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- Note -

Kinesin Superfamily Protein 5A (KIF5A) Binds to ArfGAP1, ADP-ribosylation Factor GTPase-activating Protein 1

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Kinesin-1 is a heterotetrameric protein composed of two heavy chains (KHCs, also known as KIF5s) with a motor domain and two light chains (KLCs) without a motor domain. KIF5 has three subtypes, namely, KIF5A, KIF5B, and KIF5C, which share high amino acid homology except in their carboxy (C)-terminal region. KIF5A is responsible for transporting cargo within the cell. The adaptor proteins that bind to the C-terminal region of KIF5A mediate between kinesin-1 and cargo. However, the proteins regulating the intracellular cargo transport of kinesin-1 have not yet been fully identified. In this study, we identified ADP-ribosylation factor GTPase-activating protein 1 (ArfGAP1), which is involved in the intracellular trafficking of lysosomes, as a binding partner of KIF5A. KIF5A binds to the C-terminal region of ArfGAP1, and ArfGAP1 binds to the C-terminal region of KIF5A but does not interact with KIF5B, KIF5C, kinesin light chain 1 (KLC1), or KIF3A. When co-expressed in mammalian cells, ArfGAP1 co-localized with KIF5A and co-immunoprecipitated with KIF5A, KIF5B, and KLC1, but not with KIF3B. These results suggest that kinesin-1 may be regulated by ArfGAP1 in the intracellular transport of cargo.

Key words : ArfGAP1, binding protein, intracellular transport, KIF5A, kinesin-1

Introduction

The microtubule-dependent motor protein, kinesin, transports cargo within the cells in an ATP-dependent manner [5]. Kinesins form a large superfamily with more than 45 known species [3]. Kinesin superfamily proteins (KIFs) are involved in the transport of various cargoes within cells, such as organelles and vesicles [3, 5]. Kinesin-1 is a heterotetrameric protein composed of two heavy chains (KHC) with motor activity, also known as KIF5 and two light chains (KLC) without motor activity [3]. KIF5 has several distinct domains that mediate its basic functions: a motor domain that contains ATPase motor activity and interacts with microtubules, a coiled-coil domain that is involved in binding between KIF5s and KLCs, and a carboxyl (C)-terminal domain [3]. This C-terminal domain of KIF5s have variable amino acid homology among KIF5s and mediates interactions with various cargoes transported by kinesin-1 [2, 10]. KIF5A forms homodimers or heterodimers with KIF5B or KIF5C in cells [6].

Intracellular cargo transport by the kinesin-1 can be described in three steps: 1) binding to appropriate cargo and/or adaptor proteins, 2) activation of the kinesin-1 motor and movement along microtubules, and 3) release of the cargo at the correct location to its destination [10, 13]. This intracellular cargo transport is well regulated by the modification proteins of the kinesin-1 and the adaptor proteins that connect the kinesin-1 to its cargo [9, 10, 13]. The regulatory proteins of kinesin-1, including protein kinase, protein phosphatase, and small G-proteins, regulate kinesin-1 motor activity directly through phosphorylation or dephosphorylation or indirectly through modification of adaptor proteins or the microtubule network [4, 10]. Dysfunction in the regulation of intracellular cargo transport by kinesin-1 has been implicated in several neurological diseases, including Alzheimer's disease (AD), Huntington's disease (HD), and amyotrophic later-

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al sclerosis (ALS) [2, 13].

In previous studies, conditional knockout of KIF5A resulted in various phenotypes such as anxiety-like behavior [11, 17]. This phenotype suggests that KIF5A plays a role in the intracellular trafficking of neurotransmitter receptor-containing vesicles [11]. In addition, KIF5A plays a role in the trafficking of lysosomes within the cell. Using trimethyltin chloride (TMT) treatment, reducing KIF5A protein expression impaired lysosomal trafficking [8]. Overexpression of Kif5a in cells also restored lysosomal trafficking [8]. In this study, we confirmed the interaction between KIF5A and ADP-ribosylation factor GTPase-activating protein 1 (ArfGAP1), which is involved in the regulation of vesicles trafficking in cells [1, 15]. This binding of ArfGAP1 to KIF5A suggests that ArfGAP1 may be involved in the regulation of kinesin-1 in the intracellular transport of cargo.

Materials and Methods

Plasmid constructs

Full-length mouse ArfGAP1 cDNA was purchased from Addgene (http://www.addgene.org/). The deletion constructs from KIFs were subcloned into pGEM T-easy vector (Promega Corp., Madison, WI, USA) and pLexA vector (Clontech Laboratories, Inc., Palo Alto, CA, USA).

