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- Note -

Interaction Between Prohibitin 2 and Kinesin Superfamily Protein 5A Causes Amyotrophic Lateral Sclerosis

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Kinesin superfamily protein 5A (KIF5A) is the motor protein of kinesin-1 and forms a heterotetrameric complex by binding to KIF5B or KIF5C and kinesin light chains (KLCs), a non-motor protein. Amyotrophic lateral sclerosis (ALS)-associated KIF5A single nucleotide variants are clustered in the near exon 27 and are predicted to have a change in the carboxy (C)-terminal region of KIF5A. In patients with ALS, mitochondria membrane protein prohibitin 1 (Phb1) and Phb2 were found down-regulated in the spinal cord. In this study, we confirmed that Phb2 binds to KIF5A. Phb2 bounds to the C-terminal region of KIF5A, which is the KIF5A-specific terminus, and KIF5A bounds to the C-terminal region of Phb2 but not to Phb1 in a two-hybrid assay. In human embryonic kidney-293T cells co-expressing EGFP-Phb2 and myc-KIF5A plasmids, Phb2 was co-immunoprecipitated with the motor proteins of kinesin-1, KIF5A and KIF5B, and the non-motor protein KLC1. In addition, both EGFP-Phb2 and myc-KIF5A were expressed at the same location in the cell. These results suggest that the binding of KIF5A to phb2 plays a role in mediating the association of kinesin-1 with mitochondria.

Key words : Binding protein, KIF5A, kinesin-1, mitochondria, prohibitin

Introduction

Amyotrophic lateral sclerosis (ALS) is known as the most common motor neuron disease in adults [2]. It is a type of neurological disease caused by the progressive degeneration of motor neurons in the brain and spinal cord [2, 15]. Mutations in kinesins, which are involved in axonal transport, are the cause of motor neuron disease in both humans and mice [5]. In a previous study, mutations in kinesin superfamily protein 1B (KIF1B) were found to be responsible for Charcot-Marie-Tooth disease (CMT) 2A, a disease of motor and sensory neurons [5]. In addition, mutations in KIF5A are associated with hereditary spastic paraplegia (HSP) [5, 18]. Thus, axonal transport within neurons is involved in neuronal survival and the pathogenesis of ALS [5].

In neurons, axonal transport is mediated by kinesins, which are motor proteins that move along the microtubules [6, 7]. Kinesins transport a variety of cargoes, including mitochondria and vesicles, and form a large superfamily [6, 11, 14]. Kinesin-1, the most abundant kinesin in cells, is a heterotetrameric protein composed of a heavy chain (KHC) (also known as KIF5s) and a light chain (KLC) [8, 11]. KIF5s, KIF5A, KIF5B, and KIF5C, have a three-domain structure that mediates their basic functions: a motor domain, a coiled-coil domain, and a carboxyl (C)-terminal domain [8]. The motor domain contains ATPase motor activity and interacts with microtubules, the coiled-coil domain is involved in binding to KLC, and the C-terminal domain binds cargoes transported by kinesin-1 [7, 12, 14].

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Dysfunction in the intracellular transport of cargo by kinesin-1 has been reported to be associated with several neurological diseases, including ALS [5]. Mutations in KIF5A are the cause of HSP and ALS, and the mutated residues in KIF5A are different between HSP and ALS [5]. HSP has mutated residues in the motor domain of KIF5A [15]. However, ALS-associated KIF5A mutations are located in exon 27, which corresponds to the C-terminal region of KIF5A [3, 15]. KIF5A exon 27 mutations induce a frameshift resulting in an abnormal C-terminal tail of KIF5A [15]. ALS-associated mutations in KIF5A are loss-of-function mutations that result in deletion of the C-terminal tail region, which is the cargo-binding domain (CBD) of KIF5A [3, 15].

In previous study, expression of a mutation in exon 27 of KIF5A in motor neurons resulted in the formation of large inclusion bodies in mitochondria [15]. This result showed that ALS-associated KIF5A mutations lead to mitochondrial aggregation along motor neurons. Prohibitin 2 (Phb2) is widely expressed in many tissues [10]. It is also mainly localized to mitochondria and plays a central role in the pathological cell physiology [1, 4, 10]. Phb2 plays an important role in a number of neurodegenerative diseases such as Parkinson's disease (PD) and ALS [2]. In a previous study, quantitative proteomic analysis of ALS patients showed that the Phb complex was downregulated in the spinal cord, with a greater downregulation of Phb2 [2]. This finding suggests that there may be a mitochondrial dysfunction in the cells of ALS patients.

