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Ferritin, an Iron Storage Protein, Associates with Kinesin 1 through the Cargo-binding Region of Kinesin Heavy Chains (KHCs)

Won Hee Jang[1], Young Joo Jeong[1], Won Hee Lee[2], Mooseong Kim[2], Sang-Jin Kim[3], Sang-Hwa Urm[4], Il Soo Moon[5] and Dae-Hyun Seog[1]*

[1]*Department of Biochemistry Inje University College of Medicine, Busan 614-735, Korea*
[2]*Department of Neurosurgery Inje University College of Medicine, Busan 614-735, Korea*
[3]*Department of Neurology Inje University College of Medicine, Busan 614-735, Korea*
[4]*Department of Preventive Medicine, Inje University College of Medicine, Busan 614-735, Korea*
[5]*Department of Anatomy & Dongguk Medical Institute, College of Medicine, Dongguk University, Gyeongju 780-714, Korea*

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The intracellular transport of organelles and protein complexes is mediated by kinesin superfamily proteins (KIFs). The first kinesin, kinesin 1, was identified as a molecular motor protein that moves various organelles and protein complexes along the microtubule rails in cells. Kinesin 1 is a tetramer of two heavy chains (KHCs, also called KIF5s) and two kinesin light chains (KLCs). KIF5s interact with many different proteins through their tail region, but their binding proteins have not yet been fully identified. To identify the interaction proteins for KIF5A, we performed yeast two-hybrid screening and found a specific interaction with ferritin heavy chain (Frt-h), which has a role in iron storage and detoxification. Frt-h bound to the amino acid residues between 800 and 940 of KIF5A and to other KIF5s in the yeast two-hybrid assay. The coiled-coil domain of Frt-h is essential for interaction with KIF5A. In addition, ferritin light chain (Frt-l) interacted with KIF5s in the yeast two-hybrid assay. In addition, these proteins showed specific interactions in the glutathione *S*-transferase (GST) pull-down assay. An antibody to KHC specifically co-immunoprecipitated Frt-h and Frt-l from mouse brain extracts. These results suggest the kinesin 1 motor protein may transport the ferritin complex in cells.

Key words : Cargo, ferritin, kinesin 1, microtubule motors, protein-protein interaction

Introduction

The intracellular transport of proteins is crucial for maintenance and function of a cell. Intracellular transport can be divided into two categories: fast transport and slow transport. Fast transport is responsible for transport of the organelles and vesicles. Slow transport drives the movement of cytoplasmic proteins and protein complexes [8]. Both fast transport and slow transport are powered by molecular motor proteins along microtubules [8]. Microtubules in the cell form tracks on which various cargoes can be transported by motor proteins [9]. Motor proteins fall into two superfamilies, kinesins and dyneins [8, 13]. Kinesins are main molecular motors that move toward the microtubule plus end direction, whereas dyneins move toward the microtubule minus end direction [9, 27]. Kinesin 1, a conventional kinesin, was the first identified and is the most abundant molecular motor protein [8, 23]. It is a heterotetramer composed of two kinesin heavy chains (KHCs, also called KIF5s) and two kinesin light chains (KLCs) [8]. KIF5s contain a globular motor domain that binds to microtubules, a neck linker, an α-helical stalk domain, and a tail domain that associates with various cargoes [4, 23]. The cargoes of kinesin 1 fall into six classes: mRNP protein complexes, pathogens, cytoskeleton subunits, membrane-bound organelles, signaling molecules, and protein complexes [2, 5, 7, 10, 14, 18, 22].

