ISSN (Online) 2287-3406
Journal of Life Science **2023** Vol. 33. No. 1. 1~7 DOI : https://doi.org/10.5352/JLS.2023.33.1.1

Glutamate-rich 4 Binds to Kinesin Superfamily Protein 5A

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Received September 13, 2022 /Revised October 4, 2022 /Accepted October 4, 2022

Intracellular cargo transport is mediated by molecular motor proteins, such as kinesin and cytoplasmic dynein. Kinesins make up a large subfamily of molecular motors. Kinesin-1 is a plus-end-directed molecular motor protein that moves various cargoes, such as organelles, protein complexes, and mRNAs, along a microtubule track. It consists of the kinesin superfamily protein (KIF) 5A, 5B, and 5C (also called kinesin heavy chains) and kinesin light chains (KLCs). Kinesin-1 interacts with many different binding proteins through its carboxyl (C)-terminal region of KIF5s and KLCs, but their binding proteins have not yet been fully identified. In this study, a yeast two-hybrid assay was used to identify the proteins that interact with the KIF5A specific C-terminal region. The assay revealed an interaction between KIF5A and glutamate-rich 4 (ERICH4). ERICH4 bound to the KIF5A specific the C-terminal region but did not interact with the C-terminal region of KIF5B or KIF3A (a motor protein of kinesin-2). In addition, KIF5A did not interact with another isoform, ERICH1. Glutathione S-transferase (GST) pull-downs showed that KIF5A interacts with GST-ERICH4 and GST-ERICH4-amino (N)-terminal but not with GST-ERICH4-C or GST alone. When co-expressed in HEK-293T cells, ERICH4 co-localized with KIF5A and co-immunoprecipitated with KIF5A and KLC but not KIF3B. Together, our findings suggest that ERICH4 is capable of binding to KIF5A and that it may serve as an adaptor protein that links kinesin-1 with cargo.

Key words : Adaptor protein, binding protein, glutamate-rich, kinesin-1, microtubule

Introduction

Newly generated cargoes that contain the neurotransmitter receptors or proteins then undergo microtubule-based transport to their proper destinations in neuron [3]. Microtubule dependent cargo transport is mediated by the molecular motor proteins kinesin and dynein [5]. Dynein mediates microtubule minus end-directed cargo transport [4]. In contrast, kinesin mediates microtubule plus end-directed cargo transport [4]. Kinesin-1 is the first identified member of kinesin superfamily proteins (KIFs) and involved in the transport of various cargoes, including membrane-bounded vesicles, organelles, proteins complexes, and mRNAs [2, 4]. It is a heterotetrameric protein composed of two heavy chains (KHCs), also known as KIF5s and two light chains (KLCs) [3]. KIF5s form the two distinct domains that mediate their basic function: a motor domain and a carboxyl (C)-terminal tail domain [4]. The motor domain of KIF5s contains the ATPase motor activity and interacts with microtubules. The C-terminal tail domain of KIF5s is much more variable and mediates interaction with different cargoes [4, 13].

KIF5s have been revealed to consist of three closely related subtypes: KIF5A, KIF5B, and KIF5C [6]. KIF5A and KIF5C are mostly expressed in nervous tissues while KIF5B is ubiquitously expressed [6]. KIF5A, KIF5B, and KIF5C form ho-

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modimers or heterodimers in cells [6]. To investigate the functions of KIF5A, *kif5A* knockout (KO) mice were generated [15]. *kif5A*-KO mouse was neonatal lethal but showed no apparent histological abnormalities than the wild-type mice [16]. Conditional *kif5A*-KO mice showed epileptic seizures and γ-aminobutyric acid receptors ($GABA_ARs$) trafficking to the post-synaptic terminal membrane was impaired in the KIF5A-deficient neurons [9]. But, *kif5B*-KO mouse did not show epileptic seizure [16]. Several mutations in the tail domain of *kif5A* gene have been identified to cause spastic paraplegia 10 (SPG10) [11].

KIF5A has the long C-terminal tail region that shows no homology with KIF5B or KIF5C tail region [6]. Despite this importance, relatively little is known about the function of KIF5A C-terminal tails region or the proteins it's interact with. To improve the understanding of the roles of KIF5A in intracellular cargoes transport, using the yeast two-hybrid screens, we identified the Glutamate-rich 4 (ERICH4), which encodes a small protein (136 amino acids) containing a 113 amino acids domain of unknown function (DUF4590) as a protein that interacts with kinesin-1.

