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Brain-expressed X-linked 2 Binds to Kinesin Superfamily Protein 3A

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Kinesin-2 comprises two subfamilies of the heterotrimeric or homodimeric motors found in mammalian cells. Heterotrimeric kinesin-2 consists of kinesin superfamily proteins (KIFs) 3A and 3B and kinesin-associated protein 3 (KAP3), which is a molecular motor protein that moves along microtubules. It plays diverse roles in cargo transport, including anterograde trafficking in cilia, and interacts with many different cargoes and proteins, but their binding proteins have not yet been fully identified. In this study, the yeast two-hybrid assay was used to identify the proteins that interact with the cargo-binding domain (CBD) of KIF3A, and an interaction between KIF3A and brain expressed X-linked 2 (Bex2) was found. Bex2 bound to the CBD-containing C-terminal tail region of KIF3A but did not interact with the same region of KIF3B or KIF5A (a motor protein of kinesin-1). KIF3A interacted with another isoform, Bex1, but did not interact with Bex3. In addition, glutathione *S*-transferase (GST) pull-downs showed that KIF3A specifically interacts with GST-Bex1 and GST-Bex2 but not with GST alone. When co-expressed in HEK-293T cells, Bex2 co-localized with KIF3A and co-immunoprecipitated with KIF3A and KIF3B but not KIF5B. In combination, these results suggest that Bex2 is capable of binding to heterotrimeric kinesin-2 and may serve as an adaptor protein that links heterotrimeric kinesin-2 with cargo.

Key words : Binding protein, brain expressed X-linked 2, cargo binding domain, kinesin, heterotrimeric kinesin-2

Introduction

Kinesin superfamily proteins (KIFs) are involved in diverse functions inside cells such as cargo transport and mitosis [15]. Kinesin-2 is the amino (N)-terminal motor domain member of KIFs [8, 15]. It is a distinctive subfamily of processive kinesins that contains both homodimeric and heterodimeric motors involved in microtubule (MT) plus-end directed cargo transport [15]. The mammalian heterodimeric kinesin-2 forms two genes: *kif3a*, and *kif3b* to form heterodimeric motors while expression of the kif17 gene product results in homodimeric kinesin-2, KIF17 [8, 15]. The heterotrimeric kinesin-2 consists of two distinct motor subunits (KIF3A and KIF3B) and an accessory protein, kinesin associated protein 3 (KAP3), which binds to the coiled-coil stalk domains of the motor subunits [18, 189]. It is essential for the intracellular transport of cargoes and intraflagellar transport (IFT) along cilia that is essential for the ciliary and flagellar assembly [3, 4, 15]. Also, heterodimeric kinesin-2 involved in the cilia-dependent signal transduction pathways, including the Hedgehog-signaling pathway [3, 15]. Knockout mice for KIF3A or KIF3B show the absence of nodal cilia [17], indicating the crucial role of heterotrimeric kinesin-2 in the trafficking of essential components of the ciliary axoneme, including tubulins and thus have a randomized left-right body axis [4, 8].

KIF3s are composed of an amino (N)-terminal motor domain connected to a long α-helical region that dimerizes into

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a coiled-coil stalk that ends with a carboxyl (C)-terminal domain that may interact with specific adaptor proteins for cargo linkage [9, 16]. KIF3A and KIF3B have been implicated in intracellular transport of vesicles and organelles [8, 16]. For example, in neuronal cultured cells, anti-KIF3B antibody microinjection and immunoprecipitation experiments suggested that KIF3 moves the vesicles and organelles along microtubule [16]. Previous study of KIF3A using RNA interference, and live cell imaging suggested that KIF3 mediates the intracellular transport of human immunodeficiency virus (HIV)-containing compartment along microtubule in HIV-infected cells [5].

Heterotrimeric kinesin-2 plays diverse roles in many cargos transport [7]. However, the binding proteins of heterotrimeric kinesin-2 are poorly understood. In this study, we screened for proteins that bind with the cargo-binding domain (CBD) of KIF3A, and found protein interacting with brain expressed X-linked 2 (Bex2) [10]. These results suggest that Bex2 is capable of binding to the heterotrimeric kinesin-2 and it may serve as an adaptor protein that links heterotrimeric kinesin-2 and cargo.

