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CDK2AP1, a Cyclin-Dependent Kinase 2-Associated Protein, Interacts with Kinesin-1 through Kinesin Superfamily Protein 5A (KIF5A)

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Intracellular and axonal transport is mediated by microtubule-dependent motor proteins, such as kinesins and cytoplasmic dynein. Kinesin moves along the microtubule to the positive end of the microtubule, while dynein moves to the negative end of the microtubule. Kinesin-1 was first identified as a kinesin superfamily protein (KIF) that functions in the intracellular transport of various cargoes, including organelles, neurotransmitter receptors, and mRNA-protein complexes, through interactions between the carboxyl (C)-terminal domain and the cargo. It interacts with other cargoes, but the adapter/scaffold proteins that mediate between kinesin-1 and the cargo have yet to be fully identified. In this study, a yeast two-hybrid screen was used to identify adapter proteins that interact with the C-terminal region of KIF5A. We found an association between the C-terminal region of KIF5A and the cyclin-dependent kinase 2-associated protein 1 (CDK2AP1), originally identified in malignant hamster oral keratinocytes. CDK2AP1 bound to the C-terminal region of KIF5A and did not interact with KIF3A (the motor of kinesin-2), KIF5B, KIF5C, and kinesin light chain 1 (KLC1). The C-terminal region of CDK2AP1 is essential for its interaction with KIF5A. When co-expressed in HEK-293T cells, CDK2AP1 and kinesin-1 co-immunoprecipitated and co-localized in the cells. These results suggest that the KIF5A-CDK2AP1 interaction serves as an adapter protein connecting kinesin-1 and the cargo when kinesin-1 transports cargo in cells.

Key words : Adaptor protein, cyclin-dependent kinase 2-associated protein 1, intracellular transport, kinesin-1, KIF5A

Introduction

Microtubule dependent intracellular cargo transport is the process by which organelles are moved within a cell. Microtubule dependent motor proteins, such as kinesin and cytoplasmic dynein, play a critical role in this process by using the energy from ATP hydrolysis to move along the microtubules and transport cargo [2, 4]. Kinesins are a plus end-directed motor proteins that move along microtubules in the anterograde directed-cargo transport [2, 4]. Motor proteins are involved in a variety of cellular functions, such as cell division, cell signaling, and intracellular cargo transport [2, 4]. For example, kinesin can transport various cargoes, including membrane-bound vesicles, mitochondria, protein complexes, and mRNAs within the cell [2].

Kinesin-1 is the first identified motor protein of the kinesin superfamily proteins (KIFs), which play an important role in the cargo-transport within cells [4]. It has a distinctive structure, consisting of two globular head domains and a carboxyl (C)-terminal domain [5]. The two globular motor domains of kinesin-1 contain the ATPase motor activity and interact with microtubules. The C-terminal region of kine-

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sin-1 interacts with many different cargoes [2, 5]. Kinesin-1, also known as KIF5, consists of two heavy chains (KHCs) and two light chains (KLCs) [5]. It has composed of three closely related KHC subtypes: KIF5A, KIF5B and KIF5C [5]. KIF5B is ubiquitously expressed, whereas KIF5A and KIF5C are predominantly expressed in neural tissues [5].

Among the most highly expressed KIF5s in cells, KIF5B is involved in the intracellular transport of various cargoes [2]. One of the most well-studied cargoes transported by KIF5B transport is the mitochondria. Mitochondria intracellular transport is carried out by direct binding to the Miro-Milton complex and the C-terminal region of KIF5B [1, 9]. In this case, Milton acts as a role an adaptor/scaffolding protein linking the mitochondrial outer membrane protein Miro with KIF5B [1, 9]. In previous experiments, *kif5B*-KO mice died during early embryogenesis due to impaired mitochondrial localization in cells [16]. Furthermore, the mis-localization of mitochondria in cultured cells from *kif5B*-KO mice was rescued by exogenous expression of KIF5B [16]. In a previous study, kif5A- KO mice were shown to be neonatal lethal [11]. Conditional knockout of KF5A in neurons exhibited in various neurological phenotypes such as axonal transport, neurite outgrowth, synaptic function, hyperactivity and anxiety-like behaviour [11]. These phenotypes suggest that KIF5A plays an important role in intracellular cargo transport and that disruption of KIF5A-dependent transport is associated with various diseases, including developmental and neurodegenerative disorders such as spastic paraplegia 10 (SPG10) [13].

