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The STAR RNA Binding Proteins SAM68, SLM-1 and SLM-2 Interact with Kinesin-I

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In neurons, kinesin is the molecular motor that transport cargos along microtubules. KIF5s (alias kinesin-I), are heterotetrameric motor conveying cargos, but the mechanism as to how they recognize and bind to a specific cargos has not yet been completely elucidated. To identify the interaction proteins for KIF5C, yeast two-hybrid screening was performed, and specific interaction with the Sam68-like mammalian protein 2 (SLM-2), a member of the signal transducers and activators of RNA (STAR) family of RNA processing proteins, was found. SLM-2 bound to the carboxyl (C)-terminal region of KIF5C and to other KIF5 members. The C-terminal domain of Sam68, SLM-1, SLM-2 was essential for interaction with KIF5C in the yeast two-hybrid assay. In addition, glutathione *S*-transferase (GST) pull-downs showed that SAM68, SLM-1, and SLM-2 specifically interacted to Kinesin-I complex. An antibody to SAM68 specifically co-immunoprecipitated SAM68 associated with KIF5s and coprecipitated with a specific set of mRNA. These results suggest that Kinesin-I motor protein transports RNA-associated protein complex in cells.

Key words : Kinesin-I, molecular motors, RNA, RNA binding protein

Introduction

Local protein synthesis is believed to contribute to synaptic plasticity that requires a rapid supply of new proteins to specific synaptic sites in response to appropriate stimuli [6,13,17]. The majority of mRNAs are localized to the cell body, but a subset of mRNAs is transported to dendrites and axons [6,8]. The targeting of mRNAs into synaptic terminal is an important protein sorting mechanism [1]. Live imaging studies using the green fluorescent protein (GFP) system revealed that mRNA particles are translocated in discrete transport packets of clusters [14,21]. Whereas short distance movements may involve actin filaments, the majority of long distance translocation of mRNAs into dendrites depends on microtubules [4,19,22]. Recent study, used affinity chromatography and proteomic, identified the kinesin superfamily protein (KIF) as a motor involved in the active transport RNA-containing granules into distal dendrites [4].

The first kinesin motor, (alias KIF5/kinesin-I), was identified as a major motor protein in many cell types [9]. Conventional kinesin (KIF5B) exists as a tetramer of two heavy chains (KHCs, also called KIF5s), which contain the N-terminal motor domain and the C-terminal tail, as well as two light chains (KLCs), which bind to the heavy chain tail [9,10,20]. KHCs form a highly related family (KIF5A, KIF5B, and KIF5C). KIF5B is expressed ubiquitously in many tissues, whereas KIF5A and KIF5C are specific to nerve tissue [10,30]. KIF5 initially was characterized as a motor transporting membranous organelles toward to the plus end of microtubules and forming a crossbridge between membranous organelle and microtubules [27]. Recent studies, KIF5 works as a motor for transport of mitochondria, lysosomes, tubulin oligomer, and mRNA complex toward the plus end of microtubules [9,11]. The N-terminal motor domain of KIF5s is responsible for the force-generating motor activity and for binding to microtubules [3]. The site of interaction with the cargo has been attributed to the C-terminal tail domain of KIF5s [23]. The binding proteins that bind to the C-terminal of KIF5s have suggested that there are at least two mechanisms of linkage [2,23]. KIF5s may bind directly to cargos through transmembrane proteins such as amyloid precursor protein (APP) [18] and mammalian Sundaydriver (SYD) [2], or it may bind to them indirectly, through scaffolding proteins such as glutamate receptor-interacting protein 1 (GRIP1) [23], syntabulin [24], Snytaxin [5], and β-dystrobrevin [11]. Scaffolding proteins are multifunctional proteins with several protein-protein interaction modules that can assemble large protein-protein complexes at the plasma

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membrane [23].

Despite the current knowledge of KIF5s binding partners, there are no known receptors for mRNA complex, which is dependent on KIF5s transport [11]. In addition, little is known about the regulation mechanism for KIF5s-mRNA complex recognition [11]. To improve the understanding of the role KIF5s in the mRNA complex transport, using the yeast two-hybrid screens, we identified the RNA-binding protein SLM-2 [7], as a protein that directly interacts with KIF5s.