Yeast two-hybrid positive assay

The Matchmaker yeast two-hybrid system was used for the assay according to the manufacturer's instruction (Clontech). pLexA-C-terminal region of KIFs were transformed into yeast strain EGY48 (Clontech). Transformed cells were transformed with pB42AD-ArfGAP1 and analyzed in X-gal plates.

Cell culture and transfection

Human embryonic kidney (HEK)-293T cells (American Type Culture Collection [ATCC] CRL-3216) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37℃ in a humidified 5% CO_2 incubator. Transient transfections were performed using the $CaPO_4$ precipitation method [14].

Co-immunoprecipitation and immunoblot analysis

FLAG-ArfGAP1 and myc-KIF5A constructs were transfected into HEK-293T cells to express FLAG-ArfGAP1 and myc-KIF5A. Transformed culture cells were rinsed three times with PBS buffer and lysed with lysis buffer [PBS containing 0.5% NP-40 and 1× Protease Inhibitor Cocktail Set V (Calbiochem, San Diego, CA, USA)]. The cell lysate was centrifuged and the supernatant was incubated with anti-FLAG M2 agarose beads (Sigma-Aldrich, St. Louis, MO, USA) for 3 hr at 4℃. The beads were collected by centrifugation and washed three times with cold PBS. Laemmli's loading buffer was added to the washed beads and boiled for 5 min to elute and denature the protein. Proteins were processed for SDS-PAGE and immunoblot analysis using antibodies against KIF3B, KIF5A, KIF5B, KLC1, and FLAG epitopes as described elsewhere by Nakajima et al [11].

Immunocytochemistry

The myc-KIF5A and EGFP-ArfGAP1 plasmids were transfected into HEK-293T cells grown on poly-D-lysine-coated coverslips. Twenty-four hours after transfection, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS, and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in PBS for 10 min. After washing with PBS, 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. After the cells were washed with PBS, they were mounted with Fluoromount (DAKOKorea, Seoul, Korea). The images of the cells were obtained using a Zeiss LSM510 META confocal laser scanning microscope (Carl Zeiss, Oberkochem, Germany).

Results and Discussion

ArfGAP1 interacts with KIF5A in yeast two-hybrid assay

In a previous study, KIF5A was shown to play an important role in the intracellular trafficking of lysosomes [8]. In addition, ArfGAP1 plays a critical role in maintaining the correct position of the lysosome in cells [8]. Therefore, we performed a yeast two-hybrid assay between KIF5A and ArfGAP1 to test the interaction between KIF5A and ArfGAP1. KIF5A interacted with ArfGAP1 in the yeast two-hybrid assay (Fig. 1A). As a positive control for binding to KIF5A, γ-aminobutyric acid (GABA) type A receptors associated protein (GABARAP) was used [11].

ArfGAP1 has a GTPase-activating protein (GAP) domain at the amino (N)-terminus and a coiled-coil domain [15]. To determine whether the GAP domain or the coiled-coil domain of ArfGAP1 interacts with KIF5A, we constructed a series of deletion mutants for each domain of ArfGAP1 and analyzed their interaction with KIF5A. As shown in Fig. 1B,