KIF5A has a specific C-terminal tail region that shows no homology to the C-terminal tail regions of KIF5B or KIF5C [5]. Despite the importance of this specific C-terminal region of KIF5A, little is known about the function of this KIF5A C-terminal tail region or the proteins it interacts with. To better understand the role of KIF5A in intracellular cargo transport, a yeast two-hybrid screen was used for proteins that bind to the specific C-terminus of KIF5A. We identified the cyclin-dependent kinase 2-associated protein 1 (CDK2AP1) as a protein that bind to KIF5A.

Materials and Methods

Plasmid constructs

The full-length cDNAs of mouse KIF5A (plasmid #127616) and mouse CDK2AP1 (plasmid #178030) were obtained from Addgene (http://www.addgene.org/). The C-terminal tail region (amino acids 906-1027) of KIF5A was subcloned into pGEM T-easy vector (Promega Corp, Madison, WI, USA), and pLexA vector (Clontech Laboratories, Inc., Palo Alto, CA, USA). pLexA-tail-KIF5A was used as bait plasmid this yeast two-hybrid screening.

Yeast two-hybrid positive clone screening of KIF5A

The Matchmaker yeast two-hybrid system (Clontech Laboratories, Inc., Palo Alto, CA, USA) was used for yeast two-hybrid screening. According to the manual, the pLexA-C-terminal region of KIF5A was transformed into the yeast strain EGY48, and the transformed cells were transformed with a mouse brain cDNA library [11]. Positive clones were selected on SD/-His/-Trp/-Ura/-Leu plates containing galactose, raffinose, X-gal and BU salts. Plasmids isolated from positive clones were analyzed by *Eco*RI and *Xho*I restriction digestion.

β-Galactosidase activity of CDK2AP1 and KIFs in yeast liquid culture

Liquid-cultured mid-log phase yeast cells were permeabilized with 0.1% sodium dodecyl sulphate (SDS) and chloroform. O-nitrophenyl-β-D-galactoside (ONPG) (Sigma-Aldrich, St. Louis, MO, USA) was added to the yeast lysate and the mixture was incubated at 30℃. The reaction was stopped by adding 1 M Na_2CO_3 to increase the pH to 11. The absorbance of the reaction solution was measured at 420 nm in a spectrophotometer, and the unit of enzyme activity was calculated using the following formula: units=1,000x $[(OD_{420} - 1.75 \times OD_{550})]$/ (reaction time x culture volume $\times OD_{600}$) [3].

Glutathione S-transferase (GST) pull-down assay of CDK2AP1 and KIF5A

KIF5A, CDK2AP1, CDK2AP1-amino (N) and CDK2AP1-C were cloned into pET41 or pET21 and transformed into bacterial strain BL21 (Stratagene, La Jolla CA, USA). Expression of recombinant proteins was induced with 0.5 mM isopropyl thio-β-D-galactopyranoside (IPTG) for 4 hr. Fusion proteins were purified using glutathione-agarose beads (Sigma-Aldrich). The purified fusion proteins were incubated with the His-KIF5A fusion protein at room temperature for 1 hr and then precipitated with bound glutathione beads. The precipitate was washed three times with extraction buffer (1% Triton X-100 in PBS containing 10 μg/ml aprotinin, leupeptin and pepstatin and 1 μM phenylmethanesulphonyl fluoride, respectively). Pulled-down proteins were subjected to Western blotting using an anti-KIF5A antibody [5, 11].

Cell culture and transfection of KIF5A and CDK2AP1

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 at 37℃ in a humidified 5% CO_2 incubator. Transient transfections were performed using the $CaPO_4$ precipitation method as previously reported [3].

Immunocytochemistry of KIF5A and CDK2AP1

HEK-293T cells grown on poly-D-lysine coated coverslips were transfected with myc-KIF5A and CDK2AP1-EGFP. The transfected HEK-293T cells were cultured after 24 hr and then washed with PBS. It was fixed with 4% paraformaldehyde in PBS for 5 min and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in PBS for 10 min. Transformed cells were blocked for 30 min with 5% normal goat serum in PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween- 20 (Sigma-Aldrich). After washing three times with PBS, the cells were incubated for 40 min with Dylight 594-conjugated goat anti-rabbit IgG antibody (Jackson Immuno Research Labs, West Grove, PA, USA) diluted 1:700. After washing the cells three times with PBS, the cells were mounted with Fluoromount (DAKO Korea, Seoul, Korea). Each fluorescence image was acquired on a Zeiss LSM510 META confocal laser scanning microscope (Carl Zeiss, Oberkochem, Germany).