Materials and Methods

Plasmid constructs

A previously described mouse KIF5C cDNA (NM_008449.2) was utilized as a template to amplify the region coding for amino acids 829-887 using the appropriate primers [10]. The amplified fragment was subcloned into pGEM T-easy vector (Promega Corp, Madison, WI, USA). The fragment was then *EcoR*I, *Xho*I-restricted and subcloned into the *EcoR*I, *Xho*I site of pLexA (Clontech, Palo Alto, CA, USA). Murine Sam68 (accession NM_011317.3), SLM-1 (accession AF_098796.1), and SLM-2 (accession AF_099092.1) [7] full length cDNAs were amplified by reverse transcriptase-PCR (RT-PCR) and cloned into pGEM T-easy vector.

β-actin RT-PCR

For RT-PCR analysis, beads were processed for RNA extraction using the 'Rneasy' purification kit (Qiagen, Hilden, Germany). RNA was reverse transcribed with the universal primer GGAATTC(T)17, and the resulting cDNA was amplified with the following β-actin specific primer pair: Forward 5'-GACCTGTATCCAACACA-3', Reverse 5'-TCCTCATCTGCTGAAGGT-3' [1].

Yeast Two-Hybrid Screen

The Matchmaker LexA two-hybrid system was used for screening according to the previously reported [12]. In brief, the EGY48/ p8op-lacZ yeast cells containing the KIF5C bait plasmid were transformed with the embryonic murine 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 X-gal plate. Library inserts were analyzed by restriction digestion. Library plasmids were tested for interactions of the reporter gene in yeast by the retransformation. Unique inserts were sequenced and DNA sequence analysis was performed with the BLAST algorithm at the National Center for Biotechnology Information (NCBI).

β-Galactosidase activity in liquid cultures of yeast

The strength of the interactions between SLM-2 and KIFs was assessed by measuring the β-galactosidase activity. The β-galactosidase activity of yeast was assayed as described previously [12]. In brief, mid-log phase transformed yeast cells were collected and permeabilized with 0.1% sodium dodecyl sulphate (SDS) and chloroform. An excess amount of chromogenic substrate o-nitrophenyl-β-D-galactoside was added in excess to this 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 and the cell density. The units of enzyme activity were calculated by the following equation: units=$1000\times[(OD_{420}-1.75\times OD_{550})]$/(reaction time×culture volume×OD_{600}). All experiments were independently performed at least three times.

Co-immunoprecipitation and Immunoblot analysis

Mouse brain lysate was prepared as previously described [23]. Mouse brains were homogenized in ice-cold homogenization buffer (0.32 M sucrose, 4 mM HEPES, pH 7.3) supplemented with protease inhibitors. For immunoprecipitation, the brain lysate was diluted in the same volume of 2X binding buffer (50 mM HEPES, 240 mM KCl, 2 mg/ml BSA, 0.2% Triton X-100, pH 7.4) and incubated with anti-Sam68 antibody (Santacruz Biotechnology, Santa Cruz, CA, USA) or anti-KLC antibody [10] or with control IgG overnight at 4ºC, followed by precipitation with protein-A-Sepharose (Amersham Pharmacia, Piscataway, NJ, USA). The beads were collected by brief centrifugation and washed three times with TBS-T (20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween 20). The pellets were resuspended with Laemmli's loading buffer, the proteins were eluted and denatured by boiling for 2 min and then separated by SDS-PAGE. The gel was transferred to a nitrocellulose membrane and incubated with anti-KIF5A, anti-KIF5B and anti-KIF5 antibodies [10].

Glutathione S-transferase (GST) pull-down assays

Pull-down assays using GST fusion proteins were performed as previously reported [23]. cDNAs encoding the C-terminal region of Sam68, SLM-1, and SLM-2 [7] were cloned in pET 41a, and the recombinant GST-Sam68, GST-SLM-1 and GST-SLM-2 fusion proteins were expressed in bacterial strain BL21 GOLD (Stratagene, La Jolla CA, USA) after induction with 0.5 mM isopropyl thio-β-D-galactopyranoside (IPTG) (Fisher Biotech, South Australia, Australia) for 3 hr. The fusion proteins were purified using glutathione-agarose beads (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. For pull down, protein concentration of each sample was measured in triplicate using a Bradford reagent-based assay (Bio-Rad, Hercules, CA, USA). Ten μg of each of the GST fusion proteins was then coupled to 50 μl of glutathione-agarose beads by incubating at room temperature for 1 hr, followed by rinsing several times with PBS. 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 samples were boiled for 5 min and then processed for SDS-PAGE and immunoblot analysis with anti-KIF5s antibodies and anti-KLC antibody [10].

