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CUEDC2, CUE Domain Containing Protein 2, Associates with Kinesin-1 by Binding to the C-Terminus of KIF5A

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Kinesin-1 is a motor protein identified as the first member of the kinesin superfamily (KIF), which plays a role in intracellular cargo transport by acting as microtubule-dependent motor proteins within cells. Kinesin-1 consists of two heavy chains (KHCs, also known as KIF5s) and two light chains (KLCs). The 93 amino acids in the carboxyl (C)-terminal tail region of KIF5A are not homologous to the C-terminal tail region of KIF5B or the C-terminal tail region of KIF5C. In this study, we used a yeast two-hybrid screen to identify the binding proteins that interacted with the C-terminal region of KIF5A. We found an association between KIF5A and CUE domain containing 2 (CUEDC2), which is proposed to function as an adaptor protein involved in ubiquitination pathways and protein trafficking. CUEDC2 bound to the C-terminal region of KIF5A and did not interact with KIF5B (the motor of kinesin-1), KIF3A (the motor of kinesin-2), or kinesin light chain 1 (KLC1). KIF5A specifically bound to the C-terminal region of CUEDC2. Furthermore, KIF5A did not interact with another isoform: CUEDC1. In addition, glutathione S-transferase (GST) pull-downs showed that KIF5A directly bound GST-CUEDC2 but did not interact with GST-CUEDC1 and GST alone. When myc-KIF5A and EGFP-CUEDC2 were co-expressed in HEK-293T cells, CUEDC2 co-immunoprecipitated with kinesin-1, and myc-KIF5A and FLAG-CUEDC2 colocalized in the cells. These results suggest that in intracellular cargo transport by kinesin-1, CUEDC2 serves as an adaptor protein connecting kinesin-1 and cargo by binding to KIF5A.

Key words : Adaptor protein, cargo transport, CUE domain containing 2, kinesin, kinesin-1

Introduction

The microtubule-dependent motor proteins that are responsible for the transport of intracellular cargo are kinesin and the cytoplasmic dynein [4, 6]. Kinesin motor proteins move along to the positive end of the microtubule and transport cargo from the center to the periphery of the cell [4, 9]. Kinesin-1 was first identified as a member of the kinesin superfamily (KIF) protein in the squid giant axon, which is involved in a variety of cargo transport, including mitochondria, neurotransmitter receptors, and protein complexes [4]. It has a heterotetrameric structure consisting of two heavy chains (KHCs, also known as KIF5s) and two light chains (KLCs) [4].

KIF5 has three closely related KHC subtypes: KIF5A, KIF5B and KIF5C. KIF5B is ubiquitously expressed, whereas KIF5A is predominantly expressed in the cerebral cortex and cerebellum [7]. KIF5A has motor activity by forming a heterodimer with KIF5B and KIF5C [7]. It has a structure consisting of a globular head domain, a coiled-coil domain, and a carboxyl (C)-terminal domain [7]. The head domain at the amino (N)-terminus of the KIF5A is a motor domain that has ATPase motor activity and interacts with microtubules

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[7]. The C-terminal region of KIF5A interacts with various proteins and is involved in the transport of various cargoes in cells [14].

One of the most well-studied cargoes transported by kinesin-1 is mitochondria [4, 22]. The long-distance movement of mitochondria in neurons is dependent on kinesin-1 [4, 22]. Mitochondria can bind either directly or through adaptor proteins to the C-terminal tail of KIF5s or to the tetratricopeptide repeat (TPR) domain of KLCs [10, 22]. Previous reports have suggested that syntabulin acts as an adaptor protein between kinesin and mitochondria [2, 8]. Disruption of the association of kinesin-1 and syntabulin inhibits mitochondrial anterograde transport in cells [8]. The other groups found that mitochondrial Rho (Miro) and trafficking protein kinesin-binding 1 (TRAK1) form a protein complex and play an essential role in mitochondrial intracellular trafficking in neurons and many other cell types [2, 3, 11]. TRAK 1 binds directly to KIF5s and also to the mitochondrial anchoring protein Miro [11]. Furthermore, disruption of the KIF5s-TRAK 1-Miro interaction was confirmed to stop intracellular mitochondrial trafficking [3, 11]. These results suggest that TRAK 1 acts as a motor-adaptor molecule linking kinesin-1 and mitochondria [11]. In *kif5B*-KO mice, they die during early embryogenesis [19]. This is due to impaired intracellular mitochondrial localization [19]. This mislocalization of mitochondria in cultured cells from *kif5B*-KO mice was rescued by exogenous expression of KIF5B [19]. These phenotypes suggest an important role for kinesin-1 in intracellular cargo transport involving mitochondria.