Co-immunoprecipitation and immunoblot analysis of KIF5A and CDK2AP1

HEK-293T cells were transfected with myc-KIF5A and FLAG-CDK2AP1. Cultured cells were rinsed three times with ice-cold PBS and gently rotated with ice-cold lysis buffer [PBS containing 0.5% NP-40 and 1x Protease Inhibitor Cocktail Set V (Calbiochem, San Diego, CA, USA)] 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 3 hr at 4℃. The beads were collected by centrifugation at 2,000× g for 30 sec and washed 3 times with ice-cold PBS containing 0.5% NP-40. Washed beads were resuspended in Laemmli's loading buffer to elute the proteins and denatured by boiling for 5 min. The proteins were subjected to 10% SDS-PAGE and immunoblot analysis was performed with antibodies against KIF3A, KIF5A, KIF5B, KLC1, and FLAG epitope as described by Nakajima et al. [11].

Results

Yeast two-hybrid screening of KIF5A-binding protein and confirmation of KIF5A-binding protein interaction

KIF5A, the motor protein of kinesin-1, consists of a microtubule-binding N-terminal motor domain, a coiled-coil domain, and a globular C-terminal tail region [5]. The C-terminal tail region of KIF5A has no homology to the C-terminal tail region of KIF5B or the C-terminal tail region of KIF5C [5]. To identify KIF5A-binding proteins, we performed a yeast two-hybrid screen using the KIF5A-specific C-terminal tail region as bait. A number of positive clones were obtained in a screen of 7×10^6 independent transformants. Positive clones were partially sequenced, and positive clones were found to encode the C-terminus of CDK2AP1 (Fig. 1A).

CDK2AP1 protein, a small protein of 115 amino acids protein is the only known inhibitor of CDK2, making it an important component of cell cycle regulation [17, 18]. To identify the minimum binding domain of CDK2AP1 required for binding between KIF5A and CDK2AP1, a series of CDK2AP1 deletion mutants were constructed and binding to KIF5A was analyzed by yeast two-hybrid assay. As shown in Figure 1A, the C-terminal region of CDK2AP1 was confirmed to bind to KIF5A. Various fragments of KIF5A were constructed around each domain of KIF5A, except for the motor domain, and tested for interaction with ERICH4 using a yeast two-hybrid assay. As shown in Figure 1B, it was confirmed that the C-terminal tail region of KIF5A is required for binding to CDK2AP1. Also, KIF5B and KIF5C, which are other motors of kinesin-1, did not bind to CDK2AP1 (Fig. 1B). Kinesin-1 is composed of the motor proteins KIF5A, KIF5B, and KIF5C and non-motor protein KLC [5]. Next, we investigated whether the proteins of kinesin-1 or KIF3A (motor subunit of kinesin-2) interact with CDK2AP1 using yeast two-hybrid assay. As shown in Figure 2A, KIF5A bound, but KIF5B, KIF5C, KIF3A and KLC1 did not interact with CDK2AP1. To quantify the binding affinity between CDK2AP1 and the kinesin-1 motor proteins KIF5A, KIF5B, and KIF5C, β-galactosidase activity was measured in yeast lysate. GABAAR-related protein (GABARAP) was used as a positive control [11]. As shown in Fig. 2B and Fig. 2C, the interaction of CDK2AP1 with KIF5A yielded approximately 380 units of β-galactosidase activity.

