Pcp-2 Interacts Directly with Kinesin Superfamily KIF21A Protein

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KIF21A is a member of the Kinesin superfamily proteins (KIFs), which are microtubule-dependent molecular motors, anterograde axonal transporters of cargoes. Recently, congenital fibrosis of the extracocular muscles 1 (CFEOM1) has been shown to result from a small number of recurrent heterozygous missense mutations of KIF21A. CFEOM1 results from the inability of mutated KIF21A to successfully deliver cargoes to the development of the oculo-motor neuron or neuromuscular junction. Here, we used a yeast two-hybrid system to identify a protein that interacts with the WD-40 repeat domain of KIF21A and found a specific interaction with Purkinje cell protein-2 (Pcp-2), a small protein also known as L7. Pcp-2 protein bound to the WD-40 domain of KIF21A and KIF21B but not to other KIFs in yeast two-hybrid assays. In addition, this specific interaction was also observed in the glutathione S-transferase pull-down assay. An antibody to Pcp-2 specifically co-immunoprecipitated KIF21A associated with Pcp-2 from mouse brain extracts. These results suggest that Pcp-2 may be involved in the KIF21A-mediated transport as a KIF21A adaptor protein.

Key words: Kinesin, Purkinje cell protein-2, adaptor proteins, WD-40 repeats

Introduction

Intracellular transport is fundamental for cell morphogenesis and function [9]. Microtubules serve as rails for intracellular transportation [10]. The uniform orientation of neuronal microtubules, with plus ends toward the synapse and minus ends toward the cell body, allow plus end-directed kinesins to transport anterograde cargoes to the synapse [7,9,30]. Mutation in neuronal kinesin gene recently has been linked to human neurodegenerative disease, most likely as the consequence of impaired axonal transport [7,30,41]. Genetic alteration of the KIF1B gene causes a substantial decrease in the survival of neuron and perinatal death in kif1b-/- mice as well as progressive muscle weakness in heterozygote mice [41]. A loss-of-function point mutation in the motor domain of KIF1B was detected in human patients with Charcot-Marie-Tooth disease type 2A (CMT2A), which is the most common form of inherited peripheral neuropathies [22,41]. Thus, understanding how neuronal kinesins recognize and transport cargoes has become an important question.

KIFs contain amino acid sequences that are highly conserved in motor domain regions [1,18]. Within the motor domain, there are two conserved sequences that are proximal to the ATP-binding motif and the microtubule-binding domain [1,9,10]. The remaining portions of the molecules are greatly diverged, presumably allowing association with multiple classes of cargo proteins [7,10,14,30]. Recently, it has been clearly demonstrated that several KIFs attach to specific cargoes through interactions with adaptor proteins in these binding regions [7,10,30].

KIF21A, KIF21B and KIF4 comprise one of the subgroups of KIFs [17,18]. This subgroup protein contains a highly conserved motor domain in their N-terminal region, whereas a tail domain in their C-terminal region is less well conserved [18]. KIF21A has a broader spectrum of tissue distribution and contains a domain of seven WD-40 repeats [17]. The WD-40 repeat was originally identified in the β-subunit of heterotrimeric G proteins [6]. Later, it was found in >160 functionally diverse proteins by sequence analysis. An increasing number of WD-40 repeat domain-containing proteins have been found to interact with small structural motifs with high specificity [11]. Modular protein-protein interaction domains have also been identified in other KIFs, for example, tetra-trico peptide repeats (TRP) domains in the kinesin light chain and pleckstrin homology (PH) domains in KIF1A [15,26]. Thus, the KIF21A

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may mediate interactions with their cargoes through WD-40 repeat domain.

CFEOM is a typically nonprogressive disorder of ocular motility with accompanying blepharoptosis [4]. Three distinct phenotypes, CFEOM1-3, are recognized [4]. CFEOM1 has been shown to result from heterozygous missense mutations in the kinesin motor domain encoded by KIF21A [3,39]. The mutated gene KIF21A encodes a motor protein expressed in early development and is involved in anterograde axonal transport [39]. In its mutated state, the axonal transport of a cargo critical to the development of ocular motor nerves may be disrupted, resulting in a primary neuropathy [3,39].