KIF5A has a C-terminal tail region [7]. This region is not homologous to KIF5B or KIF5C [7]. The identification of proteins that interact with the C-terminal tail region of KIF5A is important for identifying the intracellular cargo transport of kinesin-1 [14]. To better understand the role of kinesin-1 in cargo transport, we used a yeast two-hybrid screen to identify the protein that interacts with the C-terminal tail region of KIF5A. We have identified the CUE domain containing 2 (CUEDC2), which may serve as an adaptor protein that connects to kinesin-1 and cargo.

Materials and Methods

Plasmid constructs

Full-length mouse CUEDC2 cDNA (GeneBank ID: 67116) was amplified by PCR from the Marathon-Ready^{TM} cDNA library (Clontech Laboratories, Inc., Palo Alto, CA, USA) and cloned into pGEM T-easy vector (Promega Corp., Madison, WI, USA). The C-terminal tail of KIF5A and KIF5B was subcloned from pBlucript-KIF5A and pBlucript-KIF5B into pLexA vector (Clontech Laboratories, Inc.) [14]. pLexA C-terminal tail of KIF5A was used as a bait plasmid in this yeast two-hybrid screen.

Yeast two-hybrid screening for the C-terminal tail region of KIF5A

The Matchmaker yeast two-hybrid system (Clontech Laboratories, Inc.) was used to screen for KIF5A-binding proteins. According to the Matchmaker yeast two-hybrid system manual, the bait plasmid was first transformed into the yeast strain EGY48, and the transformed cells were transformed with a mouse brain cDNA library (Clontech Laboratories, Inc.) [5]. Positive clones from the two-hybrid screen were selected on SD/-His/-Trp/-Ura/-Leu plates containing galactose, raffinose, X-gal and BU salts. Plasmids isolated from the positive clones were analyzed by *Eco*RI and *Xho*I restriction digests and confirmed by re-transformation.

β-Galactosidase activity of KIF5A and CUEDC2 in yeast liquid culture

β-Galactosidase activity was estimated as previously described [5]. Briefly, mid-log phase yeast cells in liquid culture were pooled 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 lysate, the mixture was incubated at 30℃, and 1 M Na_2CO_3 was added to stop the reaction. The absorbance of the reaction solution was measured at 420 nm in a spectrophotometer, and the enzyme activity unit was calculated as previously reported [5].

Glutathione S-transferase (GST) pull-down for CUEDC2

CUEDC1, CUEDC2, and the KIF5A-tail region were cloned into pET41 or pET21, and expression of each recombinant protein was induced in bacterial strain BL21 (Stratagene, La Jolla CA, USA) with 0.5 mM isopropyl thio-β-D-galactopyranoside (IPTG) for 3 hr. Each recombinant protein was purified with glutathione-agarose beads (Sigma-Aldrich) and incubated with the His-KIF5A fusion protein for 1 hr at room temperature before precipitation 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, pepstatin, and 1 μM phenylmethanesulfonyl fluoride), and anti-KIF5A antibody [14] was used for Western blotting.

Cell culture and transfection of myc-KIF5A and FLAG-CUEDC2

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 in a humidified 5% CO_2 incubator. Transfection of each plasmid into HEK-293T was performed using the previously reported $CaPO_4$ precipitation method [5].

