Association of human kinesin superfamily protein member 4 with BRCA2-associated factor 35

Young Mi LEE and Wankee KIM[1]

Institute for Medical Sciences, School of Medicine, Ajou University, Suwon 442-749, South Korea

A large portion of human kinesin superfamily protein member 4 (KIF4) is associated with the nuclear matrix during the interphase, while a small portion is found in the cytoplasm. During mitosis, it is associated with chromosomes throughout the entire process. In the present study, we identified a protein that interacts with KIF4 using a yeast two-hybrid system, co-immunoprecipitation and co-fractionation. This protein is BRCA2-associated factor 35 (BRAF35) containing a non-specific DNA binding high-mobility-group domain and a kinesin-like coiled-coil domain. It appeared that the interaction between the two proteins occurs through their respective α-helical coiled-coil domains. The co-fractionation experiment revealed that KIF4 and BRAF35 were present in a complex of approx. 540 kDa. The composition and biological significance of this complex should be studied further.

Key words: BRCA2-associated factor 35 (BRAF35), human kinesin superfamily protein member 4 (KIF4), protein–protein interaction, yeast two-hybrid analysis.

INTRODUCTION

Microtubule-dependent movement is fundamental to many biological processes, including cell division and intracellular transport of membranous organelles in higher eukaryotic cells [1–3]. Such intracellular movement is carried out by two superfamily motor proteins called cytoplasmic dynein and kinesin. A large number of genes encoding kinesin or kinesin-like proteins have been identified in several organisms (see http://www.proweb.org/kinesin), and all those proteins have highly conserved motor domains responsible for microtubule binding and contain ATPase consensus sequences. In contrast, no significant sequence similarity is detected in the coiled-coil stalk and cargo-binding tail domains. The diversity of kinesin suggests that each kinesin may have a specific function, although many of them remain undefined.

In a previous study [4], it was found that human kinesin superfamily member 4 (KIF4) of 1232 amino acids is dominantly associated with the nuclear matrix during interphase of the cell cycle. Dominant association with the nuclear matrix raises an issue as to whether or not KIF4 is involved in the regulation of gene expression, even though the exact biological function of the nuclear matrix remains uncertain (see [5] and references therein). Since gene expression is modulated by a multi-protein complex, the involvement of KIF4 in that process could be inferred by identification of KIF4-interacting proteins. In the molecule of KIF4, all three domains have interacting potentials; the motor domain associates directly or indirectly with the microtubule, the coiled-coil domain interacts with itself for dimerization, and the tail domain binds to cargo protein.

In the present study, we exploited the yeast two-hybrid system (YTHS) using the motor domain and part of the coiled-coil domain of KIF4 as bait in search of interacting protein(s). A high-mobility-group (HMG) protein called BRCA2-associated factor 35 (BRAF35) [6], also known as human SMARCE1r (SWI–SNF-related, matrix-associated, actin-dependent regulator of chromatin subfamily E, member 1-related) [7], was identified to be associated with KIF4 through their respective coiled-coil domains.

EXPERIMENTAL

Construction of bait plasmid

The 1.2 kb KIF4 cDNA fragment encoding the motor domain, neck region and part of the stalk region was amplified by PCR using specific primers containing a *Sal*I restriction site. The fragment was ligated in frame to the *Sal*I site of pGBT9 (Clontech, Palo Alto, CA, U.S.A.), creating pGBT9-KIF4$_{1-464}$ encoding GAL4-binding domain–KIF4 fusion proteins.

Yeast two-hybrid screening

For screening with YTHS, the bait vector pGBT9-KIF4$_{1-464}$ was transformed into the yeast strain HF7c (Clontech). After confirming expression of the bait protein (results not shown), screening with the YTHS was performed using a human leukaemia cDNA library fused to the GAL4 activation domain in the pACT2 vector (Clontech), as recommended by the manufacturer. DNA sequences of the plasmids obtained from tentatively positive clones were determined by the dideoxynucleotide chain-termination sequencing method.