In this study, we confirmed that Phb2, which is mainly located in the mitochondrial membrane, interacts with KIF5A. The interaction of Phb2 with KIF5A suggests that Phb2-KIF5A may act as a mediator protein in the intracellular transport of mitochondria by kinesin-1.

Materials and Methods

Plasmid constructs

A partial length cDNA of each mouse Phb1 (GeneBank ID: NM 008831) and Phb2 (GeneBank ID: NM 007531) was amplified by PCR from the Marathon-ReadyTM cDNA library (Clontech Laboratories, Inc., Palo Alto, CA, USA) and cloned into the pLexA vector (Clontech Laboratories, Inc.). Each KIF lacking the motor domain was subcloned into the pGEM T-easy vector (Promega Corp., Madison, WI, USA), the pLexA vector, and the pB42AD vector (Clontech Laboratories, Inc.).

Yeast two-hybrid assay of KIF5A and Phbs

For the yeast two-hybrid assay of KIF5A and Phbs, the Matchmaker yeast two-hybrid system (Clontech Laboratories, Inc.) was used for analysis according to the manufacturer's instructions. Briefly, the non-motor domain of KIF5A and each KIFs was amplified by PCR and cloned into the pLexA vector. pLexA-KIFs was transformed into yeast strain EGY48 (Clontech Laboratories, Inc.). pB42AD-Phbs was transformed into EGY48/pLexA-KIFs cells and then analyzed on X-Gal plates.

β-Galactosidase activity of KIF5A and Phb2 in yeast

For the measurement of β-galactosidase activity, mid-log phase yeast cells were harvested from liquid culture and permeabilized with 0.1% sodium dodecyl sulfate (SDS) and chloroform. O-nitrophenyl-β-D-galactoside (ONPG) (Sigma-Aldrich, St. Louis, MO, USA) was added to the yeast lysates and incubated at 30℃. The reaction was stopped by the addition of 1 M Na_2CO_3. The absorbance of the reaction solution was measured at 420 nm in a spectrophotometer, and the enzyme activity unit was calculated as previously reported [16].

Cell culture and transfection of the plasmid

Human embryonic kidney (HEK)-293T cells (American Type Culture Collection (ATCC) CRL-3216) were cultured at 37℃ in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a humidified 5% CO_2 incubator as previously described [13]. As previously described [13], HEK-293T cells were transiently transfected using the $CaPO_4$ precipitation method.

Co-immunoprecipitation and immunoblot analysis

FLAG-Phb2 and myc-KIF5A were transfected in HEK-293T cells. The transformed culture cells were rinsed with PBS buffer three times and lysed with lysis buffer (PBS containing 0.5% NP-40 and 1× Protease Inhibitor Cocktail Set V (Calbiochem, San Diego, CA, USA)) as previously described [13]. Cell lysate was centrifuged. The supernatant was incubated with anti-FLAG M2 agarose beads (Sigma-Aldrich) for 3 hr at 4℃. The beads were washed three times with cold PBS, and the proteins were eluted and denatured by boiling for 5 min. Each sample was processed for SDS-PAGE and immunoblot analysis using antibodies against KIF3B, KIF5A, KIF5B, KLC1, and FLAG epitopes as previously described [13].

Immunocytochemistry

As previously reported [13], HEK-293T cells were transfected with myc-KIF5A and EGFP-Phb2 plasmids and grown on poly-D-lysine-coated coverslips for 24 hr. Transformed cells were washed with PBS, fixed with 4% paraformaldehyde in PBS, and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 10 min. The cells were incubated for 40 min with Dylight 594-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch Labs, West Grove, PA, USA) for 60 min. The cells were rinsed three times with PBS and mounted with Fluoromount (DAKOKorea, Seoul, Korea). A Zeiss LSM510 META confocal laser scanning microscope (Carl Zeiss, Oberkochem, Germany) was used to obtain each cell image.

