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KIF1Bβ2, Capable of Interacting with CHP, Is Localized to Synaptic Vesicles[1]

Norihiro Nakamura, Yoshihide Miyake, Masafumi Matsushita, Shingo Tanaka, Hiroki Inoue, and Hiroshi Kanazawa[2]

Department of Biological Science, Graduate School of Science, Osaka University, Machikaneyama, Toyonaka, Osaka 560-0043

Received June 13, 2002; accepted July 15, 2002

Kinesin family proteins are microtubule-dependent molecular motors involved in the intracellular motile process. Using a Ca^{2+}-binding protein, CHP (calcineurin B homologous protein), as a bait for yeast two hybrid screening, we identified a novel kinesin-related protein, KIF1Bβ2. KIF1Bβ2 is a member of the KIF1 subfamily of kinesin-related proteins, and consists of an amino terminal KIF1B-type motor domain followed by a tail region highly similar to that of KIF1A. CHP binds to regions adjacent to the motor domains of KIF1Bβ2 and KIF1B, but not to those of the other KIF1 family members, KIF1A and KIF1C. Immunostaining of neuronal cells showed that a significant portion of KIF1Bβ2 is co-localized with synaptophysin, a marker protein for synaptic vesicles, but not with a mitochondria-staining dye. Subcellular fractionation analysis indicated the co-localization of KIF1Bβ2 with synaptophysin. These results suggest that KIF1Bβ2, a novel CHP-interacting molecular motor, mediates the transport of synaptic vesicles in neuronal cells.

Key words: calcineurin-homologous protein, kinesin-related protein, microtubule, synaptic vesicle, vesicular transport.

Intracellular trafficking of secretory and transmembrane proteins takes place in transporting vesicles that bud from, and fuse with, sequential endomembrane compartments. The organelles and carrier vesicles are localized in association with the polarized microtubule arrays. For example, the endoplasmic reticulum and early endosomes are usually dispersed along microtubules toward the plus ends extending radially to the cell periphery, whereas the Golgi apparatus, late endosomes, and lysosomes are often clustered near the minus ends located at the cell center in non-polarized cells. The cargo-laden vesicles appear to travel along cytoskeletal tracks powered by molecular motor proteins. Thus, efficient transport through the secretory and endocytic trafficking is thought to be achieved due to the microtubule-dependent distribution (*1–4*).

Many members of the kinesin, dynein, and myosin superfamilies have been implicated as molecular motors in vesicle and organelle trafficking. Long-range vesicle transport along microtubules involves kinesin and dynein motors, whereas short-range transport is mediated by myosin motors on actin filaments (*5–7*). Kinesin and most kinesin-related proteins drive movement toward the plus ends of microtubules at the cell periphery, and dynein and some members of the kinesin family mediate it toward the minus ends (*8*). The plus-end directed kinesin-related proteins comprise over 30 members classified into eight subgroups (*9*). They share a conserved motor domain of ~350 amino acid residues, which contains binding sites for ATP and microtubules, at their amino-termini. Outside of the motor domains, their amino acid sequences differ widely, reflecting their different functions. Similar to the originally identified kinesin, most members of the kinesin family have a central α-helical region that mediates the homo-dimerization of kinesin heavy chains. The tail of kinesin is thought to bind cargo and accessory proteins. KIF1A and KIF1Bα are members of the KIF1/Unc104 family of monomeric motors, and transport membrane-bound organelles, synaptic vesicles and mitochondria, respectively (*10, 11*). Although they show a high degree of similarity in their amino-terminal motor domains, the sequences of their carboxyl termini are more divergent, which supports the view that the tails of these motors confer the cargo-selection specificity. KIF1C and KIF1D, which have been reported as the third and fourth members of the KIF1/Unc104 subfamily, possess divergent tail regions, whereas their cargo molecules/compartments remain unknown (*12–14*).

CHP/p22 (calcineurin B homologous protein) is a Ca^{2+} binding EF-hand protein of 22 kDa (*15–17*). This protein shows significant similarity to the regulatory B subunit of a heterodimeric protein phosphatase, calcineurin, which is known to participate in a number of cellular processes and Ca^{2+}-dependent signal transduction pathways (*18*). Although CHP belongs to the calcineurin B family based on

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[2] To whom correspondence should be addressed. Tel: +81-6-6850-5820, Fax: +81-6-6850-5817, E-mail: kanazawa@bio.sci.osaka-u.ac.jp

Abbreviations: CHP, calcineurin homologous protein; DMEM, Dulbecco's modified Eagles' medium; FITC, fluorescein isothiocyanate; FHA, forkhead-associated; KIF, kinesin-family; MBP, maltose-binding protein; MEM, minimal essential medium; NHE, Na^+/H^+ exchanger; PCR, polymerase chain reaction; PH, pleckstrin homology.

its primary sequence, recent studies suggested that it should be placed in a separate subfamily (*17, 18*). CHP was originally identified as a molecule required for the transcytotic trafficking of polymeric IgA receptors to the apical plasma membrane (*15*). Further studies showed that CHP regulates plasma membrane Na^+/H^+ exchangers (NHEs) by associating with the cytoplasmic domains of NHEs (*16, 17, 19*). We have recently shown that CHP interacts with not only NHEs, but also a serine/threonine protein kinase, DRAK2, involved in apoptotic cell death (*17*). Although the physiological role of CHP has not been defined, these findings raise the possibility that CHP acts as a regulator for a wide variety of target proteins. Accordingly, we searched for proteins that interact with CHP in a rat brain cDNA library using the yeast two-hybrid system, and found a kinesin-related protein. Here we describe the cloning and characterization of a new member of the KIF1/Unc104 subfamily, KIF1Bβ2, capable of interacting with CHP.

