

JSAP1 Interacts with Kinesin Light Chain 1 through Conserved Binding Segments

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A conventional kinesin, KIF5/kinesin-I, is composed of two kinesin heavy chains (KHCs) and two kinesin light chains (KLCs) and binds directly to microtubules. KIF5 motor mediates the transport of various membranous organelles, but the mechanism how they recognize and bind to a specific cargo has not yet been completely elucidated. Here, we used the yeast two-hybrid system to identify the neuronal protein(s) that interacts with the tetratricopeptide repeats (TRP) of KLC1 and found a specific interaction with JNK/stress-activated protein kinase-associated protein 1 (JSAP1/JIP3). The yeast two-hybrid assay demonstrated that the TRP 1, 2 domain-containing region of KLC1 mediated binding to the leucine zipper domain of JSAP1. JSAP1 also bound to the TRP region of KLC2 but not to neuronal KIF5A, KIF5C and ubiquitous KIF5B in the yeast two-hybrid assay. In addition, these proteins showed specific interactions in the GST pull-down assay and by co-immunoprecipitation. KLC1 and KIF5B interacted with GST-JSAP1 fusion proteins, but not with GST alone. An antibody to JSAP1 specifically co-immunoprecipitated KIF5s associated with JSAP1 from mouse brain extracts. These results suggest that JSAP1, as KLC1 receptor, is involved in the KIF5 mediated transport.

Key words – Kinesin, kinesin light chain, molecular motors, protein-protein interaction, adaptor proteins

Introduction

The intracellular organelle transport has been shown to depend on members of the kinesin superfamily of motor proteins (KIFs), which transport organelles (e.g., mitochondria, peroxisomes, and lysosomes), protein complexes (e.g., elements of the cytoskeleton and virus particles), and mRNAs to specific destinations in a microtubule- and ATP-dependent manner [15,26,34]. The original ‘conventional’ kinesin (KIF5/kinesin-I) was shown to be a tetrameric protein composed of two heavy chains and two light chains [33,34]. Electron microscopy, protease sensitivity, and primary sequence analyses showed that the kinesin heavy chain (KHC) is composed of three domains [36]. The globular N-terminal head domain contains the adenosine triphosphate (ATP)-binding motif and a microtubule-binding domain [25,34]. The head is attached via a 50-amino acid neck region to an extended α -helical stalk, which forms a coiled-coil upon dimerization with a second heavy chain.

The C-terminal tail domain is also globular in form [26,34]. KIFs contain amino acid sequences that are highly conserved among all eukaryotic cells [20]. Outside the motor domain, KIFs show few sequence similarities. Interactions with cargo molecules have been shown to occur outside the motor domain [20,26]. Recently, it has been clearly demonstrated that several KIFs attach to specific cargoes through interactions with adaptor proteins in these binding regions [27,28,31].

KIF5 proteins, which consist of two KHCs (KIF5A, KIF5B and KIF5C) and two KLCs (KLC1, KLC2), are multifunctional neuronal transporters of both axonal cargo, such as synapsin and GAP43, and dendrite cargo, such as messenger RNA and the AMPA receptor [26,28]. In mice, the KIF5 subfamily was shown to include the distinctly brain-specific KIF5A [1] and KIF5C [14], in addition to the ubiquitous KIF5B [19]. In the mouse, both the KHCs and the KLCs are encoded by different genes with distinct expression patterns [23]. KIF5A, KIF5C, and KLC1 are enriched in neural tissues, whereas KIF5B and KLC2 are ubiquitously expressed. The c-Jun NH₂-terminal kinase-interacting protein 1 (JIP1) and JIP2 dock KIF5 to vesicles via

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interaction with the reelin receptor ApoER2 [30,35]. APP was shown to interact directly with the KIF5, yet recent evidence indicates that the attachment of APP to KIF5 is not direct but may require JIPs [12,13,18]. KLC structure is highly conserved. Sequences that are most conserved include an N-terminal coiled-coil region of 107 amino acids and six modular imperfect repeats of 34 amino acids known as tetratricopeptide repeats (TRP) encompassing a total of 252 amino acids [6]. The KLC coiled-coil domain is necessary and sufficient for the interaction with KHC [2,5]. The KLC TRP motifs are highly conserved across species, and TRP motifs are known to be involved in protein-protein interactions [7]. A role for the KLC TRPs in cargo binding is supported by experiments in which an antibody to this region, when injected into squid axoplasm, dissociates organelles from MTs [29].