In mammals, there are three different KIF5s. KIF5B is expressed in all cell types, whereas KIF5A and KIF5C are only expressed in neurons [15]. To investigate the functions of KIF5s, depletion of KIF5A in mice was performed using conditionally targeting by a synapsin-promoted Cre-recombinase transgene [28]. The conditional knockout mice of KIF5A showed a reduction in neurofilament axonal transport, paralysis, and epileptic seizures [19, 28]. Mutations in motor domain of KIF5A have been identified in dominant forms of

***Corresponding author**
Tel : +82-51-890-6974, Fax : +82-51-894-5801
E-mail : daehyun@inje.ac.kr

hereditary spastic paraplegia type 10 (SPG10) [21]. Most of these are missense mutations that affect the motor domain function of KIF5A and exhibit a reduction in transport velocity [21]. Also, a mutation of KIF5A has been found in a patient with Charcot-Marie-Tooth disease type 2 (CMT2) [10]. Thus, these findings suggest that KIF5A related with SPG10, epileptic seizures, and CMT2 diseases that affect the intracellular transport within cells. In contrast to KIF5A, the depletion of KIF5C, the other neuron-specific KIF5, in mice leaded to survival with no abnormality except a reduction of brain size [15].

Although the roles of KIF5A in brain have been reported, not all cargoes have been revealed yet [10]. In addition, a little is known about the binding proteins for KIF5A. To improve the understanding of the roles of KIF5A in intracellular transport, using the yeast two-hybrid screens, we identified the ferritin heavy chain (Frt-h), a cytosolic iron storage protein as a protein that interacts with KIF5A.

Materials and Methods

Plasmid constructs

A previously described mouse KIF5A cDNA [15] was utilized as a template to amplify the region coding for amino acids 800-1027 with the appropriate primers. The amplified fragments were subcloned into pGEM T-easy vector (Promega Corp, Madison, WI, USA). The resulting recombinant plasmid was then cut with *Eco*RI and *Xho*I and the insert was subcloned into pLexA (Clontech, Palo Alto, CA, USA). Complementary DNAs for mouse Frt-h (GeneBank accession number: NM_010239) and ferritin light chain (Frt-l) (GeneBank accession number: NM_010240) were amplified by polymerase chain reaction (PCR) from Marathon-Ready™ cDNA library (Clontech). The amplified cDNA fragments were then inserted into pB42AD (Clontech).

Screening of KIF5A-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, the cDNA fragment containing the tail region of mouse KIF5A was fused to the DNA-BD region of the pLexA vector and the plasmid DNA was transformed into yeast strain EGY48 carrying the p8op-lacZ gene. EGY48 yeast cells carrying the KIF5A bait plasmid were transformed with the mouse brain cDNA library [12] 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 an SD/-His/-Trp/-Ura/-Leu plate containing galactose, raffinose, X-gal, and BU salts. Plasmids from positive colonies were isolated and rescued using *Escherichia coli* strain KC8 strain on ampicillin-resistant plates and inserts were analyzed by restriction digestion. Unique insert DNAs were sequenced and DNA sequence analysis was performed with 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 strength of the interactions between Frt-h and KIF5A was assessed by measuring the β-galactosidase activity in liquid cultures. Yeast cells were co-transformed with the expression plasmid from the positive clone and the plasmid expressing KIF5A. The β-galactosidase activity in liquid cultures of yeast was assayed as described previously [11]. 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 to the yeast lysate, 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.

Glutathione S-transferase (GST) pull-down assays

Pull-down assays using GST fusion proteins were performed as follows. Complementary DNAs encoding the full length of Frt-h and Frt-l were subcloned into pET41a. The recombinant GST-Frt-h and GST-Frt-l 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 (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. GST alone or GST fusion proteins were dialyzed for 2 hr in PBS using Slide-A-Lyzer (Pierce, Rockford, IL, USA). Ten μg of each of the GST fusion proteins was then coupled to 50 μl of glu-

tathione-agarose beads by incubating at room temperature for 1 hr, followed by rinsing several times with PBS. Mouse brain lysate was prepared as previously described [11]. 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 centrifuged at 12,000× g for 15 min and the resulting supernatant was saved. The supernatant (mouse brain lysate) was incubated overnight at 4℃ 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 Laemmli's loading buffer. The samples were boiled for 5 min and then separated by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and subjected to immunoblot analysis with anti-KIF5A and anti-KIF5B antibodies [12].