EXPERIMENTAL PROCEDURES

Cell Culture—Embryonic hippocampal cell cultures were prepared from the hippocampi of 18-day-old fetal rats as described previously (*20*). The hippocampi were treated with 0.25% trypsin at 37°C for 10 min, and then incubated in the presence of 10 μg/ml DNaseI for 5 min. The dissociated neuronal cells were washed with DMEM containing 10% fetal calf serum (FCS), plated at 2×10^5 cells/cm^2 on polyethyleneimine-coated coverslips, and then cultured in the medium containing 10 μM cytosine-β-D-arabinofuranoside hydrochloride. The cells were fed once every 7 days. Neuro2A and NB-1 cells were cultured in MEM and RPMI-1640, respectively, supplemented with 10% FCS. All media contained non-essential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Yeast Two-Hybrid Screening—The entire coding sequence of rat CHP was subcloned into the *Bam*HI-*Sal*I sites of the bait vector pGBTK (*17*) to generate pGBTK-CHP. This placed the CHP sequence in frame with the DNA binding domain of the Gal4 transcriptional activator. Bait strains were generated by transforming pGBTK-CHP into *Saccharomyces cerevisiae* strains HF7c (*MAT***a** *ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2::GAL1-HIS3 URA3::8(GAL4 17mers)3-CYC1-lacZ*) and SFY526 (*MAT***a** *ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3,112 gal4-542 gal80-538 URA3::GAL1-lacZ*) according to the manufacturer's instructions (CLONTECH). HF7c cells were transformed with a rat brain cDNA library constructed on pGAD10 (CLONTECH), and then plated onto a synthetic minimal medium containing 2.5 mM 3-aminotriazole, but lacking histidine, tryptophan, and leucine (1×10^6 transformants screened). The colonies were restreaked onto fresh selective plates containing 5 mM 3-aminotriazole. Candidate His^+ clones were further assayed for β-galactosidase activity with the HF7c and SFY526 strains according to the manufacturer's instructions (CLONTECH). The prey plasmid was isolated from the cells and the nucleotide sequence of the cDNA was determined using standard methods (*21, 22*).

cDNA Cloning—Colonies (6.4×10^5) of *Escherichia coli* cells carrying the rat brain cDNA library were replica-plated onto Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech), and then screened by hybridization with the ^{32}P-labeled cDNA clone originally isolated on the two-hybrid screening. Hybridizing clones were isolated, purified, and sequenced using standard methods (*21*).

Northern Blotting—Total RNAs (20 μg) from rat tissues were electrophoresed and then blotted onto a Hybond-N^+ membrane. A ^{32}P-labeled DNA fragment of rat KIF1Bβ2 cDNA (3074–3759 nt, numbered from the first letter of the initiation codon) was used as a probe.

Antibodies—The monoclonal antibody for synaptophysin (mouse clone SY38) was purchased from Chemicon. Alexa-546 and FITC-conjugated secondary antibodies were from Molecular Probes and American Qualex (San Clemente, CA), respectively. Two kinds of KIF1Bβ2 antisera were raised by immunizing rabbits. One (CBD antibody) was generated against a bacterially expressed rat KIF1Bβ2 fragment (aa 301–554) tagged with 6x histidine. The other was raised against a synthetic KIF1Bβ2 peptide (aa 396–435; DDYSGSGGKYLKDF) conjugated to keyhole limpet hemocyanin. The antibodies were affinity-purified using the KIF1Bβ2 fragment (aa 301–554) fused with maltose-binding protein (MBP) (*23*). Anti-rat CHP antibodies were as described previously (*17*).

Immunofluorescence Microscopy—Fixed cells were permeabilized (*24*), incubated with the antibodies against KIF1Bβ2, synaptophysin, or CHP, and then reacted with fluorescently labeled secondary antibodies. For double staining with mitochondria, cells were pre-incubated with 100 nM MitoTracker Red CMXRos (Molecular Probes) at 37°C for 15 min prior to fixation (*25*). Samples were filled in VectaShield (Vector Laboratories) and then observed under an Olympus BX51 Microscope.

Subcellular Fractionation—A whole rat brain was rinsed with phosphate-buffered saline, and then homogenized in a buffer comprising 150 mM potassium acetate, pH 7.4, 1 mM $MgCl_2$, 1 mM ethyleneglycol bis(β-aminoethylether)-tetraacetic acid, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 2 mM phenylmethylsulfonyl fluoride. The postnuclear supernatant obtained on centrifugation at 460 ×*g* was layered on the buffer containing 2 M sucrose, and then spun at 100,000 ×*g* for 30 min. The subcellular membranes at the interface between the 0 and 2 M sucrose layers were collected, resuspended in the buffer containing 1.8 M sucrose, and then laid at the bottom of a sucrose gradient composed of eight 1 ml layers of the following: 0.1, 0.2, 0.3, 0.4, 0.8, 1.0, 1.2, and 1.5 M sucrose in the buffer. The gradient was centrifuged at 170,000 ×*g* for 24 h, and then fractionated from the top into 19 fractions (0.5 ml each). The proteins in each fraction were precipitated with ice-cold trichloroacetic acid, and then subjected to Western blotting analysis.