Results and Discussion

Phb2 interacts with KIF5A

The homeostasis of the neuron is highly dependent on the mitochondria due to its high energy requirements [6]. Phb2 is highly expressed in the mitochondria of neurons [1, 9]. It was downregulated in the spinal cord in ALS [2]. In this study, a yeast two-hybrid assay was applied to test the interaction between KIF5A (the motor protein of kinesin-1) and Phb2. Fig. 1A shows KIF5A bound to Phb2 (Fig. 1A). In order to quantify the binding affinity between KIF5A and

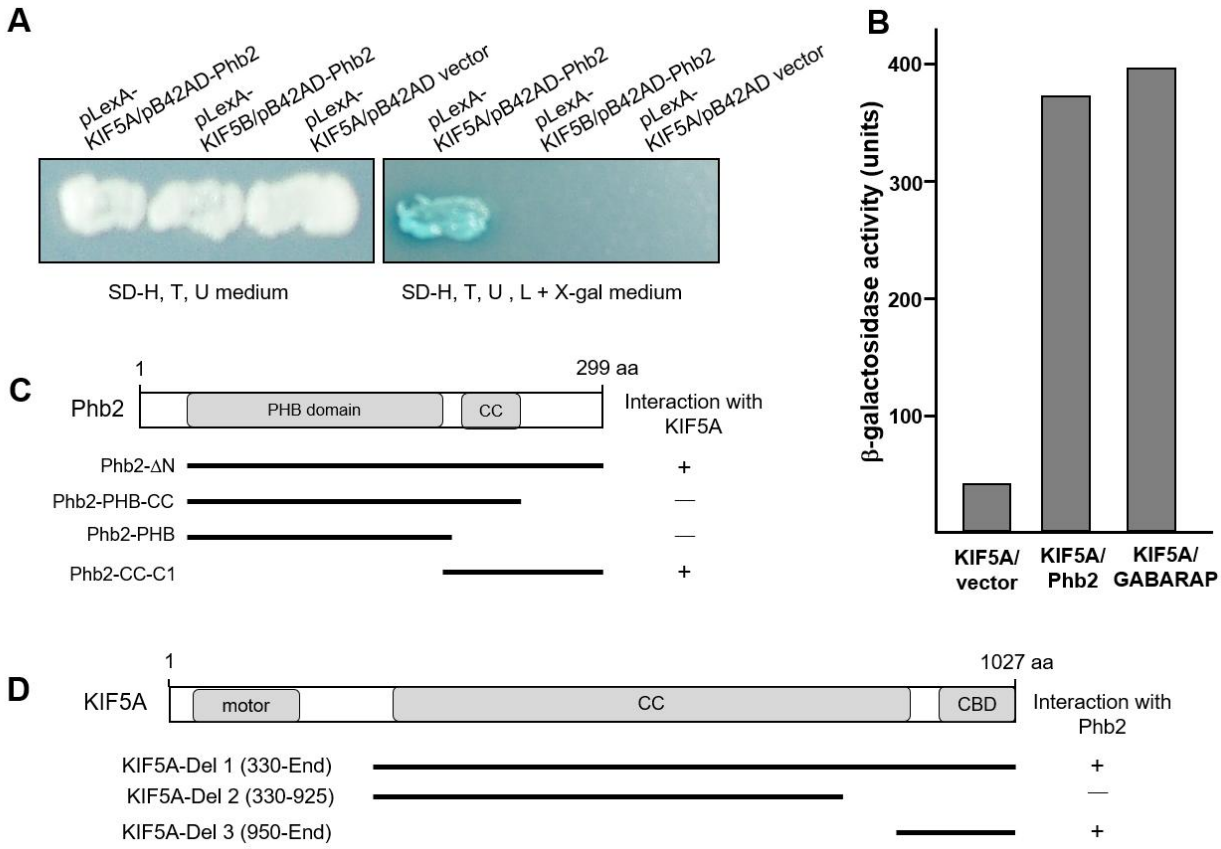

Fig. 1. KIF5A interacts with Phb2 in a yeast two-hybrid assay. (A) KIF5A interaction with Phb2. pB42AD-Phb2 plasmid was transformed into yeast strain EGY48/pLexA-KIF5A or EGY48/pLexA-KIF5B. Transformed cells were grown in SD-selective medium or SD-selective medium containing X-gal. (B) The strength of the interaction between KIF5A and Phb2 was quantitatively examined using β-galactosidase activity in yeast. (C) The minimal binding region of Phb2 for KIF5A. There are two domains in Phb2: the PHB domain, and CC, shown in gray. In a yeast two-hybrid assay, the different truncations of Phb2 were tested for binding to KIF5A. (D) The minimal binding region of KIF5A for Phb2. There are three domains in KIF5A: the motor domain, the CC, and CBD, shown in gray. In a yeast two-hybrid assay, the different truncations of KIF5A were tested for interaction with Phb2. +, interaction; -, no interaction; KIF5A, kinesin superfamily protein 5A; Phb2, prohibitin 2; GABARAP, γ-aminobutyric acid receptor-associated protein; CC, coiled-coil; SD, synthetic-defined; CBD; cargo-binding domain; H, histidine; T, tryptophan; U, uracil; L, leucine; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; aa, amino acids.

Phb2 in yeast, the activity of β-galactosidase was measured. γ-aminobutyric acid (GABA) type A receptor-associated protein (GABARAP) was used as a binding positive control for KIF5A [13]. The β-galactosidase activities of KIF5A and Phb2 were determined to be approximately 381 units (Fig. 1B).

Phb2 has a prohibitin (PHB) domain at the amino (N)-terminus and a coiled-coil domain in the middle [1]. To determine whether the PHB, coiled-coil, or C-terminal region of Phb2 interacts with KIF5A, we constructed a series of several fragments for each domain of Phb2 and analyzed their interaction with KIF5A. The C-terminal region, excluding the PHB domain of Phb2 and including some coiled-coil domains, is the minimal binding domain that interacts with KIF5A (Fig. 1C). KIF5A has an N-terminal motor domain, a coiled-coil domain, and a CBD that binds to proteins or cargos [8]. We constructed several fragments based on the motor domain, the coiled-coil domain, and the CBD of KIF5A and tested their interaction with Phb2 using a yeast two-hybrid assay. The CBD, which contains the coiled-coil domain of KIF5A, is required for the binding of KIF5A to Phb2 (Fig. 1C).

