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Kinesin Superfamily KIF5 Proteins Bind to βIII Spectrin

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The kinesin proteins (KIFs) make up a large superfamily of molecular motors that transport cargo such as vesicles, protein complexes, and organelles. KIF5 is a heterotetrameric motor that conveys vesicles and plays an important role in neuronal function. Here, we used the yeast two-hybrid system to identify the neuronal protein(s) that interacts with the tail region of KIF5 and found a specific interaction with βIII spectrin. The amino acid residues between 1394 and 1774 of βIII spectrin were required for the interaction with KIF5C. βIII spectrin also bound to the tail region of neuronal KIF5A and ubiquitous KIF5B but not to other kinesin family members in the yeast two-hybrid assay. In addition, these proteins showed specific interactions, confirmed by GST pull-down assay and co-immunoprecipitation. βIII spectrin interacted with GST-KIF5 fusion proteins, but not with GST alone. An antibody to βIII spectrin specifically co-immunoprecipitated KIF5s associated with βIII spectrin from mouse brain extracts. These results suggest that KIF5 motor proteins transport vesicles or organelles that are coated with βIII spectrin.

Key Words: Kinesin, Spectrin, Molecular motors, Microtubule, Adaptor proteins

INTRODUCTION

Early light microscopic studies of living nerve axons and biochemical studies of axonal transport revealed membranous organelles moving by fast flow (Hirokawa, 1998; Kamal et al, 2000). This active intracellular 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 (Karcher et al, 2002; Vale et al, 2003; Seog et al, 2004). Since the kinesin was first characterized, many related proteins have been discovered, including conventional kinesins and proteins belonging to the growing KIF superfamily (Aizawa et al, 1992; Hirokawa, 1998; Miki et al, 2001; Seog et al, 2004). In a search of the total human genome database, a total of 45 KIFs have been identified (Miki et al, 2001).

Three major types of KIFs have been identified according to the position of the motor domain: NH2-terminal motor domain type (N-type), middle motor domain type (M-type), and COOH-terminal motor domain type (C-type). N-type kinesins (KIF1, KIF3A, KIF3B, KIF4, KIF5, KIF10, KIF12, KIF13, KIF14, KIF15, KIF16, KIF17, KIF18, KIF20, KIF23, and KIF26) and M-type kinesin (KIF2) move exclusively toward the plus-ends of microtubules, such as toward the cell periphery or synaptic terminals, whereas C-type kinesins (KIFC1, KIFC2, and KIFC3) move toward the minus-ends (Miki et al, 2001; Seog at al, 2004). Rotary shadowing and biochemical analyses have distinguished five conformational types: monomers, homodimers, heterodimers, heterotrimers, and heterotetramers (Scholey et al, 1989; Hirokawa, 1998). The original 'conventional' kinesin (KIF5B/kinesin-I) was shown to be a tetrameric protein composed of two heavy chains and two light chains (Vale et al, 1997; Vale, 2003). Electron microscopy, protease sensitivity, and primary sequence analyses showed that the kinesin heavy chain (KHC) is composed of three domains (Yang et al, 1989). The globular N-terminal head domain contains the adenosine triphosphate (ATP)-binding motif and a microtubule-binding domain (Scholey et al, 1989; Vale et al, 2003). The head is attached via a 50-amino acids 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 (Seog et al, 2004). KIFs contain amino acid sequences that are highly conserved among all eukaryotic cells (Miki et al, 2001). Within the motor domain, there are two conserved sequences that are proximal to the ATP-binding motif and the microtubule-binding domain (Scholey et al, 1989). Outside the motor domain, KIFs show few sequence similarities. Interactions with cargo molecules have been shown to occur outside the motor domain (Miki et al, 2001; Seog et al, 2004). Recently, it has been clearly demon-