Materials and Methods

Plasmid constructs

The full-length cDNAs of mouse Bex 1 (GeneBank ID: 19716) and Bex 3 (GeneBank ID: 12070) were amplified by PCR from Marathon-Ready™ cDNA library (Clontech Laboratories, Inc.) and cloned into pGEM T-easy vector (Promega Corp, Madison, WI, USA). The CBD (aa 585-701) of KIF3A were subcloned from pCAGGS-KIF3A obtained from Prof. Kaibuchi K. Nagoya University, Nagoya, Japan [14] into the *EcoR*I and *Xho*I restriction sites of the pLexA. The resulting recombinant plasmid, pLexA-CBD-KIF3A, was used as bait plasmid this yeast two-hybrid screening. The series of deletion mutants of mouse KIF3A were subcloned from pCAGGS-KIF3A.

Screening of KIF3A binding protein

The Matchmaker LexA two-hybrid system was used for screening according to the manufacturer's manual (Clontech). In brief, pLexA-CBD of KIF3A was transformed into yeast strain EGY48 carrying the p8op-lacZ gene. Transformed cells were transformed with the mouse brain cDNA library [16] 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 clone was performed on an SD/-His/-Trp/-Ura/-Leu plate containing galactose, raffinose, X-gal, and BU salts. Plasmid from positive clone was analyzed by *EcoR*I and *Xho*I restriction digestion. Unique insert was sequenced and protein sequence analysis was performed with the BLAST algorithm at the National Center for Biotechnology Information (NCBI).

β-Galactosidase activity in liquid cultures of yeast

The β-galactosidase activity of yeast was assayed as described previously [16]. 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) (Sigma-Aldrich, St. Louis, MO, USA) was added to yeast lysate, and the mixture was incubated for each time 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=1,000x [(OD_{420} - 1.75× OD_{550})]/(reaction time x culture volume x OD_{600}) [2].

Glutathione S-transferase (GST) pull-down assays

The full length of Bex1, and Bex2 was cloned in pET41a. The recombinant GST-Bex1, and Bex2 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 2.5 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 pulled-down proteins were analyzed by Western blotting with anti-KIF3A antibody [16]. The animal study was approved by the institutional review board (IRB), and the approval number was 20-12 of Inje University animal center.

Cell culture and transfection

Human embryonic kidney (HEK)-293T [American Type

Culture Collection (ATCC) CRL-3216] cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37℃ in a humidified 5% CO_2 incubator. Transient transfections were performed using the $CaPO_4$ precipitation method [13].

Immunocytochemistry

HEK-293T cells grown on poly-D-lysine-coated coverslips were transfected with enhanced green fluorescent protein (EGFP)-Bex2 constructs. Twenty-four hours after transfection, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 5 min, and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in PBS for 10 min. After blocking with 5% normal goat serum in PBS for 30 min, cells were incubated overnight at 4℃ with anti-KIF3A antibody [13] in PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween-20 (Sigma-Aldrich). After washing 3 times with PBS, cells were incubated for 40 min with Dylight 594-conjugated goat anti-rabbit IgG antibody (Jackson Immuno Research Labs, West Grove, PA, USA) diluted 1:800. After washing 3 times with PBS, the cells were mounted with Fluoromount (DAKOKorea, Seoul, Korea). Fluorescence images were acquired on Zeiss LSM510 META confocal laser scanning microscope (Carl Zeiss, Oberkochem, Germany).

