

Sequences and Phylogenetic Analysis of Squid New Kinesin Superfamily Proteins (KIFs)

Sang-Jin Kim² and Dae-Hyun Seog^{1*}

¹Departments of Biochemistry, ²Neurology, College of Medicine, Inje University, Busan 614-735, Korea

Received December 26, 2011 / Revised January 17, 2012 / Accepted January 17, 2012

The movement of vesicles from the neuronal cell body to specific destinations requires molecular motors. The squid giant axon represents a powerful model for studies of the axonal transport mechanism because the axoplasm can readily be separated from the sheath by simple extrusion. In a previous study, vesicular movements in the axoplasm of the squid giant axon were inhibited by the kinesin antibody. In the present study, we cloned and sequenced the cDNAs for squid brain KIFs. Amplification of the conserved nucleotide sequences of the motor domain by polymerase chain reaction (PCR) using first-strand cDNAs of the squid optic lobe identified six new KIF proteins. Motif analysis of the motor domains revealed that the squid KIFs are homologous to the consensus sequences of the mouse KIFs. The phylogenetic tree generated by using the maximum parsimony (MP) method, the neighbor-joining (NJ) method, the minimum evolution (ME) method, and the maximum likelihood (ML) method showed that squid KIFs are closest to mouse KIFs. These data prove the phylogenetic relationships between squid KIFs and mouse ones.

Key words : Kinesin-I, molecular motors, giant axon, phylogenetic tree

Introduction

After protein synthesis, proteins are transported to specific destinations within the neuron as membranous organelle or vesicle [4]. The movement of organelle and vesicle from cell body to specific destinations requires molecular motor proteins. Kinesin is a molecular motor protein that generates ATP-dependent movements along microtubules of organelle and vesicle [5].

The squid giant axon provides a very simple preparation for the collection of bulk quantities of transport vesicles, and this greatly facilitates intracellular transport study of the vesicles. Thus, the squid KIFs are excellent candidates for modeling neuronal vesicle transport. The first kinesin (Kinesin-1) was identified in a biochemical fraction of squid giant axon and mammalian nervous tissue for proteins that generate microtubule-based motility *in vitro* [13,14]. Kinesin-1 is a heterotetramer composed of two kinesin heavy chains (KHC) and two kinesin light chain (KLC). The KHC (KIF5A, KIF5B, and KIF5C) contains an amino (N)-terminal motor domain, a long coiled-coil stalk interrupted by a central hinge, and a globular tail domain [5,9]. Kinesin-2 (KIF3A, and KIF3B) was first identified biochemically in sea

urchin eggs and found to contain two distinct motor-containing polypeptide chains that come together to form a heterodimer [5]. Bound to this motor tail domain is tightly associated subunit (KAP3) with an armadillo repeat domain that is known to mediate protein-protein interactions. Another Kinesin-2 gene (KIF17), which current evidence indicates encodes a protein that forms homodimers [4]. Kinesin-3 (KIF1A, KIF1B, and KIF1C) motor was discovered in a mutant screen in *C. elegans*, where null mutations cause paralysis due to a failure to transport synaptic vesicles to the pre-synaptic terminals of motor neurons [3]. Kinesin-3 is predominantly monomeric, in contrast to other kinesins, which are dimeric or tetrameric [4]. Kinesin-4 (KIF4, KIF21A, and KIF21B) has been implicated in chromosome segregation during the mitotic phase. In the mitotic stage, Kinesin-4 was localized to mitotic spindles [1]. Kinesin-14 (KIFC1, KIFC2, and KIFC3) forms a homodimer without associated polypeptides. Kinesin-14 has a motor domain in the COOH-terminal region. This family members exhibit a microtubule minus end-directed motility [5].

Molecular biological approaches were instrumental in identifying members of KIFs [1,8,9]. The amino acid motifs in the N-terminal motor domain of KIFs, such as IFAYGQT, DLAGSE, and SSRSH, are highly conserved among species [1]. Fragments flanked by these sequences can therefore be amplified by PCR using degenerate oligonucleotide primers.