Results

Identification of KIF5C interacting proteins by yeast two-hybrid screening

KIF5C was strong expressed in motor neuron and transport numerous cargos [10]. Earlier studies identified in the tail coiled-coil region of 51 residues that is highly conserved among KIF5s [11]. This region is essential for localizing tagged KIF5s constructs to cargos destined for secretion at the cell terminal region [10]. To elucidate the binding proteins that interact with KIF5C, the yeast two-hybrid screening was performed and used this region

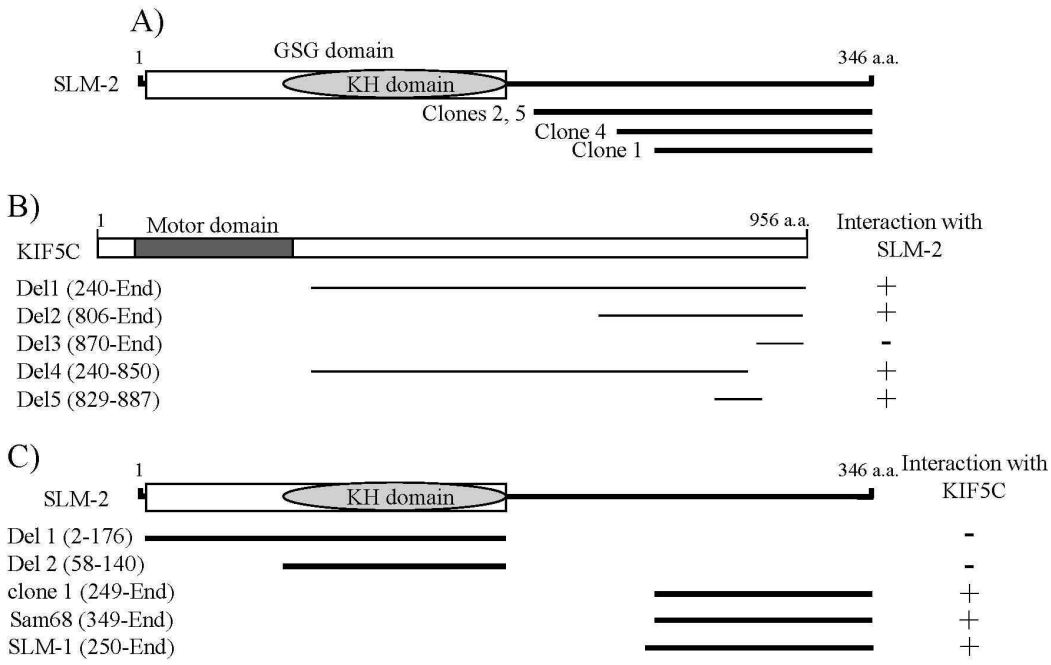

Fig. 1. Identification of the proteins interacting with KIF5C by yeast two-hybrid screening. (A) Domain structure of SLM-2 illustrates that the clones 1, 2, 4, and 5 overlap in the C-terminal region corresponding to amino acids 249-346. The GSG domain and the KH domain are near the N-terminus. aa, the number of amino acid residue. (B) Minimal SLM-2 binding region in KIF5C. KIF5C has motor domain and the C-terminal domain. Different truncations of KIF5C were constructed by PCR. Several truncated forms of KIF5C were tested in the yeast two-hybrid assays for interaction with SLM-2. +, interaction with SLM-2; -, no interaction with SLM-2. (C) Minimal KIF5C binding region in SLM-2. Different truncations of SLM-1, SLM-2 and Sam68 were constructed by PCR. Several truncated forms of SLM-1, SLM-2, and Sam68 were tested in the yeast two-hybrid assays for interaction with KIF5C. +, interaction with KIF5C; -, no interaction with KIF5C.

as bait. A bait construct encoding a fusion protein containing the C-terminal region (aa 829-887) of murine KIF5C was used to screen a murine brain pB42AD-cDNA library. In screen of 1×10^7 independent transformants, 6 positive clones were obtained. These clones were sequenced and subjected to further yeast two-hybrid retransformation assay to confirm the interactions. Four positive clones (clones 1, 2, 4, and 5) were turned out cDNA fragments containing SLM-2 and overlapped in only the C-terminal region of SLM-2 corresponding to amino acids 187-346 (Fig. 1A). To identify the region of KIF5C required for the interaction with SLM-2, a series of deletion mutants of KIF5C was constructed and analyzed their interactions with SLM-2 using the yeast two-hybrid assay (Fig. 1B). This experiment demonstrated that the minimal domain required for binding was critically dependent on the tail region of the KIF5C.