Co-immunoprecipitation and immunoblot analysis

HEK-293T cells were transfected with myc-KIF3A and FLAG-Bex2 constructs. Cells were rinsed with ice-cold PBS twice and lysed with ice-cold lysis buffer [PBS containing 0.5% NP-40 and 1x protease inhibitor cocktail set V (Calbiochem, San Diego, CA, USA)] by gentle rotation for 30 min. Lysates were centrifuged at 16,000x *g* for 10 min at 4℃. The supernatant was incubated with anti-FLAG M2 agarose beads (Sigma-Aldrich) for 2 hr at 4℃ with constant shaking. The beads were collected by centrifugation at 2,000× *g* for 30 sec and washed 5 times with ice-cold PBS containing 0.5% NP-40. The washed beads were resuspended with Laemmli's loading buffer and the proteins were eluted and denatured by boiling for 5 min. The proteins were processed for 10% SDS-PAGE and immunoblot analysis with antibodies against KIF3A, KIF3B, KIF5B, FLAG, and myc epitope as described elsewhere by Nakajima et al. [13].

Results

Identification of KIF3A interacting proteins by yeast two-hybrid assay

Heterotrimeric kinesin-2 consists of the two motor subunits, KIF3A/ KIF3B, and kinesin associated protein 3 (KAP3) [19]. To improve the understanding of heterotrimeric kinesin-2-dependent transport events, it was necessary to identify the interacting partners of KIF3s. A yeast two-hybrid screening was performed using the KIF3A-CBD (amino acids 585-701) as bait and isolated positive clone from a mouse brain cDNA library. From 5×10^6 colonies screened, we obtained positive clone. The positive clone turned out to the full legnth of Bex2 (Fig. 1B). Previously study identified that the CBD of KIF3A has three phosphorylation sites, S689, T694, and S698 [9]. Interestingly, these phosphorylation sites were located on the CBD of KIF3A. The phosphorylation of KIF3A directly facilitates the binding activity of the N-cadherin containing-vesicles to KIF3A and intracellular transport in cells [9]. Next, we examined the effects of the C-terminal phosphorylation sites of KIF3A on binding with Bex2 and KIF3A. We constructed three deletion mutants of the C-terminal phosphorylation sites of KIF3A and tested the interaction with Bex2 and the three deletion mutants mof KIF3A. As shown in Fig. 1A, the three deletion mutants of the C-terminal phosphorylation sites of KIF3A interact with Bex2. This data suggests that the phosphorylation sites of KIF3A do not directly contribute to the interaction of KIF3A with Bex2.

Bex2 has a BEX-domain [10]. To identify the binding domain of Bex2 required for the interaction with KIF3A, a series of deletion mutants of Bex2 was constructed and analyzed their interactions with KIF3A using the yeast two-hybrid assay. Only the full length of Bex2 interacted with KIF3A in the yeast two-hybrid assay (Fig. 1B). This experiment demonstrated that the full length of Bex2 binds with the C-terminal region of KIF3A.

Next, we investigated whether Bex2 interacts with KIF3B, KAP3, or KIF5A, KIF5B, a motor subunits of kinesin-1. Bex2 did not interact with KIF3B, KIF5A, KIF5B and KAP3 (Fig. 2A). This data indicats that Bex2 specifically interacts with KIF3A. Subsequently, we investigated whether KIF3A interacts with the other Bex2 isoforms, Bex1 and Bex3. As shown in Fig. 2B, KIF3A interact with Bex1 and Bex2. However, KIF3A did not interact with Bex3. Calcium-calmodulin-dependent kinase II a (CaMKIIa), known to interact with

