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Kinesin Superfamily-associated Protein 3 (KAP3) Mediates the Interaction between Kinesin-II Motor Subunits and HS-1-associated Protein X-1 (HAX-1) through Direct Binding

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Kinesin-II, a molecular motor, consists of two different motor subunits, KIF3A and KIF3B, and one large kinesin superfamily-associated protein 3 (KAP3), forming a heterotrimeric complex. KAP3 is associated with the tail domains of motor subunits. However, its exact role remains unclear. Here, we demonstrated KAP3 binding to the carboxyl (C)-terminal tail region of HS-associated protein X-1 (HAX-1). HAX-1 bound to the C-terminal region of KAP3, but not to KIFs (KIF3A, KIF3B, and KIF5B) and the kinesin light chain (KLC) in the yeast two-hybrid assays. The interaction was further confirmed in the glutathione *S*-transferase (GST) pull-down assay and by co-immunoprecipitation. Anti- HAX-1 antibody as well as anti-KIF3A antibody co-immunoprecipitated KIF3B and KAP3 from mouse brain extracts. These results suggest that KAP3 could mediate the interaction between Kinesin-II and HAX-1.

Key words : Microtubule motors, kinesin-II, kinesin superfamily-associated protein 3 (KAP3), HS-associated protein X-1 (HAX-1), adaptor proteins, protein-protein interaction

Introduction

Kinesins are motor proteins that utilize ATP hydrolysis to drive the transport of cargoes along microtubules [2]. Kinesin-II is the amino (N)-terminal motor domain member of Kinesin superfamily (KIF) [9]. It is a heterotrimeric complex of three dissimilar subunits, two motor subunits (KIF3A and KIF3B) and a larger non-motor protein (kinesin superfamily-associated protein 3, KAP3) [7]. KIF3A and KIF3B were shown to bind to each other through the coiled-coil stalk domains, while KAP3 was proposed to bind to the tail region of the motor subunits [8]. Kinesin-II is a versatile motor involved in multiple different transports involving a large variety of cargoes [7]. Kinesin-II is ubiquitously expressed, with abundant expression in nerve tissue [8]. Microinjection and immunoprecipitation by anti-KIF3B antibody revealed that Kinesin-II is thought to play a role in anterograde axonal transport in neuron [22].

In the nerve axons, the formation of cilia and flagella needs the transport of various proteins to the nerve terminal along microtubules. *Chlamydomonas* KIF3 homolog FLA10 was localized in flagella and highly concentrated around the flagellar basal bodies [15]. *Caenorhabditis elegans* KIF3 homolog Osm3 is essential for the assembly of the cilia of sensory neurons [16]. Kinesin-II in sea urchin has been localized in the connecting cilia. Kinesin-II was shown to be involved in ciliogenesis in the sea urchin embryo. Microinjection of anti-Kinesin-II antibody disrupted the formation of cilia [21]. Thus, Kinesin-II plays important roles in anterograde intraflagellar transport for the flagella and cilia formation in many species and cell types [8]. Interestingly, knockout mice for Kinesin-II genes showed the randomization of left-right asymmetry [8] which is implicated in certain chronic human disorders such as Bardet-Biedl syndrome [4] and polycystic kidney disease [13].

Previous genetic studies suggest that KAP3 plays an important role in Kinesin-II functions. Mutation in the *FLA3*, a *Chlamydomonas KAP3* homolog, was shown to cause loss of the anterograde intraflagellar transport [12, 15]. In *Drosophila*, mutation in the *DmKAP3* eliminated the sensory cilia of neurons [18]. Similarly, mutation in the *Caenorhabditis elegans KAP3* was shown to affect the Kinesin-II mediated transport into the sensory cilia [16]. However, a study with recombinant KIF3A/KIF3B and KAP3 suggested that KAP3 has no effect on the motor activity of Kinesin-II [23]. Human KAP3 was shown to mediate the regulation of the interaction of small G proteins implicated in vesicle transport with

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membranes [19]. Therefore, KAP3 may control the binding of Kinesin-II to vesicular cargoes through the interaction with small G proteins located at membranes. On the basis of these observations, it could be speculated that KAP3 may function as a cargo adapter for Kinesin-II.