CDK2AP1 is interacted with kinesin-1 in cells

To confirm the binding of KIF5A to CDK2AP1 at the pro-

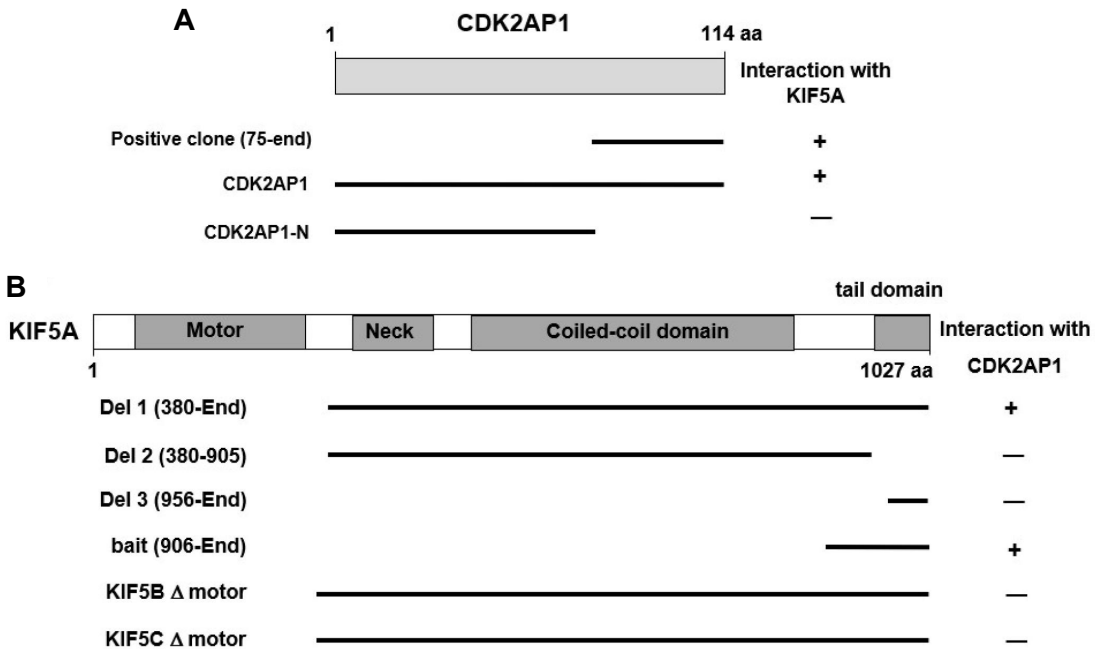

Fig. 1. Identification of KIF5A-binding proteins by yeast two-hybrid screening. (A) Schematic diagram of CDK2AP1 and the KIF5A binding region of CDK2AP1. Positive clones isolated from yeast two-hybrid screening contain the ORF for CDK2AP1. The different truncations of CDK2AP1 were tested in a yeast two-hybrid assay for interaction with KIF5A. (B) CDK2AP1 binding region of KIF5A. KIF5A has a motor domain, neck domain, coiled-coil domain and KIF5A-specific tail region shown in gray. The Different truncated forms of KIF5A were assessed in a yeast two-hybrid assay for their interaction with CDK2AP1. +, interaction; -, no interaction; KIF5A, kinesin superfamily protein 5A; KIF5B, kinesin superfamily protein 5B; KIF5C, kinesin superfamily protein 5C; CDK2AP1, cyclin-dependent kinase 2-associated protein 1; aa, amino acids.

tein level, we analyzed the interaction between KIF5A and CDK2AP1, CDK2AP1-N, or CDK2AP1-C using GST pull-down. Recombinant His-KIF5A, GST-CDK2AP1, GST-CDK2AP1- N or GST-CDK2AP1-C were expressed in bacteria and then pulled down. As shown in Fig. 2D, immunoblotting results showed that KIF5A bound to GST-CDK2AP1 and GST-CDK2AP1-C, but did not interact with GST and GST-CDK2AP1-N. This result confirmed that CDK2AP1 binds to KIF5A at the protein level.

Kinesin-1 forms a heterotetrameric complex with two motor subunits between KIF5A, KIF5B, and KIF5C and two KLCs [5]. To determine whether CDK2AP1 binds only KIF5A or whether CDK2AP1 binds kinesin-1, myc-KIF5A and FLAG-CDK2AP1 were transfected into HEK-293T cells and co-immunoprecipitated with anti-FLAG antibody. As shown in Fig. 3A, the motor proteins of Kinesin-1, KIF5A, and KIF5B, and the non-motor protein, KLC1 were precipitated by anti-FLAG antibody. However, KIF3A did not precipitate with FLAG-CDK2AP1 (Fig. 3B). These results suggest that CDK2AP1 interacts with kinesin-1 through KIF5A.

To confirm whether KIF5A and CDK2AP1 are co-expressed in cells, EGFP- CDK2AP1 was expressed together with myc-KIF5A in HEK-293T cells. As shown in Fig. 3B, KIF5A and CDK2AP1 were expressed overlapping in the same region in the cells. This result suggests that CDK2AP1 interacts with KIF5A, the motor protein of kinesin-1, in cells.