The mechanism how KIF21A interacts with the specific proteins has not yet been elucidated. In this study, we identified Purkinje cell protein-2 (Pcp-2) as a protein that interacts with KIF21A in vitro and in vivo.

Materials and Methods

Plasmid constructs

The coding region of Pcp-2 was cloned into T-vector (Invitrogen, Carlsbad, CA, USA) using by reverse transcriptase polymerase chain reaction (RT-PCR). After EcoRI digestion, the Pcp-2 fragment was inserted into the EcoRI site of plG4-5 (Clontech, Palo Alto, CA, USA). Mouse KIF21A was utilized as a template to amplify the region coding for amino acids (a.a) 1235-1573 using appropriate primers. The amplified fragment was sub-cloned into T-vector. The fragment was then EcoRI-digested and sub-cloned into the EcoRI site of pLexA. The correct orientation of the cDNA inserts was verified by restriction enzyme analysis, and sequence analysis was used to check that they were in-frame. Other molecular procedures were performed according to the standard protocols [29].

Screening of KIF21A-binding proteins by the yeast two-hybrid assay

The Matchmaker LexA two-hybrid system was used for screening according to the manufacturer's manual (Clontech). In brief, a part of the kif21A gene (a.a) 1235-1573) was fused to the DNA-8D region of the pLexA vector, and the plasmid DNA was transformed into yeast strain EGY48 carrying the p8op-lacZ gene. Transformed EGY48 yeast strains containing the KIF21A bait plasmid were transformed with a mouse brain cDNA library [15,35] and the cells were grown on synthetic dextrose (SD) plates supplemented with glucose but with no histidine, tryptophan, or uracil (SD/-His/-Trp/-Ura). The selection of positive clones was performed on an SD/-His/-Trp/-Ura/-Leu plate containing galactose, raffinose, and X-gal. Library plasmids from positive colonies were isolated and rescued using Escherichia coli (E. coli) strain ampicillin-resistant plates. Library inserts were analyzed by restriction enzyme digestion. Unique inserts were sequenced, and DNA sequence analysis was performed with the BLAST algorithm at the National Center for Biotechnology Information (NCBI). After isolation of the plasmids encoding the library clones, these plasmids were tested for interactions of the reporter gene in yeast by the retransformation.

β-Galactosidase activity in liquid cultures of yeast

The strength of the interactions between Pcp-2 and KIF21A was assessed by measuring the β-galactosidase activity in liquid cultures. Yeast was co-transformed with the expression plasmid of the positive clone and the plasmid expressing KIF21A (described above) or other KIFs. Plasmids expressing the tails of KIF1A (aa 400 to the C-terminus), KIF3B (aa 413 to the C-terminus), KIF5B (aa 810 to the C-terminus), KIF17 (aa 939 to the C-terminus) and KIF21B (aa 1301 to the C-terminus), were tested for binding with Pcp-2. The β-galactosidase activity in liquid cultures of yeast was assayed as described previously [15]. In brief, mid-log phase transformed yeast cells were collected and permeabilized with 0.1% sodium dodecyl sulphate (SDS) and chloroform. An excess amount of chromogenic substrate o-nitrophenyl-β-D-galactoside was added in excess to this lysate, and the mixture was incubated at 30°C, and then the reaction was stopped by increasing pH to 11 by the addition of 1 M Na2CO3. 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 and the cell density.

Preparation of antibody

A KIF21A-glutathione S-transferase (GST) fusion protein constructs which contained amino acids 1120-1350 was generated in pET41. This fusion protein was produced in BL21 GOLD (Stratagene, La Jolla CA, USA) bacteria by inducing its expression with 1 mM isopropyl thio-β-D-galactopyranoside (IPTG) (Fisher Biotech, South Australia, Australia), and purified using glutathione-agarose beads.
(Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s protocol. Purified KIF21A-GST fusion protein was used to produce polyclonal rabbit anti-sera against KIF21A. Anti-KIF21A antibody was affinity-purified using Affigel-bound antigen.