Antibodies

Purified mouse polyclonal antiserum against KIF4 and BRAF35 were prepared as described previously [4,7]. Anti-FLAG M2 monoclonal antibody and anti-FLAG M2–agarose were purchased from Sigma (St. Louis, MO, U.S.A.). Anti-c-Myc mouse monoclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Glutathione S-transferase

Abbreviations used: BRAF, BRCA2-associated factor; β-gal, β-galactosidase; GST, glutathione S-transferase; HMG, high-mobility group; IPTG, isopropyl β-D-thiogalactoside; KAP3, kinesin superfamily-associated protein 3; KIF, kinesin superfamily; KLCC, kinesin-like coiled coil; ORF, open reading frame; SMARCE1r, SWI–SNF-related, matrix-associated actin-dependent regulator of chromatin subfamily E, member 1-related; YTHS, yeast two-hybrid system.

[1] To whom correspondence should be addressed (e-mail wkim@ajou.ac.kr).

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(GST) was detected using anti-GST polyclonal mouse serum produced in our laboratory.

Preparation of cell extracts

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 1 % (v/v) penicillin/streptomycin and 10 % (v/v) heat-inactivated foetal calf serum at 37 °C in a 5 %-CO_2 incubator. For preparation of cell extracts, cells were washed three times with PBS and were resuspended in lysis buffer [20 mM Tris/HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl and 0.2–1 % (v/v) Triton X-100]. The cell suspension was left on ice for 30 min and was centrifuged at 18 000 ***g*** for 30 min at 4 °C. The supernatant was used for immunoprecipitation experiments.

Mapping of interaction domains

A series of truncated fragments of the KIF4 and BRAF35 were constructed by performing PCR. The amplified fragments were cloned in frame into pGBT9 and the GST vector, pGEX4T-1 (Amersham Biosciences, Picastaway, NJ, U.S.A.), for KIF4 mapping (see Figure 2) or into pACT2 for BRAF35 mapping (see Figure 3A). The resulting KIF4 and BRAF35 plasmids were used for mapping of the interacting domain with $BRAF35_{156-317}$ and $KIF4_{1-464}$ respectively, exploiting YTHS and GST pull-down assay.

GST pull-down assay

GST-fusion constructs were used to transform *Escherichia coli* strain BL21(DE3). The expression of GST-fusion proteins was induced by adding IPTG (isopropyl β-D-thiogalactoside) for 3 h at 37 °C. Bacteria were harvested, resuspended in lysis buffer [20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA and 0.5 % (v/v) Nonidet P40] and sonicated on ice. GST-fusion proteins were purified from bacterial lysates with glutathione–Sepharose 4B beads (Amersham Biosciences). Beads were washed five times with lysis buffer and twice with binding buffer [20 mM Tris/HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl and 1 % (v/v) Triton X-100]. Equal amounts of GST or GST-fusion proteins bound to glutathione–Sepharose 4B beads were incubated for 4 h at 4 °C with 2 mg of HeLa cell extracts in binding buffer. GST-fused cyclin E was used as a negative control. After beads were washed three times with the binding buffer and resuspended in 2× gel-loading buffer, bead-bound proteins were resolved by SDS/PAGE and were subjected to Western blot analysis.

Co-immunoprecipitation

The DNA fragments corresponding to amino acid residues 8–1232 of KIF4 and amino acid residues 1–317 of BRAF35 were amplified by PCR. After the sequences were verified, they were directionally cloned in frame into the pCMV-Myc (Clontech) and pFlag-CMV2 (Eastman Kodak, New Haven, CT, U.S.A.) vectors respectively. For co-immunoprecipitation experiments, HeLa cells were seeded in 100 mm-diameter dishes at a density of 1×10^6 cells/dish. After culture for 16 h, the cells were co-transfected with the indicated constructs or control plasmids, using GenePORTER1 transfection reagent (Gene Therapy Systems, San Diego, CA, U.S.A.). At 48 h after transfection, transfected cells were washed three times with PBS, scraped, and spun down in a microcentrifuge tube. The cell pellet was resuspended in lysis buffer [20 mM Tris/HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl and 0.2 % (v/v) Triton X-100]. The cell suspension was left on ice for 30 min and centrifuged at 18 000 ***g*** for 30 min at 4 °C to prepare the supernatant. For immunoprecipitation, 2 mg of the cell extracts was incubated with 10 μl of anti-FLAG M2–agarose. After 4 h at 4 °C, anti-Flag M2-agarose was washed three times with 500 μl of lysis buffer and immunoprecipitated proteins were eluted from the agarose with 2× gel-loading buffer, resolved by SDS/PAGE and subjected to Western blot analysis.