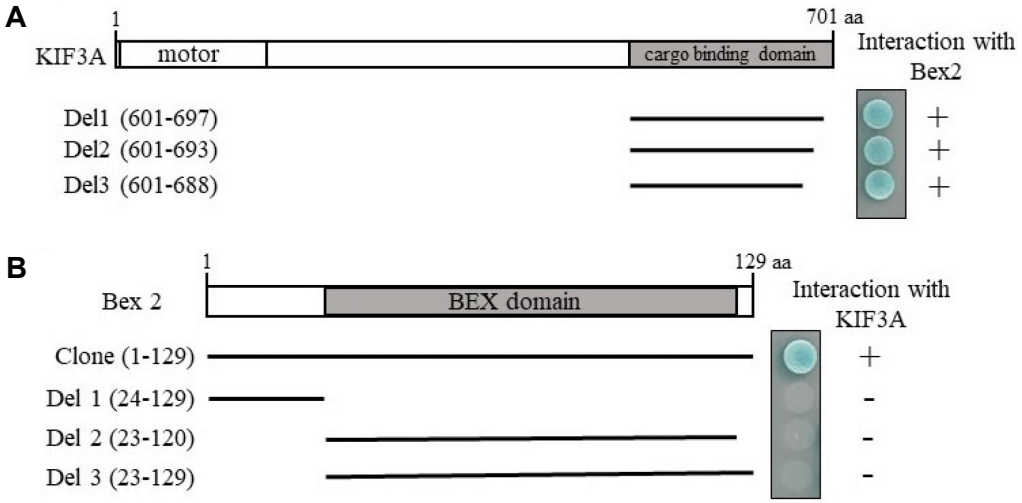

Fig. 1. Identification of the proteins interacted with KIF3A by yeast two-hybrid screening. (A) Bex2 binding region in KIF3A. KIF3A has the motor domain and cargo binding domain, indicated in gray. The truncated forms of KIF3A were assayed in the yeast two-hybrid assay for interaction with Bex2. (B) KIF3A binding region in Bex2. The positive clone isolated from the yeast two-hybrid screening. Bex2 has BEX domain, indicated in gray. The truncated forms of Bex2 were assayed in the yeast two-hybrid assay for interaction with KIF3A. +, interaction; -, no interaction; KIF3A, kinesin superfamily protein 3A; Bex2, brain expressed X-linked 2; aa, amino acids.