Co-immunoprecipitation

For immunoprecipitation, the mouse 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-KHC antibody (Merck Millipore, Darmstadt, Germany) or with control IgG overnight at 4℃, 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, and the proteins were eluted, denatured by boiling for 2 min, and then processed for SDS-PAGE and immunoblot analysis with antibodies against KLC1, Frt-h, and Frt-l (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Results

Identification of KIF5A interacting proteins by yeast two-hybrid screening

Previous studies suggested that the carboxyl (C)-terminal tail region of KIF5s recognize and bind to the various cargoes [10, 23]. Especially, KIF5A has 73 amino acids that have no homology with KIF5B and KIF5C. Using the C-terminal tail region containing fragment (800-1,027 aa) of KIF5A as a bait, 2 positive clones were obtained from 0.7×10^6 in-

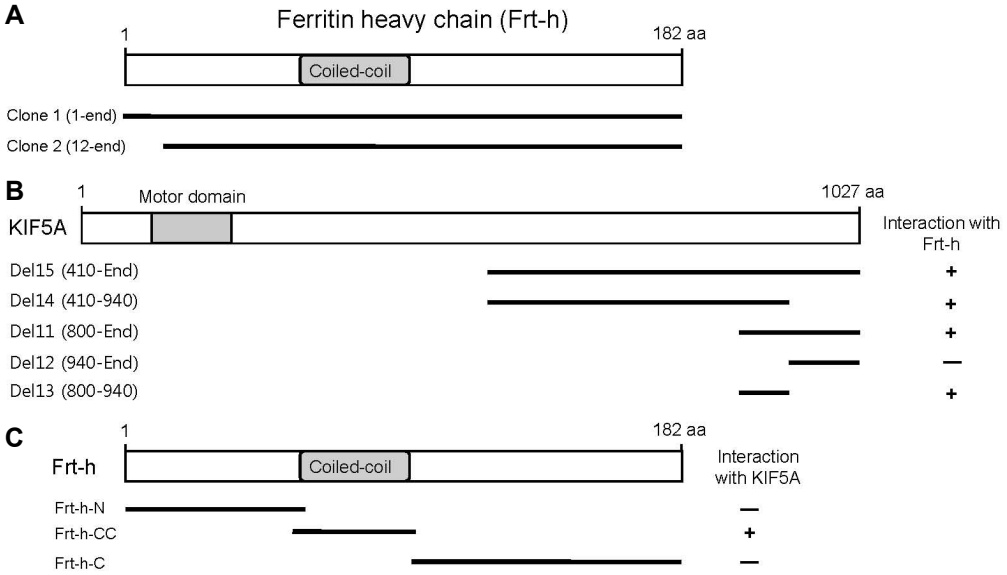

Fig. 1. Identification of the proteins interacting with KIF5A by yeast two-hybrid screening. (A) Schematic diagram of Frt-h. The gray box corresponds to the coiled-coil domain of Frt-h. Clone 1 and 2 isolated from the yeast two-hybrid screen were overlapped at the open reading frame of Frt-h. (B) Minimal Frt-h binding region in KIF5A. KIF5A has motor domain, coiled-coil domain, and tail region. Motor domain is indicated in gray. Several truncated forms of KIF5As were constructed by PCR and tested in the yeast two-hybrid assay for interaction with Frt-h. aa, the amino acid residue number. +, interaction with Frt-h; -, no interaction with Frt-h. (C) Minimal KIF5A binding region in Frt-h. The coiled-coil domains of Frt-h are indicated in gray. Several truncated forms of Frt-h were constructed by PCR and tested in the yeast two-hybrid assay for interaction with KIF5A. aa, the amino acid residue number. +, interaction with KIF5A; -, no interaction with KIF5A.