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ABBREVIATIONS: KIF, Kinesin superfamily proteins; KHC, kinesin heavy chain; KLC, kinesin light chain; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate

strated that several KIFs attach to specific cargoes through interactions with adaptor proteins in these binding regions (Takeda et al, 2000; Setou et al, 2000; Setou et al, 2002). KIF5 proteins, which consist of two KHCs and two kinesin light chains (KLC), are multifunctional neuronal transporters of both axonal cargo, such as synapsin and GAP43, and dendrite cargo, such as messenger RNA and the AMPA receptor (Setou et al, 2002; Seog et al, 2004). In mice, the KIF5 subfamily was shown to include the distinctly brain-specific KIF5A (Aizawa et al, 1992) and KIF5C (Kanai et al, 2000), in addition to the ubiquitous KIF5B (Meng et al, 1997). Although both KIF5A and KIF5C are expressed in neurons, they have different distribution patterns; the expression of KIF5A was observed at similar levels in various kinds of neurons, whereas KIF5C expression was more intense in motor neurons (Karcher et al, 2002). Recent studies have demonstrated that KIF5C knockout mice have smaller brains and a loss of motor neurons relative to sensory neurons (Kanai et al, 2000). Understanding how KIF5C becomes linked to particular cargoes and deciphering the regulatory mechanisms for vesicle transport remain to be major unsolved research questions.

In this study, we screened for neuronal proteins that interacted specifically with KIF5 family isoforms, and found in the yeast two-hybrid assay that βIII spectrin interacted with the tail domain of KIF5C, KIF5A and KIF5B. Using extracts of mouse brain tissues, this finding was confirmed by glutathione-S-transferase (GST) pull-down assays and co-immunoprecipitation with an antibody to βIII spectrin. These results suggest that KIF5s acts as motor proteins for the transport of βIII-spectrin-associated vesicles and organelles.

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, Palo Alto, CA, USA), and mRNA was isolated from this preparation with the mRNA Separator kit (Clontech). A cDNA library was prepared using the SuperScript Choice system (GibcoBRL, Grand Island, NY, USA) 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, KJ Venlo, NA, Netherlands). General recombinant DNA techniques were performed according to standard protocols (Sambrook et al, 1989).

Screening of KIF5C-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 KIF5C heavy chain gene (amino acids [aa] 810-883) 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 KIF5C bait plasmid, were transformed with the mouse brain cDNA library and the cells were grown on SD plates supplemented with glucose, but 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 (III spectrin and KIF 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 tail domains of KIF5 (described above) or other KIFs. Plasmids expressing the tails of KIF1A (400 amino acids to the carboxy-terminus) (Okada et al, 1995), KIF1B (280 amino acids to the carboxy-terminus) (Nangaku et al, 1994), KIF3A (280 amino acids to the carboxy-terminus) (Kondo et al, 1994), KIF3B (280 amino acids to the carboxy-terminus) (Nonaka et al, 1998), and KIF17 (939 amino acids to the carboxy-terminus) (Setou et al, 2000) were tested for binding with βIII spectrin.

The β-galactosidase activity in liquid cultures of yeast was assayed as described previously (Takeda et al, 2000). 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 in excess was added 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 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.

GST pull-down assays

Pull-down assays using GST fusion proteins were performed as follows. cDNAs encoding the tail domains of KIF5A, KIF5B, and KIF5C were cloned in pGEX4T-1, and the recombinant GST-KIF fusion proteins were expressed in bacterial strain BL21 GOLD (Stratagene, La Jolla, CA, USA) after induction with 1 mM isopropyl thio-β-D-galactopyranoside (Fisher Scientific, NJ, USA). The fusion proteins were purified, using glutathione-agarose beads (Sigma, St. Louis, MO, USA) according to the manufacturer's protocol. GST alone or GST fusion proteins were dialyzed for 2 h in PBS using Slide-A-Lyzer (Pierce Chemical, IL, USA). Ten μg of each of the GST fusion proteins were then coupled to 50 μl of glutathione-agarose beads for each reaction by incubating at room temperature for 1 h, 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 of 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 Western blotting with anti-βIII spectrin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Subcellular fractionation and co-immunoprecipitation

Subcellular fractionation was performed as previously described (Takeda et al, 2000; Setou et al, 2000). 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×g for 10 min, followed by centrifugation at 1000×g for 10 min, producing a pellet (P1) and supernatant (S1). The S1 supernatant was centrifuged again at 12,000×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-β III spectrin antibody (Santa Cruz Biotechnology) or with control IgG overnight at 4°C, followed by precipitation with protein-A Sepharose (Amersham Pharmacia, Piscataway, NJ, USA). The beads were washed five times in binding buffer (1X), and the immunoprecipitates were analyzed by SDS-PAGE and Western immunoblotting with antibodies to KIF5B (Kanai et al, 2000), KIF5C (Kanai et al, 2000), KIF1A (Okada et al, 1995), KIF1B (Nangaku et al, 1994), KIF3A (Kondo et al, 1994), and KIF17 (Setou et al, 2000).