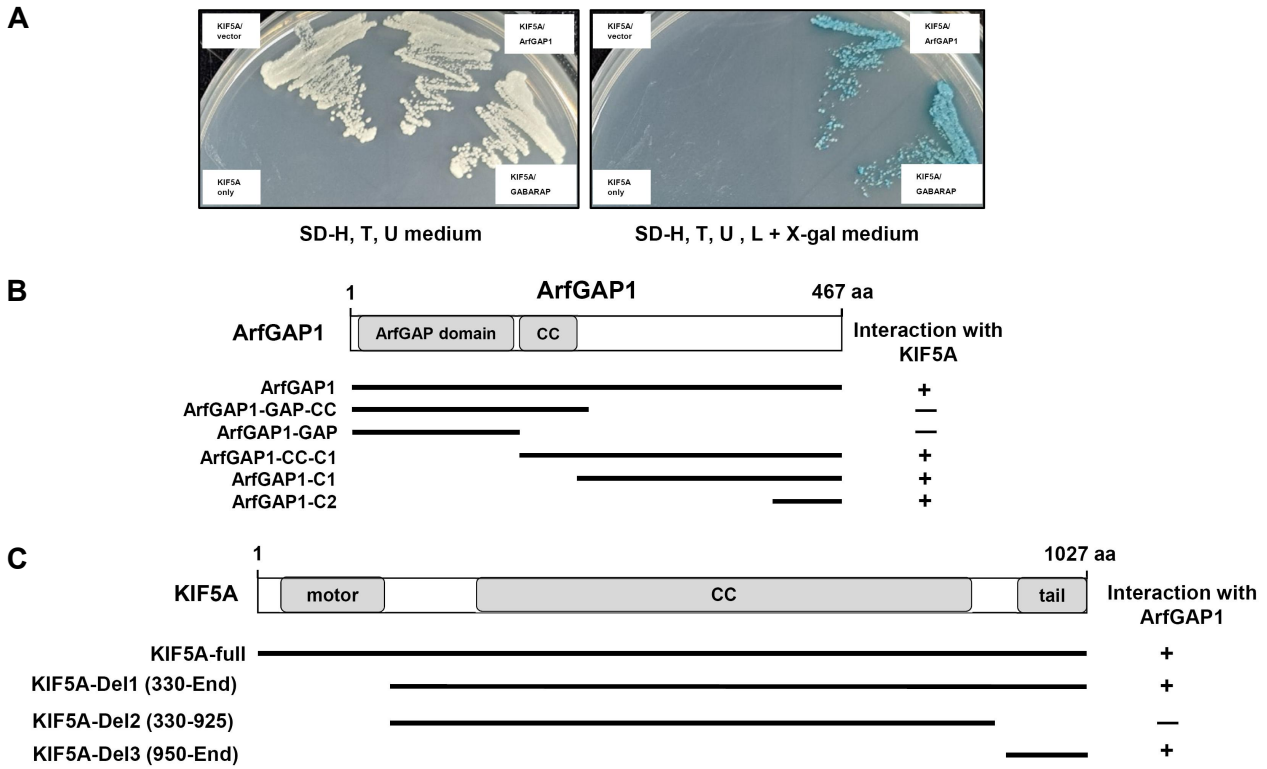

Fig. 1. KIF5A interacts with ArfGAP1 in a two-hybrid assay. (A) ArfGAP1 binding to KIF5A. pLexA-KIF5A and pB42AD-ArfGAP1 plasmids were transformed into yeast strain EGY48. Transformed cells were grown on SD medium and X-gal medium plates. (B) The minimum binding region of ArfGAP1 for KIF5A. In a yeast two-hybrid assay, the different truncations of ArfGAP1 were tested for binding to KIF5A. (C) The minimum binding region of ArfGAP1 for KIF5A. KIF5A has three domains: The motor domain, the coiled-coil domain, and the C-terminal tail domain, shown in gray. In a yeast two-hybrid assay, the different truncations of KIF5A were tested for interaction to ArfGAP1. +, interaction; -, no interaction; KIF5A, kinesin superfamily protein 5A; ArfGAP1, ADP-ribosylation factor GTPase-activating protein 1; CC, coiled-coil; SD, synthetic-defined; H, histidine; T, tryptophan; U, uracil; L, leucine; X-gal, 5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside; aa, amino acids.

the results showed that ArfGAP1 is a minimal binding domain in which the C-terminal region, excluding the N-terminal GAP domain and the coiled-coil domain, interacts with KIF5A.

KIF5A consists of a motor domain that binds to microtubules and an ATPase activity in the N-terminal region, a long coiled-coil domain that interacts with KLCs, and a C-terminal region that binds to various binding proteins or cargo [6]. We constructed different fragments based on the motor domain, coiled-coil domain and C-terminal region of KIF5A and tested their interaction with ArfGAP1 using yeast two-hybrid assay. The C-terminal region of KIF5A is required for ArfGAP1 binding (Fig. 1C).

ArfGAP1 interaction with KIF5A in cells

Next, we examined whether ArfGAP1 interacts with other KIFs, including KIF3A (the motor subunit of kinesin-2), KIF5B, KIF5C, and KLC1. As shown in Fig. 2A, ArfGAP1 interacted with KIF5A. KIF3A, KIF5B, KIF5C and KLC1 did not interact in the yeast two-hybrid assay. There are 16 known types of ArfGAPs known in mammals [12]. The ArfGAP domain shares 85% amino acid identity [15, 16]. We have tested whether SMAP1, which has a high degree of amino acid homology to ArfGAP1, interacts with KIF5A. KIF5A did not interact with SMAP1 in the yeast two-hybrid assay (Fig. 2B). This result is not surprising because ArfGAPs have no similarity in their amino acid sequence except for the ArfGAP domain [15]. These data suggest that among the ArfGAPs, KIF5A specifically interacts with ArfGAP1.