dependent mouse brain cDNA library. These two clones were individually isolated, sequenced, and subjected to further yeast two-hybrid filter assay to confirm the interactions. Two positive clones turned out to be the cDNA of Frt-h (Fig. 1A). To identify the region of KIF5A required for the interaction with Frt-h, a series of deletion mutants of KIF5A were constructed and analyzed their interactions with Frt-h using the yeast two-hybrid assay (Fig. 1B). This yeast two-hybrid assay demonstrated that the minimal binding region for Frt-h was located in a small region of KIF5A corresponding to amino acids 800-940, in which a coiled-coil domain exists [15]. Frt-h contains the coiled-coil domain, which seems to interact with various binding proteins [1]. To identify the region of Frt-h required for the interaction with KIF5A, a series of deletion mutants of Frt-h were constructed and analyzed their interactions with KIF5A using the yeast two-hybrid assay. Only the coiled-coil domain of Frt-h interacted with KIF5A (Fig. 1C). This result indicates that the binding domain for KIF5A was located in the small coiled-coil domain of Frt-h.

To clarify whether Frt-h interacts with only KIF5A or with other kinesin 1 subunits, the C-terminal tails of KIF5A, KIF5B, and KIF5C, and the full length KLC1 were tested for binding with Frt-h. Frt-h interacted with the tail domains of the KIF5A, KIF5B, and KIF5C in the yeast two-hybrid system (Fig. 2A). There was no detectable binding between KLC1 and Frt-h (Fig. 2A). This result was not surprising in view of the fact that the KIF5B and KIF5C also have the coiled-coil domain similar to that of KIF5A [15]. Frt-l, other subunit of ferritin contains the two coiled-coil domain in their primary structure [1]. To clarify whether kinesin 1 subunits interact with the coiled-coil domain of Frt-l, the C-terminal tails of KIF5s and the full length KLC1 were tested for binding with Frt-l. As shown in Fig. 2B, KIF5s interacted with Frt-l but KLC1 did not bind to Frt-l. These data indicate that the coiled-coil domain of Frt-h and Frt-l binds to KIF5s. To quantify the binding affinity of KIF5A to Frt-h or Frt-l, the KIF5A bait plasmid and the Frt-h or Frt-l expression plasmids were transformed to yeast and the β-galactosidase activity was measured in liquid cultures. The interaction of KIF5A with Frt-h or Frt-l yielded approximately 360 units of β-galactosidase activity (Fig. 2C).

Frt-h and Frt-l directly interact with KIF5A at the protein level

As an additional demonstration for the interaction between KIF5s and ferritin complex at the protein level, direct interaction between KIF5s and Frt-h or Frt-l was assayed using a GST pull-down experiment. Recombinant GST-Frt-h and GST-Frt-l fusion proteins were expressed in *E. coli*. The purified GST fusion proteins are allowed to interact with mouse brain extracts. Immunoblot analyses revealed that both the GST-Frt-h and the GST-Frt-l interacted with KIF5A and KIF5B (Fig. 3A).

To examine whether the interaction between kinesin 1 and ferritin complex takes place *in vivo*, co-immunoprecipitation analyses were performed. Lysates from mouse brain were incubated with an anti-KHC antibody. Protein G-agarose