Recombinant KIF1 and CHP Proteins—PCR fragments encoding the CHP-binding regions of rat KIF1Bβ2 (aa 301–554) with and without the 40 amino acid insertion, mouse KIF1A (aa 295–508), and rat KIF1C (aa 294–518) were introduced into the *Bam*HI-*Sal*I sites of pMAL-cRI (New England BioLabs) to generate expression plasmids for in frame fusion proteins with MBP. The entire coding sequence of rat CHP was inserted into the *Nde*I–*Hin*dIII sites of pET21b (Novagen). This placed the CHP sequence in frame with 6xhistidine tag. The MBP-KIF1 and CHP-6xHis proteins were expressed in *E. coli*, and then affinity-purified using amylose-resin (New England BioLabs) and Ni-NTA-resin (Qiagen), respectively.

In Vitro Binding Assay—The recombinant CHP (10 μg) was incubated with the MBP-KIF1 fusion proteins (5 μg) immobilized on 25 μl of amylose-resin in 20 mM Tris-HCl, pH 7.4, containing 200 mM NaCl, 1 mg/ml bovine serum albumin, and either 1 mM ethylenediaminetetraacetic acid or 2 mM $CaCl_2$ for 30 min at 4°C. After washing the resins with the buffer, the proteins were eluted by incubation in SDS-sample buffer at 50°C, subjected to SDS-polyacrylamide gel electrophoresis, and then visualized by Coomassie brilliant blue staining.

Materials—Cell culture reagents were obtained from Sigma and GIBCO BRL. Other chemicals were from Sigma, unless otherwise specified.

RESULTS

Identification of a CHP-Binding Protein on Yeast Two-Hybrid Screening—To identify protein(s) binding to CHP, we employed the yeast two-hybrid system (*26*). We screened a rat brain cDNA library using rat CHP cDNA as a bait, and found one positive clone. The cDNA fragment encodes 254 amino acid residues highly similar to regions of KIF1B proteins, KIF1Bα, KIF1Bβ (aa 295–508), and KIF1Bp204 (aa 301–554) (*10, 27, 28*), but lacking both initiation and termination codons (see Fig. 1A). This cDNA clone contained an insertion sequence of 40 amino acids in length, which is conserved in KIF1Bp204. The homology of the sequences of the rat clone and mouse KIF1B without the insertion was 98%. The sequence similarity to other members of the KIF1 family, mouse KIF1A (*11*), human KIF1C (*13*), and rat KIF1D (*12*), was 83, 71, and 69%, respectively.

Cloning of the Entire Sequence of the Kinesin-Related Protein Capable of Interacting with CHP—We cloned cDNAs containing the full-length open reading frame of the KIF1B-related protein from the rat brain library, which was probed with the cDNA isolated on the two-hybrid screening. The cDNA cloned encoded a single protein consisting of 1816 amino acids, with a predicted molecular weight of 204 kDa (Fig. 1A). The deduced amino acid sequence is highly similar to those of mouse KIF1Bp204 (99% identity) and KIF1Bβ (98% identity) (*27, 28*). The insertion sequence of 6 amino acids in length found in the mouse KIF1Bp204 was conserved in this rat clone (aa 289–294). The amino-terminal region (aa 1–706) containing the motor domain was quite similar to that of mouse KIF1B (98 % identity) (Fig. 1, B and C). The P-loop sequence (Gly-97–Ser-104) characteristic of ATP- or GTP-binding proteins (*29–31*) and the nucleotide binding motif of kinesins (*32, 33*) were conserved in the motor domain. Like other kinesin-related proteins, this gene product is capable of binding