RESULTS

Identification of KIF5C interacting proteins by yeast two-hybrid screening

To examine which neuronal protein(s) was bound to KIF5C, we screened a mouse brain cDNA library in the yeast two-hybrid assay, using a portion of KIF5C tail domain as bait. From 6×10^6 colonies screened, we obtained ten positive clones which were cDNA fragments containing β III spectrin (two unique overlapping clones, with a total of five duplicate clones) (Fig. 1a) and 14-3-3 proteins (five clones). The two interacting βIII spectrin clones (clones 3 and 6) overlapped at only a small repeat region corresponding to amino acids 1394-1774 of βIII spectrin (Fig. 1a). To identify the region of KIF5C required for the interaction with βIII spectrin, we constructed a series of deletion mutants of KIF5C and analyzed their interactions with βIII spectrin using the yeast two-hybrid assay (Fig. 1b). This experiment demonstrated that the minimal binding domain was located in a small region of KIF5C corresponding to amino acids 806-883.

βIII spectrin also interacted with the tail domains of the KIF5A and KIF5B heavy chains in the yeast two-hybrid

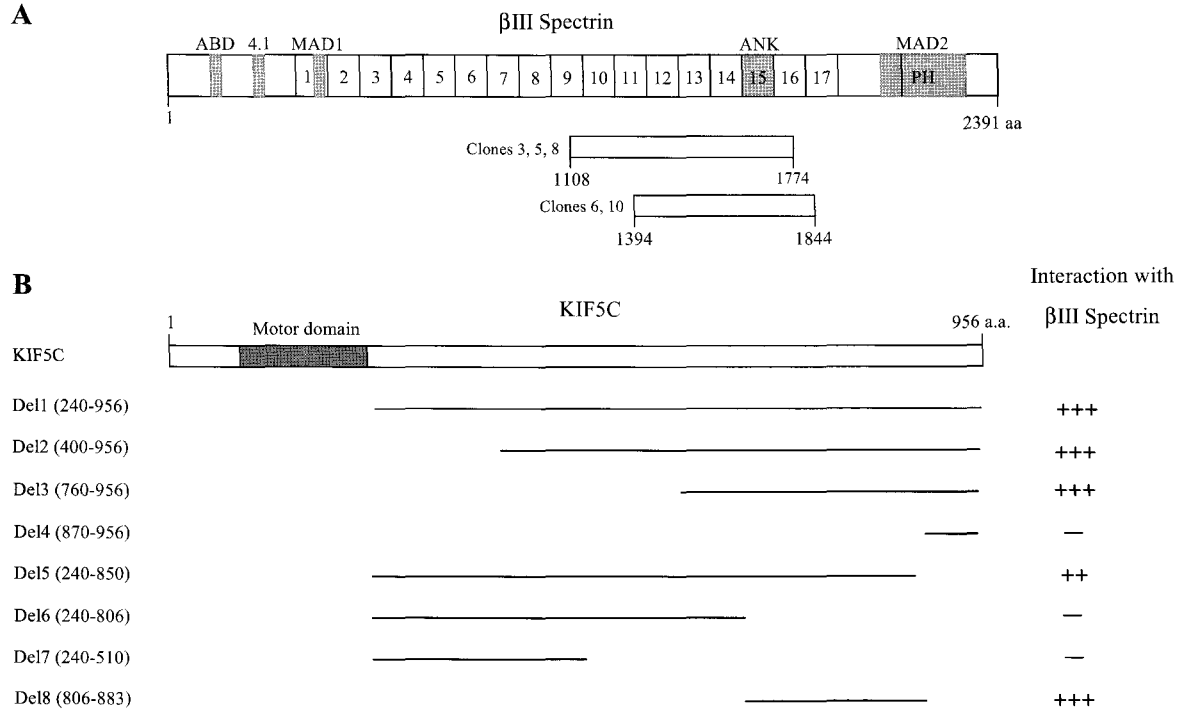

Fig. 1. Identification of the proteins interacting with KIF5C by yeast two-hybrid screening. (A) The domain structure of βIII spectrin illustrates that clones 3, 5, and 8, and clones 6 and 10, overlap at a small spectrin repeat region corresponding to amino acids 1394-1774. The actin-binding domain (ABD) and protein-4.1-binding domain (4.1) are near the N-terminus. They are followed by 17 tandem spectrin repeats. Repeats 1, 15, and 17 contain a potential membrane-association domain (MAD1), ankyrin-binding domain (ANK), and spectrin self-association site, respectively. Near the C-terminus are a second membrane-association domain (MAD2) and the PH domain. aa, the amino acid residue number. (B) Several truncated forms of KIF5C were tested in the yeast two-hybrid assay for interaction with βIII spectrin. Amino acid numbers are indicated on the left. +, interaction with βIII spectrin; −, no interaction with βIII spectrin.