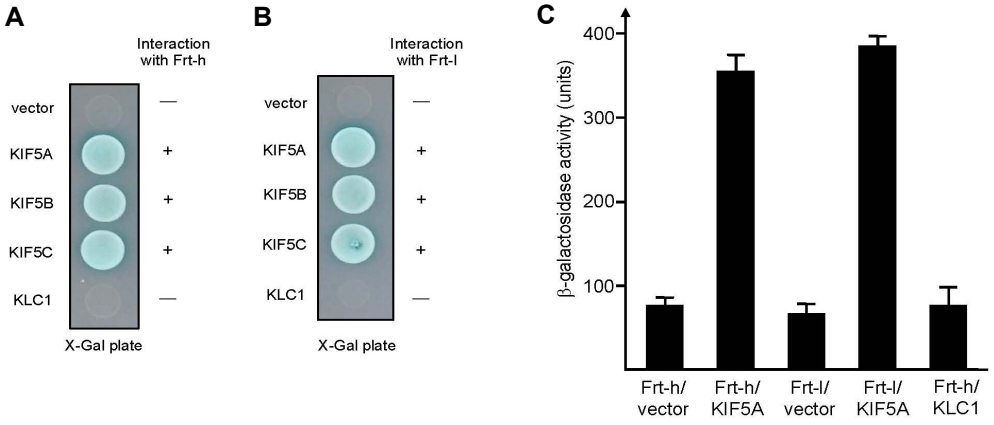

Fig. 2. Interaction between KIF5A and Frt-h. The tail region of each KIF5 and the full length KLC1 were fused to the pLexA DNA binding domain. (A) Frt-h specifically interacted with KIF5s but not with KLC1. +, interaction with Frt-h; -, no interaction with Frt-h. (B) Frt-l specifically interacted with KIF5s but not with KLC1. +, interaction with Frt-l; -, no interaction with Frt-l. (C) The strength of interactions between Frt-h or Frt-l and KIF5A was examined quantitatively using β-galactosidase activity in yeast two-hybrid reporter assay.

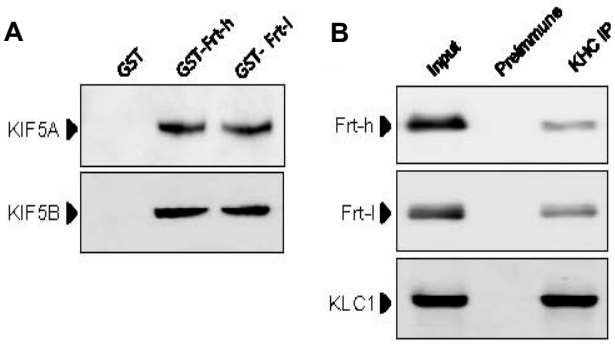

Fig. 3. Association of KIF5A with Frt-h in the GST pull-down assay and co-immunoprecipitation. (A) Proteins in the mouse brain lysate were allowed to bind to GST alone, GST-Frt-h fusion protein, or GST-Frt-l fusion protein. The elution fractions were resolved by SDS-PAGE and immunoblotting was performed using antibodies against KIF5A or KIF5B. (B) Mouse brain lysates were immunoprecipitated with an anti-KHC antibody or preimmune serum, and then the precipitates were immunoblotted with anti-Frt-h, Frt-l, or KLC1 antibodies. Input, 10% of the mouse brain lysates used for each co-immunoprecipitation assay.

beads selectively precipitated the immuno-complexes, which were subsequently separated by SDS-PAGE and immunoblotted with anti-Frt-h, Frt-l, and KLC1 antibodies. As shown in Fig. 3B, Frt-h, Frt-l, and KLC1 were co-immunoprecipitated with KHC. These results demonstrate that ferritin complex is a specific binding partner of kinesin 1 *in vivo*.

Discussion

Mutations in KIF5A are linked to various neurological diseases such as CMT [10], hereditary spastic paraplegia (HSP) [21], and epileptic seizures [19], but the specific function of KIF5A are incompletely understand. In this study, we show that kinesin 1 can interact with ferritin complex. Using the C-terminal domain of KIF5A as bait, we identified Frt-h in a yeast two-hybrid assay of a mouse brain cDNA library. The coiled-coil domain of Frt-h interacted with KIF5s. Furthermore, using a combination of GST-pull down and co-immunoprecipitation, we confirmed that kinesin 1 interacted with ferritin complex. Taking all of these results together, we hereby propose a model that kinesin 1 transports the ferritin complex in cells.

Several kinesin 1 cargo molecules have been shown to interact with KIF5s [10, 23]. The C-terminal tail region of KIF5s is known as a protein-interacting domain, functioning as a physical linker between kinesin 1 and various proteins such as glutamate receptor-interacting protein 1 (GRIP1), huntingtin-associated protein 1 (HAP1), and $GABA_A$ receptor-associated protein (GABARAP) [18, 19, 24]. GRIP1 and HAP1 have been reported to interact with KIF5s. However, GABARAP has been identified to interact with only KIF5A. Our data showed that Frt-h and Frt-l interact with all three KIF5s.