Co-immunoprecipitation and immunoblot analysis of KIF5A and CUEDC2

HEK-293T cells were transfected with myc-KIF5A and FLAG-CUEDC2. Co-immunoprecipitation and immunoblot were performed as previously reported [5]. Briefly, transfected cultured cells were rinsed three times with PBS and gently rotated with lysis buffer [PBS containing 0.5% NP-40 and 1x Protease Inhibitor Cocktail Set V (Calbiochem, San Diego, CA, USA)] for 30 min. The lysates were centrifuged at 16,000 × g for 10 min at 4℃, and 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 the washed beads were resuspended in Laemmli's loading buffer. Immunoblot analysis was performed using antibodies against KIF3A, KIF5A, KIF5B, KLC1 and FLAG epitopes [14].

Immunocytochemistry of KIF5A and CUEDC2

HEK-293T cells transfected with myc-KIF5A and EGFP-CUEDC2. After culturing the transfected HEK-293T cells for 24-36 hr, the cells were fixed with 4% paraformaldehyde in PBS for 5 min. The cells were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 10 min followed by 30 min in 5% normal goat serum in PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween-20 (Sigma-Aldrich). The cells were blocked while incubated with Dylight 594-conjugated goat anti-rabbit IgG antibody (Jackson Immuno Research Labs, West Grove, PA, USA) diluted 1:700 for 40 min. After washing the cells were washed three times with PBS and mounted with Fluoromount (DAKO Korea, Seoul, Korea). Fluorescence imaging was performed with a Zeiss LSM510 META confocal laser scanning microscope (Carl Zeiss, Oberkochem, Germany).

Results

Screening and identification of the KIF5A binding protein

Kinesin-1 forms a heterotetrameric structure composed of two KHCs and two KLCs, and the KHCs include KIF5A, KIF5B, and KIF5C [7]. According to previous study, the C-terminal tail regions of KIF5A, KIF5B, and KIF5C have regions with no mutual amino acid homology, and it was speculated that kinesin-1 binding proteins would bind to these C-terminal regions of KIF5s [7, 14]. To identify of the kinesin-1 binding proteins, we performed a yeast two-hybrid screen using the KIF5A-specific C-terminal region, which has no homology to KIF5B, and KIF5C as bait (Fig. 1A). The mouse brain cDNA library was transformed to generate $6×10^6$ independent transformants from which a number of positive clones were obtained in this screen. Plasmids were isolated from the positive clones and DNA sequences were analyzed. One positive clone was found to encode the C-terminal region of CUEDC2 (Fig. 1B).

CUEDC2, a protein containing a CUE domain, has been proposed to function as a scaffold for complexes involved in trafficking and ubiquitination pathways [1]. The CUE domain is a motif of approximately 40 amino acids found in a large number of eukaryotic proteins [1]. This CUE domain plays a dual role not only in ubiquitin interactions but also in the regulation of protein stability via ubiquitination of specific substrates [18]. To determine whether the CUE domain of CUEDC2 is involved in binding to KIF5A, we constructed a series of CUEDC2 deletion mutants and analyzed their binding to KIF5A using a yeast two-hybrid assay. The CUE domain and the N-terminal region of CUEDC2 did not bind to KIF5A. However, only the C-terminal region of CUEDC2 bound to KIF5A (Fig. 1B).

KIF5A consists of an N-terminal motor domain, a coiled-coil domain, and a C-terminal domain (Fig. 1A). To confirm the interaction domain of KIF5A between KIF5A and CUEDC2, we constructed different fragments on each domain of KIF5A and tested the interaction with yeast two-hybrid assay. As shown in Figure 1C, the interaction of KIF5A and CUEDC2 confirmed that CUEDC2 interacts with the C-terminal tail region of KIF5A.