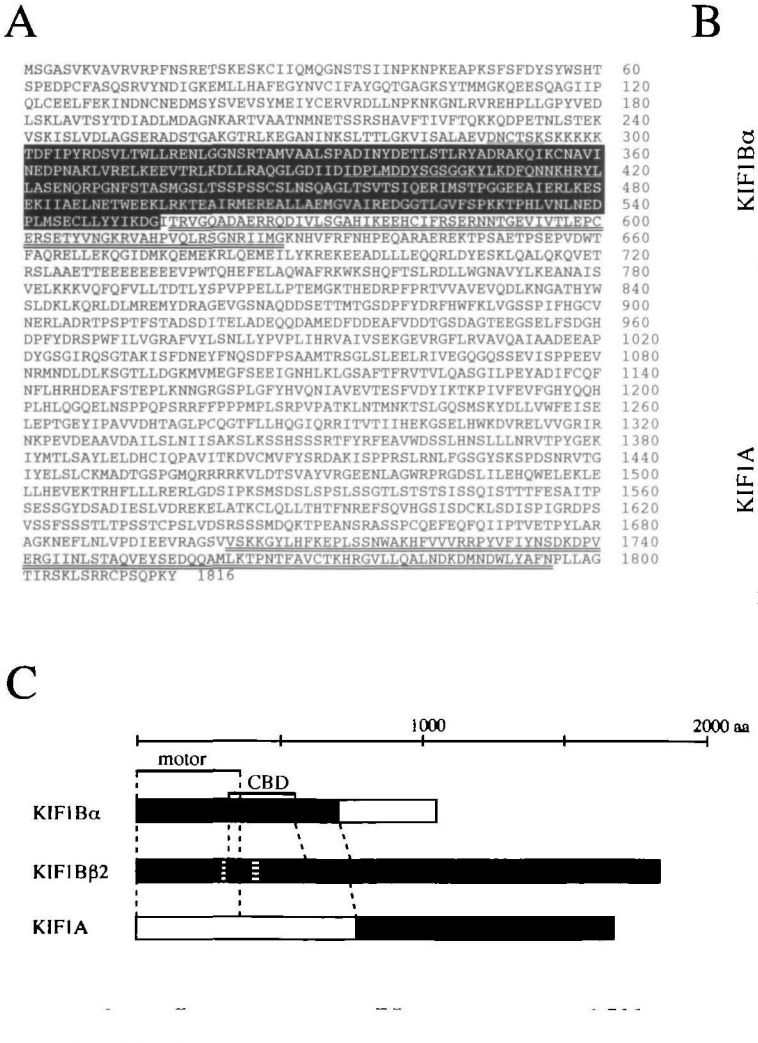

Fig. 1. **Sequence and domain structure of rat KIF1Bβ2.** (A) The amino acid sequence of rat KIF1Bβ2. The CHP-binding domain, and insertions of 6 and 40 amino acids in length are inverted and underlined, respectively. The FHA and PH domains are double-underlined. (B) Harr plot analyses of rat KIF1Bβ2 (horizontal) compared with mouse KIF1Bα and KIF1A (vertical). The intensities of the slanting bars correspond to the degrees of similarity between two proteins. (C) Domain structures of KIF1Bβ2, KIF1A, and KIF1Bα. Regions similar in KIF1A and KIF1Bα are indicated by gray and closed bars, respectively. The 6 and 40 amino acid insertions in KIF1Bβ2 are indicated by stripes. The motor and CHP binding (CBD) domains are also indicated.

to microtubules *in vitro* (data not shown). The CHP-binding region (Thr-301–Gly-554) is located at the carboxyl-terminal end of the motor domain (Fig. 1C). Close to the motor domain, the sequence contains a forkhead-associated (FHA) domain (Thr-556–Gly-627, Fig. 1A), which is also found in the other kinesins belonging to the KIF1/Unc104 family (*34*). The carboxyl-terminal region (aa 707–1816) exhibits significantly high homology to that of KIF1A (63% identity) rather than that of KIF1Bα (11% identity) (Fig. 1, B and C). The pleckstrin homology (PH) domain (*35*) found in the C-terminal tail domain of KIF1A (Val-1581–Gln-1674), and proteins involved in cellular signalling such as phospholipase C-γ (*36–38*) and Bruton's tyrosine kinase (*39, 40*) was conserved in the deduced sequence (Val-1702–Gln-1795, Fig. 1A). We designated this molecule as KIF1Bβ2 to distinguish it from KIF1Bα and KIF1Bβ.

Interaction of CHP with KIF1 Proteins—To confirm the interaction between KIF1Bβ2 and CHP, *in vitro* association was examined using recombinant proteins. A bacterially expressed CHP was incubated with MBP (maltose binding protein)-fusion proteins of rat KIF1Bβ2 (aa 301–554) with or without the 40 amino acid insertion, mouse KIF1A (aa 301–514), or rat KIF1C (aa 294–518) in the presence and absence of Ca^{2+}. The proteins recovered on affinity binding of MBP to amylose-resin were analyzed by SDS-PAGE (Fig. 2A). CHP specifically associated with KIF1Bβ2 in the presence of Ca^{2+}, but not with KIF1A or KIF1C. Interestingly,

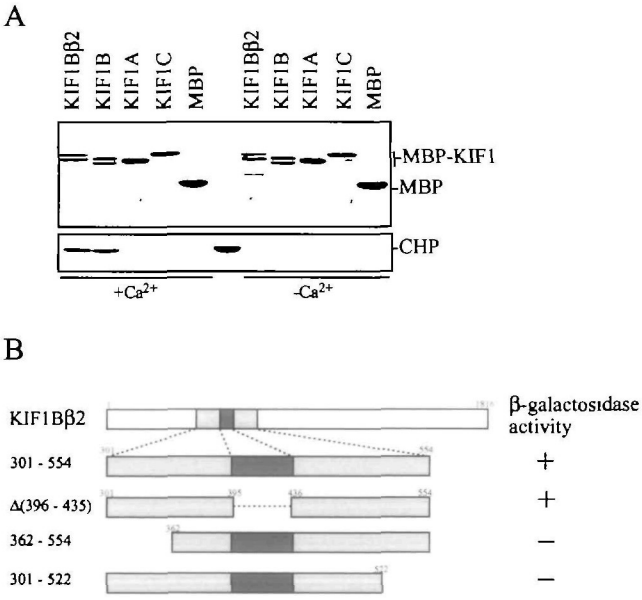

Fig. 2. **Interaction of CHP with KIF1B proteins.** (A) *In vitro* interaction of CHP with KIF1 family proteins. The recombinant CHP (10 μg) was incubated with KIF1-family proteins fused with MBP or MBP (5 μg) immobilized amylose-resin. After washing, the proteins were subjected to gel electrophoresis, followed by staining with Coomassie Brilliant Blue. The regions of KIF1-family proteins used were: mouse KIF1A (aa 295–508), rat KIF1Bβ2 and KIF1B (aa 301–554), and rat KIF1C (aa 294–518). The insertion of 40 amino acids in length is deleted in MBP-KIF1B. (B) Two-hybrid analysis of the interaction of CHP with KIF1Bβ2. The entire length of CHP was fused with the DNA binding domain of Gal4p, and the regions of KIF1Bβ2 indicated by bars were fused with the activation domain of Gal4p. The results of β-galactosidase assaying are shown as plus or minus symbols.