Co-fractionation

Crude nuclei were prepared according to the method of Evan and Hancock [8]. Briefly, confluent HeLa cells were washed three times with cold PBS, resuspended in low-salt lysis buffer [20 mM Tris/HCl, pH 8.0, 5 mM KCl, 5 mM $MgCl_2$, 0.1 mM PMSF, 0.5 % (v/v) Nonidet P40] at 4 °C and gently homogenized on ice using six strokes of a glass tissue grinder. Crude nuclei were pelleted by centrifugation at 1000 ***g*** for 5 min at 4 °C, and the cytoplasmic extract was removed. The crude nuclei were then resuspended in lysis buffer [20 mM Tris/HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl and 0.2 % (v/v) Triton X-100], incubated on ice for 30 min and centrifuged at 18 000 ***g*** for 30 min at 4 °C. The supernatants were fractionated on a Superose 6 column (Amersham Biosciences) for FPLC. Proteins from each fraction were resolved by SDS/PAGE and subjected to Western blot analysis.

Western blot analysis

The samples were transferred on to a nitrocellulose membrane at 50 V for 2 h at 4 °C in 0.1 M glycine and 12.5 mM Tris/HCl. Blots were blocked by incubation in TNE buffer [10 mM Tris/HCl, pH 7.5, 2.5 mM EDTA, 50 mM NaCl and 0.1 % (v/v) Tween 20] containing 5 % (v/v) non-fat dried milk for 1 h at room temperature (25 °C) before probing with specific antibodies. After incubation with antisera, the blots were washed five times with TNE buffer and then incubated with the peroxidase-conjugated goat anti-rabbit (diluted 1:10000) (Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.) or rabbit anti-mouse immunoglobulins (diluted 1:4000) (Advanced Biochemicals, Chonju, South Korea). After washing, bound antibodies were visualized using the ECL® (enhanced chemiluminescence) Western blotting detection system (Amersham Biosciences).

RESULTS

Identification of KIF4-interacting protein using YTHS

We were originally interested in the identification of cellular protein(s) that interact with the motor domain of KIF4. The bait used for YTHS included the coiled-coil region (amino acid residues 303–464) in addition to the entire motor domain (amino acid residues 1–302) with an aim to yield dimeric motors by analogy from a previous finding that murine KIF4 is dimerized through the coiled-coil domain [9]. Even though it is not evident whether or not dimerization is required for proper conformation of KIF4, it was postulated that the dimer form of the motor domain would maintain as natural a conformation as possible and would help to recognize interacting protein(s), if any. Without confirming dimer formation of manipulated KIF4 in the yeast cell, we proceeded to identify the protein(s) interacting with a part of KIF4 using YTHS.

When the cDNA library constructed from human T-cell leukaemia was screened with pGBT-$KIF4_{1-464}$ as bait, 17 of the 1×10^7 clones screened were isolated as tentatively positive

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A

pGBT9 fusion	pACT2 fusion	X-gal filter assay results
Vector	Vector	White
$KIF4_{1-464}$	Vector	White
Vector	$BRAF35_{156-317}$	White
$KIF4_{1-464}$	$BRAF35_{156-317}$	Blue
$BRAF35_{156-317}$	$KIF4_{1-464}$	Blue
pVA3	pTD1	Blue

