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Interaction of Ras-GTPase-activating Protein SH3 Domain-binding Proteins 2, G3BP2, With the C-terminal Tail Region of KIF5A

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Vesicles and organelles are transported along microtubule and delivered to appropriate compartments in cells. The intracellular transport process is mediated by molecular motor proteins, kinesin, and dynein. Kinesin is a plus-end-directed molecular motor protein that moves the various cargoes along microtubule tracks. Kinesin 1 is first isolated from squid axoplasm is a dimer of two heavy chains (KHCs, also called KIF5s), each of which is associated with the light chain (KLC). KIF5s interact with many different binding proteins through their carboxyl (C)-terminal tail region, but their binding proteins have yet to be specified. To identify the interacting proteins for KIF5A, we performed the yeast two-hybrid screening and found a specific interaction with Ras-GTPase-activating protein (GAP) Src homology3 (SH3)-domain-binding protein 2 (G3BP2), which is involved in stress granule formation and mRNA-protein (mRNP) localization. G3BP2 bound to the C-terminal 73 amino acids of KIF5A but did not interact with the KIF5B, nor the KIF5C in the yeast two-hybrid assay. The arginine-glycine-glycine (RGG)/Gly-rich region domain of G3BP2 is a minimal binding domain for interaction with KIF5A. However, G3BP1 did not interact with KIF5A. When co-expressed in HEK-293T cells, G3BP2 co-localized with KIF5A and was co-immunoprecipitated with KIF5A. These results indicate that G3BP2, which was originally identified as a Ras-GAP SH3 domain-binding protein, is a protein that interacts with KIF5A.

Key words : Binding protein, kinesin, microtubule, Ras signaling, SH3 domain

Introduction

Many proteins, vesicles, and organelles move to appropriate destinations after their synthesis in the cell body. Motor proteins, kinesin and dynein play important roles during cargo trafficking to appropriate destinations [13]. Kinesin motors are primarily involving in the transport of various cargoes from cell body to cell periphery, while dynein motors mediate the movement of cargoes from cell periphery toward the cell body along microtubules [12].

Kinesin 1 is the first identified member of kinesin superfamily proteins (KIFs) as major KIFs in squid axoplasm and involved in the transport of various cargoes, including membrane-bounded vesicles, organelles, proteins complexes, and mRNAs [12, 13]. It is a heterotetrameric protein composed of two heavy chains (KHCs), also known as KIF5s and two light chains (KLCs) [18]. The KIF5s possess a motor domain at the amino (N)-terminal region connected by a long stalk domain to a carboxyl (C)-terminal tail domain [12]. The motor domain, which binds to microtubules, is responsible for the motor activity of kinesin 1, and the C-terminal tail domain is responsible for interacting to cargoes [9, 12]. The cargoes that bind to the C-terminal region of KIF5s fall into six classes: membrane-bound organelles, cytoskeleton subunits, pathogens, RNA macromolecular complexes (mRNPs), protein complexes, and signaling molecules [6, 10, 17, 24, 28]. KIF5s have been revealed to consist of three closely related subtypes: KIF5A, KIF5B, and KIF5C [18]. KIF5B is expressed ubiquitously in many tissues, whereas KIF5A and KIF5C are expressed in nervous tissue [18]. In nervous tis-

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sue, KIF5A can form homodimer or heterodimer with KIF5B, or KIF5C [18].

To investigate the functions of each KIF5s, *kif5* knockout (KO) mice were generated [29]. *kif5B*-KO mice died during early embryonic development because of impaired transport of multiple essential organelles, including mitochondria and lysosomes [29]. Furthermore, the abnormal localization of mitochondria in yolk cultured cells from a *kif5B* null mice was rescued by exogenous expression of KIF5A, or KIF5C [29]. This finding suggested that the members of KIF5s have functionally similar role(s) in cells. *kif5C*-KO mice were normal in their appearance but showed a smaller brain size [18]. *kif5A*-KO mice were neonatal lethal and showed no apparent histological abnormalities in their brains [31]. Conditional *kif5A*-KO mice showed epileptic seizures [25]. The γ-aminobutyric acid receptors ($GABA_A$Rs) trafficking to the neuronal surface was impaired in the KIF5A-deficient neurons [25]. *kif5B*-KO mice and *kif5C*-KO mice did not show epileptic seizure [25]. Several mutations in the *kif5A* gene have been identified to cause spastic paraplegia 10 (SPG10) [26].