Discussion

KIF5A has a highly conserved motor domain and a globular C-terminal tail domain that is not homologous to the C-terminal region of KIF5B and KIF5C [5]. Kinesin-1 transports various cargoes in cells, and the KIF5A-specific C-terminal region is expected to play an important role of the intracellular cargo transport in cells [2, 11]. We isolated a protein that binds to the KIF5A C-terminal region using the yeast two-hybrid screening. In this study, we found that CDK2AP1 binds to the C-terminal region of KIF5A. And, we confirmed that the C-terminal region of KIF5A also directly interacts with CDK2AP1 at the protein level. When co-immunoprecipitation was performed by expressing myc-

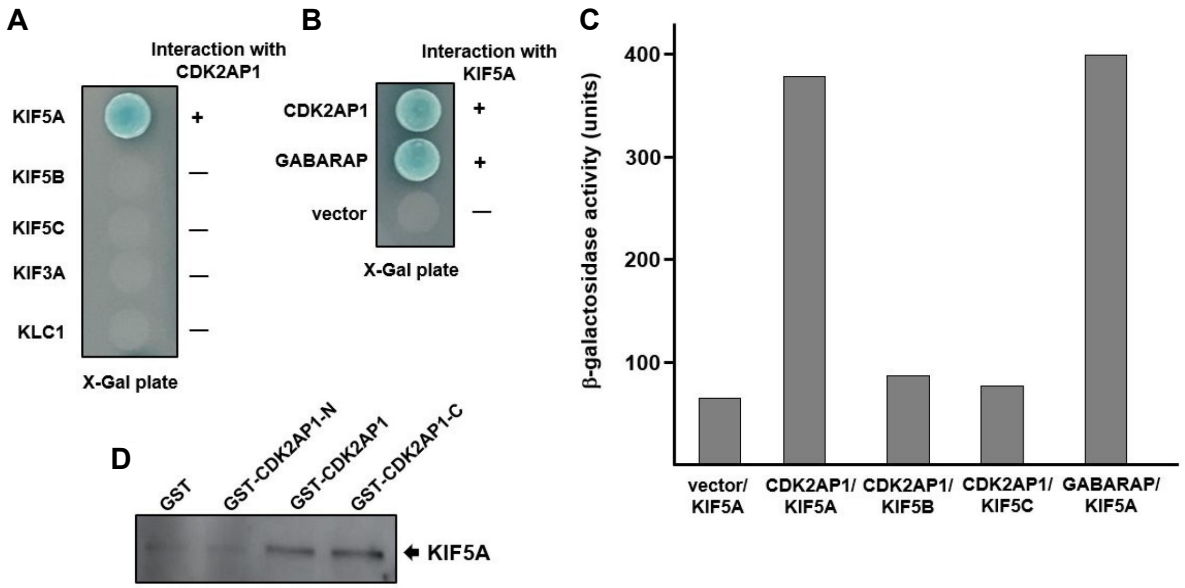

Fig. 2. Identification of the interaction KIF5A and CDK2AP1. (A, B) The tail region of KIF5s and KIF3A, the full length KLC1, and CDK2AP1 were tested for the interaction. CDK2AP1 interacted with KIF5A but not with KIF3A, KIF5B, KIF5C or KLC1. KIF5A also bound with CDK2AP1. GABARAP was used as a positive control for interaction with KIF5A. (C) The strength of the interaction between CDK2AP1 and KIF5A, KIF5B, or KIF5C was examined quantitatively using β- galactosidase activity in yeast two-hybrid reporter assay. (D) Direct binding of CDK2AP1 to KIF5A in a GST pull-down assay using the recombinant GST-fused CDK2AP1s. Precipitates were immunoblotted with anti-KIF5A antibody. +, interaction; -, no interaction; KIF5, kinesin superfamily protein 5; KIF3A, kinesin superfamily protein 3A; KLC1, kinesin light chain 1; CDK2AP1, cyclin-dependent kinase 2-associated protein 1; GABARAP, γ-aminobutyric acid receptor-associated protein; GST, glutathione S-transferase; X-gal, 5-Bromo-4-Chloro-3-Indolyl-β-D-galactoside.