Understanding how KIF5 becomes linked to particular cargoes and deciphering the regulatory mechanisms for vesicle transport remain major unsolved research questions. In this study, we screened for neuronal proteins that interact specifically with KLC1. We found that JNK/stress-activated protein kinase-associated protein 1 (JSAP1/JIP3) interacted with the TRP domains of KLC1 and KLC2 in the yeast two-hybrid assay. This finding was confirmed by glutathione-S-transferase (GST) pull-down assays and co-immunoprecipitation with an antibody to JSAP1 using extracts of mouse brain tissues. These results suggest that JSAP1 may play a role as a KLC1 receptor protein.

Materials and Methods

Construction of cDNA libraries for yeast two-hybrid screening

Mouse total brain RNA was prepared from 5-day-old ICR pups using the Total RNA Separator kit (Clontech), and mRNA was isolated from this preparation with the mRNA Separator kit (Clontech). A cDNA library was prepared using the SuperScript Choice system (GibcoBRL) with random hexamer primers. The cDNA library was ligated with *EcoRI/NotI/SalI* adaptors and inserted into the pB42AD expression vector (Clontech). ElectroMAX DH10B cells (GibcoBRL) were transformed with the expression library constructs, generating 6×10^6 independent clones. The plasmids were purified using the QIAGEN Plasmid kit (QIAGEN). General recombinant DNA techniques were performed according to standard protocols [24].

Screening of KLC1-binding proteins by yeast two-hybrid assay

The Matchmaker LexA two-hybrid system was used for screening according to the manufacturer's manual (Clontech). In brief, a part of the KLC1 gene (amino acids [aa] 250-500) was fused to the DNA-BD region of the pLexA vector using the PCR method and the plasmid DNA was transformed into yeast strain EGY48 carrying the p8op-lacZ gene. Transformed EGY48 yeast strains containing the KLC1 bait plasmid were transformed with the mouse brain cDNA library and the cells were 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. The bait plasmids were recovered from the positive clones, and the reproducibility of the observed interactions was confirmed by retransformation into yeast. We repeated these procedures several times and analyzed the sequences for which interaction was reproducibly ascertained.

β -Galactosidase activity in liquid cultures of yeast

The strength of the interactions between JSAP1 and KLC1 constructs was assessed by measuring the β -galactosidase activity in liquid cultures or using the two-hybrid system. Yeast was co-transformed with the expression plasmids of the positive clones and the plasmids expressing the TRP domains of KLC1 (described above) or KIF5s. Plasmids expressing the C-terminal tails of KIF5A (aa 808 to the carboxy-terminus), KIF5B (aa 808 to the carboxy-terminus), and KIF5C (aa 808 to the carboxy-terminus) [14] were tested for binding with JSAP1. The β -galactosidase activity in liquid cultures of yeast was assayed as described previously [31]. In brief, mid-log phase transformed yeast cells were collected and permeabilized with 0.1% sodium dodecyl sulphate (SDS) and chloroform. The chromogenic substrate o-nitrophenyl- β -D-galactoside was added in excess to this lysate, the mixture was incubated at 30°C, and the reaction was stopped by increasing the pH to 11 by the addition of 1 M Na₂CO₃. 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.