Understanding how Kinesin-II binds to its specific cargo is an important question. In this study, we screened for proteins that bind to KAP3 and found an interaction with HS-1-associated protein X-1 (HAX-1). HAX-1 was originally identified as a 35 kDa protein that interacts with HS-1, a Src kinase substrate [5]. The KAP3 and HAX-1 interaction suggests that Kinesin-II may transport HAX-1 containing cargo along microtubules in cells.

Materials and Methods

Plasmid constructs

Full-length mouse *HAX-1* (accession NM_011826.3), and *KAP3* (accession NM_010629.2) were amplified by polymerase chain reaction (PCR) from Marathon-Ready™ cDNA library (Clontech, Palo Alto, CA, USA) and cloned into pGEM T-easy vector (Promega Corp, Madison, WI, USA). The C-terminal region of KAP3 was utilized as a template to amplify the region coding for amino acids 320-772 using the specific primers [23]. The amplified fragment was cloned into pGEM T-easy vector (Promega). The resulting recombinant plasmid was then cut with *EcoR*I and *Xho*I and the insert was subcloned into pLexA (Clontech), pJG4-5 (Clontech), and pET41 (Novagen, San Diego, CA, USA).

Screening of KAP3-binding proteins by yeast two-hybrid system

The Matchmaker LexA two-hybrid system was used for screening according to the manufacturer's manual (Clontech). In brief, the yeast strain EGY48 carrying the p8op-*lacZ* reporter plasmid was transformed with pLexA-KAP3. The yeast cells were subsequently transformed with the mouse brain cDNA library [22] 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 clones were analyzed by digestion with *EcoR*I and *Xho*I. Unique inserts were sequenced and protein sequence analysis was performed with the BLAST algorithm at the National Center for Biotechnology Information (NCBI). Sequence-verified clones were tested again for interactions of with the bait in yeast by retransformation.

β-Galactosidase activity in liquid cultures of yeast

The β-galactosidase activity of yeast was assayed as described previously [11, 22]. Mid-log phase yeast cells were collected and permeabilized with 0.1% sodium dodecyl sulphate (SDS) and chloroform. An excess amount of o-nitrophenyl-β-D-galactoside (ONPG) was added to yeast 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. The units of enzyme activity were calculated by the following equation: units=1000 × [(OD_{420} - 1.75 × OD_{550})] / (reaction time × culture volume × OD_{600}). All experiments were independently performed at least four times [1].

Co-immunoprecipitation and Immunoblot analysis

Mouse brains were homogenized in ice-cold homogenization buffer (0.32 M sucrose, 4 mM HEPES, pH 7.3) supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). For immunoprecipitation, mouse brain lysate was diluted in the same volume of 2X binding buffer (50 mM HEPES, 200 mM KCl, 0.2% Triton X-100, pH 7.0) and incubated with anti-HAX-1 antibody (Santacruz Biotechnology, Santa Cruz, CA, USA), anti-KIF3A antibody [22], or 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, 0.2 M NaCl, 0.1% Tween 20). The washed beads were resuspended with Laemmli's loading buffer and the proteins were eluted and denatured by boiling for 2 min and then separated by SDS-PAGE. The proteins were transferred from the gel to a nitrocellulose membrane and incubated with anti-KAP3 [23], anti-KIF3B [23], and anti-HAX-1 antibodies.

Glutathione S-transferase (GST) pull-down assays

cDNA encoding the C-terminal region of HAX-1 was cloned into pET41a. The recombinant GST-HAX-1 fusion protein was expressed in bacterial strain BL21 GOLD (Stratagene, La Jolla CA, USA) after induction with 0.5 mM isopropyl thio-β-D-galactopyranoside (IPTG) for 3 hr. The

fusion proteins were purified using glutathione-agarose beads (Sigma-Aldrich) according to the manufacturer's protocol. The mouse brain S2 fraction 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 pulled-down proteins were analyzed by immunoblotting with anti-KIF3A, anti-KIF3B, anti-KIF5B, and anti-KAP3 antibodies [10, 23].