Subcellular fractionation, co-immunoprecipitation and Western blot analysis

Subcellular fractionation was performed as previously described [31,35]. Mouse brains were homogenized in ice-cold homogenization buffer (0.32 M sucrose, 4 mM HEPES, pH 7.3) supplemented with protease inhibitors. The homogenate was clarified by centrifugation at 900× g for 10 min followed by centrifugation at 1000× g for 10 min, producing a pellet (P1) and supernatant (S1). The S1 supernatant was centrifuged again at 12,000× g for 15 min, and the resulting supernatant (S2) was saved. For immunoprecipitation of the S2 fraction, the samples were diluted in the same volume of 2× binding buffer (50 mM HEPES, 240 mM KCl, 2 mg/ml BSA, 0.2% Triton X-100, pH 7.4) and incubated overnight with an anti-Pcp-2 antibody (Abgent Inc, San Diego, CA, USA) [25] or with control IgG overnight at 4°C, followed by precipitation with protein-A Sepharose (Amersham Pharmacia, Piscataway, NJ, USA). The beads were collected by brief centrifugation and washed three times with TBST (20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% tween 20). The pellets were eluted and denatured by boiling for 2 min in Laemmli’s loading buffer and then resolved by SDS-PAGE. The gel was transferred to a nitrocellulose membrane and incubated with antibodies against the KIF1A [26], KIF5B [13], KIF17 [32] and KIF21A. Rabbit horseradish-linked secondary antibody (Sigma-Aldrich, St. Louis, MO, USA) was used at a final dilution of 1:2,000, and immunoreactivities were detected using the ECL Western blotting system.

GST pull-down assays

Pull-down assays using GST fusion protein were performed as follows. cDNA encoding of Pcp-2 was cloned in pET41, and the recombinant GST-Pcp-2 fusion protein was expressed in bacterial strain BL21 GOLD (Stratagene) after induction with 1 mM IPTG (Fisher Biotech). The fusion protein was purified using glutathione-agarose beads (Sigma-Aldrich) according to the manufacturer’s protocol. GST alone or GST-Pcp-2 fusion protein were dialyzed for 2 h in PBS using Slide-A-Lyzer (Pierce, Rockford, IL, USA).

Ten μg of each of the GST fusion proteins was then coupled to 50 μl of glutathione-agarose beads for each reaction by incubating at room temperature for 1 hr, followed by rinsing several times with PBS. The mouse brain S2 fraction was incubated overnight at 4°C 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 bound proteins were eluted from the glutathione beads with 100 μl of SDS sample buffer. The samples were boiled for 5 min and then processed for SDS-PAGE and Western blotting with antibody to KIF21A.

Results

Identification of KIF21A interacting proteins by yeast two-hybrid screening

KIF21A has a cluster of negatively charged amino acids of unknown function within stalk domain and seven consensus WD-40 repeats in C-terminal region (Fig. 1A). WD-40 repeats have been found in numerous functionally unrelated proteins and are believed to be involved in protein-protein interactions [5,23,33]. Thus, WD-40 domains of KIF21A play a key role in the recognition and binding of their cargoes. To identify proteins that interact with KIF21A, we screened for proteins that interact with the

![Fig. 1. Identification of the proteins interacting with KIF21A by yeast two-hybrid screening. (A) The domain structure of KIF21A. KIF21A has a WD-40 repeat domain, which was used as a bait in this study. aa, the amino acid residue number. (B) Minimal Pcp-2 binding region in KIF21A. Different truncations of KIF21A were constructed by PCR, and tested in yeast two-hybrid assays for interaction with Pcp-2. *, interaction with Pcp-2; -, no interaction with Pcp-2.](image-url)
WD-40 repeats domain of KIF21A through the yeast two-hybrid system. A screen of approximately 6×10^6 independent transformants on histidine-, tryptophan-, uracil- and leucine-deficient medium yielded two cDNA clones coding for the same protein. These clones were individually isolated, sequenced and subjected to further yeast two-hybrid filter assay to confirm the interactions. We obtained two positive clones which were turned out to be Pcp-2 (Fig. 1B).