Materials and Methods

Plasmid constructs

The full-length cDNAs of mouse ERICH1 (GeneBank ID: 234086) and ERICH4 (GeneBank ID: 632778) were amplified by PCR from Marathon-Ready™ 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 region (amino acids 906-1027) of KIF5A was subcloned from pBlucript-KIF5A obtained from Kanai *et al*. [6]. The resulting recombinant plasmid, pLexA-tail-KIF5A, was used as bait plasmid this yeast two-hybrid screening.

Screening of yeast two-hybrid positive clone

The Matchmaker yeast two-hybrid system was used for screening according to the manufacturer's manual (Clontech). In brief, pLexA-tail region of KIF5A was transformed into yeast strain EGY48. Transformed cells were transformed with the mouse brain cDNA library [16]. The selection of positive clones 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 *Eco*RI and *Xho*I restriction digestion.

β-Galactosidase activity in liquid cultures of yeast

Mid-log phase yeast cells were collected and permeabilized with 0.1% sodium dodecyl sulfate (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 reaction product 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=1,000× $[(OD_{420} - 1.75 \times OD_{550})]$/ (reaction time × culture volume × OD_{600}) [14].

Glutathione S-transferase (GST) pull-down assays

The full length of ERICH4, ERICH4-amino (N), and ERICH4-C were cloned in pET41a. The recombinant GST-ERICH4, ERICH4-N and ERICH4-C fusion protein was expressed in bacterial strain BL21 (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). The mouse brain lysate 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). The pulled-down proteins were analyzed by Western blotting with anti-KIF5A antibody [9]. The animal study was approved by the institutional review board (IRB), and the approval number was 21-08 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 [9].

Immunocytochemistry

HEK-293T cells grown on poly-D-lysine-coated coverslips were transfected with enhanced green fluorescent protein (EGFP)-ERICH4 constructs. Twenty-four hours after transfection, cells were washed with PBS, fixed with 4% paraf-

ormaldehyde 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-KIF5A antibody [9] 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 1:700. After washing 3 times with PBS, the cells were mounted with Fluoromount (DAKO*Korea*, 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-KIF5A and FLAG-ERICH4 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 16,000× *g* for 10 min at 4℃. The supernatant was incubated with anti-FLAG M2 agarose beads (Sigma-Aldrich) for 3 hr at 4℃ with constant shaking. The beads were collected by centrifugation at 2,000 x *g* for 30 sec and washed 3 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 KIF3B, KIF5A, KLC, and FLAG epitope as described elsewhere by Kanai et al. [6].

Results

Identification of KIF5A interacting proteins by yeast two-hybrid screening

KIF5A is composed of a motor domain in the N-terminal region, coiled-coil domain, and the C-terminal tail region [6]. The 73 amino acids tail region of KIF5A has no homology with KIF5B-tail region or KIF5C-tail region [6]. To identify KIF5A binding proteins, we screened a mouse brain cDNA library by yeast two-hybrid system using the C-terminal tail region of KIF5A as bait. In screen of 7×10^6 CFU/ml independent transformants, one positive clone was obtained. After confirmation and partial sequencing, positive clone was found to encode the nearly full length of ERICH4 (Fig. 1A). Various fragments of KIF5A were constructed and tested for interaction with ERICH4 using a yeast two-hybrid assay. These results indicate that the C-terminal tail region of KIF5A are required for specific binding to ERICH4 (Fig. 1B).

ERICH proteins have not been well characterized and their functions are presently unknown [1, 7]. ERCIH4 have a 113 amino acids domain of unknown function (DUF) domain in the N-terminal region (Fig. 1A) [10]. To identify the minimal binding domain of ERICH4 required for the binding with KIF5A, a series of deletion mutants of ERCIH4 was constructed and analyzed for their interactions with KIF5A. Results indicate that the DUF domain of ERICH4 interacted with KIF5A in the yeast two-hybrid assay (Fig. 1C). Mammalian possess three KIF5: KIF5A, KIF5B, and KIF5C. Next, we investigated whether KIF5s, and KIF3A (a motor subunit of kinesin-2) interact with ERICH4. As shown in Fig. 2A, KIF5B, KIF5C, KIF3A, and KLC1 did not interact with ERICH4 in the yeast two-hybrid assay.