the 40 amino acid insertion was dispensable for the interaction, indicating that CHP is capable of interacting with KIF1Bs (Fig. 2, A and B). *N*-Myristoylated CHP, bacterially co-expressed with myristoyl CoA:protein *N*-myristoyltransferase, showed the ability to interact with KIF1Bβ2 and KIF1Bs, but not with KIF1A or KIF1C (data not shown). Deletion of 61 and 32 amino acid residues from the N- and C-terminal ends, respectively, prevented the interaction with CHP, as observed on two-hybrid analysis (Fig. 2B), suggesting that the entire length of this region is essential for the interaction.

Tissue Distribution of KIF1Bβ2—We examined the tissue distribution of KIF1Bβ2 transcripts by Northern blotting, using a probe corresponding to a C-terminal region (3074–3759 nt from the initiation codon) to distinguish KIF1Bβ2 transcripts from KIF1Bα and KIF1A. A transcript (>10 kb) was detected in all rat tissues examined, but was significantly abundant in brain (Fig. 3).

Immunoblotting analysis with antibodies against the CHP-binding domain of KIF1Bβ2 revealed two bands corresponding to 200 and 190 kDa, with the highest expression in brain (Fig. 4A), in which KIF1Bβ2 was found in all sub-regions in the nervous system examined (Fig. 4B). Lower expression of the 190 kDa form was found in liver and testis (Fig. 4A). The 200 and 190 kDa forms would be splicing isoforms varying in the 40 amino acid insertion, since antibodies against the 40 amino acid insertion (394–433 aa) of KIF1Bβ2 only detected the 200 kDa protein in a brain lysate (Fig. 4D). The specificity of the antibodies on Western blotting was confirmed by means of competition analysis using the antigenic recombinant KIF1Bβ2 protein (data not shown), and immunobloting analysis using NB-1 cells (*41*), a human embryonic neurocarcinoma cell line, lacking the KIF1B locus on chromosome 1p36.2–p36.3 (Fig. 4, C and D). The tissue distribution of the 190 kDa isoform is considerably different from that in mouse, in which the 190 kDa protein is ubiquitously expressed except in brain (*28*). The expression of KIF1Bβ2 is restricted to specific tissues, especially brain. We examined the expression of KIF1Bβ2 in cultured rat hippocampal neurons and mouse neuroblastma cells, Neuro2A. The two forms, 200 and 190 kDa, were predominantly detected in both rat and mouse cells, and were expressed significantly more (>10-fold) than the 130 kD isoform, KIF1Bα (Fig. 4C).

Association of KIF1Bβ2 with Synaptic Transport Vesicles—We observed the intracellular localization of KIF1Bβ2 on immunochemical staining. The antibody against KIF1Bβ2 stained numerous small compartments in cultured rat hippocampal neurons. They were abundant in the perinuclear region and distributed in the neurites (Fig. 5A).

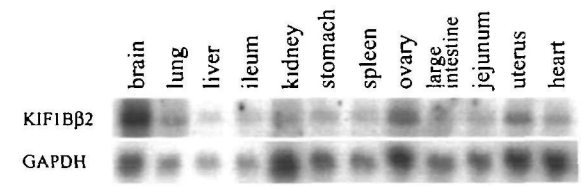

Fig. 3. **Tissue distribution of the KIF1Bβ2 transcript.** Total RNAs (20 μg) from various rat tissues were processed for Northern blotting using a probe corresponding to the C-terminal tail domain of KIF1Bβ2 (3074–3759 nt from the initiation codon). GAPDH was used as an internal control.