Cytosolic ferritin is playing an essential role in iron homeostasis of the cell [16]. Ferritin is made up of two components, Frt-h and Frt-l, which combine to form the 24-subunit protein [17]. Frt-l is required for the long-term storage of iron, while Frt-h has ferroxidase activity in cells [16, 17]. The proportion of Frt-h and Frt-l composing ferritin multimeric complexes is regulated in a tissue-specific manner. Immunohistochemical study of ferritin in brain showed that neurons express predominantly Frt-h, microglia express Frt-l, and both Frt-h and Frt-l are found in oligodendrocytes [3]. In this study, although we did not show the interaction of kinesin 1 with Frt-h and Frt-l in a tissue-specific manner, our observations suggest a mechanism that kinesin 1 is linked directly to ferritin complex and could transport to ferritin complex in the cell.

Ferritin has typically been reported as a cytoplasmic protein [6]. However, several reports have found the presence of ferritin in nucleus [6, 20, 26]. Ferritin that is present in the nucleus is comprised of the same ferritin found in the cytoplasm [26]. A previous study reported that the phosphorylation of Frt-h is important for the specific nuclear translocation of ferritin [25]. These finding suggest that iron may be necessary for the activity of nuclear enzymes for DNA repair or RNA transcription [26]. However, the transport mechanism of ferritin from cytoplasm to nucleus is still unclear. To address this issue, it would be worth to identify the specific transport motor protein for ferritin from cytoplasm to nucleus. In this study, it is proposed that ferritin is a new cargo of kinesin 1. The direct interaction between Frt-h and KIF5s sheds new light on the mechanisms of ferritin complex transport to nucleus from cytoplasm, giving at the same time one more example of kinesin 1 cargo.

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초록 : 철 저장 단백질 ferritin과 kinesin 1 결합 규명

장원희[1] · 정영주[1] · 이원희[2] · 김무성[2] · 김상진[3] · 엄상화[4] · 문일수[5] · 석대현[1]*

([1]인제대학교 의과대학 생화학교실, [2]인제대학교 의과대학 신경외과학교실, [3]인제대학교 의과대학 신경과학교실, [4]인제대학교 의과대학 예방의학교실, [5]동국대학교 의과대학 해부학교실)

세포내소기관과 단백질 복합체의 운반은 kinesin superfamily proteins (KIFs)에 의해 매개된다. 처음으로 밝혀진 kinesin인 kinesin 1은 motor단백질로서 세포 내에서 미세소관을 따라 이동하며, 다양한 세포내 소기관이나 단백질복합체를 운반한다. Kinesin 1은 장쇄(KHC, 또한 KIF5s로도 통칭) 2분자와 단쇄(KLCs) 2분자로 구성된 4합체(tetramer) 구조를 가진다. KIF5s의 운반체 결합영역을 포함하는 말단영역은 다수의 운반체와 결합하지만, 결합운반체에 관하여 아직 충분히 밝혀지지 않았다. 본 연구에서 KIF5A의 결합 단백질을 동정하기 위하여 효모 two-hybrid screening을 수행하였고 철 저장 및 해독 기능을 하는 단백질인 ferritin heavy chain (Frt-h)을 찾아내었다. Frt-h은 KIF5A의 아미노산 800번과 940번 사이의 부위와 결합하며, 다른 KIF5s와도 결합함을 효모 two-hybrid assay로 확인하였다. 또한 Frt-h의 coiled-coil 도메인이 KIF5A와의 결합에 필수영역임을 밝혔다. 한편, ferritin light chain (Frt-l) 또한 KIF5s와 결합함을 효모 two-hybrid assay로 확인하였다. 이러한 단백질간의 결합을 glutathione *S*-transferase (GST) pull-down assay를 통하여 검증하였다. 추가적으로 생쥐의 뇌 파쇄액을 항 KHC 항체로 면역침강을 행한 결과, KLC1뿐만 아니라 Frt-h와 Frt-l도 같이 침강하였다. 이러한 결과들은 세포 내에서 kinesin 1이 ferritin 복합체를 운반함을 시사한다.