To identify KIF5A binding proteins, using the yeast two-hybrid screens, we found that Ras-GTPase-activating protein (GAP) SH3-domain-binding protein 2 (G3BP2) interacted with KIF5A in cells.

Materials and Methods

Plasmid constructs

Full length cDNA clones of KIF5s were previously obtained by Kanai *et al*. [18]. The mouse KIF5A cDNA fragment corresponding to the cargo-binding domain (amino acids, 906-1,027) was amplified by polymerase chain reaction (PCR) using the appropriate primers (forward primer: 5′-TAC AAG AGC TCC GGC AAG CGG GGC-3′, reverse primer: 5′-TTA GCT GGC TGC TGT CTC TTG GTG-3′). The amplified fragment was cloned into pLexA (Clontech Laboratories, Inc., Palo Alto, CA, USA). The plasmid, pLexA-cargo-binding domain-KIF5A, served as bait plasmid. The full-length cDNAs of mouse G3BP1 (GeneBank accession number: NM_013716) and G3BP2 (GeneBank accession number: NM_011816) were amplified by PCR from Marathon-Ready™ cDNA library (Clontech Laboratories, Inc.) and cloned into pGEM T-easy vector (Promega Corp, Madison, WI, USA).

Screening of KIF5A-binding proteins by yeast two-hybrid screening

The Matchmaker LexA two-hybrid system was used for screening according to the manufacturer's manual (Clontech Laboratories, Inc.). pLexA-cargo-binding domain-KIF5A was transformed into yeast strain EGY48 carrying the p8op-lacZ gene. The transformed EGY48 yeast cells containing pLexA-cargo-binding domain-KIF5A were transformed with the mouse brain cDNA library (Clontech Laboratories, Inc.) [15]. The selection of positive clones was performed on an SD/-His/-Trp/-Ura/-Leu plate containing galactose, raffinose, BU salts, and X-gal. Plasmids from positive clones were analyzed by restriction digestion with *Eco*RI and *Xho*I. The insert DNAs were sequenced and DNA sequence analysis was performed using the BLAST algorithm at the National Center for Biotechnology Information (NCBI). Sequence-verified clones were tested again for interaction with bait in yeast by retransformation.

β-Galactosidase activity in liquid cultures of yeast

The β-galactosidase activity of yeast was assayed as described previously [15, 16]. In brief, 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°C, 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 and the cell density. The units of enzyme activity were calculated by the following equation: units=1,000× [(OD_{420} − 1.75× OD_{550})]/[reaction time (min) x culture volume (ml) × OD_{600}].

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

For immunocytochemistry analysis, HEK-293T cells grown on poly-D-lysine-coated coverslips were transfected with KIF5A and EGFP-G3BP2 constructs. Twenty-four hours after transfection, cells were washed with phosphate-buffered sal-

ine (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 overnight at 4°C with anti-KIF5A antibody [25] diluted 1:500 in PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween-20. After washing 3 times with PBS, cells were incubated for 40 min with Dylight 594-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch Labs, West Grove, PA, USA) diluted 1:800. After washing 3 times with PBS, the cells were mounted with Fluoromount (DAKOKorea, Seoul, Korea). Fluorescence images were acquired on Zeiss LSM510 META confocal laser scanning microscope (Carl Zeiss, Oberkochem, Germany).

