin cytoskeleton in spine morphogenesis and dendrite development in neurons. Curr. Biol. *17*, 1752-1758.

Weirich, C.S., Erzberger, J.P., and Barral, Y. (2008). The septin family of GTPases: architecture and dynamics. Nat. Rev. Mol. Cell Biol. *9*, 478-489.

Wu, S.H., Arevalo, J.C., Sarti, F., Tessarollo, L., Gan, W.B., and Chao, M.V. (2009). Ankyrin Repeat-rich Membrane Spanning/Kidins220 protein regulates dendritic branching and spine stability *in vivo.* Dev. Neurobiol. *69*, 547-557.

Xie, Y., Vessey, J.P., Konecna, A., Dahm, R., Macchi, P., and Kiebler, M.A. (2007). The GTP-binding protein Septin 7 is critical for dendrite branching and dendritic-spine morphology. Curr. Biol. *17*, 1746-1751.

Zhou, Q., Xiao, J., and Liu, Y. (2000). Participation of syntaxin 1A in membrane trafficking involving neurite elongation and membrane expansion. J. Neurosci. Res. *61*, 321-328.