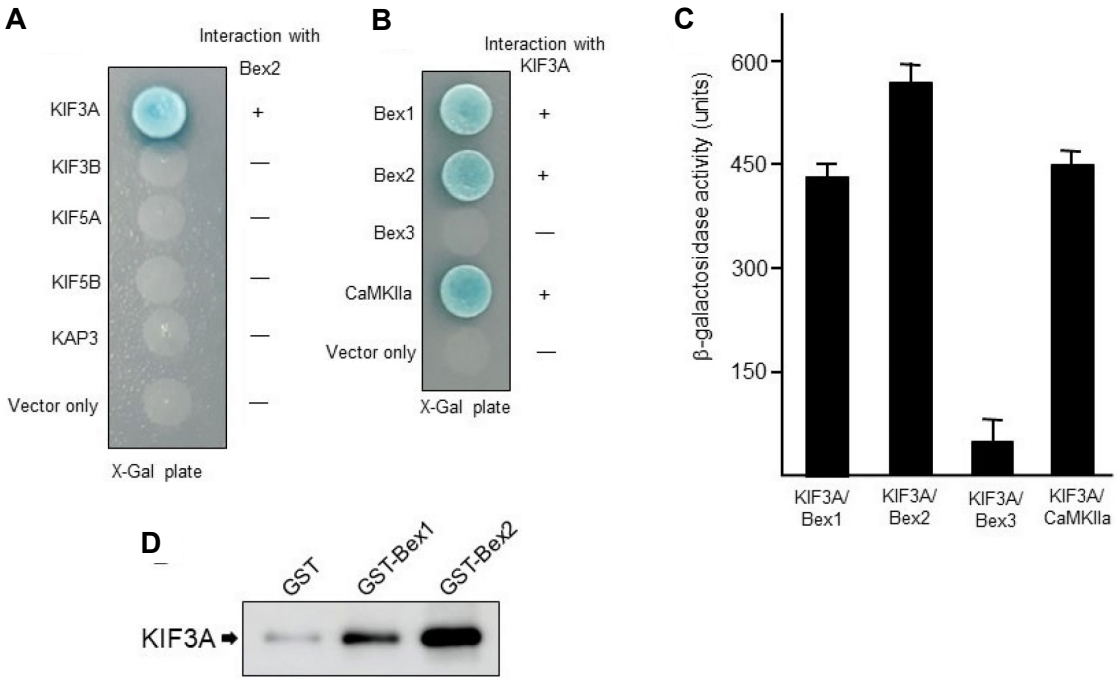

Fig. 2. Interaction of KIF3A with Bex2. (A) The tail region of each KIFs and the full length KAP3 were tested for the interaction with Bex2 in the yeast two-hybrid system. Bex2 specifically interacted with KIF3A, but not with KIF3B, KIF5A, KIF5B, and KAP3. +, interaction; -, no interaction. (B) Bex1, Bex2, or Bex3 were tested for the interaction with KIF3A in the yeast two-hybrid system. KIF3A interacted with Bex1, and Bex2, but not with Bex3. CaMKIIa served as a positive control for interaction. +, interaction; -, no interaction. (C) The strength of interactions between Bexs and KIF3A was examined quantitatively using β-galactosidase activity in the yeast two-hybrid reporter assay. Values are presented as the mean ± standard deviation. (D) Direct binding of KIF3A to Bex2 in a GST pull-down assay using purified GST-Bex1, GST-Bex2, and GST only. KIF3A, kinesin superfamily protein 3A; KIF3B, kinesin superfamily protein 3B; KIF5A, kinesin superfamily proteins 5A; KIF5B, kinesin superfamily proteins 5B; KAP3, kinesin superfamily-associated protein 3; Bex, brain expressed X-linked; GST, glutathione S-transferase; CaMKIIa, Calcium-calmodulin-dependent kinase II a.

KIF3A [9], served as a positive control. This result is not surprising because Bex1 and Bex2 share high identity in their amino acid sequences (Bex1 and Bex2 are 87% identical, while Bex2 and Bex3 are 30% identical) [10, 12].

To quantify the binding affinity of Bexs to KIF3A, Bexs, or the C-terminal region of KIF3A expression plasmids were transformed to yeast and the β-galactosidase activity was measured in liquid cultures. CaMKIIa served as a positive control [9]. The interaction of Bex2 with KIF3A yielded approximately 570 units of β-galactosidase activity (Fig. 2C). Together, these results show that Bex2 specifically interacts with KIF3A.

To confirm the KIF3A and Bex2 interaction at the protein level, the interaction between KIF3A and Bex1, or Bex2 was assayed using a GST pull-down experiments. Recombinant GST-Bex1, or GST-Bex2 fusion proteins were expressed in *E. coli*. The purified GST fusion proteins are allowed to interact with mouse brain lysates. Immunoblotting analyses revealed that KIF3A interacted with GST-Bex1, and GST-Bex2 but not with GST (Fig. 2D). This result indicates that Bex1 and Bex2 interact with KIF3A at protein level.

Heterotrimeric kinesin-2 is associated with Bex2 in cells

Heterotrimeric kinesin-2 is composed of two motor proteins, KIF3A/KIF3B, and KAP3 [8, 19]. To address the question whether KIF3A mediates the interaction of Bex2 and heterotrimeric kinesin-2, we performed co-immunoprecipitation from HEK-293T cells that were transfected with myc-KIF3A and FLAG-Bex2. Anti-FLAG antibody precipitated KIF3A and endogenous KIF3B; however, KIF5B (a motor protein of kinesin-1) did not (Fig. 3A). Also, anti-myc antibody precipitated Bex2 and endogenous KIF3B, but not KIF5B (Fig. 3B). These results indicate that Bex2 interacts with KIF3A bound to KIF3B.

Bex proteins have different subcellular localization [1]. Bex1 primarily localized to the nucleus while Bex2 localized to the nucleus and cytoplasmic [1]. In order to address whether KIF3A and Bex2 co-localize in cells, KIF3A was co-expressed with EGFP-Bex2 in HEK-293T cells. KIF3A and