Kinesin-1 is composed of two KIF5s and two KLCs, which form a heterotetrametric complex [6]. To confirm the kinesin-1 and ArfGAP1 interaction at the protein level in cells, we performed co-immunoprecipitation from cells that were transfected with FLAG-ArfGAP1 and myc-KIF5A. As a re-

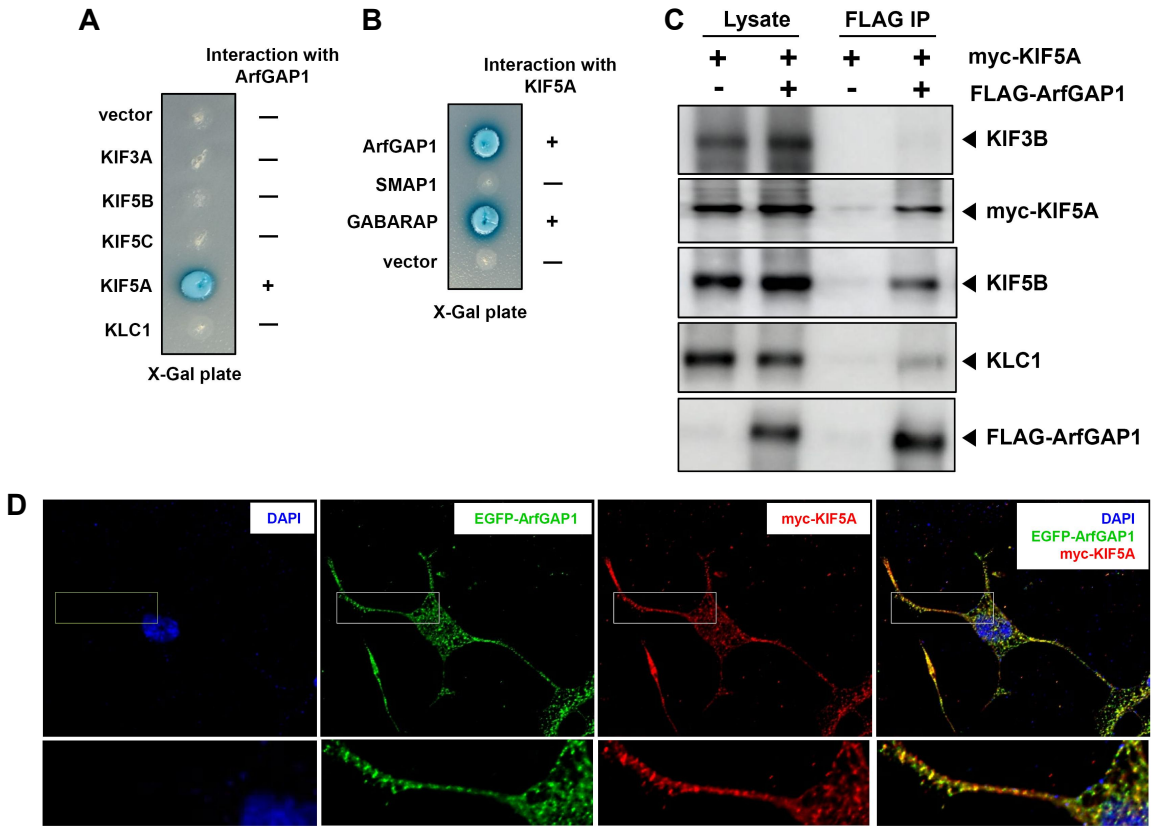

Fig. 2. ArfGAP1 interaction with kinesin-1. (A, B) KLC1 and C-terminal region of KIF5s and KIF3A and ArfGAP1 were tested for interaction. ArfGAP1 interacted with KIF5A, but did not interact with KIF3A, KIF5B, KIF5C, or KLC1. Also, KIF5A interacted with ArfGAP1. GABARAP was used as a positive control for the interaction with KIF5A. (C) HEK-293T cells were transiently transfected with FLAG-ArfGAP1 and myc-KIF5A plasmids as indicated. Cell lysates were immunoprecipitated with monoclonal anti-FLAG antibody. Precipitates were immunoblotted with anti-KIF5A, KIF3B, KIF5B, KLC1, and FLAG antibodies. ArfGAP1 co-precipitated myc-KIF5A, KIF5B and KLC1. (D) HEK-293T cells were transiently transfected with EGFP-ArfGAP1 and myc-KIF5A plasmids. Twenty-four hours after transfection, cells were subjected to immunofluorescence with anti-KIF5A antibody. ArfGAP1and KIF5A are seen in the same subcellular region in the cells. +, interaction; -, no interaction; KIF5, kinesin superfamily protein 5; KIF3A, kinesin superfamily protein 3A; KIF3B, kinesin superfamily protein 3B; KLC1, kinesin light chain 1; ArfGAP1, ADP-ribosylation factor GTPase-activating protein 1; GABARAP, γ-aminobutyric acid receptor-associated protein; X-gal, 5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside; DAPI, 4′,6-diamidino-2-phenylindole.