There are six isoforms of ERICH, from ERICH1 to ERICH6 [10]. Each isoform has a DUF domain [10]. Next, we investigated whether KIF5A interacts with another isoform, ERICH1. KIF5A did not interact with ERICH1 in the yeast two-hybrid assay (Fig. 2B). This result is not surprising because ERICH proteins has no similarity in their amino acid sequence in DUF domain [7, 10]. This data suggests that KIF5A specifically interacts with ERICH4. To quantify the binding affinity of KIF5s to ERICH4, the KIF5A or KIF5B expression plasmids were transformed into yeast and the β-galactosidase activity was measured in liquid cultures. $GABA_AR$-associated protein (GABARAP), served as a positive control [9]. The interaction of KIF5A with ERICH4 yielded approximately 310 units of β-galactosidase activity (Fig. 2C).

ERICH4 is associated with KIF5A in cells

To confirm the KIF5A and ERICH4 interaction at the protein level, the interaction between KIF5A and ERICH4, ERICH4-N, or ERICH4-C was assayed using a GST pull-down. Recombinant GST-ERICH4, GST-ERICH4-N, or GST-ERICH4-C fusion proteins were expressed in bacteria. The purified GST fusion proteins are allowed to interact with mouse brain lysates. Immunoblotting analyses revealed that KIF5A interacted with GST-ERICH4, and GST-ERICH4-N but not with GST, and GST-ERICH4-C (Fig. 3A). This data indicates that KIF5A associates with ERICH4 at protein level.

Kinesin-1 consists of two motor subunits KIF5s and two KLCs, forming a heterotetrametric complex [6]. To address the question whether KIF5A mediates the interaction of