GST pull-down assays

Pull-down assays using GST fusion proteins were performed as follows. cDNA encoding the leucine zipper domain of JSAP1 was cloned in pGEX4T-1, and the recombinant GST-JSAP1 fusion protein was expressed in bacterial strain *E. coli* BL21 GOLD (Stratagene) after induction with 1 mM isopropyl thio-β-D-galactopyranoside (Fisher Scientific). The fusion proteins were purified using glutathione-agarose beads (Sigma) according to the manufacturer's protocol. GST alone or GST fusion proteins were dialyzed for 2 hr in PBS using Slide-A-Lyzer (Pierce Chemical). Ten μg of each of the GST fusion proteins was then coupled to 50 μl of glutathione-agarose beads for each reaction by incubating at room temperature for 1 hr, followed by rinsing several times with PBS. 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 SDS sample buffer. The samples were boiled for 5 min and then processed for SDS-PAGE and immunoblotting with anti-KLC1 antibody and anti-KIF5B antibody [28].

Subcellular fractionation and co-immunoprecipitation

Subcellular fractionation was performed as previously

described [28,31]. Mouse brains were homogenized in ice-cold homogenization buffer (0.32 M sucrose, 4 mM HEPES, pH 7.3) supplemented with protease inhibitors. The homogenate was clarified by centrifugation at 900 x g for 10 min followed by centrifugation at 1000 x g for 10 min, producing a pellet (P1) and supernatant (S1). The S1 supernatant was centrifuged again at 12,000 x g for 15 min, and the resulting supernatant (S2) was saved. For immunoprecipitation of the S2 fraction, the samples were 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-JSAP1 antibody [28] or with control IgG overnight at 4°C, followed by precipitation with protein-A Sepharose (Amersham Pharmacia). The beads were washed five times in binding buffer (1X), and the immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with antibodies to KIF5A [14], KIF5B [14], KIF5C [14], KIF3B [22], and KIF17 [28].

Results

Identification of KLC1 interacting proteins by yeast two-hybrid screening

To examine KLC1-interacting proteins, we screened a mouse brain cDNA library through yeast two-hybrid assays using a portion of KLC1 TRP domains as bait (Fig. 1A). From 1 x 10⁷ colonies screened, we obtained five positive clones which were cDNA fragments encoding JSAP1

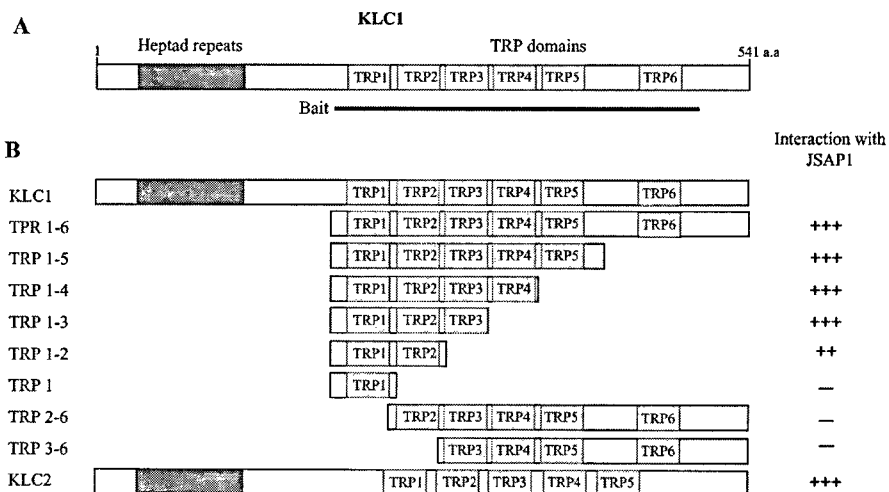


Fig. 1. Identification of the minimal binding domain of KLC1 by yeast two-hybrid assays. (A) The domain structure of KLC1. KLC1 has six TRP domains. Heptad repeats and TRP domain are indicated in gray. aa, the amino acid residue number. (B) Minimal JSAP1 binding region in KLC1. Different truncations of KLC1 were constructed by PCR. Several truncated forms of KLC1 were tested in the yeast two-hybrid assay for interaction with JSAP1. +, interaction with JSAP1; -, no interaction with JSAP1.