SLM-2 contains a KH domain, an RNA-binding motif that was originally identified in the heterogeneous nuclear ribonucleoprotein (hnRNP) K [7,29]. KH domain is embedded in a conserved domain of about 200 amino acids called the GSG domain (GRP33, Sam68, GLD-1) [28]. GSG domain-containing proteins share several properties, including RNA binding (Fig. 1A) [16]. To identify the minimal binding domain of SLM-2 required for the interaction with KIF5C, a series of deletion mutants of Sam68, SLM-1, and SLM-2 was constructed and analyzed their interactions with KIF5C using the yeast two-hybrid assay. The C-terminal domain of Sam68, SLM-1, and SLM-2 interacted with KIF5C in the yeast two-hybrid assay (Fig. 1C). This result was not surprising in view of the fact that the sequence identity between Sam68, SLM-1, and SLM-2 was in the minimal binding domain: 52% between Sam68 and SLM-2, 66% between SLM-1 and SLM-2 [7,16]. This experiment demonstrated that the minimal binding domain was located in the tail region.

To clarify whether SLM-2 interacts specifically with KIF5C or with other KIFs, the C-terminal regions of KIF1A, KIF3B, KIF5A, KIF5B, KIF5C, KIF17, and KLC1 were constructed and the interaction of KIFs were tested for binding with SLM-2. There was no detectable binding between SLM-2 and KIF1A, KIF3B, KIF17, and KLC1. SLM-2 interacted with KIF5s in the yeast two-hybrid assay (Fig. 2A). This data was not surprising in view of the fact that the KIF5A, KIF5B, and KIF5C share extensive similarity in their amino acid sequence (81%-83% identity in the C-terminal region) [10]. To estimate the strength of the interaction between KIF5s and SLM-2, the bait plasmid of KIFs and SLM-2 was co-transformed to yeast and was measured the β-galactosidase activity. The interaction of KIF5s with SLM-2 yielded approximately 328-397 units of β-galactosidase activity (Fig. 2B). These results indicate that SLM-2 binds specifically to KIF5s.

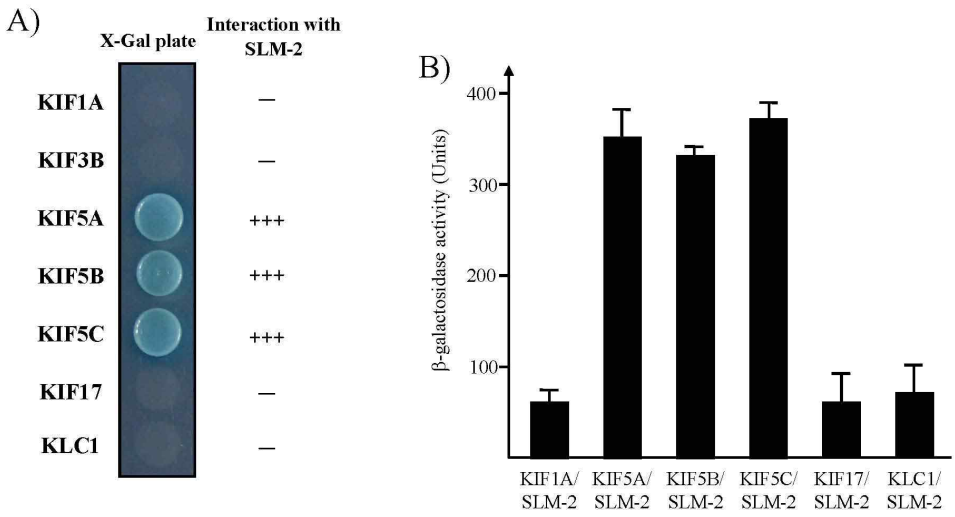

Fig. 2. Interaction between KIFs and SLM-2. (A) The C-terminal regions of each KIF protein or KLC1 were fused to the pLexA DNA binding domain. SLM-2 specifically interacted with KIF5s but not with KIF1A, KIF3B, KIF17, and KLC1. (B) The strength of interaction of KIF1A, KIF5s, KIF17, or KLC1, and SLM-2 was examined quantitatively using β-galactosidase activity in yeast two-hybrid reporter assay.