*Corresponding author

Tel : +82-51-890-6974, Fax : +82-51-894-5801

E-mail : daehyun@inje.ac.kr

This strategy identified many KIFs in various organisms, such as *Drosophila*, mouse, *Xenopus*, yeast, and rat [7,8]. Although KIFs have been cloned from several organisms, only two genes have been identified within squid [7]. To improve the understanding of the role KIFs in the giant axon of the squid, based on PCR approach using degenerate oligonucleotide primers, six new members of KIF were identified in the squid. In this report the partial amino acid sequences of these new KIFs are presented with the classification based on molecular evolutionary analysis.

Materials and Methods

Animal

Adults squids (*Loligo forbesi*) obtained by trawling at the Marine Biological Research Laboratory (Tokyo University, Tokyo, Japan) were kept in a large rectangular tank with circulating sea water (12°C) and used within 3 days capture. Squid optic lobes were dissected and snap frozen in liquid nitrogen until use in RNA extraction.

mRNA purification and cDNA synthesis

To isolate mRNA for the amplification of the cDNA fragments, 5 g of squid optic lobe was homogenized in 10 ml Trizol (Gibco-BRL, Gaithersburg, MD, USA). Aliquots were frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated from the homogenate according to the manufacturer's instructions. mRNA was isolated with the PolyAtract mRNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions and used to synthesize double-stranded DNA with the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA, USA).

PCRs to identify new KIFs

The primers were designed according to the conserved amino acid sequences IFAYGQT and DLAGSE in the motor domain of mouse KIFs. The oligonucleotide primers used in this study are listed in Table 1. For all PCRs, AmpliTaq

DNA polymerase (Perkin - Elmer) and GeneAmp PCR system 9600 (Perkin - Elmer) were used. Primers IFAYGQT and DLAGSE were used to amplify squid optic lobe first-strand cDNA; the reaction program was as follows: 30 cycles at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 1 min. The reaction mixture contained 0.5 µl of cDNA solution (directly used from cDNA synthesis after heat inactivation), 1 µl of 10 mM dNTP, 5 units of AmpliTaq DNA polymerase, 100 pmol of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM MgCl₂ in a total volume of 50 µl. Single band appeared when separated by agarose gel electrophoresis and recovered by QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). DNA fragment was directly ligated into the T/A overhang of the pGEM-T vector (Promega) according to manufacturer's instructions. The ligations were electroporated into TB1 *Escherichia coli* and plated on IPTG, X-Gal, and ampicillin-containing agar plates. Sequencing was performed by using the Dyeamic ET primer and Deza sequencing kit (Amersham Pharmacia, Piscataway, NJ, USA) and an Applied Biosystems 377 DNA sequencer (Perkin-Elmer).

Sequence alignment and molecular evolutionary analysis

All KIFs, the amino acid sequences between IFAY and LAGSE motifs were subjected to phylogenetic analysis. The motor domain of new cloned KIFs was aligned with CLUSTALW software by the neighbor-joining method. A phylogenetic tree of the motor domain sequences of the KIF was generated using the Maximum Parsimony (MP) method [2], the Neighbor-Joining (NJ) method [11], the Minimum Evolution (ME) method [10], and the Maximum likelihood (ML) method [6] based on the JTT matrix-based model. The confidence limits on the tree were calculated by performing bootstrap sampling 1,000 times. The region between IFAYGQT and DLAGSE was used for the alignment. Mouse KIFs sequences were obtained from the NCBI database.

Table 1. Degenerate oligonucleotide primers used in this study

Primer	Sequence
IFAYGQT	5'-AT(T/C/A),TT(T/C),GCL,TA(T/C),GGI,CA(A/G),AC-3'
DLAGSE	5'-(C/T)TC,I(A/C)(A/T),ICC,IGC,IAG,(A/G)TC-3'
DLAGSE-1	5'-CTC,(A/G)CT,ICC,IGC,(C/T)A(A/G),(A/G)TC,IA-3'
DLAGSE-2	5'-CTC,(A/G)CT,ICC,IGC,(A/G)AG,(A/G)TC,IA-3'
DLAGSE-3	5'-CTC,IAG,ICC,IGC,(C/T)A(A/G),(A/G)TC,IA-3'