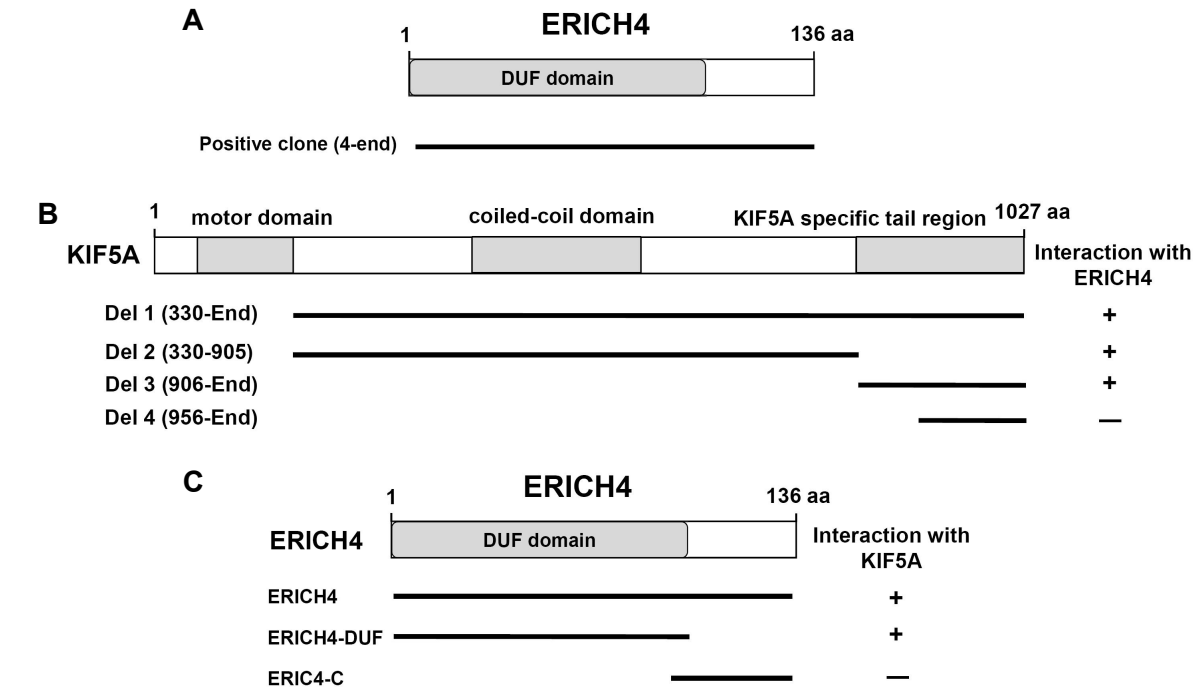

Fig. 1. Identification of the protein interacted with KIF5A by yeast two-hybrid screening. (A) Schematic diagram of ERICH4. The positive clone isolated from the yeast two-hybrid screening possesses the cDNA for ERICH4. (B) ERICH4 binding region in KIF5A. KIF5A has the motor domain, coiled-coil domain, and KIF5A-specific tail region, indicated in gray. The different truncated forms of KIF5A were assessed in the yeast two-hybrid assay for interaction with ERICH4. (C) KIF5A binding region in ERICH4. The different truncations of ERICH4 were tested in the yeast two-hybrid assay for interaction with KIF5A. +, interaction; -, no interaction; KIF5A, kinesin superfamily protein 5A; ERICH4, Glutamate-rich 4; DUF, unknown function; aa, amino acids.

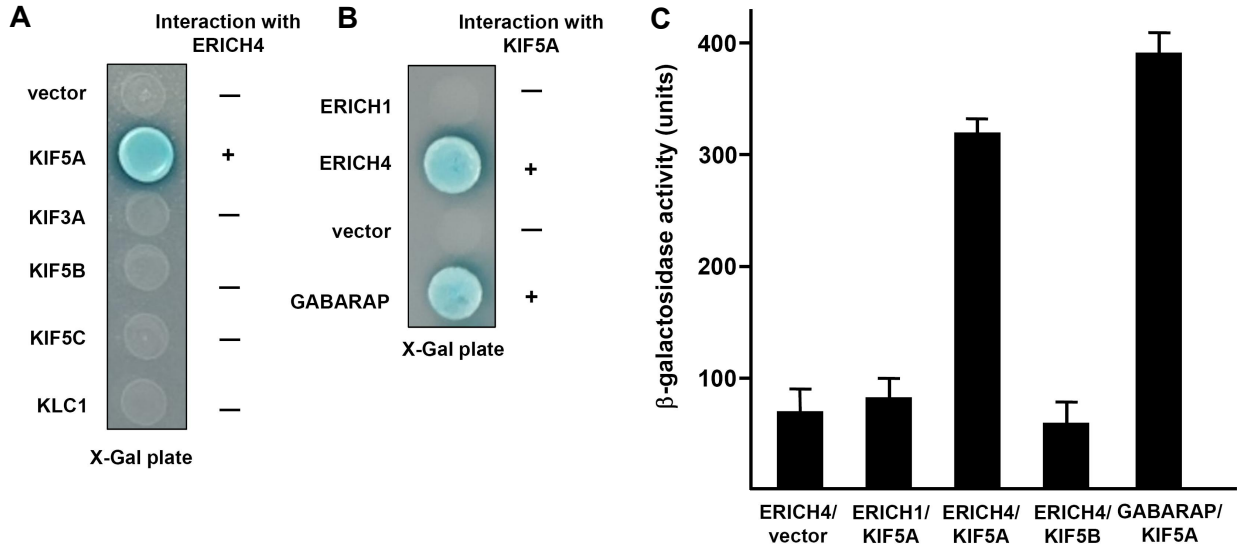

Fig. 2. Interaction of ERICH4 with KIF5A. (A, B) The tail region of KIF5s and KIF3A, the full length KLC1, and ERICH4 were tested for the interaction. ERICH4 specifically interacted with KIF5A but not with KIF3A, KIF5B, KIF5C or KLC1. Also, KIF5A specifically interacted with ERICH4. GABARAP served as a positive control for interaction with KIF5A. (C) The strength of interaction between ERICH4 and KIF5A or KIF5B was examined quantitatively using β-galactosidase activity in yeast two-hybrid reporter assay. +, interaction; -, no interaction; KIF5, kinesin superfamily protein 5; KIF3A, kinesin superfamily protein 3A; KLC1, kinesin light chain 1; ERICH1, Glutamate-rich 1; ERICH4, Glutamate-rich 4; GABARAP, γ-aminobutyric acid receptor-associated protein; X-gal, 5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside.