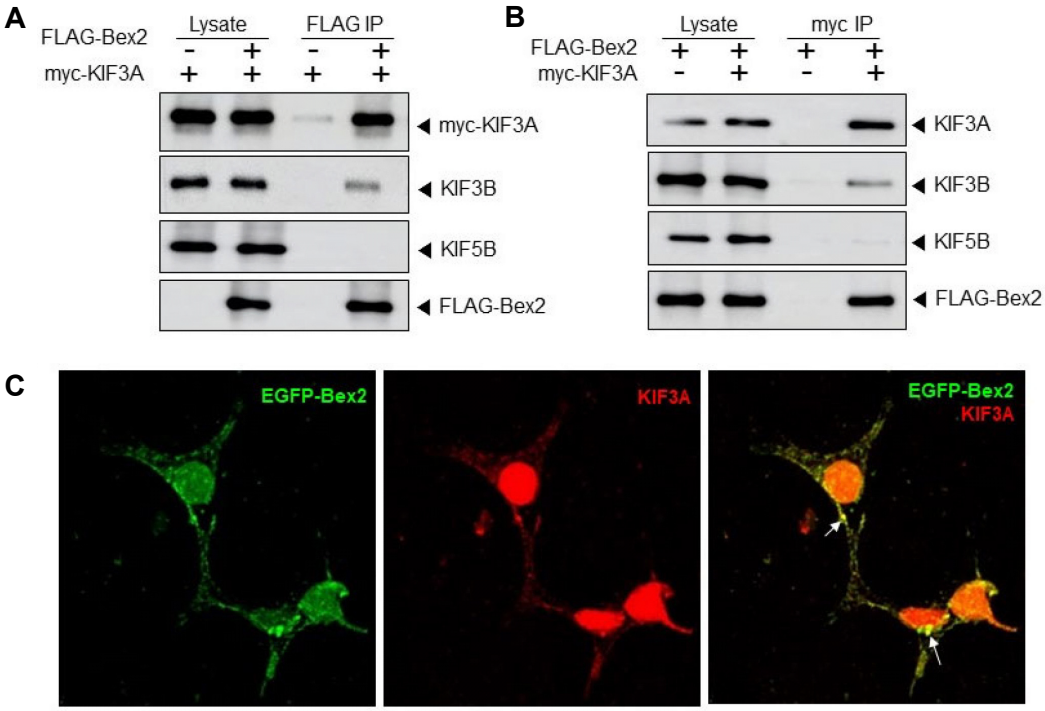

Fig. 3. Co-immunoprecipitation and co-localization of KIF3A and Bex2 in mammalian cells. (A and B) HEK-293T cells were transiently transfected with FLAG-Bex2 and myc-KIF3A plasmids as indicated. Cell lysates were immunoprecipitated with (A) monoclonal anti-FLAG antibody or (B) anti-myc antibody. Precipitates were immunoblotted with anti-KIF3A, KIF3B, KIF5B and FLAG antibodies. Bex2 specifically co-precipitated KIF3A and KIF3B, but not with KIF5B. (C) HEK-293T cells were transiently transfected with EGFP-Bex2 and KIF3A plasmids. Twenty-four hours after transfection, cells were subjected to immunofluorescence with anti-KIF3A antibody. KIF3A and Bex2 were observed in the same subcellular region in cells (allow). KIF, kinesin superfamily proteins; Bex2, brain expressed X-linked 2; EGFP, enhanced green fluorescent protein; IP, immunoprecipitation.

Bex2 overlapped at the cytoplasmic region and nucleus in cells (Fig. 3C, allow). Taken together, these results indicate that Bex2 is a binding partner of heterotrimeric kinesin-2 through the binding with KIF3A.

Discussion

In this study, we show that KIF3A interacts with Bex2. Using the CBD of KIF3A as bait, we identified Bex2 in a yeast two-hybrid assay. The BEX domain-containing region of Bex2 interacted with the CBD of KIF3A. Furthermore, using a co-immunoprecipitation, we showed that heterotrimeric kinesin-2 can be co-precipitated with Bex2. When co-expressed in HEK-293T cells, Bex2 co-localized with KIF3A in nucleus and cytoplasmic. Taking all of these results together, we hereby suggest that Bex2 is a new binding partner for heterotrimeric kinesin-2 and it may have role the scaffold to the cargo transport of heterotrimeric kinesin-2.