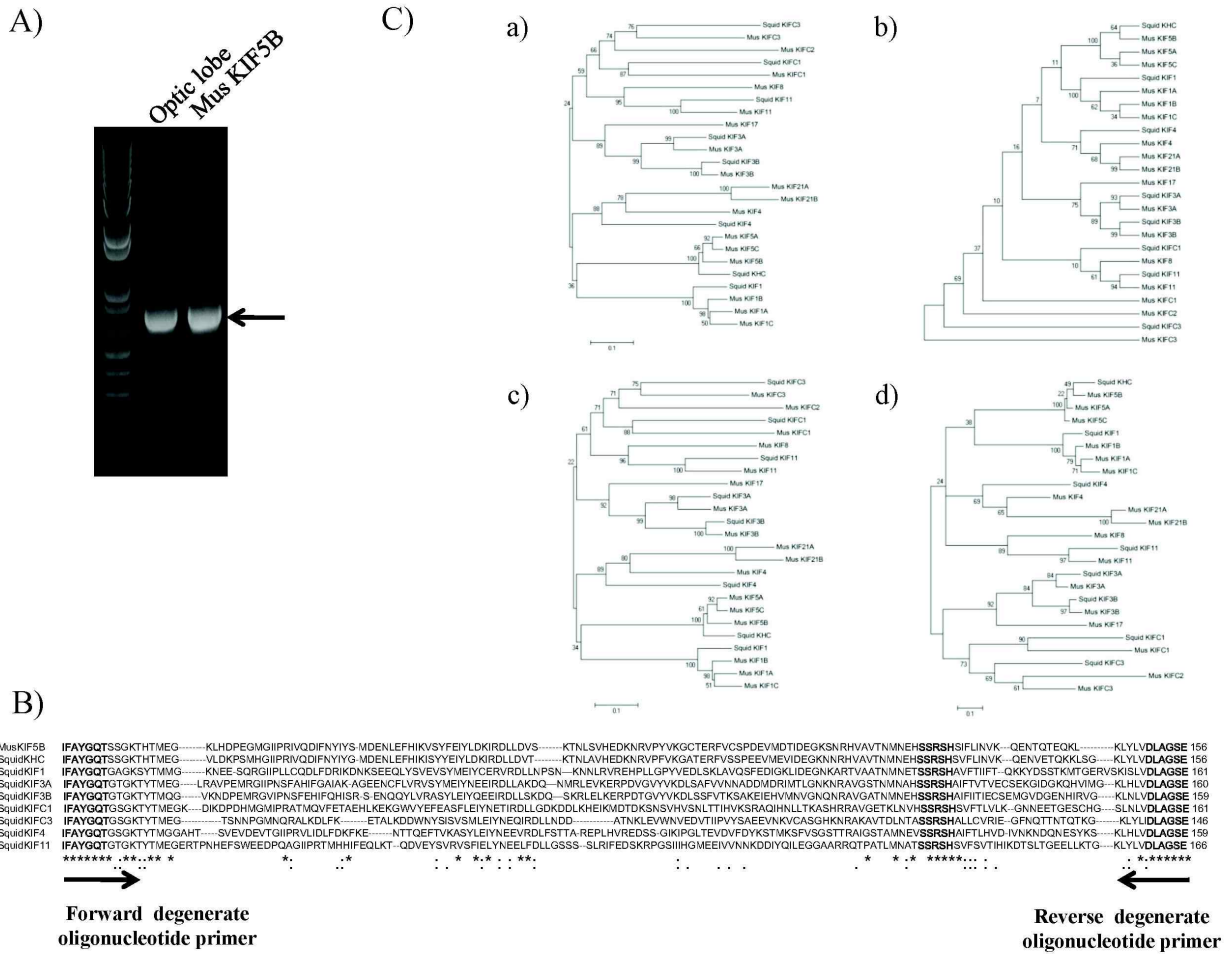


Fig. 1. Comparison of partial amino acid sequences of squid new KIFs. (A) PCR amplification of squid KIFs. PCR was conducted by using squid optic lobe first-strand cDNA and degenerate primers for the conserved amino acid sequences IFAYGQT and DLAGSE in the motor domain. Arrow indicates PCR amplified DNA. (B) Comparison of squid KIFs. Partial amino acid sequence from IFAYGQT to DLAGSE in motor domain of mouse KHC and squid KIFs are aligned. Arrows indicate the positions of the primers. Asterisks are identical amino acids. Dots are similar amino acids. Amino acid residues conserved among all the members are bolded. (C) Phylogenetic tree shows the relative evolutionary divergence of squid KIFs and mouse KIFs. The amino acid sequence between IFAYGQT and DLAGSE was used to align the sequences. The amino acid sequence of mouse KIFs were from GenBankTM. The evolutionary history was inferred by using the MJ method (a), the NP method (b), the ME method (c), and the ML method (d). The confidence limits on the tree were calculated by performing bootstrap sampling 1,000 times. The tree with the highest log likelihood (-4116.9175) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Results and Discussion