sult of immunoprecipitation with anti-FLAG antibody from cells expressing FLAG-ArfGAP1 and myc-KIF5A, myc-KIF5A, KIF5B, and KLC1, which are the constituent proteins of kinesin-1, were immunoprecipitated together. However, KIF3B, the motor protein of kinesin-2, was not immunoprecipitated (Fig. 2C). This result suggests that kinesin-1 is interacting with ArfGAP1. To determine whether KIF5A and ArfGAP1 are expressed at the same location in cells, EGFP-ArfGAP1 and myc-KIF5A were coexpressed. KIF5A and ArfGAP1 were found to overlap in the same cytoplasmic region (Fig. 2D). These data suggest that the binding of ArfGAP1 to kinesin-1 is through the binding of KIF5A.

Arfs are Ras-related GDP/GTP-binding proteins that are regulators of intracellular vesicle trafficking in cells [15]. Arfs are inactive when bound to GDP and active when bound to GTP [15]. ArfGAPs inactivate Arfs by regulating Arf GTPase activity and ArfGAP1 has many biological functions, such as an effector protein that recruits cargo proteins as a component of coat complexes to form vesicles [7, 18], promotes AP-2-dependent endocytosis, actin remodeling, and intracellular vesicle trafficking. This vesicle trafficking is involved in the trafficking of COPI-coated vesicles between the endoplasmic reticulum and the Golgi apparatus [1]. ArfGAP1 was also found to be involved in the regulation of the trafficking of proteins to the lysosomes [8].

In this study, we show for the first time that ArfGAP1

interacts with kinesin-1 through KIF5A. The C-terminal region of KIF5A interacts with the C-terminal region of ArfGAP1. When FLAG- ArfGAP1 and myc-KIF5A were expressed in mammalian cells, they co-immunoprecipitated and co-localized in cells. Although we did not determine the intracellular transport of lysosomes by the interaction of kinesin-1 and ArfGAP1, the available data of in this study suggest that ArfGAP1 may play a role in regulating in the intracellular transport of lysosomes by kinesin-1. Future studies are needed to determine how kinesin-1 regulates intracellular trafficking mechanisms.

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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 : Kinesin Superfamily Protein 5A (KIF5A)와 ADP-ribosylation Factor GTPase-activating Protein 1 (ArfGAP1)의 결합

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키네신-1은 모터 도메인이 있는 두 개의 중쇄(KHC 또는 KIF5)와 모터 도메인이 없는 두 개의 경쇄(KLC)로 구성된 이형사량체 단백질이다. KIF5에는 KIF5A, KIF5B 및 KIF5C의 세 가지 subtype이 있으며, 카르복실(C)-말단 영역을 제외하고는 아미노산 상동성이 높다. KIF5A는 세포 내에서 화물을 운반하며, KIF5A의 C-말단 영역에 결합하는 매개 단백질은 키네신-1과 화물 사이를 연결한다. 키네신-1의 세포내 수송을 조절하는 단백질은 아직 충분히 확인되지 않았다. 본 연구는 리소좀의 세포 내 수송에 관여하는 ADP-ribosylation GTPase-activating protein 1 (ArfGAP1)과 KIF5A와의 결합을 확인하였다. KIF5A는 ArfGAP1의 C-말단 영역에 결합하고, ArfGAP1은 KIF5A의 C-말단 영역에 결합하지만 KIF5B, KIF5C, 키네신 경쇄 1 (KLC1) 또는 KIF3A와는 결합하지 않았다. ArfGAP 도메인을 가진 다른 동질형인 SMAP1과는 결합하지 않았다. 세포에서 KIF5A는 ArfGAP1과 같은 위치에서 발현하며, KIF5A, KIF5B 및 KLC1와 같이 면역 침전하였다. 그러나, KIF3B와는 같이 면역 침전하지 않았다. 이러한 결과들은 키네신-1은 세포내 화물 수송에서 ArfGAP1에 의해 조절될 수 있음을 시사한다.