ERICH4 and kinesin-1, we performed co-immunoprecipitation from HEK-293T cells that were transfected with myc-KIF5A and FLAG-ERICH4. Anti-FLAG antibody precipitated with KIF5A and KLC. However, KIF3B (a motor protein of kinesin-2) did not (Fig. 3B). This result indicates that ERICH4 interacts with kinesin-1. In order to address whether KIF5A and ERICH4 co-localize in cells, EGFP-ERICH4 was co-expressed with myc-KIF5A in HEK-293T cells. ERICH4 and KIF5A were overlapped at the same cytosolic region in cells (Fig. 3C). These data suggest that ERICH4 is a new binding partner of kinesin-1 through the binding with KIF5A C-terminal region.

Discussion

KIF5s have a high conserved motor domain and stalk domain [6]. However, their tail domains that mediate the interaction with various binding proteins have no homology [6, 9]. In this study, we found that ERICH4 interacts only with KIF5A. We identified that the C-terminal region of KIF5A interacts with ERICH4 at the protein level. Furthermore, when FLAG-ERICH4 and myc-KIF5A were expressed in mammalian cells, they co-immunoprecipitated and co-localized in cells. Taking all of these data, we hereby suggest that ERICH4 is new binding protein to KIF5A and may serve as an adaptor protein that links kinesin-1 with cargo.

ERICH proteins have one DUF domain and the mostly uncharacterized protein [7]. In previous study, loss of ERICH3 causes abnormally short cilia and accumulated at intraflagellar transport complex proteins in the ciliary tip [1]. This finding suggests that ERICH3 may be play as adaptor protein in intraflagellar transport in cilia [1]. Previous proteomic and biochemical studies have shown that ERICH3 is linked to clathrin heavy chain that is involved in vesicular function [10]. In addition, ERICH3 showed a cytoplasmic localization as well as localization to vesicle-like structures [7].

What would the interaction between the C-terminal region of KIF5A and ERICH4 mean? We are able to suggest one possibility is that ERICH4 may be role an adaptor protein that links kinesin-1 and various cargoes. Kinesin-1 plays role