RNA binding proteins are associated with Kinesin-I

The high degree of sequence similarity between the GSG domains of SLM-1, SLM-2, and Sam68 suggest that the Sam68/SLM family members might from heteromultimers as well as homomultimers [7,16,28]. To clarify whether the RNA binding proteins interact specifically with KIF5s or whether the interaction includes KLC at the protein level, direct interaction between the RNA binding proteins and KIF5s was assayed suing a GST pull-down experiments. Recombinant GST-Sam68, GST-SLM-1, or GST-SLM-2 fusion proteins were expressed in *E. coli*. The purified GST fusion proteins are allowed to interact with brain lysates. Immunoblotting analyses revealed that Kinesin-I efficiently precipitated with GST-Sam68, GST-SLM-1, and GST-SLM-2, but not with GST (Fig. 3).

To address the question whether the complete Kinesin-I complex interacts with RNA binding proteins, immunoprecipitation analyses were performed. Lysates from mouse brain were incubated with an anti-Sam68 or anti-KLC antibodies. Protein G-agarose beads selectively precipitated the immuno-complexes, which were then subsequently separated by SDS-PAGE and immunoblotted with anti-KIF5A, anti-KIF5B and anti-KIF5C antibodies (Fig. 4A). As shown in Fig. 4A, antibody against Sam68 and KLC efficiently precipitated the KIF5s.

To investigate the possibility that the immunoprecipitated of KIF5C from mouse brain lysate may be associated with RNAs, assayed the presence of β-actin mRNA. After reverse transcription of the coprecipitated mRNA under non-denaturing conditions, primers specific for the β-actin mRNA allowed amplification of a band displaying the length expected for that mRNA, and comigrating with an amplification product obtained from the input lysate (Fig 4B). This data indicated that KIF5C is associated with a specific set of mRNAs containing protein complex. These results indicate that RNA containing protein complex and Kinesin-I motor form a complex *in vivo*.

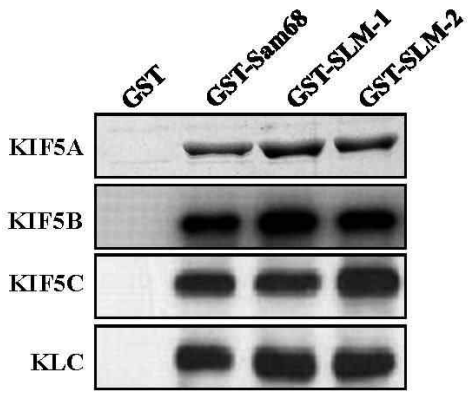

Fig. 3. Association of KIF5s with RNA binding proteins in the GST pull-down assay. Proteins in the mouse brain lysate were allowed to bind to GST alone, GST-Sam68, GST-SLM-1, and GST-SLM-2 fusion proteins. The elution fractions were resolved by SDS-PAGE, and immunoblotting was performed using an antibody to KIF5s, KLC, or Sam68.

Discussion

In this study, using a combination of yeast two-hybrid, GST pull-down and immunoprecipitation experiments, demonstrate that KIF5s directly interacts with RNA binding proteins, Sam68, SLM-1, and SLM-2. First, KIF5s directly interacts with the C-terminal region of Sam68, SLM-1, and SLM-2 in the yeast two-hybrid system (Fig. 1C). Secondly,

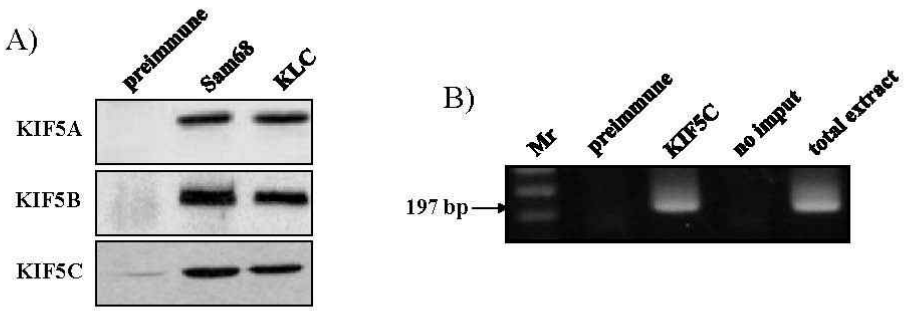

Fig. 4. Association of KIF5s with RNA binding proteins with RNA in the co-immunoprecipitation. (A) Mouse brain lysates were immunoprecipitated with an anti-Sam68 antibody, anti-KLC antibody, or preimmune serum, and then the precipitates were immunoblotted with anti-KIF5s antibodies. (B) Binding of immunoprecipitated KIF5C from mouse brain lysate with β-actin mRNA. RT-PCR with specific β-actin primers was carried out with anti-KIF5C antibody.