Co-immunoprecipitation and immunoblot analysis

To examine co-immunoprecipitation of KIF5A with G3BP2, myc-KIF5A and FLAG-G3BP2 were transfected to HEK-293T cells. 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, San Diego, CA, USA)] by gentle rotation for 30 min. Lysates were centrifuged at 16,000x *g* for 10 min at 4°C. The supernatant was incubated with anti-FLAG M2 agarose beads (Sigma-Aldrich) for 2 hr at 4℃ 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 washed beads were resuspended with Laemmli's loading buffer and the proteins were eluted and denatured by boiling for 5 min. The proteins were processed for 10% SDS-PAGE and immunoblot analysis with antibodies against KIF5A, KIF3A, KLC, and FLAG epitope as described elsewhere by Nakajima et al. [25].

Results

Identification of KIF5A interacting proteins by yeast two-hybrid screening

To identify KIF5A specific interacting proteins, we screened a mouse brain cDNA library by yeast two-hybrid system using the cargo-binding domain (amino acids 906-1027) of KIF5A as bait (Fig. 1A). In screen of 1×10^7 independent transformants, two positive clones were obtained. After confirmation and partial sequencing, two clones were found to encode the same C-terminal region of G3BP2 (Fig. 1B). KIF5A is composed of a motor domain in the N-terminal region, stalk domain, and the C-terminal tail domain [18]. KIF5A has the C-terminal tail region of 73 amino acids that shows no homology with KIF5B or KIF5C [18]. Various fragments of KIF5A were constructed and tested for interaction with G3BP2 using a yeast two-hybrid assay. The result indicates that the C-terminal 73 amino acids of KIF5A are required for binding to G3BP2 (Fig. 2A). G3BP2 was originally identified as Ras-GAP SH3 domain-binding protein, which previously had been shown to be essential in the assembly of stress granules and is a multi-domain protein comprised of a nuclear transport factor 2 (NTF2) in N-terminal region, acid rich domain, a hydrophobic PxxP motif, RNA recognition motif (RRM), and arginine-glycine-glycine (RGG)/Gly-rich region domain in the C-terminal tail region. (Fig. 1B) [5, 14, 20, 23]. To identify the domain of G3BP2 required for the interaction with KIF5A, a series of deletion mutants of G3BP2 was constructed and analyzed for their interactions

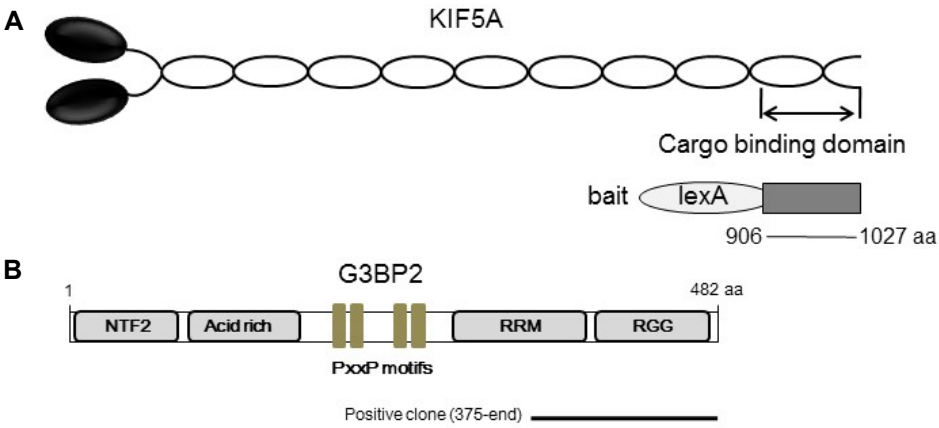

Fig. 1. Identification of the proteins interacted with KIF5A by yeast two-hybrid screening. (A) Schematic diagram of KIF5A. The cargo-binding domain of KIF5A used for the yeast two-hybrid screen. (B) Schematic diagram of G3BP2. The positive clone isolated from the yeast two-hybrid screening possesses the cDNA for G3BP2.