Results

Identification of KAP3 interacting proteins by yeast two-hybrid system

To identify KAP3 interacting proteins, we screened a mouse brain cDNA library by yeast two-hybrid system using the C-terminal region (aa 320-772) of KAP3 as bait. From 5×10^6 colonies screened, we obtained 4 positive clones. Two clones (clone 1 and 3) of the 4 clones were identical and possessed cDNA fragments corresponding to the C-terminal region of HAX-1 (Fig. 1A). HAX-1 was isolated as HS-1 interacting protein and suggested to be involved in B cell signal transduction [20]. HAX-1 contains several protein-protein interaction domains, the putative two Bcl-2 homology (BH) domains and one proline, glutamic acid, serine, threonine (PEST) domain. The C-terminus of HAX-1 has the putative transmembrane domain (TMD) [5]. To determine the minimal binding domain of HAX-1 that is required for the interaction with KAP3, we constructed several deletion mutants of HAX-1. Yeast two-hybrid assays showed that the minimal domain required for binding was located in the C-terminal region of HAX-1 (Fig. 1A). Unexpectedly, KAP3 did not interact with the putative protein-protein interaction domains within the N-terminal region of HAX-1. Next, we determined the minimal binding domain of KAP3. We constructed several deletion mutants of KAP3 and tested the interaction with HAX-1 by yeast two-hybrid assays. The interaction with HAX-1 was dependent on the C-terminal region of KAP3 (Fig. 1B). Together, these results show that the interaction between KAP3 and HAX-1 is mediated through their C-terminal regions.

Next we investigated whether HAX-1 interacts with

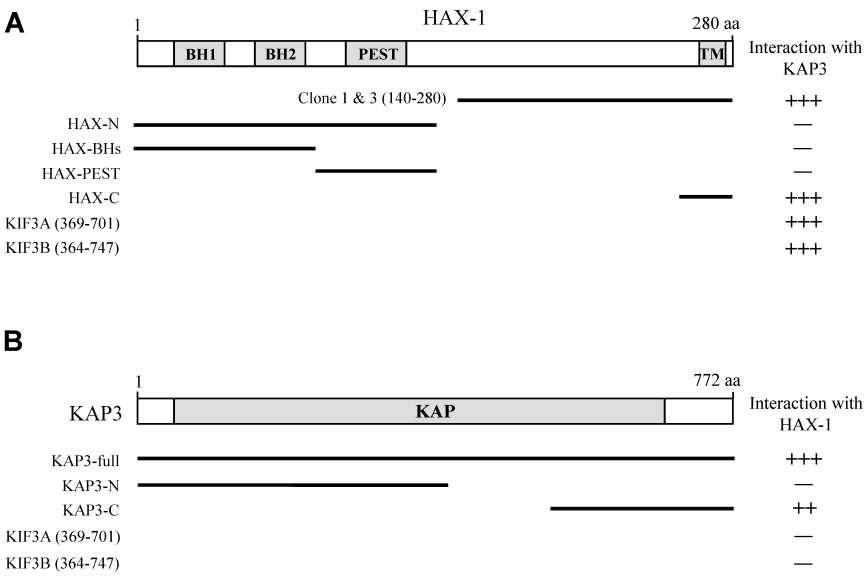

Fig. 1. Identification of the proteins interacting with KAP3 by yeast two-hybrid screening. (A) The domain structure of HAX-1 illustrating that clones 1 and 3 corresponds to the C-terminal fragment of HAX-1. The putative BH, PEST, and TM domains are indicated in gray. To determine minimal KAP3 binding region and binding specificity of HAX-1, several truncated forms of HAX-1, KIF3A, and KIF3B were constructed by PCR and tested in the yeast two-hybrid assay for interaction with KAP3. +++, interaction with KAP3; -, no interaction with KAP3. aa, the amino acid residue number. (B) The C-terminal region of KAP3 mediates interaction with HAX-1. KAP3 has a KAP domain, depicted as the gray box. Several truncated forms of KAP3, KIF3A, and KIF3B were constructed by PCR and tested in the yeast two-hybrid assay for interaction with HAX-1. ++ or +++, interaction with HAX-1; -, no interaction with HAX-1. aa, the amino acid residue number.