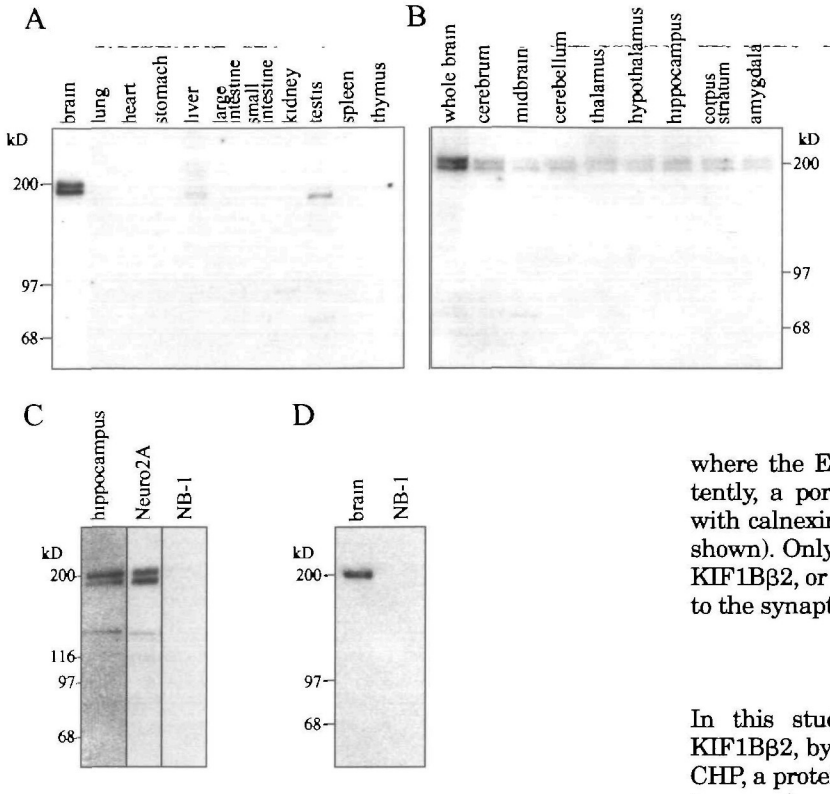

Fig. 4. **Distribution of the KIF1Bβ2 protein in tissues and cell lysates.** (A, B) Total proteins from rat tissues (A) and brain subtissues (B) were subjected to SDS-PAGE, followed by Western blotting using affinity-purified antibodies against the CHP-binding domain (CBD) of KIF1Bβ2. (C, D) Lysates of cultured rat hippocampal neurons, Neuro2A, NB-1 cells, and rat whole brain were analyzed by Western blotting using KIF1Bβ2 antibodies against CBD (C) or the 40 amino acid insertion (D).

Most of the KIF1Bβ2-containing compartments were also stained by antibodies against synaptophysin, a marker protein for synaptic transport vesicles (*42–46*) (Fig. 5, D–F). In contrast, essentially no staining of mitochondria by the anti-KIF1Bβ2 antibody was observed in cultured hippocampal neurons, whereas KIF1B was shown to be associated with mitochondria (Fig. 5, G and H). These results of immunofluorescence staining are well-consistent with those on pull-down assaying and electron microscopic observation (*28*), in which 10% of total KIF1Bβ is associated with synaptic vesicles in mouse brain. Immunofluorescence labelling of CHP revealed only limited co-localization with synaptophysin in the neurites (Fig. 5, I–K). The distribution of CHP is largely different from that of KIF1Bβ2.

The association of KIF1Bβ2 and CHP with the synaptophysin-containing synaptic transport vesicles was examined by subcellular fractionation analysis involving isopycnic sucrose density centrifugation in the range of 0.1 to 1.5 M sucrose. The postnuclear supernatant of the rat brain homogenate was spun at 100,000 ×*g*, and the resulting pellet was resolved in a 0.1–1.5 M discontinuous sucrose gradient by 170,000 ×*g* centrifugation. The fractions obtained were subjected to immunoblotting for KIF1Bβ2, CHP, synaptophysin, Golgi and ER markers, GM130 and calnexin, respectively. Portions (~25%) of KIF1Bβ2 and synaptophysin were co-fractionated into 9–11 fractions with a density of 1.107–1.137 g/ml, which is slightly but clearly different from in the cases of GM130 and calnexin (Fig. 6). These results confirm the association of KIF1Bβ2 with synaptic vesicles suggested by our immunofluorescence staining and the Nycodenz gradient flotation assay by Zhao *et al.* (*28*). CHP (29% of total) was fractionated in lower density fractions (fractions 8–10) than KIF1Bβ2 and synaptophysin, where the ER marker, calnexin, was fractionated. Consistently, a portion of CHP was observed to be co-localized with calnexin on immunofluorescence microscopy (data not shown). Only a small portion of CHP may co-function with KIF1Bβ2, or CHP may interact with KIF1Bβ2 not localized to the synaptophysin-containing vesicles.

DISCUSSION

In this study, we identified a kinesin-related protein, KIF1Bβ2, by screening for the possible catalytic subunit of CHP, a protein homologous to calcineurin B. As observed on immunofluorescence microscopy and subcellular fractionation, KIF1Bβ2 is associated with synaptic vesicles in neurons. The overall intracellular distribution of the binding protein CHP is largely different from that of KIF1Bβ2, suggesting the dynamic and/or transient interaction between these molecules in the cell. Although evaluation of the physiological relevance of the association of CHP with KIF1Bβ2 and KIF1Bs is still in progress, the association of kinesin-related motor proteins with a Ca^{2+}-binding protein, CHP, is a novel and important finding, and may provide new insights into the regulation of kinesin-related molecular motors.

Over 30 members of the kinesin family, which possess a motor domain in the N-terminus, have been identified and classified into eight subgroups based on their primary sequences (*9*). KIF1Bβ2 belongs to the KIF1/Unc104 subgroup, and has a chimeric structure comprising KIF1B and KIF1A as the N- and C-halves, respectively. Since the C-terminal tail domain of kinesin-related proteins plays a key role in the recognition and binding of their cargo, KIF1Bβ2 is expected to have functions physiologically different from those of KIF1Bα. Immunochemical and subcellular fractionation analyses showed that KIF1Bβ2 is associated with synaptic transport vesicles in neurons. This observation suggests that KIF1Bβ2 is involved in the transport of the synaptic vesicles to the plus ends of microtubules at nerve terminals. Recently, Zhao *et al.* (*28*) reported mouse KIF1Bβ, which is localized to the synaptic vesicles. The deletion of the genes encoding KIF1Bs causes a defect in the transport of synaptic vesicle proteins to the distal region in the axon, suggesting a transporting function of KIF1Bβ to the nerve terminal. The results we obtained on immunocytochemical and cell fractionation analyses are well consistent with their immunoelectron microsopic and immunopurification analyses results. KIF1A is known to