To identify the region of KIF21A required for the interaction with Pcp-2, we constructed deletion mutants of KIF21A and analyzed their interactions with Pcp-2 using the yeast two-hybrid assay. WD-40 repeat domain of KIF21A interacts with Pcp-2 in the yeast two-hybrid assay, as shown in Fig. 1B. This experiment demonstrated that the binding domain was located in the C-terminal WD-40 repeat domain of KIF21A corresponding to amino acids 1233-1573.

To quantify the binding affinity of KIF21A to Pcp-2, we measured β-galactosidase activity in liquid cultures of yeast co-transformed with the KIF21A bait plasmid. The interaction of Pcp-2 with KIF1Bβ yielded approximately 250 U of β-galactosidase activity (data not shown), reflecting a binding strength that is sufficient to mediate molecular sorting in vivo [15,35].

To clarify whether Pcp-2 interacts specifically with KIF21A or with other KIFs, we performed yeast two-hybrid assay. Thus, the tails of KIF1A, KIF3B, KIF5B, KIF17, KIF21A and KIF21B were tested for binding with Pcp-2 (Fig. 2). However, there was no detectable binding between Pcp-2 and the tail domains of other major neuronal KIFs (KIF1A, and KIF17) and ubiquitous KIFs (KIF3B, and KIF5B). Pcp-2 interacted with the tail domains of the KIF21A and KIF21B in the yeast two-hybrid system (Fig. 2). This result was not surprising in view of the fact that the KIF21A and KIF21B have seven consensus WD-40 repeats in their C-terminal region [17]. These data indicate that Pcp-2 binds specifically to WD-40 repeats domain of KIF21A.

Pcp-2 is associated with KIF21A at the protein level

In order to investigate the interaction between KIF21A and Pcp-2, we generated the polyclonal KIF21A antibody in rabbits. However, because the KIF21A and KIF21B show a very high degree of homology in their entire length, we thus generated antibody using recombinant KIF21A corresponding to the low-homology region (aa, 1120-1350). The antibody was subjected to immunoblotting on filters loaded with 10 μg of brain crude extract (Fig. 3A). The antibody revealed a single band corresponding to the predicted molecular weight (~180 kDa) [15].

Fig. 2. Interaction between KIFs and Pcp-2. The C-terminal regions of each KIF protein were fused to the pLexA DNA binding domain, and tested for interaction with Pcp-2. Note specific interaction of Pcp-2 with KIF21A but not with KIF1A, KIF3B, KIF5B, or KIF17 (+, +++, interaction with Pcp-2; −, no interaction with Pcp-2).

Fig. 3. Association of KIF21A with Pcp-2 in the GST pull-down assay and co-immunoprecipitation. (A) Specificity of anti-KIF21A antibody. Ten micrograms of crude extracts from an adult mouse brain were loaded on each lane. (B) Proteins in the mouse brain lysate were allowed to bind to GST alone, and GST-Pcp-2 fusion proteins. The elution fractions were resolved by SDS-PAGE, and Western blotting was performed using an antibody to KIF21A. (C) Mouse brain lysates were immunoprecipitated with an anti-Pcp-2 antibody or preimmune serum, and the precipitates were immunoblotted with anti-KIFs antibodies. Input: 10% of the mouse brain lysates used for each co-immunoprecipitation assay.
As an additional demonstration of interaction between KIF21A and Pcp-2 interaction, we performed GST pull-down experiments. This, recombinant GST alone or GST-Pcp-2 fusion proteins were expressed in E. coli, and the purified GST fusion proteins were allowed to interact with mouse brain extracts. This experiment revealed that KIF21A interacted with GST-Pcp-2, but not with GST alone, consistent with the results of yeast two-hybrid assays (Fig. 3B). This result suggests that KIF21A interacts with Pcp-2 at the protein level.

To determine whether the interaction between KIF21A and Pcp-2 occurs in vivo, we performed co-immunoprecipitation experiments using mouse brain extracts. Thus, lysates from mouse brain were incubated with an anti-Pcp-2 antibody, and the immunocomplexes were selectively precipitated with protein G-agarose beads, which were then subsequently separated by SDS-PAGE and immunoblotted with anti-KIFs antibodies (Fig. 3C). As shown in Fig. 3C, Pcp-2 was co-immunoprecipitated with KIF21A but not with KIF1A, KIF5B and KIF17. These results are consistent with the result from yeast two-hybrid assays, and indicate that Pcp-2 is a specific binding partner of KIF21A in vivo.