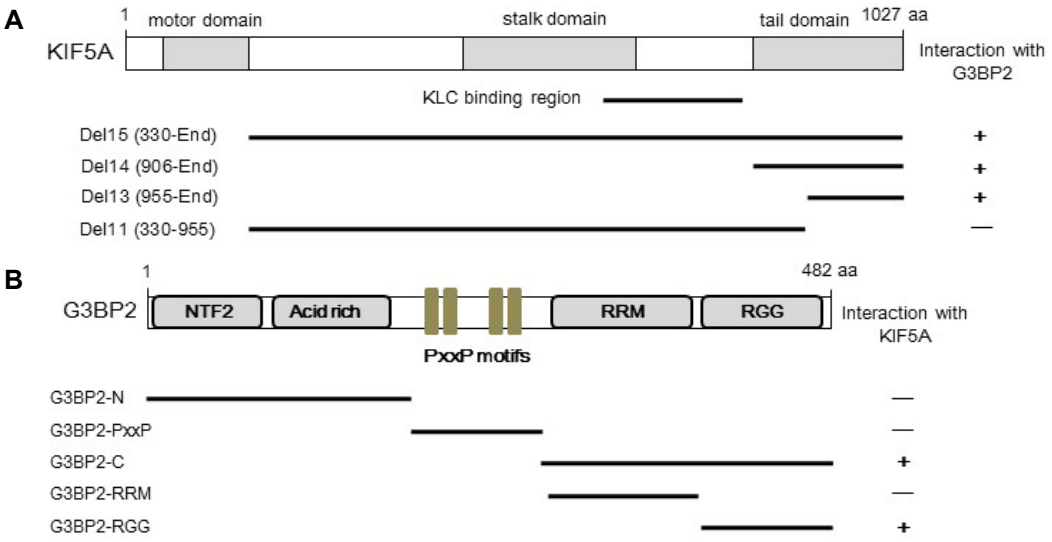

Fig. 2. Minimal G3BP2 binding region in KIF5A. (A) G3BP2 binding region in KIF5A. KIF5A has the motor domain, stalk domain, and tail domain, indicated in gray. The truncated forms of KIF5A were assessed in the yeast two-hybrid assay for interaction with G3BP2. (B) KIF5A binding region in G3BP2. Different truncations of G3BP2 were tested in the yeast two-hybrid assay for interaction with KIF5A. +, interaction; -, no interaction; KIF5A, kinesin superfamily protein 5A; G3BP2, Ras-GTPase-activating protein (GAP) Src homology3 (SH3) domain-binding protein 2; NTF2, nuclear transport factor 2 (NTF2)-like domain; Acid rich, acid rich domain; RRM, RNA recognition motif; RGG, arginine-glycine-glycine (RGG)/Gly-rich domain; KLC, kinesin light chain; aa, amino acids.

with KIF5A. Results exhibited that only the C-terminal RGG/Gly-rich region G3BP2 interacted with KIF5A (Fig. 2B).

Subsequently, we investigated whether G3BP2 interact with KIF5B, KIF5C, KLC1, and KIF3A, a motor subunit of kinesin 2. As shown in Fig. 3A, KIF5B, KIF5C, KIF3A, and KLC1 did not interact with G3BP2. The main difference between G3BP1 and G3BP2 is found in the number of PxxP motifs and the C-terminal region of the protein [20]. Next, we investigated whether KIF5A interacts with the other iso-