Phb2 interaction with kinesin-1

Next, we examined whether Phb2 interacts with other KIFs other than KIF5A, namely KIF3A (kinesin-2 motor protein), KIF5B, KIF5C, or KLC1. KIFs other than KIF5A and KLC1 did not interact with Phb2 in yeast two-hybrid plate assays (Fig. 2A). These results show that Phb2 is only able to interact directly with KIF5A. Phbs are a type of proteins that was first discovered as inhibitors of cell proliferation, and Phb1 and Phb2 have been identified in mammals [2]. Phb1 and Phb2 function as a heterodimeric complex in the mitochondrial membrane, but they can also function independently of each other [1, 2]. We performed a test for interaction between Phb1 and KIF5A. Phb1 did not interact with KIF5A in the yeast two-hybrid plate assay (Fig. 2B). This result is not surprising as the overall amino acid sequence similarity between Phb1 and Phb2 is 54%, specifically 74% in the PHB domain, but the amino acid similarity of the minimal binding domain required for binding to KIF5A is very low [1, 10]. These data showed that KIF5A only interacts with Phb2.

To determine whether Phb2 interacts with kinesin-1 in cells or only with KIF5A, we performed co-immunoprecipitation with anti-FLAG antibody transfected with FLAG-Phb2 and myc-KIF5A. As shown in Fig. 2C, when immunoprecipitation was performed using anti-FLAG antibody, Phb2 was immunoprecipitated together with KIF5B and KLC1, which are components of kinesin-1, including KIF5A. However, there was no immunoprecipitation of KIF3B, the motor protein of kinesin-2 (Fig. 2C). This result suggests that Phb2 interacts with kinesin-1 through KIF5A. Furthermore, Phb2 and KIF5A were found to have overlapping expression in the same region within the cell (Fig. 2D). These data suggest that the binding of Phb2 to kinesin-1 is mediated by KIF5A.

Mitochondrial genetic mutations and physiological dysfunction have been identified as important in the development of various neurological diseases [2, 5]. Mitochondrial-related neurological diseases include ALS, CMT, and PD [2]. These neurological diseases are thought to be caused by a dysfunctional mitochondrial energy metabolism. Previous studies have shown that Phbs may play a role in regulating and maintaining mitochondrial function in cells [9]. Phb1 and Phb2 are located in the mitochondrial membrane and function independently and in a heterodimeric complex [1]. Phb2 is a gene essential for mitochondrial homeostasis and has been shown to function as a key mitochondrial molecule that regulates proliferation during cell differentiation [9, 10]. At the proteomic level, the expression level of Phb2 was downregulated in the spinal cord of ALS patients, suggests an intracellular mitochondrial imbalance caused by the protein interactome of Phb2 [2].