system (Fig. 2). This result was not surprising in view of the fact that the KIF5A, KIF5B, and KIF5C heavy chains share extensive similarity in their primary structure (89% identity in the minimal binding domain) (Kanai et al, 2000). In addition, the sequence of the binding site of the KIF5 proteins overlapped with the cargo-binding domain of a fungal kinesin, which lacks the light chains (Seiler et al, 2000). We also quantified the binding affinity of βIII spectrin to KIF5A, KIF5B, and KIF5C by measuring β-galactosidase activity in liquid cultures of yeast transformed with the appropriate constructs. The interaction of the KIF5s with βIII spectrin yielded approximately 180 U of β-galactosidase activity (Fig. 2), reflecting a binding strength that is sufficient to mediate molecular sorting *in vivo* (Setou et al, 2000; Takeda et al, 2000). βIII spectrin is widely expressed, but at varied levels; it is concentrated in the brain and is moderately expressed in kidneys, liver, and testes, as well as in the prostate, pituitary, adrenal, and salivary glands (Stankewich et al, 1998). We next examined whether βIII spectrin interacted with other KIFs (Fig. 2). Therefore, the tails of KIF1A (Okada et al, 1995), KIF1B (Nangaku et al, 1994), KIF3A (Kondo et al, 1994), KIF3B (Nonaka et al, 1998), and KIF17 (Setou et al, 2000) were tested for βIII spectrin-binding by the yeast two-hybrid assay (Fig. 2). There was no detectable binding between βIII spectrin and the tail domains of the other major neuronal KIFs, such as KIF1A and KIF17 (which are bound to the mLin10 PDZ domain), nor with the ubiquitous KIFs, such as KIF1B, KIF3A, and KIF3B. These data indicate that βIII spectrin binds specifically to the tail domains of the KIF5s.

KIF5s are associated with βIII spectrin in vitro and in vivo

To biochemically confirm the KIF5s-βIII spectrin interaction, the direct interaction between KIF5s and βIII spectrin was assayed using the GST pull-down assay. GST fusion proteins with the tail domains of KIF5A, KIF5B, and KIF5C were expressed in *E. coli* and purified by affinity chromatography. βIII spectrin was found to interacted with GST-KIF5A, GST-KF5B, and GST-KIF5C, but not with GST alone (Fig. 3), indicating that the KIF5s interact with βIII spectrin at the protein level *in vitro*.

To determine whether KIF5s and βIII spectrin associate *in vivo*, we performed co-immunoprecipitation analyses with mouse brain extracts. After immunoprecipitation of clarified brain homogenates with anti-βIII spectrin antibody, the samples were analyzed by Western blotting with antibodies to KIF5B, KIF5C, KIF1A, KIF1B, KIF3A, and KIF17 (Kanai et al, 2000; Okada et al, 1995; Nangaku et al, 1994; Kondo et al, 1994; Setou et al, 2000). As shown in Fig. 4, βIII spectrin co-immunoprecipitated with KIF5B and KIF5C, but not with KIF1A, KIF1B, KIF3A, or KIF17, suggesting that βIII-spectrin is a specific binding partner of KIF5s in neurons *in vivo*.

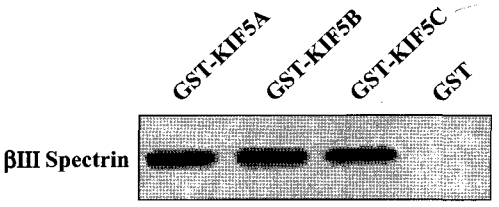

Fig. 3. Association of KIF5s with βIII spectrin in the GST pull-down assay. Proteins in the mouse brain lysate were allowed to bind to GST alone or to GST-KIF5 fusion proteins, containing the tail domains of KIF5A, KIF5B, and KIF5C. The elution fractions were resolved by SDS-PAGE, and Western blotting was performed using an antibody to βIII spectrin.