Kinesin-1 consists of motor protein KHCs and non-motor protein KLCs [5]. Next, we investigated whether the KLC of kinesin-1 or KIF3A (motor subunit of kinesin-2) interacts with CUEDC2 by yeast two-hybrid assay. As shown in Fig. 2A, KLC1 and KIF3A did not interact with CUEDC2. We next examined whether KIF5A interacts with CUEDC1, another CUEDC2 isoform. KIF5A did not interact with CUEDC1 [Fig. 2B]. This result is not surprising as the amino acid sequences of CUEDC2 and CUEDC1 share low identity

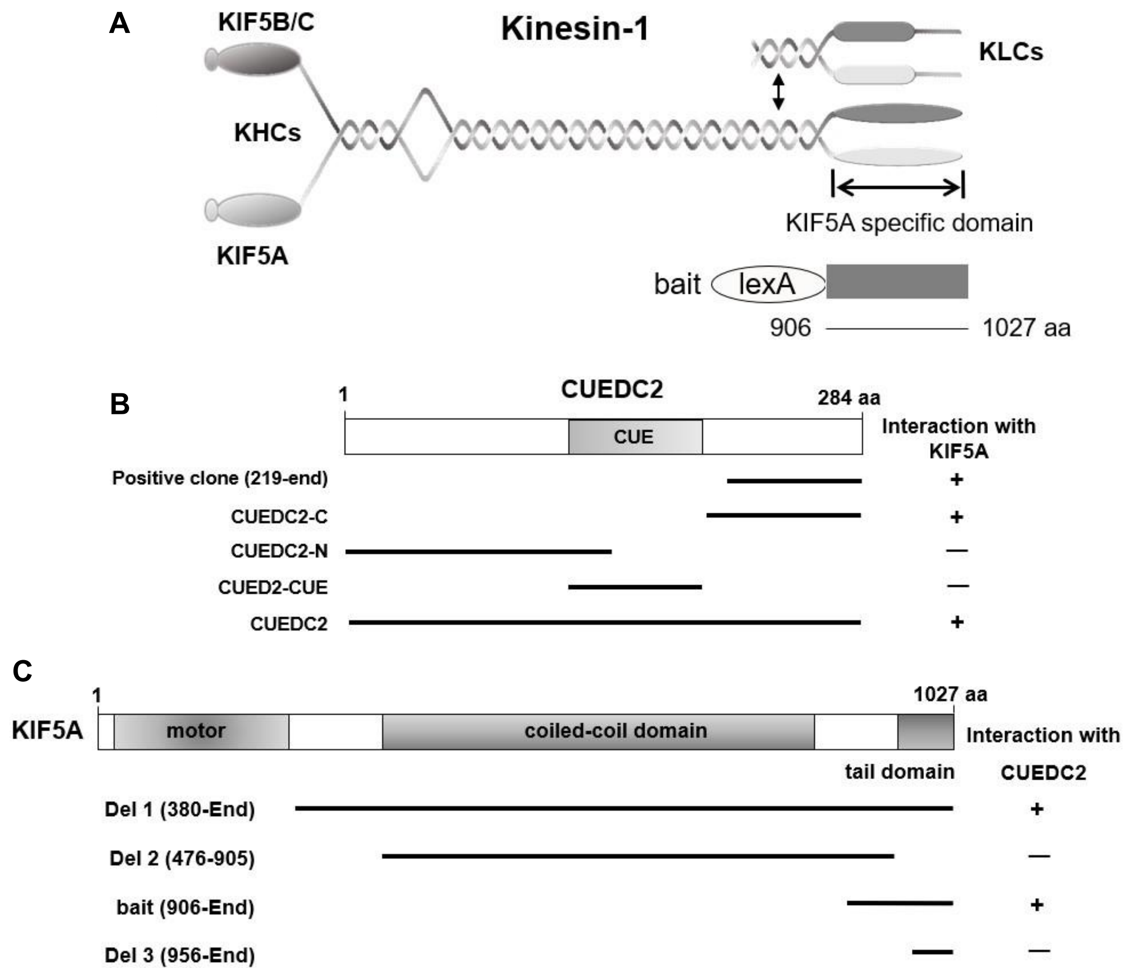

Fig. 1. Screening and dentification of KIF5A binding proteins. (A) Schematic diagram of kinesin-1. A yeast two-hybrid screen was performed using the C-terminal region of KIF5A. Kinesin-1 consists of KHCs, also known as KIF5s and KLCs. (B) Schematic diagram of CUEDC2 and the KIF5A binding region of CUEDC2. Yeast two-hybrid positive clone containing the ORF for CUEDC2. CUEDC2 truncations were tested for interaction with KIF5A in a yeast two-hybrid assay. (C) The CUEDC2 binding region of KIF5A. KIF5A has a motor domain, a coiled-coil domain, and a tail domain. These domains are shown in gray. The different truncations of KIF5A were tested in a yeast two-hybrid assay for their ability to interact with CUEDC2. +, interaction; -, no interaction; KIF5A, kinesin superfamily protein 5A; KIF5B, kinesin superfamily protein 5B; KIF5C, kinesin superfamily protein 5C; KHC, kinesin heavy chain; KLC, kinesin light chain; CUEDC2, CUE domain-containing protein 2; aa, amino acids.