RNA binding proteins interacts with Kinesin-I heterotetrameric proteins in GST pull-down assay and co-immunoprecipitation study (Fig. 3, Fig. 4A). Furthermore, in this study also demonstrated that Kinesin-I can be coprecipitated with a specific set of mRNA (Fig. 4B). Thus, these results suggest that Kinesin-I transports RNA by directly interaction of different RNA binding proteins.

In neuron, mRNA is localized to dendrites and that translation of mRNA is regulated to neuronal activity [25]. Using radioactive uridine precursors, demonstrated that mRNA was transported into dendrites of cultural neurons [14,21]. Also, *in situ* hybridization studies revealed the presence of specific mRNA encoding cytosolic proteins, cytoskeletal proteins, and integral membrane proteins in dendritic layers of the hippocampus and at postsynaptic dendrites of hippocampal neurons *in vivo* and *in vitro* [26]. Previously report, unbiased approaches to amplify mRNA from purified postsynaptic dendrites have generated lists of localized mRNAs numbering in the hundreds [6,25]. The population of dendritically localized mRNAs is likely to be regulated by development [13]. Thus, many mRNAs localized to dendritic growth cones of developing neurons. Other mRNAs appear to become dendritically localized in response to activity [15,26]. Early study showed that localized mRNA is transported in the form of large protein complex particles containing mRNAs, in a rapid and microtubule dependent manner [4]. These observations suggested a critical role for mRNA-binding proteins as molecular adaptors between mRNAs and microtubule-based transport machinery.

Recent studies presented evidence that Kinesin-I are responsible for axonal and dendritic transport [11,23]. By purifying a specific subpopulation of messenger ribonucleoprotein particles (mRNPs) associated with Kinesin-I, numerous mRNA binding proteins were identified such as staufen1 and Purα [11]. What is the direct binding partner for Kinesin-I in mRNA transporting complex? In this report, SLM-2 directly interacts with KIF5s and associates with mRNA in brain extract (Fig. 4B). SLM-2 is mammalian proteins that share the basic organization of Sam68. SLM-1, SLM-2, and Sam68 share an about 70% sequence identity in their GSG domains [7]. The high degree of sequence similarity between the GSG domain of SLM-1, SLM-2, and Sam68 suggest that the Sam68/SLM family members form heteromultimers as well as homomultimers [7,16,28]. Also, the GSG family members including GRPP33, KEP1, Sam50, GDL-1, Qk1, and Who/How form heteromultimer complex [28,29]. Although this study did not show the interaction of KIF5s with other GSG family proteins, one appealing model proposes that Kinesin-I transport RNA via GSG family proteins complex. In this report, the direct interaction between Kinesin-I and SLM-2 sheds new light on the study of RNA transport.

Acknowledgments

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초록 : Kinesin-I과 직접 결합하는 STAR RNA 결합 단백질인 SAM68, SLM-1과 SLM-2의 규명

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키네신은 신경세포에서 미세소관 위를 따라 소포들을 운반하는 분자 motor 단백질로 4개의 단백질로 구성되어있다. 신경세포내에서 발현하는 KIF5C가 세포 내에서 어떤 특정소포를 이동시키는가는 신경세포성장에서 중요문제이다. 이에 본연구는 KIF5C와 결합하는 단백질을 동정하기 위하여 효모 two-hybrid 방법을 사용하여 KIF5C와 특이적으로 결합하는 Sam68-like mammalian protein 2 (SLM-2)을 확인하였다. Signal Transducers and Activators of RNA (STAR) family의 한 종류이며 RNA processing에 관여하는 RNA 결합단백질인 SLM-2는 KIF5s의 C-말단과 결합하며, 또한 SLM-2의 C-말단은 KIF5s와 결합하는데 필수영역이였다. 이러한 단백질간의 결합은 Glutathione *S*-transferase (GST) pull-down assay를 통하여 SAM68, SLM-1, SLM-2은 특이적으로 Kinesin-I과 결합함을 확인하였으며, SAM68의 항체로 면역침강한 결과 KIF5s와 mRNA는 같이 침강하였다. 신경세포의 말단에는 돌기형성에 필요한 단백질들의 주형인 mRNA가 다수 존재하며, 이러한 mRNA는 세포의 중앙에서 세포의 말단쪽으로 이동하여야 하는데, 이번 연구 결과는 Kinesin-I이 특이적으로 mRNA을 운반할 것으로 예상된다.