In this study, we first show that Phb2 interacts with kinesin-1 through KIF5A. The CBD of KIF5A interacted with the C-terminal region excluding the PHB domain and the coiled-coil domain of Phb2. When FLAG-Phb2 and myc-KIF5A were expressed in cells, they co-immunoprecipitated and co-localized in cells.

What does the interaction between Phb2 and kinesin-1 identified in this study mean? One possibility is that the KIF5A-Phb2 interaction serves as an adaptor protein that mediates the kinsin-1-mitochondria interaction. In a previous study, *kif5*-KO mice died during early embryogenesis due to impaired mitochondrial localization in cells [17]. This mislocalization of mitochondria in the cells was rescued by exogenous expression of KIF5s [17]. In zebrafish, mutations in kif5A resulted in larval lethality and sensorimotor dysfunction similar to that seen in human ALS patients [3, 15]. In the peripheral axons of zebrafish with kif5a mutations, mitochondrial transport is defective and the axons degenerate [3, 15]. The kif5s genes are generally known to be redundant in function [8]. However, other Kif5s, Kif5B or Kif5C, cannot substitute for Kif5A in zebrafish, and these results suggest that the only Kif5A mediates mitochondrial axonal transport in the peripheral sensory neuron [15]. Although we have not shown direct data in this study on the interaction of KIF5A