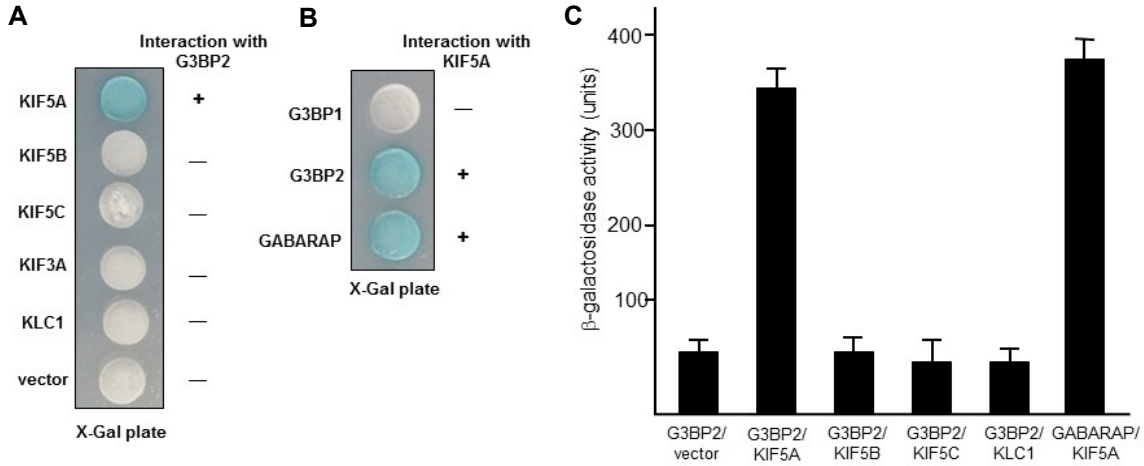

Fig. 3. Interaction of KIF5A with G3BP2. (A, B) The tail region of KIF5s and KIF3A, the full length KLC1, and the full length G3BPs were tested for the interaction in the in the yeast two-hybrid assay. G3BP2 specifically interacted with KIF5A but not with KIF5B, KIF5C, KIF3A or KLC1. KIF5A specifically interacted with G3BP2. GABARAP served as a positive control for interaction with KIF5A. (C) The strength of interaction between G3BP2 and KIF5s or KLC1 was examined quantitatively using β-galactosidase activity in yeast two-hybrid reporter assay. +, interaction; -, no interaction; KIF5, kinesin superfamily protein 5; KIF3A, kinesin superfamily protein 3A; KLC1, kinesin light chain 1; G3BP2, Ras-GTPase-activating protein (GAP) Src homology3 (SH3) domain-binding protein 2; GABARAP, γ-aminobutyric acid receptor-associated protein.

form, G3BP1. KIF5A did not interact with G3BP1 (Fig. 3B). This data suggest that KIF5A specifically interacts with G3BP2. To quantify the binding affinity of KIF5s to G3BP2, the KIF5s or KLC1 expression plasmids were transformed into yeast and the β-galactosidase activity was measured in liquid cultures. $GABA_AR$-associated protein (GABARAP), known to interact with KIF5A [25], served as a positive control. The interaction of KIF5A with G3BP2 yielded approximately 340 units of β-galactosidase activity (Fig. 3C).

G3BP2 is associated with KIF5A in cells

The KIF5A-G3BP2 interaction was also assessed in mammalian cells. When the lysate of HEK-293T cells transiently expressing myc-KIF5A and FLAG-G3BP2 was immunoprecipitated with anti-FLAG antibody, KLC and KIF5A, but not KIF3A were co-precipitated with G3BP2 (Fig. 4A). This result indicates that G3BP2 associates with kinesin 1. In order to further confirm whether KIF5A and G3BP2 co-localize in cells, KIF5A was co-expressed with EGFP-G3BP2 in HEK-293T cells. KIF5A and G3BP2 co-localized at the same region in cells (Fig. 4B). Taken together, these results indicate that G3BP2 interacts with KIF5A in cells.

Discussion

In this study, we show that KIF5A interacts with G3BP2. Using the cargo-binding domain of KIF5A as bait, we identified G3BP2 in a yeast two-hybrid assay. The RGG/Gly-rich region of G3BP2 interacted with the C-terminal 73 amino acids of KIF5A. Furthermore, when KIF5A and G3BP2 were expressed in mammalian cells, they co-immunoprecipitated and co-localized in cells. Taking all of these results together, we hereby suggest that kinesin 1 transports G3BP2-associated stress granules or mRNPs in cells.