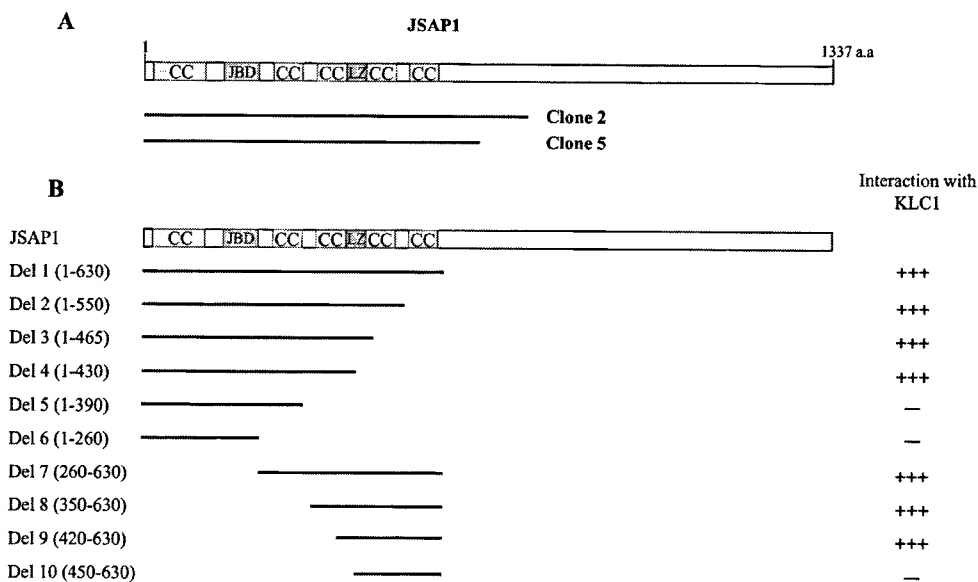


Fig. 2. Identification of the proteins interacting with KLC1 by yeast two-hybrid assays. (A) The domain structure of JSAP1 illustrating that clones 2 and 5 overlap in the small N-terminal region. The Coiled-coil domain (CC), JNK binding domain (JBD) and leucine zipper (LZ) are in the N-terminus. aa, the amino acid residue number. (B) Minimal KLC1 binding region in JSAP1. Different deletion mutants of JSAP1 were constructed by PCR. Several deletion mutants of JSAP1 were tested in the yeast two-hybrid assay for interaction with KLC1. +, interaction with KLC1; -, no interaction with KLC1.

(two overlapping clones) (Fig. 2A) and 14-3-3 proteins (three clones). The two interacting JSAP1 clones (clones 2 and 5) overlapped at the N-terminal region of JSAP1 (Fig. 2A). KLC1 has six TRP domains (Fig. 1A). To determine the TRP domain of KLC1 that is required for the interaction with JSAP1, we constructed a series of deletion mutants of KLC1 and analyzed their interactions with JSAP1 using yeast two-hybrid assays. This result indicated that the minimal domain required for binding was critically dependent on the TRP1 and TRP2 containing region of the KLC1. JSAP1, moreover, interacted with KLC2 (Fig. 1B). This result was not surprising in view of the fact that KLC1 and KLC2 share extensive sequence similarity (71.1% identity in the full length). JSAP1 has a predicted coiled-coil domain in the N-terminal region. This region also contained a predicted leucine zipper (Fig. 2A). To identify the region of JSAP1 required for the interaction with KLC1, we constructed deletion mutants of JSAP1 and analyzed their interactions with KLC1 using yeast two-hybrid assays (Fig. 2B). This results indicated that the minimal binding domain was located in a small region of JSAP1 corresponding to the leucine zipper domain. We also quantified the binding affinity of JSAP1 to KLC1 by measuring β -galactosidase activity in liquid cultures of yeast transformed with the appropriate constructs. The interaction of

the JSAP1 with KLC1 yielded approximately 360 U of β -galactosidase activity (Fig. 3), indicating a sufficient strength to mediate molecular sorting *in vivo* [28,31]. We next examined whether JSAP1 directly interacted with KIF5s. The C-terminal tails of KIF5A, KIF5B, and KIF5C were tested for JSAP1-binding by yeast two-hybrid assays (Fig. 3). There was no detectable binding between JSAP1