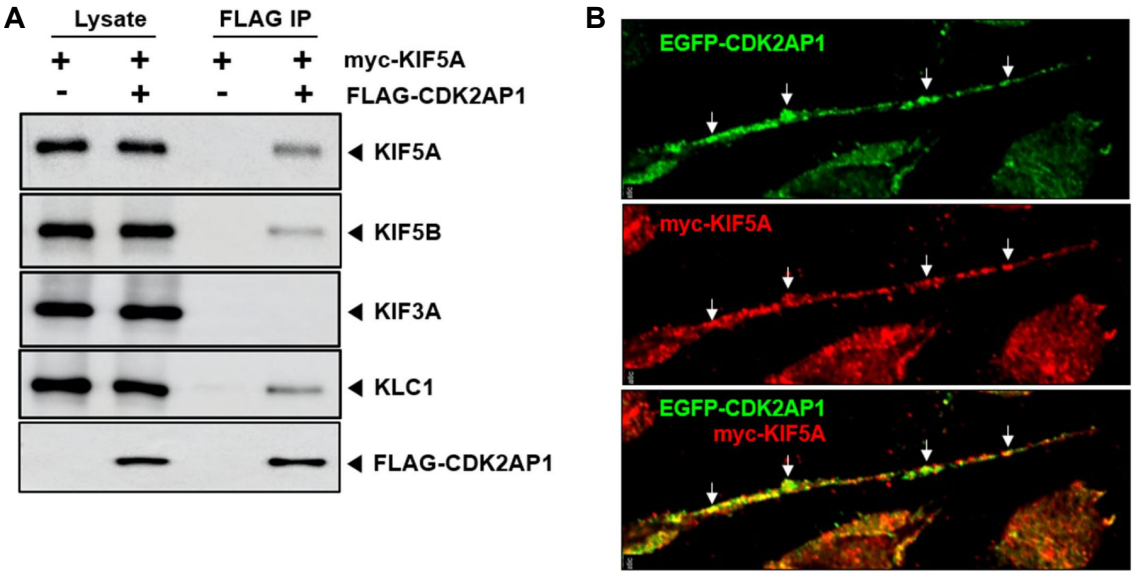

Fig. 3. Immunoprecipitation and subcellular localization of KIF5A and CDK2AP1. (A) HEK-293T cells were transiently transfected with myc-KIF5A and FLAG-CDK2AP1 plasmids. Cell lysates were immunoprecipitated with a monoclonal anti-FLAG antibody. Precipitates were immunoblotted with anti-KIF3A, KIF5A, KIF5B, KLC1 and FLAG antibodies. CDK2AP1 co-precipitated with KIF5A, KIF5B and KLC1. (B) HEK-293T cells were transiently transfected with EGFP-CDK2AP1 and myc-KIF5A. 24 hr after transfection, the cells were immunofluoresced with an anti-KIF5A antibody. CDK2AP1 and KIF5A are seen in the same subcellular region of the cell (arrow). KIF5, kinesin superfamily protein 5; KIF3A, kinesin superfamily protein 3A; KLC1, kinesin light chain 1; CDK2AP1, cyclin-dependent kinase 2-associated protein 1.

KIF5A and FLAG-CDK2AP1 in HEK-293T cells, CDK2AP1 co-precipitated together with kinesin-1. Furthermore, the intracellular expression distributions of CDK2AP1 and KIF5A were expressed at the same location in the cell. Based on these data, we propose that CDK2AP1 is a novel binding protein that binds to the C-terminus of KIF5A and may serve as an adaptor protein to mediate kinesin-1 and its cargo.

CDK2AP1 was first identified as a growth suppressor and a prognostic marker for various cancers [8, 17]. It is multifunctional protein that plays an important role in cell cycle regulation, autophagy, DNA repair, and protein trafficking. CDK2AP1 acts as a tumour suppressor by regulating the cell cycles [8]. It inhibits CDK2-mediated phosphorylation of the retinoblastoma (Rb), leading to the arrest of cell proliferation and is also associated with apoptosis in cells [8]. In previous studies, CDK2AP1 has been reported to interact with various proteins, such as CDK2, Beclin-1, a protein involved in autophagosome formation, DNA repair proteins, and protein trafficking proteins include clathrin and dynamin [8]. In this study, we have now shown that CDK2AP1 also directly interacts with kinesin-1 via KIF5A.