(CUEDC1 and CUEDC2 are 14% identical based on the KIF5A binding domain) [24]. As a positive control, GABAAR-associated protein (GABARAP) was used [14]. To quantify the binding affinity between KIF5A and CUEDC2, we measured β-galactosidase activity in yeast lysate. The interaction of KIF5A and CUEDC2 resulted in approximately 315 units of β-galactosidase activity (Fig. 3C).

CUEDC2 interacts with cellular kinesin-1

Next, we analyzed the interaction between KIF5A and GST-CUEDC1 or GST-CUEDC2 using a GST pull-down assay to confirm the direct binding of KIF5A and CUEDC2 at the protein level. Recombinant His-KIF5A, GST-CUEDC1, or GST-CUEDC2 was expressed in bacteria. It was purified and then pulled down. Immunoblotting with anti-KIF5A antibody showed that KIF5A does not interact with GST and GST-CUEDC1 (Fig. 3A). However, it binds to GST-CUEDC2 (Fig. 3A). These results suggest that KIF5A and CUEDC2 bind directly at the protein level and are consistent with the results of the yeast two-hybrid assay.

Kinesin-1 forms a heterotetrameric complex consisting of the KHCs (KIF5A, KIF5B, and KIF5B), and the KLCs (KLC1, and KLC2) [7]. To confirm that CUEDC2 associates with kinesin-1 by binding to KIF5A, we transfected myc-