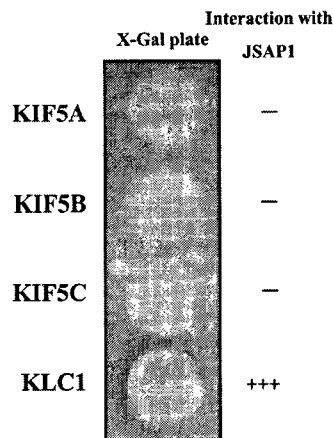


Fig. 3. Interaction between KIF5s and JSAP1. The indicated amino acid residues of each KIF5 proteins and KLC1 were fused to the pLexA DNA binding domain. JSAP1 specifically interacted with KLC1 but not with KIF5A, KIF5B, or KIF5C (+++, interaction with JSAP1; -, no interaction with JSAP1).

and the tail domains of major neuronal KIFs, such as KIF5A and KIF5C, nor with the ubiquitous KIF, such as KIF5B. These data indicate that JSAP1 directly binds TRP domains of the KLC1.

KLC1 are associated with JSAP1 in vitro and in vivo

To further confirm the KLC1-JSAP1 interaction, GST pull-down assays were performed using a GST fusion protein with the N-terminal region of JSAP1 which was expressed in *E. coli* and purified by affinity chromatography. As shown in Fig. 4, KLC1 interacted with GST-JSAP1, but not with GST alone, indicating that the KLC1 interacts with JSAP1 at the protein level *in vitro*.

To determine whether KLC1 and JSAP1 associate *in vivo*, we performed co-immunoprecipitation analyses with mouse brain extracts. After the immunoprecipitation of clarified brain homogenates with anti-JSAP1 antibody, the samples were analyzed by immunoblotting with antibodies to KIF5A, KIF5B, KIF5C, KIF3B, and KIF17 [14,18]. As shown in Fig. 5, JSAP1 was coimmunoprecipitated with KLC1, KIF5A, KIF5B, and KIF5C, but not with KIF3B, or KIF17 (Fig. 5). This result suggests that JSAP-1 is a specific binding partner of KLC1 in neurons *in vivo*.

Discussion

The findings of this study provide biochemical evidence that JSAP1 is a candidate molecular receptor of KLC1. First, we showed that KLC1 directly interacts with JSAP1 in the yeast two-hybrid system (Fig. 1, and 2). Secondly, we demonstrated that KLC1 interacts with JSAP1 *in vitro*

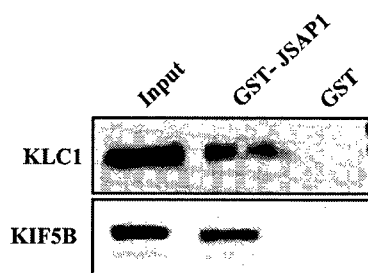


Fig. 4. Association of KLC1 with JSAP1 in the GST pull-down assay. Proteins in the mouse brain lysate were allowed to bind to GST alone or to GST-JSAP1 fusion proteins containing the leucine zipper domain of JSAP1. The elution fractions were resolved by SDS-PAGE, and Western blotting was performed using an antibody to KLC1 or KIF5B.

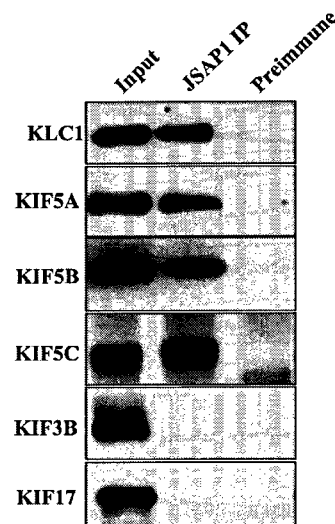


Fig. 5. Co-immunoprecipitation of KLC1 and JSAP1 from brain extracts. Mouse brain lysate was immunoprecipitated with anti-JSAP1 antibody or preimmune serum, and the precipitates were immunoblotted with anti-KIF antibodies. Input: 5% of the mouse brain lysates used for each co-immunoprecipitation assay.