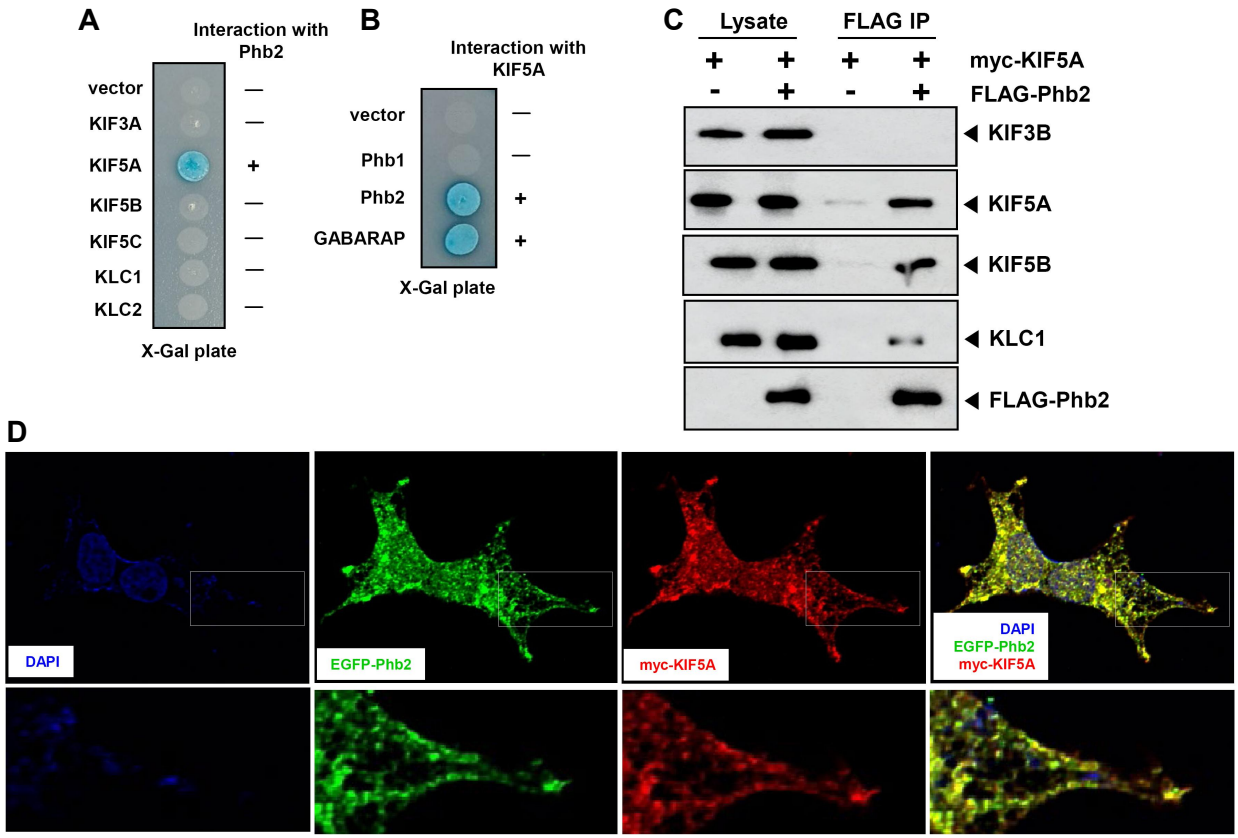

Fig. 2. Phb2 interaction with kinesin-1. (A, B) The C-terminal region of KIF5s and KIF3A and Phb2 were tested for interaction. In a yeast two-hybrid assay, Phb2 interacted only with KIF5A, but not with KIF3A, KIF5B, or KIF5C. KIF5A also only interacted with Phb2. GABARAP was used as a positive control for the interaction with KIF5A. (C) HEK-293T cells were transiently transfected with FLAG-Phb2 and myc-KIF5A plasmids as indicated. Immunoprecipitation was performed with a anti-FLAG antibody. Immunoprecipitates were probed with anti-KIF3B, KIF5A, KIF5B, KLC1 and FLAG antibodies. FLAG-Phb2 co-precipitated KIF5A, KIF5B and KLC1. (D) HEK-293T cells were transiently transfected with EGFP-Phb2 and myc-KIF5A plasmids. Twenty-four hours after transfection, cells were subjected to immunofluorescence with anti-KIF5A antibody. KIF5A and Phb2 co-localize in the same subcellular region of the cell. +, interaction; -, no interaction; KIF5, kinesin superfamily protein 5; KIF3A, kinesin superfamily protein 3A; KLC1, kinesin light chain 1; KLC2, kinesin light chain 2; Phb1, prohibitin 1; Phb2, prohibitin 2; GABARAP, γ-aminobutyric acid receptor-associated protein; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; DAPI, 4′,6-diamidino-2-phenylindole.

and Phb2 in the intracellular transport of mitochondria by kinesin-1. However, the data presented in this study suggest a novel model in which Phb2 interacts with kinesin-1 for intracellular mitochondrial transport in cells. Future studies are needed to determine the mechanism of intracellular mitochondrial transport of kinesin-1 mediated by KIF5A interacting with Phb2.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : Prohibitin 2와 근위축성 측삭 경화증 원인 단백질인 Kinesin Superfamily Protein 5A의 결합

김명훈[1] · 표세영[2] · 정은주[3] · 강미리[3] · 정영주[4] · 박성우[5,6] · 서미경[5,6] · 이원희[2] · 엄상화[7] · 김무성[2] · 석대현[4,5,8]*

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Kinesin superfamily protein (KIF5A)은 키네신-1의 운동 단백질이며 KIF5B 혹은 KIF5C와 결합하고, 또한 비운동 단백질인 키네신 경쇄(KLC)와 결합하여 이종사량체 복합체를 형성한다. 근위축성 측삭 경화증(ALS) 원인 유전자인 KIF5A의 단일 뉴클레오타이드 변이는 엑손 27 근처에 클러스터링되어 있으며, 이 영역은 키네신 5A의 카르복시(C)-말단 영역에 해당된다. 근위축성 측삭 경화증 환자에서 미토콘드리아 막 단백질인 prohibitin 1 (Phb1)과 Phb2는 척수에서 발현량이 감소한다. 본 연구에서는 Phb2는 KIF5A와 결합한다는 것을 확인하였다. Phb2는 KIF5A의 C-말단 영역에 결합하였고, KIF5A는 Phb2의 C-말단 영역에 결합하지만, KIF5A는 Phb1과는 결합하지 않았다. 인간배아신장 세포-293T에서 EGFP-Phb2와 myc-KIF5A 플라스미드를 공동 발현한 결과, Phb2는 키네신-1의 모터 단백질인 KIF5A와 KIF5B, 그리고 KLC1과 공동 면역침강하였다. 또한, EGFP-Phb2와 myc-KIF5A는 세포 내의 동일한 위치에서 발현하였다. 이러한 결과는 KIF5A는 Phb2와 결합하며, 키네신-1과 미토콘드리아의 결합을 매개하는 역할을 시사한다.