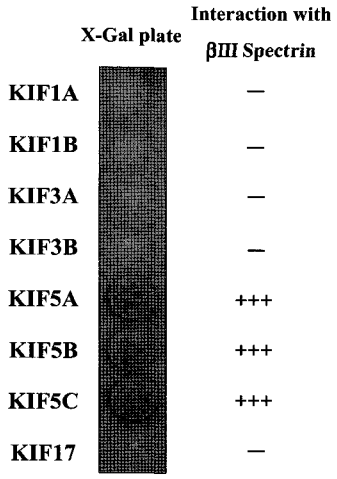

Fig. 2. Interaction between KIFs and βIII spectrin. The indicated amino acid residues of each KIF protein were fused to the pLexA DNA binding domain. βIII spectrin specifically interacted with KIF5 proteins but not with KIF1A, KIF1B, KIF3A, KIF3B, or KIF17 (+++, interaction with βIII spectrin; —, no interaction with βIII spectrin).

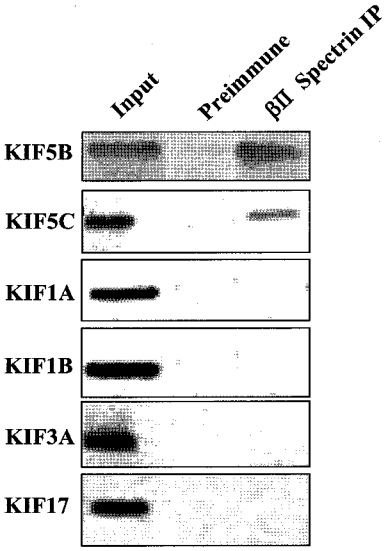

Fig. 4. Co-immunoprecipitation of KIF5s and βIII spectrin from brain extracts. Mouse brain lysate was immunoprecipitated with anti-βIII spectrin antibody or preimmune serum, and the precipitates were immunoblotted with anti-KIF antibodies. Input: 5% of the mouse brain lysates used for each co-immunoprecipitation assay.

DISCUSSION

In mammalian cells, spectrin functions in controlling plasma membrane shape, organization, and stability, and has best been characterized in erythrocytes (Beck et al, 1998; De Matteis et al, 2000). Plasma membrane spectrin is a heterotetramer consisting of high molecular weight α -chains and β-chains that form long flexible proteins which interact with actin, as well as with other proteins including ankyrin (De Matteis et al, 2000). The association of spectrin with membranes is a multivalent process involving several protein-protein interactions and at least two membrane association domains, as well as a C-terminal PH domain that binds phosphatidylinositol 4,5-bisphosphate (De Matteis et al, 2000). Recently, several isoforms of spectrin (αI, αII, βI, βII, βIII, and βIV) were identified on Golgi membranes (Winkelmann et al, 1993; Beck et al, 1994; Devarajan et al, 1996; Beck et al, 1997; Devarajan et al, 1997; Fath et al, 1997; Tse et al, 2001), lysosomal membranes (Hoock et al, 1997), and intracellular vesicles in cerebellar neurons (Malchiodi-Albedi et al, 1993). Spectrin has also been shown to associate with centractin, a subunit of the dynactin complex (Devarajan et al, 1997; Holleran et al, 1997) which, in association with the microtubule-based motor cytoplasmic dynein, plays a major role in the anterograde transport of vesicles in the secretory pathway from the vesiculotubular compartment to the Golgi (Presley et al, 1997; Lippincott-Schwartz, 1998). βIII spectrin is a 270-kDa polypeptide with conserved actin-, protein-4.1-, and ankyrin-binding domains, and has been postulated to function in maintaining the Golgi structure, coating cytoplasmic transport vesicles, and associating with the cytoplasmic membrane organelles (Beck et al, 1994; Stankewich, 1998). A role for βIII spectrin in the post-Golgi transport of trafficking vesicles-lysosomes and endocytic vesicles-possibly in association with kinesin-based motors has also been proposed (De Matteis et al, 1998; Hirokawa, 1998; Lippincott-Schwartz, 1998).

Although we did not show the interaction of KIF5s with other spectrin isoforms, our observations suggest a novel mechanism by which βIII spectrin could mediate interactions between the kinesin motor proteins and βIII spectrin-coated vesicles. In this work, we have provided a clue to the mechanism by which KIF5s transport βIII spectrin-coated vesicles.

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