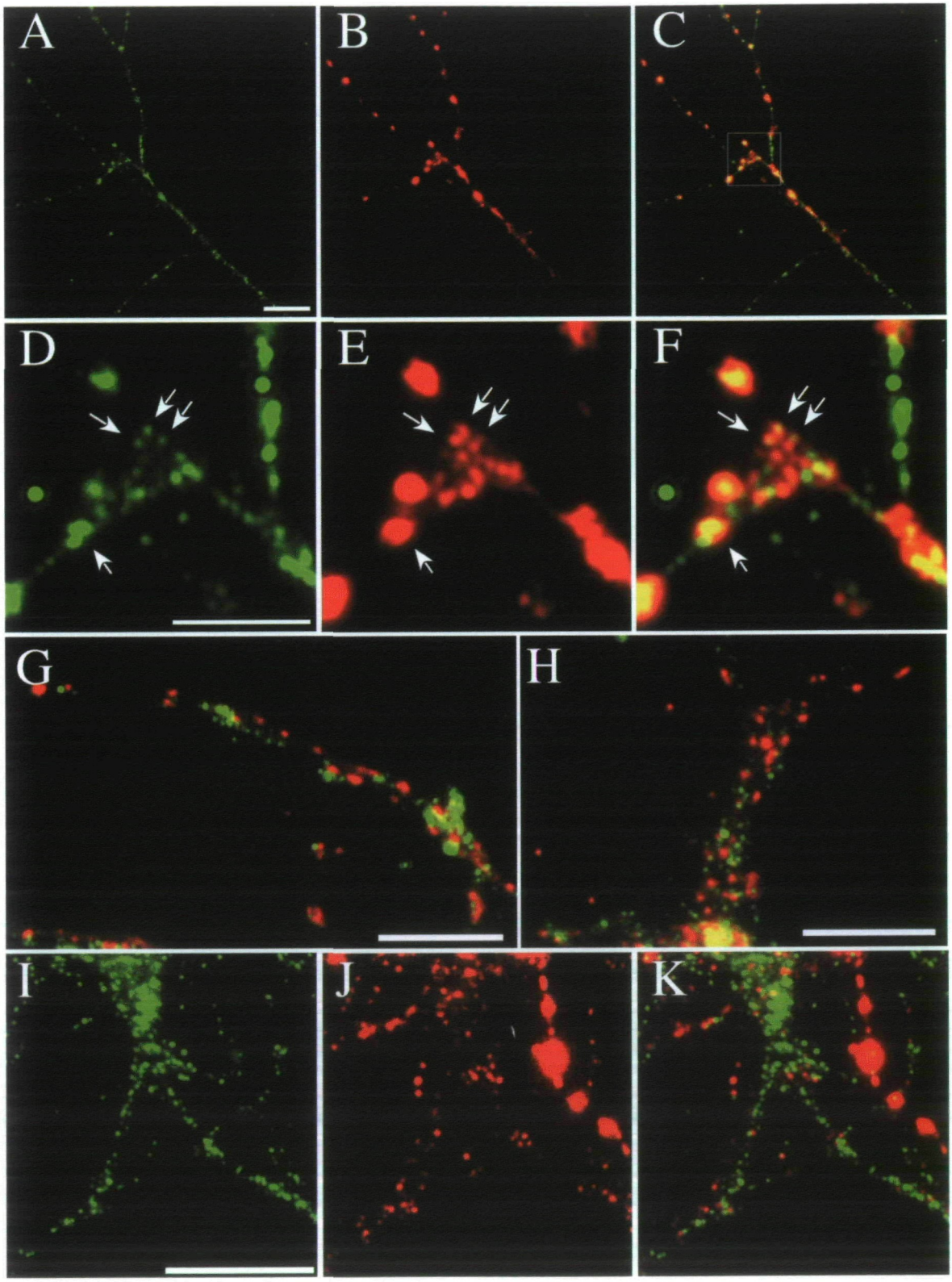

Fig. 5. **Intracellular localization of KIF1Bβ2 in neurons.** Cultured rat hippocampal neurons were fixed, permeabilized, and stained with KIF1Bβ2 (A, D, G, H) and CHP (I) antibodies, followed by incubation with Alexa488-labeled anti-rabbit IgG before processing for fluorescence microscopy. The cells were counter-stained with anti-synaptophysin antibodies, and visualized with Alexa546-labeled anti-mouse IgG (B, E, J) and MitoTracker Red (G, H). The signals were merged: C, for A and B; F, for D and E; K, for I and J. The region indicated by an open square in C is magnified in D–F. Arrows in D–F indicate the regions of co-localization. Bars indicate 10 μm (A, G, I) and 5 μm (D).