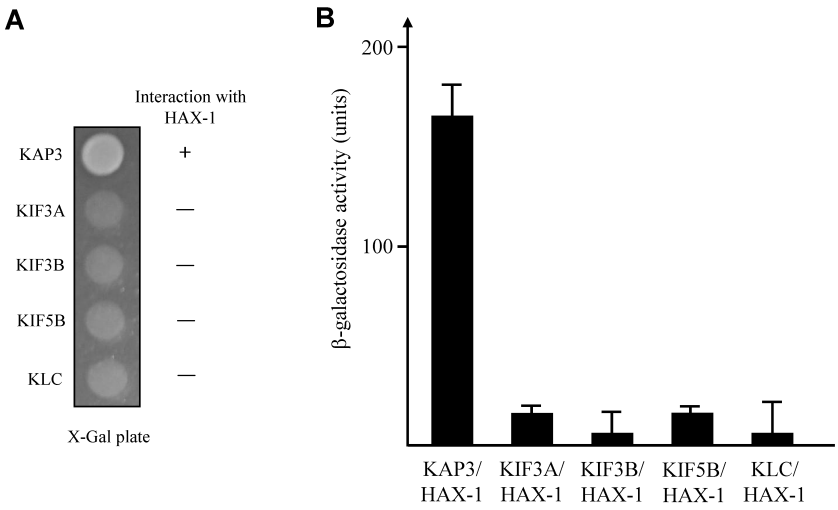

Fig. 2. Interaction between KAP3 and HAX-1. (A) The C-terminal regions of each KIF, KAP3, and KLC were fused to the pLexA DNA binding domain. HAX-1 specifically interacted with KAP3 but not with KIFs and KLC. +, interaction; -, no interaction. (B) The strength of interactions between KIFs, KAP3, or KLC and HAX-1 were examined quantitatively using β-galactosidase activity in yeast two-hybrid reporter assay.

KIF3A and KIF3B subunits of Kinesin-II and KIF5B and kinesin light chain 1 (KLC1) subunits of Kinesin-I. As shown in Fig. 1B, 2A, and 2B, there was no detectable binding of HAX-1 with KIF3A, KIF3B, KIF5B, and KLC1. A quantitative β-galactosidase assay showed that HAX-1 directly bound to KAP3 but not to KIFs (Fig. 2B). These data indicate that the interaction of KAP3 with HAX-1 is specific.

HAX-1 is associated with Kinesin-II

Kinesin-II is composed of a KIF3A/KIF3B heterodimer and KAP3, forming a heterotrimeric complex [23]. Therefore, we next determined whether HAX-1 interacts specifically with KAP3 and whether the interaction includes Kinesin-II at the protein level using GST pull-down experiments. Recombinant GST-HAX-1 fusion protein was expressed in *E. coli*. The purified GST fusion protein was allowed to interact with mouse brain lysates. Immunoblotting analyses revealed that KAP3 interacted with GST-HAX-1, but not with GST. In addition, KIF3A and KIF3B, which bind to the KAP3, were efficiently precipitated with GST-HAX-1 (Fig. 3A). This indicates that HAX-1 interacts with Kinesin-II.

To further confirm whether the binding of KAP3 to HAX-1 mediates the interaction between Kinesin-II and HAX-1, we performed co-immunoprecipitation analyses. Lysates from mouse brain were incubated with anti-HAX-1 antibody or anti-KIF3A antibody. Protein A-Separose beads precipitated the immuno-complexes, which were then subsequently separated by SDS-PAGE and immunoblotted with

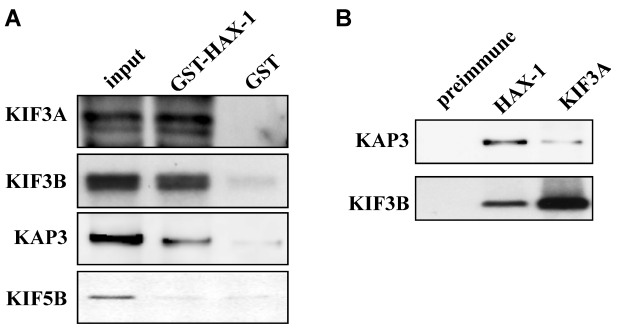

Fig. 3. Association of Kinesin-II with HAX-1 in the GST pull-down assay and co-immunoprecipitation. (A) Proteins in the mouse brain lysate were allowed to bind to GST alone or GST-HAX-1 fusion proteins. The elution fractions were resolved by SDS-PAGE and analyzed by immuoblotting using anti-KIF3A, KIF3B, KAP3, or KIF5B antibodies. (B) Mouse brain lysates were immunoprecipitated with anti-KIF3A, HAX-1 antibodies, or preimmune serum, and then the precipitates were immunoblotted with anti-KAP3, or KIF3B antibodies.

anti-KAP3 and anti-KIF3B antibodies. As shown in Fig. 3B, both anti-HAX-1 and anti-KIF3A antibodies efficiently precipitated the Kinesin-II complex, KIF3A/KIF3B heterodimer and KAP3. These results suggest that the interaction of HAX-1 with Kinesin-II is mediated by KAP3.