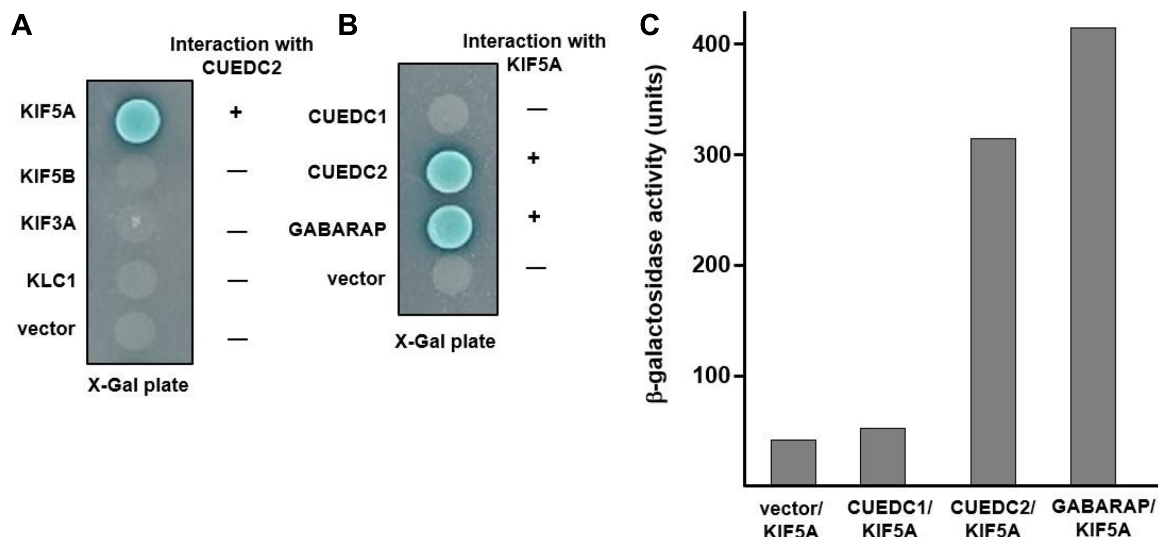

Fig. 2. The identification of a specific interaction between KIF5A and CUEDC2. (A) Interaction between the tail region of KIF5A, KIF5B, KIF3A, full-length KLC1, and CUEDC2 was tested by yeast two-hybrid assay. CUEDC2 interacted with KIF5A, but did not interact with KIF3A, KIF5B or KLC1. (B) KIF5A was tested for the interaction with CUEDC1 or CUEDC2 by a yeast two-hybrid assay. The positive control for the interaction with KIF5A was GABARAP. (C) The strength of the interaction between KIF5A and CUEDC1, or CUEDC2 was quantitatively examined using β-galactosidase activity in yeast. +, interaction; -, no interaction; KIF3A, kinesin superfamily protein 3A, KIF5, kinesin superfamily protein 5; KLC1, kinesin light chain 1; CUEDC, CUE domain-containing protein; GABARAP, γ-aminobutyric acid receptor-associated protein; X-gal, 5-Bromo-4-Chloro-3-Indolyl-β-D-galactoside.

KIF5A and FLAG-CUEDC2 into HEK-293T cells and co-immunoprecipitated them with anti-FLAG antibody. When CUEDC2 was precipitated with anti-FLAG antibody, it was found to co-precipitate with myc-KIF5A, KIF5B, and KLC1 of kinesin-1. However, KIF3A did not co-precipitate with CUEDC2 (Fig. 3B). These results suggest that CUEDC2 associates with kinesin-1 through its binding to KIF5A. When myc-KIF5A and EGFP-CUEDC2 were expressed in HEK-293T cells, it was determined whether KIF5A and CUEDC2 were co-localized in the cells. As shown in Fig. 3C, KIF5A and CUEDC2 were found to co-localized in the same region of the cytoplasm in the cells. This result is consistent with an interaction between KIF5A and CUEDC2 in the cells.

Discussion

The KHCs of kinesin-1 have a highly conserved N-terminal motor domain, a coiled-coil domain, and a non-homologous globular tail domain at the C-terminus [7]. In this study, to identify the cargo transported by kinesin-1, we used yeast two-hybrid screening to identify proteins that bind to the C-terminal region of KIF5A. We found that CUEDC2 binds to the C-terminal region of KIF5A. In addition, it was confirmed that KIF5A directly interacts with CUEDC2 at the protein level. When myc-KIF5A and FLAG-CUEDC2 were expressed in HEK-293T cells and then immunoprecipitated with anti-FLAG antibody, CUEDC2 co-precipitated with kinesin-1. Furthermore, CUEDC2 expression was at the same cytoplasmic location in the cells as KIF5A. These results suggest that CUEDC2 is a novel binding protein that specifically binds to the C-terminus of KIF5A, associates with kinesin-1, and may act as an adaptor protein mediating between kinesin-1 and cargo.

CUEDC2 was originally identified in the regulation of progesterone receptor degradation by the ubiquitin proteasome pathway in cancer [25]. It is widely expressed in tissues and organs, including the brain, heart and testis, and has been shown to play a central role in many cellular events, including cell cycle regulation, inflammation and carcinogenesis [12]. CUEDC2 has a CUE domain that serve as a scaffold for complexes that are involved in the trafficking and ubiquitination pathways [22]. In this study, we showed that KIF5A does not bind to the CUE domain of CUEDC2. Instead, KIF5A interacts with the C-terminal region of CUEDC2.