In mouse, three different KIF5s, KIF5A, KIF5B, and KIF5C have been identified [18]. Each KIF5 has a high conserved motor domain and stalk domain [18]. However, their tail domain that mediate the interaction with various binding partners or cargoes has low similarity [18, 19]. We found that G3BP2 interacts only with KIF5A. G3BP1 and G3BP2 have 59% amino acid similarity and share similar domain architecture [14, 20]. However, the RGG domain of G3BP2 has no homology with G3BP1 [20]. In this study, we found that KIF5A interacts only with G3BP2.

The exposure of heat shock or hypoxia to cells, cells acti-

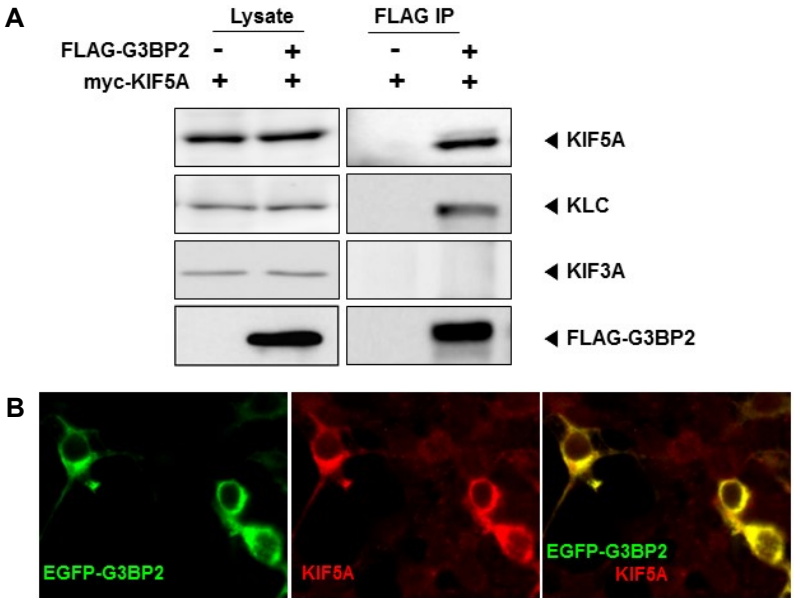

Fig. 4. Co-immunoprecipitation and co-localization of KIF5A and G3BP2 in mammalian cells. (A) HEK-293T cells were transiently transfected with FLAG-G3BP2 and myc-KIF5A plasmids as indicated. Cell lysates were immunoprecipitated with monoclonal anti-FLAG antibody. Precipitates were immunoblotted with anti-KIF5A, KLC, KIF3A, and FLAG antibodies. G3BP2 co-precipitated KLC and KIF5A, but not KIF3A. (B) HEK-293T cells were transiently transfected with EGFP-G3BP2 and KIF5A plasmids. Twenty-four hours after transfection, cells were subjected to immunofluorescence with anti-KIF5A antibody. KIF5A and G3BP2 are seen at the same subcellular region in cells. KIF5A, kinesin superfamily protein 5A; KIF3A, kinesin superfamily protein 3A; KLC, kinesin light chain; G3BP2, Ras-GTPase-activating protein (GAP) Src homology3 (SH3) domain-binding protein 2.

vate protective mechanisms including the formation of stress granules [2, 7]. Such stress granule contains various RNA-binding proteins, such as G3BPs, eukaryotic initiation factors (eIFs), and poly-A binding protein (PABP) [2, 3, 30]. G3BPs were composed of five domains, the NTF2-like domain, the acidic rich domain, the PXXP motif, and two RNA-binding domains, RRM domain and RGG domain [20]. The NTF2-like domain is required for stress granule formation, as evidenced by the fact that knockdown of G3BPs reduces the stress granule formation in cells [30]. PxxP motifs interact with Ras-GAP and the acid rich domain contains the conserved Ser149 that is phosphorylated in a Ras-GAP-dependent manner [30]. A previous study showed that the acid rich domain of G3BP1 showed inhibitory activity for stress granule formation [30]. RRM domain and RGG domain interact with 40S ribosomal subunits and are required for G3BP-mediated stress granule formation [8, 19]. In this study, we have identified that KIF5A interacted with the RGG domain of G3BP2.