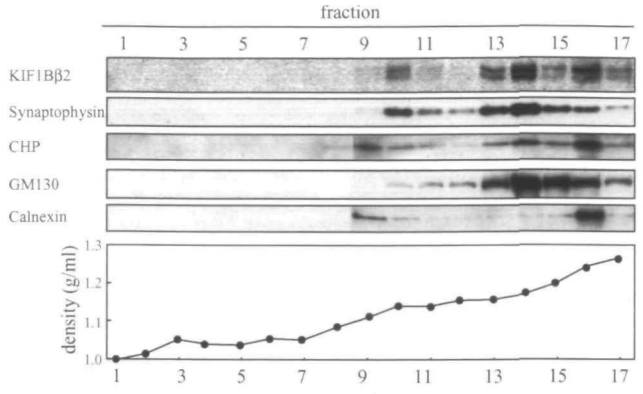

Fig. 6. **Association of KIF1Bβ2 with synaptophysin-containing synaptic transport vesicles.** The postnuclear supernatant of a rat brain lysate was centrifuged at 100,000 ×*g* for 30 min, and the resulting membrane fraction was laid at the bottom of a discontinuous sucrose gradient. After centrifugation at 170,000 ×*g* for 24 h, fractions were collected from the top of gradient, and then subjected to Western blotting with anti-KIF1Bβ2, CHP, synaptophysin, GM130, or calnexin antibodies. The density (g/ml) of each fraction is indicated at the bottom.

participate in the synaptic vesicle transport (*11*), whereas CHP interacts specifically with KIF1Bβ2, but not with KIF1A. It would be of great interest to determine whether and how these two KIF1 motors are distinctively regulated in synaptic vesicle transport, and whether CHP is involved in the specific regulation.

The pleckstrin homology (PH) domain is found at the C-terminus of KIF1Bβ2. This sequence is conserved in KIF1A and a variety of signalling proteins peripherally associated with cellular membranes (*47–49*). Crystal structure analysis of the PH domain showed that it comprises one α-helix and seven β-sheets forming a β-sandwich structure, in which the positively charged face binds to the head groups of phosphoinositide lipids. This binding allows the PH domain to act as an anchor that recruits and tethers the molecule to the cellular membrane (*35*). The amino acid residues forming hydrogen bonds with inositol-phosphate ligands are well conserved in the PH domain of KIF1Bβ2. Thus, the PH domain of KIF1Bβ2 may be involved in the interaction with the membrane lipids of synaptic vesicles.

CHP interacts with the region adjacent to the motor domains of KIF1Bβ2 and KIF1B in a calcium-dependent manner. This is the first observation of the possible involvement of a Ca^{2+}-binding protein and its functional interaction with kinesin-related motor proteins in mammals. Calmodulin, an EF-hand Ca^{2+}-binding protein, is known to interact with myosins (*50, 51*). This protein tightly associates with the neck region of myosins connecting the motor head and tail domain. The precise role of this association remains controversial, but it is suggested that calmodulin mediates the regulation of ATPase activity and motility on actin filaments of myosin by calcium. Similar to the roles of calmodulin in the myosin function, CHP may participate in regulation of the motor function of the KIF1Bβ2 and KIFB proteins. We do not have direct evidence that CHP regulates KIF1B motor activity, because an active recombinant KIF1B protein has not been obtained using bacterial and insect expression systems. However, the recombinant CHP does not affect on the binding ability of rat brain KIF1β2 as to taxol-treated rat brain microtubules (our unpublished data). Although CHP is capable of interacting with both KIF1Bβ2 and KIF1B *in vitro*, very little association of CHP with synaptic vesicles and mitochondria was observed on microscopy and subcellular fractionation analysis. Changes in the intracellular calcium concentration due to calcium ionophore, which would affect the interaction of these proteins, did not alter the subcellular distribution of KIF1Bs and CHP (data not shown). Together with these observations, we did not detect a stable complex of CHP and KIF1Bβ2 in a rat brain lysate on co-immunoprecipitation analysis (data not shown). These results suggest that CHP interacts with KIF1B proteins transiently or under limited conditions *in vivo*. Further biochemical analyses are necessary to obtain clues as to the calcium regulation of KIF1B motor proteins.

The CHP-binding domains of KIF1Bs, NHE1 (*19*), and DRAK2 (*17*) show only limited similarity: the domain of rat KIF1Bβ2 (aa 301–554) exhibits 16 and 12% identity with those of human NHE1 (aa 510–540) and rat DRAK2 (aa 227–293), respectively, and the domain of NHE1 is 3% identical to that of DRAK2, suggesting that the determinant of recognition and/or binding is their three-dimensional structures. Calcineurin B is known to interact with the domain forming the α-helix in the calcineurin A subunit, as judged on crystallography (*52, 53*). The CHP-binding domains of these proteins have different lengths, from 30 to 214 amino acids, but have been predicted to form an α-helix consisting of amphiphilic clusters of charged and hydorophobic residues with the Chou-Fasman algorithm (*54*). These α-helical structures may be important for the interaction with CHP.

Finally, it is noteworthy that CHP interacts with multiple proteins including KIF1Bβ2 and KIF1B, Na^+/H^+ exchangers (*17, 19*), apoptosis-inducing protein kinase (*17*), calcineurin complex (*16*), NFAT transcriptional regulatory factor (*16*), and the microtubule cytoskeleton (*55*). These proteins seem to have diverse functions however, suggesting a novel calcium regulation mechanism integrated by CHP. Further studies on the CHP function would provide new insights into the molecular mechanism of intracellular regulation by calcium.

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