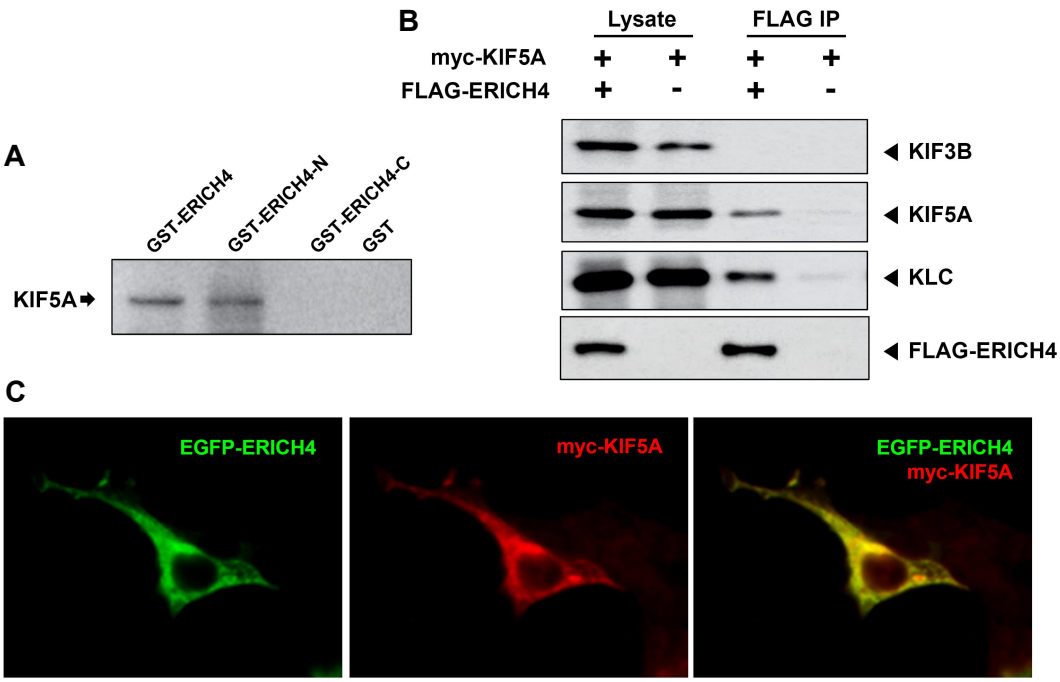

Fig. 3. Interaction and subcellular localization of KIF5A and ERICH4. (A) Direct binding of ERICH4 to KIF5A in a GST pull-down assay using purified GST-fused ERICH4. Precipitates were immunoblotted with anti-KIF5A antibody. (B) HEK-293T cells were transiently transfected with FLAG-ERICH4 and myc-KIF5A plasmids as indicated. Cell lysates were immunoprecipitated with monoclonal anti-FLAG antibody. Precipitates were immunoblotted with anti-KIF3B, KIF5A, KLC, and FLAG antibodies. ERICH4 co-precipitated KLC and KIF5A. (C) HEK-293T cells were transiently transfected with EGFP-ERICH4 and myc-KIF5A plasmids. Twenty-four hours after transfection, cells were subjected to immunofluorescence with anti-KIF5A antibody. ERICH4 and KIF5A are seen at the same subcellular region in cells. KIF3B, kinesin superfamily protein 3B; KIF5A, kinesin superfamily protein 5A; KLC, kinesin light chain; ERICH4, Glutamate-rich 4; GST, glutathione S-transferase.

of various different cargo transport along the microtubule in cells [4]. In some cases, these cargoes bind to soluble adaptor proteins/scaffolding proteins that mediate the attachment to kinesin-1 [12]. The role of these adaptor proteins/scaffolding proteins effectively controls the intracellular cargo transport of kinesin-1 [12]. The first adaptor protein identified to bind to kinesin-1 was the c-Jun NH_2-terminal kinase (JNK)-interacting protein 1 (JIP1, also known as JSAP1) [8]. JIP1 is connecting the kinesin-1 to cargo receptors such as β-amyloid precursor protein (APP) [4, 8]. In many cases, kinesin-1 bind to cargo through it's the C-terminal region of KIF5s and adaptor protein [12]. Previously study had shown that the γ-aminobutyric acid (GABA) type A receptor-associated protein (GABARAP) binds with the C-terminal region of KIF5A and acts as an adaptor protein that links kinesin-1 to GABA type A receptor containing vesicles [9].

Here, we found that the ERICH4 interacts with kinesin-1 through the C-terminal region of KIF5A. Our findings suggest that ERICH4 may serve as an adaptor protein that links kinesin-1 and the clathrin coated vesicles or/and the intraflagellar complex proteins. Future studies are needed to determine the precise intracellular transport mechanisms of the ERICH4 containing cargo by kinesin-1.

Acknowledgment

This research was supported by Basic Science Research Program though the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2022R1F1A1064272).

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 : Glutamate-rich 4와 kinesin superfamily protein 5A와의 결합

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세포내 운반체는 kinesin과 dynein과 같은 미세소관 분자 모터단백질에 의하여 운반된다. Kinesin은 분자 모터단백질의 큰 그룹을 형성하며, kinesin-1은 미세소관 위를 정방향으로 세포내 소기관, 단백질 복합체, 그리고 mRNAs을 운반한다. Kinesin-1은 kinesin superfamily protein (KIF) 5A, 5B, 그리고 5C (또 다른 명칭으로 kinesin장쇄) 그리고 kinesin 단쇄로 구성되어져 있다. Kinesin-1은 KIF5s의 carboxyl (C)-말단 부위를 통하여 다양한 단백질과 결합한다는 사실은 알려져 있지만, 결합단백질에 대하여서는 아직 충분히 밝혀지지 않았다. 본 연구에서는 KIF5A의 C-말단 특정영역과 결합하는 단백질을 효모 two-hybrid system을 사용하여 탐색한 결과, Glutamate-rich 4 (ERICH4)를 분리하였다. ERICH4는 KIF5A의 C-말단 특정영역과 결합하지만, KIF5B와 KIF3A (kinesin-2의 모터단백질)와는 결합하지 않았다. 그리고 KIF5A는 ERICH4의 다른 isoform인 ERICH1과는 결합하지 않았다. 또한 KIF5A은 GST-ERICH4, GST-ERICH4-amino (N)-말단과는 결합하지만 GST-ERICH4-C말단과 GST와는 결합하지 않았다. HEK-293T 세포에 ERICH4와 KIF5A을 발현시켰을 때 ERICH4와 KIF5A는 세포 내의 같은 부위에서 발현하며, ERICH4을 면역침강한 결과 KIF5A와 KLC은 같이 침강하였다. 이러한 결과들은 ERICH4는 kinesin-1이 운반하는 수송체와 KIF5A와의 결합에 매개단백질로의 역할의 가능성을 시사한다.