What would be the meaning of the interaction between KIF5A and G3BP2? G3BP2 may play as a scaffolding protein that links kinesin 1 and stress granules and/or mRNPs [11, 18]. G3BPs are involved in several cellular functions including stress granule formation and localization [14] and mRNPs localization [4]. Therefore, the interaction of KIF5A and G3BP2 may be involved in the localization of mRNPs and stress granules localization in cells. Previous study indicated that G3BP2 form a homo-multimer and a hetero-multimer with G3BP1 [25]. Moreover, like G3BP1, the overexpression of G3BP2 induced stress granule formation in cells [25]. G3BP1 and G3BP2 are components interacting with the neuronal stress granules and stabilizing mRNA [1]. *G3BP1*-KO mice show abnormal synaptic plasticity in neuron [22]. Interestingly, previous results indicated that KIF5s interacted with mRNP granules consisting of various mRNA binding proteins including RNA-binding protein with multiple splicing (RBPMS), an interacting protein of G3BPs [18]. In cultured neurons, the localization of G3BP1 overlapped with KIF5A in large stress granules [11]. In many cases, various cargoes interact with adaptor proteins/scaffolding proteins that mediate the attachment to kinesin 1 [12, 27]. Thus, G3BP2 may serve as a scaffolding protein that links kinesin 1 and stress granules and/or mRNPs. Taken together, we suggest that the KIF5A-G3BP2 interaction may play crucial role(s) in intracellular transport of stress granules or mRNPs.

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초록 : Ras-GTPase-activating protein SH3 domain-binding proteins 2, G3BP2와 KIF5A C-말단 꼬리 영역과의 결합

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세포 내 소기관들과 소포들은 세포 내에서 미세소관을 따라 적절한 구획으로 수송된다. 이러한 세포 내 수송 과정은 분자 모터단백질인 kinesin과 dynein에 의하여 이루어진다. Kinesin 1은 오징어 축삭돌기 세포질로부터 처음 분리되었으며 2개의 중쇄단위체(KHCs, 또는 KIF5s) 및 이와 결합하는 경쇄단위체(KLCs)의 복합체를 형성한다. KIF5s는C-말단 고리 영역을 통해 많은 다양한 단백질과 결합하는데, 아직 그 결합단백질들은 충분히 밝혀지지 않았다. 본 연구에서는 KIF5A 결합단백질을 분리하기 위하여 효모 two-hybrid 탐색을 수행하여 스트레스 과립 형성과 mRNP 위치결정에 관여하는 Ras-GTPase-activating protein (GAP) Src homology3 (SH3)-domain-binding protein 2 (G3BP2)를 분리하였다. G3BP2는 KIF5A의 C-말단 고리 영역에 존재하는 73개 아미노산을 포함하는 영역과 결합하였다. 그러나 G3BP2는 KIF5B, KIF5C, KLC1, KIF3A와는 결합하지 않았다. KIF5A는 G3BP2의 arginine-glycine-glycine (RGG)/Gly-rich 도메인과 결합하지만 G3BP1과는 결합하지 않았다. HEK-293T세포에 G3BP2와 KIF5A를 발현하여 면역침강한 결과 G3BP2와 KIF5A는 같이 침강하였다. 또한 HEK-293T 세포 내의 전체에서 두 단백질은 같은 부위에 존재하였다. 이러한 결과들은 세포 내에서 G3BP2는 KIF5A와 결합하는 결합단백질로 확인 되었다.