(Fig. 4). Finally, co-immunoprecipitation study showed that JSAP1 is a specific binding partner of KLC1 in cells (Fig. 5). Our result also demonstrated that the TRP1 and 2 domains of KLC1 are required for the interaction with the leucine zipper domain of JSAP1 (Fig. 1B, Fig. 2B).

In mammals, three major groups of the mitogen-activated kinase (MAPK) have been identified, i.e. extracellular regulated kinase, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 MAPK cascades [21]. JNK is activated in response to a variety of extracellular and intracellular stimuli, and it plays crucial roles in cellular processes that include cell proliferation, differentiation, apoptosis, and migration. JSAP1 is a member of a family of scaffold factors for the MAPK cascades, and it also forms a complex with focal adhesion kinase [11]. Nearly 20 proteins have been so far proposed as scaffold proteins for mammalian MAPK signaling pathways [21]. JSAP1 is exclusively expressed in the brain [17,32]. JSAP1-deficient mice show various development deficits in the brain, including axon guidance defects of the corpus callosum, suggesting importance of JSAP1 in mice development [8,16]. JSAP1 is different from other JIP family members in its primary structure, being consistent with the fact that it has a distinct role from that of JIP1. Genetic analysis of model organisms indicates that orthologs of JSAP1 in *Drosophila* (*Sunday driver*) and *C. elegans* (*unc-16*) are re-

quired for normal axonal transport [3,4]. Mutant worms and flies lacking JSAP1 mislocalize and accumulate vesicular cargo within axon and dendritic processes. For example, *unc-16* mutant worms mislocalize both synaptic vesicles and glutamate receptors, suggesting that UNC-16 is required for transport of synaptic vesicles or synaptic vesicle precursors [4]. JSAP1 therefore may function as an adaptor protein that mediates KIF5-dependent cargo transport in neurons. Although we did not show the interaction of the COOH terminal of KLC1 with other adaptor proteins or cargoes, our observations suggest JSAP1 may play a role as a KIF5 receptor protein. The fact that KIF5 associates with various binding proteins or organelles, suggests a redundancy mechanism for a physiologically important cargo [9,10]. But the mechanism how they recognize and bind to a specific cargo has not yet been completely elucidated. Future anatomical and biochemical studies are needed to address these issues.

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초록 : JSAP1과 Kinesin Light Chain 1의 결합 및 결합부위 규명

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KIF5는 2분자의 kinesin heavy chain (KHC)과 2분자의 kinesin light chain (KLC)으로 구성되며 미세소관과 직접 결합한다. KIF5는 여러가지 세포 내 소기관을 이동시키나 KIF5가 이동시키는 운반체가 어떻게 특이적으로 결합하는지는 아직 밝혀지지 못하였다. 본 연구에서 효모 two-hybrid system을 사용하여 KLC1의 tetratricopeptide repeats (TRP) 부위와 결합하는 세포 내의 단백질을 분리하였다. 결과 KLC1와 특이적으로 결합하는 JNK/stress-activated protein kinase-associated protein 1 (JSAP1/JIP3)을 분리하였다. 이러한 결합은 KLC1의 TRP1, 2 영역과 JSAP1의 leucine zipper 영역이 결합에 관여하며, 또한 효모 two-hybrid assay에서 JSAP1은 KLC2와 결합하지만 신경세포에서 발현하는 KIF5A, KIF5C 그리고 모든 세포에서 발현하는 KIF5B와는 결합하지 않았다. 단백질간의 결합을 pull-down assay로 확인한 결과 KLC1은 glutathione S-transferase (GST)와는 결합하지 않으나 GST결합 JSAP1과는 결합하였다. 또한 생쥐의 뇌 파쇄 액으로부터 JSAP1 항체로 면역침강을 행한 결과 KLC1은 JSAP1과 같이 침강하였다. 이러한 결과들은 KLC1은 JSAP1과 결합하며, JSAP1은 KLC1의 수용체로 세포 내 KIF5의 수송의 매개 단백질로 작용함을 시사한다.