Bex2 is expressed in wide range of tisses and plays diverse roles in cells [10, 11]. It displays diverse function in cells and shows a nucleus, and cytoplasmic distribution despite the presence of a putative nuclear localization signal (NLS) in the amino acid sequences of Bex2 [12]. Bex2 regulates signals from different cell surface receptors and lead to enhancement of transcriptional activity which is required for cell development [12]. Also, It promotes breast cancer cell growth and survival by modulating the mitochondrial apoptotic pathway and G1 cell cycle [10]. In this process, Bex2 has cross-talk with the NF-κB, c-Jun/JNK and ErbB2 [10]. In addition, Bex2 is highly expressed in the embryonic brain and can interact with transcriptional factor Lin-11, lsl-1, Mec-3 (Lim) domain only-2 (LMO2) to regulate transcriptional activity [6]. The Bex2 protein was identified by its ability to interact with olfactory marker protein (OMP) [11]. Bex1 and Bex2 have both been characterized as OMP partners by biochemical, coimmunoprecipitation, and structural studies [11].

What would the interaction between KIF3A and Bex2 mean? We are able to suggest one possibility is that Bex2 may be role an adaptor protein that links heterotrimeric kinesin-2 and cargo. In previous report, heterotrimeric kinesin-2 plays a role in anterograde transport of membrane-bound organelles to plasma membrane [16]. In many cases, kinesins bind to various cargoes through it's the C-terminal tail region and a variety of different adaptor proteins/scaffolding proteins [7]. Over 20 adaptor proteins to kinesins have been identified [7]. Adaptor proteins that interact with kinesin-1 to mediate transport of diverse cargoes [7]. The gamma-aminobutyric acid (GABA) type A receptor-associated protein (GABARAP) acts as an adaptor protein that links KIF5A motor to GABA type A receptor [13]. The growing number of identified adaptor proteins to kinesins is thought to reflect the specificity of cargo selection or delivery to particular subcellular destinations in cells [7]. Our findings suggest that Bex2 may serve as an adaptor protein that links heterotrimeric kinesin-2 and cargo is important for the nucleus localization of transcriptional regulating proteins including c-Jun and p65. Future studies are needed to determine the precise mechanisms of associating with both Bex2 associated protein complex and heterotrimeric kinesin-2, and delivery to nucleus in cells.

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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 : Brain-expressed X-linked (Bex) 2와 heterotrimeric kinesin-2의 KIF3A와의 결합

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Kinesin-2는 heterotrimeric kinesin-2와 homodimeric kinesin-2의 두종류가 존재한다. 세포내 미세소관을 따라 이동하는 분자 모터 단백질인 heterotrimeric kinesin-2는 motor 활성을 가진 kinesin superfamily protein (KIF) 3A와 3B (KIF3A와 KIF3B) 그리고 kinesin associated protein 3 (KAP3)로 구성되어져 있다. 특히 섬모내에서 heterotrimeric kinesin-2는 미세소관의 plus방향으로 운반체를 운반하는 역할을 수행한다. Heterotrimeric kinesin-2는 다양한 운반체 그리고 단백질들과의 결합이 알려져 있지만 heterotrimeric kinesin-2와 결합하는 결합단백질에 대하여서는 아직 충분히 밝혀지지 않았다. 본 연구에서는 KIF3A의 C-말단 영역과 결합하는 단백질을 효모 two-hybrid system을 사용하여 탐색한 결과, brain expressed X-linked 2 (Bex2)를 분리하였다. Bex2는 KIF3A의 cargo-binding domain (CBD)을 포함하는 C-말단 영역과 결합하지만, KIF3B와 kinesin-1의 motor 단백질인 KIF5A와는 결합하지 않았다. 그리고 KIF3A는 Bex2의 다른 isoform인 Bex1과는 결합하지만, Bex3와는 결합하지 않았다. KIF3A은 GST-Bex1, GST-Bex2와는 결합하지만 GST와는 결합하지 않았다. HEK-293T세포에 Bex2을 발현시켰을 때 Bex2와 KIF3A는 세포 내의 같은 부위에 발현하며, Bex2 그리고 KIF3A을 면역침강한 결과 KIF3A와 KIF3B도 같이 침강함을 확인하였다. 이러한 결과들은 Bex2는 heterotrimeric kinesin-2와 운반체를 매개하는 단백질로의 가능성을 시사한다.