What does the interaction between KIF5A and CKD2AP1 identified in this study mean? One possibility is that the interaction of KIF5A-CKD2AP1 serves as an adaptor protein that mediate between kinsin-1 and the cargo. An important role of adaptor/scaffolding proteins linking kinesins and cargos is to efficiently regulate cargo binding, cargo release, and intracellular transport between kinesins and cargos [6, 12, 14]. For example, γ-aminobutyric acid receptors ($GABA_ARs$) associate-protein (GABARAP) is an adaptor protein that regulates the transport of $GABA_ARs$ by KIF5A [11]. Milton is another adaptor protein that mediates between kinesin-1 and mitochondria, and regulates the localization of mitochondria [1, 9]. Previous studies linked CDK2AP1 to epigenetic regulation and stem cell biology [8]. CDK2AP1 has been identified as a core subunit of the nucleosome remodeling and histone deacetylation complex (NuRD) associated with methyl CpG binding domain proteins 2 and 3 (MBD2 and MBD3) [7, 15]. Based on the ability of KIF5A to bind CDK2AP1, we favor a model in which the interaction of KIF5A-CDK2AP1 plays as an adaptor/scaffolding between kinesin-1 and cargo.

Another possibility is that the interaction of KIF5A-CDK2AP1 is involved in various signaling within the cells. CDK2AP1 interacts with CDK2 and inhibits the activity of the CDK2-cyclin complex, leading to cell cycle arrest in the G1 phase [8, 17]. In additionally, CDK2AP1 interacts with the Beclin-1 complex and helps initiate autophagy, which is important for maintaining cellular homeostasis [8]. CDK2AP1 has also been reported to interact with the TRAIL receptor, suggesting its involvement in diverse cellular processes [8]. Previous studies have shown that kinesin-I binds to JIP, an adaptor protein for the JNK signaling pathway [10]. Activation of the JNK signaling pathway plays an important role in the cellular homeostasis [10]. Our findings provide the possibility that kinesin-1 transports CDK2AP1-associated signaling molecules through the interaction of kinesin-1 and CDK2AP1. Future studies are needed to determine the cargo of kinesin-1 linked by CDK2AP1 and the accurate intracellular cargo transport mechanism.

Acknowledgment

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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 : Cyclin-dependent kinase 1 결합 단백질 CDK2AP1은 kinesin superfamily protein 5A (KIF5A)을 매개로 Kinesin-1와 결합

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세포 내 수송 및 축삭 수송은 kinesin 및 cytoplasmic dynein과 같은 미세소관 의존성 모터단백질에 의해 운반된다. Kinesin은 미세소관을 따라 미세소관의 플러스 쪽 끝으로 이동하고, dynein은 미세소관의 마이너스 쪽 끝으로 이동한다. Kinesin-1은 kinesin superfamily protein (KIF)중에서 처음으로 확인된 kinesin으로, 카복실(C)-말단 영역과 cargo간 결합을 통해 세포내 소기관, 신경전달물질 수용체 및 mRNA-단백질 복합체를 포함한 다양한 cargo의 세포내 수송 기능을 수행한다. Kinesin-1은 다양한 cargo들을 수송하지만, kinsin-1과 cargo 사이를 매개하는 어댑터/스캐폴더 단백질은 아직 완전히 확인되지 않았다. KIF5A의 C-말단 영역과 상호 작용하는 어댑터 단백질을 규명하기 위해 효모 2-하이브리드 스크리닝을 하여, cyclin-dependent kinase 2-associated protein 1 (CDK2AP1)를 확인하였다. CDK2AP1은 KIF5A의 C-말단 영역에 결합하고 KIF3A, KIF5B, KIF5C 및 kinesin light chain 1 (KLC1)과는 결합하지 않았다. CDK2AP1의 C-말단 영역은 KIF5A와의 결합에 필수적이었다. HEK-293T 세포에 CDK2AP1 및 kinesin-1은 동시 발현하여 면역침강하면 CDK2AP1 및 kinesin-1은 같이 면역침강하였다. 그리고 CDK2AP1 및 kinesin-1은 세포내에서도 같은 위치에 발현하였다. 이러한 결과들은 KIF5A-CDK2AP1결합은 kinesin-1이 cargo를 운반할 때 kinesin-1과 cargo 사이를 연결하는 어댑터 단백질 역할을 시사한다.