What does this interaction between KIF5A and CUEDC2 mean? One possibility is that CUEDC2 acts as an adaptor protein. It mediates kinesin-1 to its cargo by interacting with KIF5A. The C-terminal tail of KIF5s and the tetratricopeptide

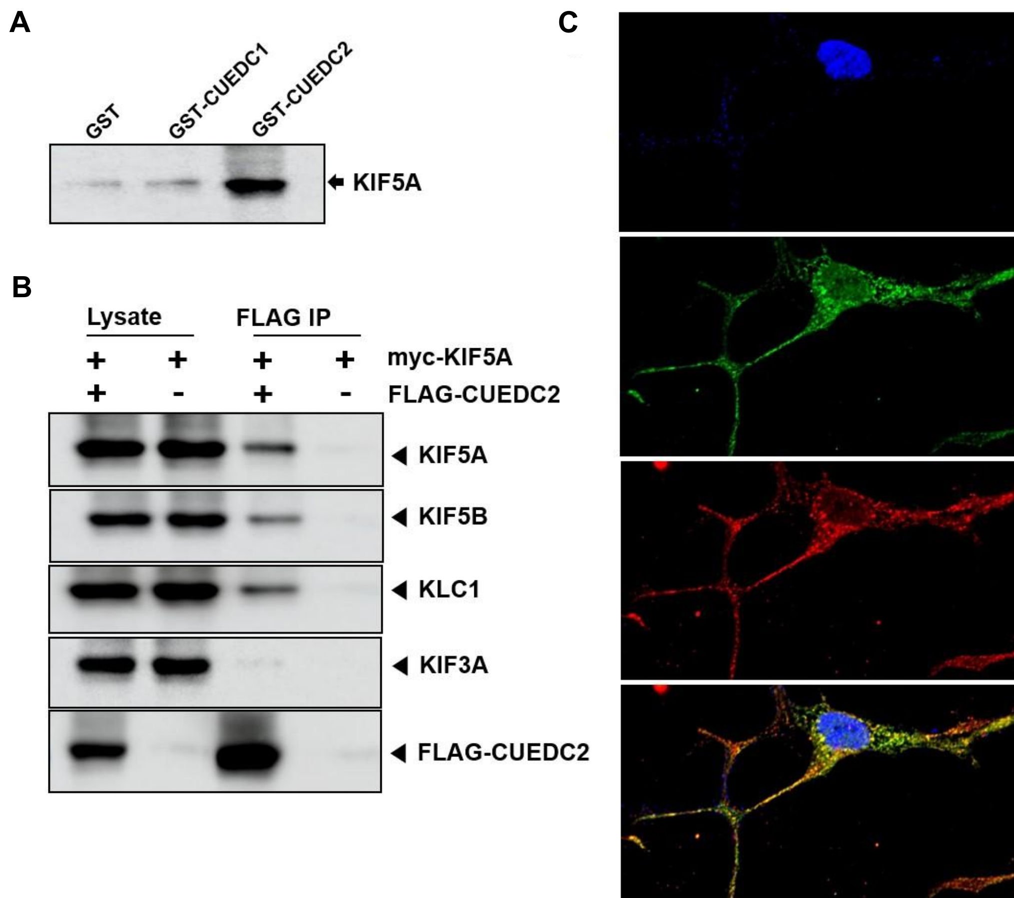

Fig. 3. Immunoprecipitation of CUEDC2 and kinesin-1 and subcellular localizing with KIF5A. (A) Direct binding of CUEDC2 to KIF5A in a GST pull-down assay using the recombinant GST-CUEDC1, and GST-CUEDC2. The precipitates from the GST pull-down were immunoblotted with an anti-KIF5A antibody. (B) The myc-KIF5A and FLAG-CUEDC2 plasmids were transiently transfected into HEK-293T cells. Cell lysates were immunoprecipitated with an anti-FLAG monoclonal antibody. Precipitates were subjected to immunoblotting with anti-KIF5A, anti-KIF5B, anti-KIF3A, anti-KLC1 and anti-FLAG antibodies. CUEDC2 co-precipitated with kinesin-1. (C) EGFP-CUEDC2 and myc-KIF5A were transiently transfected into HEK-293T cells. At 24 hr after transfection, the cells were analyzed by confocal microscopy using DAPI (blue), EGFP (green), and anti-KIF5A (red). CUEDC2 and KIF5A are seen colocalized in the cell cytoplasm. KIF5, kinesin superfamily protein 5; KIF3A, kinesin superfamily protein 3A; KLC1, kinesin light chain 1; CUEDC2, CUE domain-containing protein 2; GST, glutathione S-transferase.