**Discussion**

The findings of our study provide evidence that Pcp-2 is a candidate molecule receptor of KIF21A. First, we showed that Pcp-2 interacts directly with KIF21A in the yeast two-hybrid system (Fig. 1). Secondly, we demonstrated the interaction of Pcp-2 with KIF21A in GST-pull down assay and co-immunoprecipitation study (Fig. 3). Furthermore we demonstrated that the WD-40 repeat domain of KIF21A is required for the interaction with Pcp-2 (Fig. 1B).

In neuron, newly synthesized proteins are sorted in the Golgi apparatus of the cell body and are then transported to synaptic terminus. Linking of cargoes with the appropriate KIFs must occur with a high degree of specificity. Increasing evidence suggests that KIFs-cargo interaction is mediated by adaptor proteins [8,30]. For examples, syntaxin containing vesicle is attached to the KIF5a by an adaptor protein, syntabinulin, for their transport to synapse; KIF17 forms a complex with mLin10 in the transportation of N-methyl-D-aspartate receptor NR2B subunits to the synapse; KIF3 forms a complex with CAP3 in the transporting of fordrin coated vesicle to the cell periphery; and β1-adap-ter subunit of the AP-1 complex binds to KIF13A to transport the mannose-6-phosphate receptor containing vesicle [21,32,34,35]. Therefore, KIFs recognize and bind to the adaptor proteins to transport membrane cargoes that contain functional membrane proteins. Adaptor proteins are multifunctional proteins with several protein-protein interaction modules that can assemble large protein-protein complexes at the plasma membrane. The use of adaptor proteins appears to be one of the basic mechanisms for the recognition and transport of cargoes [8,30].

Pcp-2 was first identified as a gene candidate to account for the axiathaxia and spontaneous pcd ( Purkinje cell degeneration) in autosomal recessive pcd mice [20,24]. Pcp-2 is abundant in retinal bipolar neurons, cerebellar Purkinje cells, and expression coincides with Purkinje cell development after birth [38]. It is an abundant protein with message levels similar to those of actin and tubulin [25]. Pcp-2 is widely distributed throughout the cytosol including axonal and dendritic compartments [2]. In addition, this protein is developmentally regulated during times of peak synaptogenesis [24,40]. Previous studies are shown that Pcp-2 stimulates neurite formation and modulates neuronal differentiation [40]. The function of Pcp-2 remains still unknown. No gross anatomical or behavioral abnormalities were observed in Pcp-2-knockout mice [12,19,37]. In a recent study, PCP-2 was demonstrated to bind to a Gα2, of heterotrimeric G-proteins in yeast two-hybrid system [16]. This interaction was confirmed in vivo by co-immunoprecipitation from mouse cerebellum and eyes, and co-localized in the distal processes Purkinje cells including axonal endings and dendritic spines [16,27]. Gα2 also directly or indirectly modulates potassium channels and calcium channels in sensory neurons [28]. Gα2 knockout mice have impaired motor control, and shorten survival [28,36]. The simplest model for Pcp-2 action is that it is an adaptor protein in KIF21A dependent trafficking of potassium channel or calcium channel containing vesicle pools. To address this issue, it would be worth to study whether potassium channel or calcium channel can co-immunoprecipitate with KIF21A. Although we did not identify the cargo of KIF21A, it is possible that the mutated gene KIF21A may be disrupted to the axonal transport of a Pcp-2 mediated-cargo critical to the development of ocular motor nerves, resulting in CFEOM1.

Taken together, in this study provides new insights into an important interaction of Pcp-2 as an adaptor protein in-
volved in KIF21A-dependent anterograde transport. It is not yet known where KIF21A binds to Pcp-2. Future biochemical and morphological studies are needed to address this issue.

References


초록: Kinesin superfamily KIF21A와 직접 결합하는 Pcp-2의 규명

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