To obtain sequences of squid KIFs, PCR was conducted by using squid optic lobe first-strand cDNAs and degenerate primers. Upstream primer sequences were derived from a putative ATP-binding motif and downstream primers from a conserved region 5' to the second microtubule binding site (Table 1). For most of the known mouse KIFs, the nucleotide sequences between IFAYGQT and DLAGSE correspond to

~450 bp [1,9]. The motor domain primers specific for mouse KIF5B allowed amplification of ~450 bp length expected for that cDNA (Fig. 1A). The single band around 450 bp was cut out and subcloned into cloning vector. From a total 341 PCR fragments that were cloned in the cloning vector, 75 clones were found to encode members of KIFs by sequence analysis (Table 2). The remaining 266 clones were not related to KIF. The KIFs presented here were identified by the following criteria: conservation of upstream Walker A

Table 2. Summary of searching the new KIFs

No. of clones sequenced	Identification
24	KIF3B
20	KIF3A
15	KHC
5	KIF4
5	KIF1
2	KIFC1
2	KIF11
2	KIFC3

ATP-binding motifs and a LAGSE or similar sequence \approx 150-200 amino acid residues down-stream, an YXXXXDL motif where X is any amino acid, and a SSRSH motif located between the Walker A and LAGSE sequences. The PCR fragments encoding the KIF fell into eight classes (Table 2). Each class encoded a predicted protein that was homologous to KHC in the region between forward primer and reverse primer (Fig. 1B).

Fig. 1B shows the alignment of the amino acid sequences translated from the PCR products together with the previously identified ones. Squid KIF1 is 79% identical in amino acid sequence to mouse KIF1A, and is 46% identical to mouse KIF5B. Thus squid KIF1 is considered to be a mouse counterpart of KIF1A. Squid KHC is 85% identical in amino acid sequence to mouse KIF5B. Squid KIF3A is 83%, and 67% identical in amino acid sequence to mouse KIF3A, and mouse KIF3B, respectively. Also, Squid KIF3B is 92%, and 69% identical in amino acid sequence to mouse KIF3B, and mouse KIF3A, respectively. Squid KIF11 is 67% identical in amino acid to mouse KIF11. Squid KIF4 is 53%, 40%, and 38% identical in amino acid to mouse KIF4, mouse KIF21A, and mouse KIF21B, respectively. Squid KIFC1 is 46% identical in amino acid sequence to mouse KIFC1. And, Squid KIFC3 is 53% identical in amino acid sequence to mouse KIFC3.

To classify the newly identified KIFs, molecular evolutionary analysis was performed. Phylogenetic trees were obtained by the MJ method (Fig. 1C, a), the NP method (Fig. 1C, b), the ME method (Fig. 1C, c), and the ML method (Fig. 1C, d). A phylogenetic tree of the amino acid sequences of squid KIFs versus mouse KIFs revealed the level of similarity of the sequences of these two organisms of KIFs (Fig. 1C). These results allow the new information to assess whether a newly identified squid KIF is a homologue of previously identified motors in other organisms.

Until now, only two kinds of KIF, have been identified in the squid optic lobe. In this study, six new KIFs were identified. And they shared homologous domains which include a putative ATP-binding site and a microtubule-binding site of KHC [7,14]. It was clearly demonstrated that the conserved motor domain of KHC was both necessary and sufficient to move along microtubules in the presence of ATP *in vitro* [12,13]. Taking these data together, the squid axon contains at least eight KIFs. The squid KIFs have properties that make it an excellent candidate for modeling neuronal vesicle transport in axon. Future studies on biochemical characterization are necessary to answer the mechanism of axoplasmic KIFs.

Acknowledgment

This work was supported by an Inje University Research Grant for 2010.