repeat (TPR) domain of KLCs are required for interaction with cargo proteins [4, 17]. Kinesin-1 often uses specific adaptor proteins to transport its cargo [4, 16, 17]. The interaction between kinesin-1 and adaptor proteins may serve to regulate cargo association and dissociation [9]. For example, JNK-interacting protein (JIP) 1 binds to KLCs, and JIP1 also binds to a wide variety of other proteins [20]. Thus, JIP1 acts as an adaptor protein that connects JIP1-binding cargoes, including amyloid precursor protein (APP), apolipoprotein E receptor 2 (ApoER2), and kinesin-1, allowing the movement of specific cargoes [13, 15, 21]. CUEDC2 was previously been reported to be an important adaptor protein that regulates protein degradation, cell signaling pathway, and maintenance of cellular homeostasis [24]. The CUE domain of CUEDC2 interacts with the tumor suppressor protein p53, cyclin A, cyclin E, which are critical for cell cycle regulation and cell proliferation [24]. Based on the interaction between KIF5A and CUEDC2, we favor the model in which CUEDC2 functions as an adaptor protein between kinesin-1 and its cargo. Future studies are needed to determine the cargoes of kinesin-1 that are linked by CUEDC2 or to determine the mechanism regulating intracellular cargo trafficking.

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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 : CUE 도메인 포함 단백질인 CUEDC2는 KIF5A의 C-말단과 결합을 통하여 Kinesin-1와 결합

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Kinesin-1은 kinesin superfamily (KIF) 단백질 중에서 처음으로 확인된 모터 단백질로 세포내 미세소관 의존하여 세포내 cargo를 수송한다. Kinesin-1은 두 개의 중쇄(KHC, 또는 KIF5)와 두 개의 경쇄(KLC)로 구성된다. KIF5A의 C-말단의 93개 아미노산은 KIF5B와 KIF5C의C-말단 꼬리 영역과는 상동성이 없다. 본 연구에서 우리는 KIF5A의 C-말단 영역과 특이적으로 결합하는 단백질을 분리하기 위해 효모 2-하이브리드 스크리닝을 하였다. 본 연구에서 우리는 KIF5A와 결합하는 단백질로 유비퀴틴화 경로 및 단백질 수송에 관여하는 어댑터 단백질로 기능하는 CUE 도메인을 가진 CUEDC2를 확인하였다. CUEDC2는 KIF5A의 C-말단 영역과 결합하지만, KIF5B, KIF3A 및KLC1과는 결합하지 않았다. KIF5A는 CUEDC2의 C-말단 영역과 특이적으로 결합하였지만, CUEDC2의 다른 isoform인 CUEDC1과는 결합하지 않았다. 또한, KIF5A와 CUEDC2의 결합은 글루타티온 S-트랜스퍼라제(GST) 풀다운으로 단백질간 결합을 확인하였다. HEK-293T 세포에서 myc-KIF5A와 FLAG-CUEDC2을 공동 발현되었을 때, CUEDC2는 kinesin-1과 공동 면역 침전되었고, myc-KIF5A와 EGFP-CUEDC2는 세포내의 같은 위치에서 발현하였다. 이러한 결과들은 kinesin-1에 의한 세포내 화물 수송에서 CUEDC2는 KIF5A에 결합하여 kinesin-1과 화물을 연결하는 어댑터 단백질 역할을 시사한다.