Discussion

In the search for proteins interacting with KAP3 we have shown that HAX-1, a multifunctional protein can associate

with KAP3. Using the C-terminal region of KAP3 as bait, we identified HAX-1 in yeast two-hybrid assay of a mouse brain cDNA library. The C-terminal region of HAX-1 interacted with KAP3. Furthermore, using a combination of GST pull-down assay and co-immunoprecipitation, we confirmed that KAP3 interacted with HAX-1 at the protein level. Moreover, we showed that Kinesin-II complex can be co-precipitated with HAX-1. Although we did not identified the HAX-1 containing cargoes, this result suggest that Kinesin-II transports HAX-1 containing cargo through the interaction between KAP3 and HAX-1.

HAX-1 was first identified as a partial cDNA for mRNAs induced upon exposure of macrophages to silica. HAX-1 is ubiquitously expressed and a multifunctional protein [20]. Recent studies have shown that HAX-1 interacts with a number of cellular and viral proteins [5]. HAX-1 interacts with HtrA2 (high temperature requirement protein A2) and Parl (presenilin-associated, rhomboid-like) within mitochondria. This interaction was shown to be required to suppress apoptosis in lymphocytes and neurons [3]. In other study, it was suggested that HAX-1 is involved in the formation of cell-matrix contacts through its binding to the polycystic kidney disease protein PKD2 as well as to cortactin, an F-actin -associated protein [6]. In additional study, HAX-1 was shown to bind the integrin β_6 subunit and regulate the internalization of the integrin $\alpha_v\beta_6$ [17]. Reduction of HAX-1 expression level by siRNA showed that integrin dependent cell migration was reduced through down regulation of endocytosis via a clathrin [17]. A recent study revealed that HAX-1 interacts with the HIV-1 Rev protein and facilitates the export of viral mRNAs. In HAX-1 and Rev co-expressing cells, Rev was transported from the nucleus to the cytoplasm, where it co-localized with HAX-1 in the cytoplasm [14].

As described in this study, KAP3 directly interacted with HAX-1. These data suggested that KAP3 may link Kinesin-II and HAX-1. We propose one possibility to illustrate how Kinesin-II can mediate the transport of many cargoes. Different cargoes may use different adaptor proteins that mediate the attachment of Kinesin-II to proteins or membrane-bound cargoes. Based on the ability of KAP3 to bind both KIF3A/KIF3B and HAX-1, associated with various cellular and viral proteins, we favor the model that HAX-1 may function as an adaptor and mediate the attachment of many different cargoes to Kinesin-II. In this report, we describe that the C-terminal of HAX-1 mediates interaction with KAP3. This interaction may link Kinesin-II to many different cargoes.

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초록 : Kinesin superfamily-associated protein 3 (KAP3)를 통한 HS-1-associated protein X-1 (HAX-1)과 Kinesin-II의 결합

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Kinesin-II는 다양한 운반체들을 미세소관을 따라 운반하는 motor 단백질의 하나이다. Kinesin-II는 두 개의 motor 단백질 KIF3A와 KIF3B, 그리고 motor 단백질의 말단에 결합하는 kinesin superfamily-associated protein 3 (KAP3)로 구성되어 있다. KAP3는 Kinesin-II의 기능에 중요한 역할을 하는 것으로 알려져 있으나 명확한 기능은 아직 밝혀지지 않았다. 본 연구에서 KAP3와 결합하는 단백질을 분리하기 위하여 효모 two-hybrid system을 사용하여 탐색한 결과 HS-1-associated protein X-1 (HAX-1)을 분리하였다. KAP3은 HAX-1의 C-말단 부위와 결합하며, HAX-1은 KAP3의 C-말단부위와 결합함을 효모 two-hybrid assay로 확인하였다. 그러나, HAX-1는 KIF3A, KIF3B, KIF5B, 그리고 kinesin light chain (KLC)과는 결합하지 않았다. KAP3와 HAX-1의 단백질 결합은 glutathione *S*-transferase (GST) pull-down assay와 공동면역침강으로 추가 확인하였다. 생쥐의 뇌 파쇄액을 HAX-1 항체와 KIF3A 항체로 면역침강을 행한 결과 Kinesin-II의 구성단백질인 KIF3B와 KAP3가 같이 침강하였다. 이러한 결과들은 KAP3가 Kinesin-II와 HAX-1의 결합을 매개한다는 것을 시사한다.