Refernces

- Aizawa, H., Y. Sekine, R. Takemura, Z. Zhang, M. Nangaku, and N. Hirokawa. 1992. Kinesin family in murine central nervous system. *J. Cell Biol.* **119**, 1287-1296.
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**, 783-791.
- Hall, D. H and E. M. Hedgecock. 1991. Kinesin-related gene *unc-104* is required for axonal transport of synaptic vesicles in *C. elegans*. *Cell* **31**, 837-847.
- Hirokawa, N., S. Niwa, and Y. Tanaka. 2010. Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease. *Neuron* **68**, 610-638.
- Hirokawa, N. 1998. Kinesin and dynein superfamily proteins and the mechanism of organelle transport. *Science* **279**, 519-526.
- Jones, D. T., W. R. Taylor, and J. M. Thornton. 1992. The rapid generation of mutation data matrices from protein sequences. *Comput. Appl. Biosci.* **8**, 275-282.
- Lawrence, C. J., R. K. Dawe, K. R. Christie, D. W. Cleveland, S. C. Dawson, S. A. Endow, L. S. Goldstein, H. V. Goodson, N. Hirokawa, J. Howard, R. L. Malmberg, J. R. McIntosh, H. Miki, T. J. Mitchison, Y. Okada, A. S. Reddy, W. M. Saxton, M. Schliwa, J. M. Scholey, R. D. Vale, C. E. Walczak, and L. Wordeman. 2004. A standardized kinesin nomenclature. *J. Cell Biol.* **167**, 19-22.
- Miki, H., M. Setou, K. Kaneshiro, and N. Hirokawa. 2001. All kinesin superfamily protein, KIF, genes in mouse and human. *Proc. Natl. Acad. Sci. USA* **98**, 7004-7011.
- Nakagawa, T., Y. Tanaka, E. Matsuoka, S. Kondo, Y. Okada, Y. Noda, Y. Kanai, and N. Hirokawa. 1997. Identification and classification of 16 new kinesin superfamily (KIF) pro-

- teins in mouse genome. *Proc. Natl. Acad. Sci. USA* **94**, 9654-9659.
10. Rzhetsky, A. and M. Nei. 1992. A simple method for estimating and testing minimum evolution trees. *Molecular Biology and Evolution* **9**, 945-967.
 11. Saitou, N., and M. Nei. 1987 The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406-425.
 12. Tanaka, Y., Y. Kanai, Y. Okada, S. Nonaka, S. Takeda, A. Harada, and N. Hirokawa. 1998. Targeted disruption of mouse conventional kinesin heavy chain, kif5B, results in abnormal perinuclear clustering of mitochondria. *Cell* **93**, 1147-1158.
 13. Vale, R. D., T. S. Reese, and M. P. Sheetz. 1985. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell* **42**, 39-50.
 14. Yang, J. T., W. M. Saxton, R. J. Stewart, E. C. Raff, and L. S. Goldstein. 1990. Evidence that the head of kinesin is sufficient for force generation and motility *in vitro*. *Science* **249**, 42-47.

초록 : 오징어과의 Kinesin Superfamily Proteins (KIFs)의 유전자분석 및 계통분석

김상진¹ · 석대현*

(인제대학교 의과대학 생화학교실, ¹신경과학교실)

분자 운동 단백질은 신경세포 내의 세포체에서 특정 목적지까지 소포를 이동시키는데 관여한다. 오징어의 거대 축삭은 간단한 제거조작으로 축삭을 분리 가능하기 때문에 신경세포내 물질이동기전 연구의 좋은 모델로 활용 가능하다. 이전연구에서 오징어 거대축삭의 소포들은 미세소관을 따라 이동하는 키네신 항체에 의하여 운반됨이 확인되었다. 본 연구는 오징어 뇌에 존재하는 키네신들을 크로닝하고, 분리된 유전자의 분석을 행하기 위하여 키네신 운동 도메인에서 잘 보존된 아미노산 배열에 해당되는 영역에 DNA primer를 이용하여 새로운 6종류의 키네신을 분리하였다. 오징어의 키네신들과 생쥐의 키네신들의 motor 영역의 아미노산분석에서 보존된 영역이 존재하며, Maximum Parsimony (MP) 방법, Neighbor-Joining (NJ) 방법, Minimum Evolution (ME) 방법, 그리고 Maximum likelihood (ML) 방법을 기초로 한 계통분석에서 생쥐의 키네신과 높은 상동성을 나타내었으며, 또한 계통수에서도 높은 상관관계가 확인되었다.