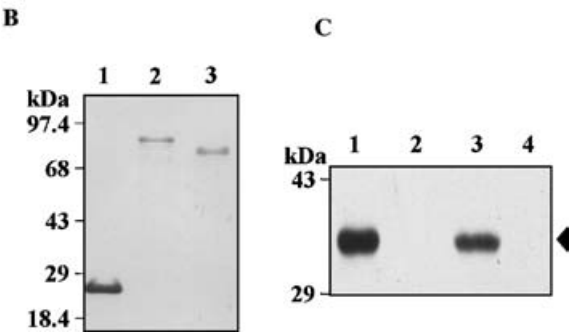

Figure 1 Binding between KIF4 and BRAF35 using YTHS

(**A**) β-Gal filter assay. $KIF4_{1-464}$ and $BRAF35_{156-317}$ fused to GAL4 DNA-binding (pGBT9) and GAL4-activation (pACT2)-derived vectors were β-gal assayed for interaction. pVA3 encodes murine p53–GAL4 DNA-binding domain hybrid in pGBT9. pTD1 encodes an SV40 (simian virus 40) large T-antigen–GAL4 activation-domain hybrid in pGAD3F. (**B**) Expressed bacterial proteins for immunoprecipitation. Lane 1, GST; lane 2, GST–$KIF4_{1-464}$; lane 3, GST–cyclin E_{ORF}. (**C**) Detection of immunoprecipitated BRAF35. The HeLa cell extract was precipitated with bacterial proteins bound to glutathione–Sepharose 4B beads, followed by Western blot analysis with anti-BRAF35 antibody. Lane 1, HeLa cell extract (40 μg); lane 2, GST; lane 3, GST–$KIF4_{1-464}$; lane 4, GST–cyclin E_{ORF}.

clones after a β-galactosidase (β-gal) assay and 14 of them were found to contain BRAF35 inserts, all of which harboured single complete KLCC (kinesin-like coiled coil) domains at the C-terminus of the molecule, the termination codon, and the 3′ untranslated region along with an ORF (open reading frame) of 105–290 amino acid residues. A repeated selection of BRAF35 clones indicated a high probability of interaction between KIF4 and BRAF35. To verify the YTHS results further, the DNA fragments covering 1–464 residues of KIF4 and 156–317 residues of BRAF35 were PCR-cloned into pACT2 and pGBT9 respectively. When the β-gal assay was performed, positive β-gal activity was observed in a reverse direction (Fgure 1A).

KIF4 binds to BRAF35 *in vitro*

As an initial step for confirmation of interaction between KIF4 and BRAF35, an *in vitro* binding assay was performed. The DNA fragments covering residues 1–464 of KIF4 and an entire cyclin E were cloned in frame into the pGEX-4T-1 vector to produce GST-fusion proteins. After induction with IPTG, GST and GST-fusion proteins were purified using glutathione–Sepharose 4B beads and their purity was determined by SDS/PAGE (Figure 1B). When the HeLa cell extract was incubated, pulled down with GST or GST-fusion proteins, and subjected to Western blot analysis with mouse anti-BRAF35 polyclonal antibody, GST–$KIF4_{1-464}$ was found to bring down a BRAF35 of 36 kDa from the extract (Figure 1C).

Determination of interacting regions

To determine the domain of KIF4 that interacts with BRAF35, several internal deletions of KIF4 were made from pGBT-$KIF4_{1-464}$ (Figure 2A). When co-transformed with the activation-domain vectors, including pACT2-$BRAF35_{156-317}$, and assayed for β-gal activity, the interacting domain of KIF4 with BRAF35 was narrowed down to amino acid residues 359–440 of KIF4 (Figure 2B). This region contains a α-helical coiled-coil domain, but not the motor domain.

To confirm further the interaction of $KIF4_{359-440}$ with BRAF35, each KIF4 DNA fragment of the above deleted mutants was cloned into the GST vector, expressed in bacteria, and purified for the GST pull-down assay (Figure 2C, upper panel). After the HeLa cell extract was incubated and pulled down with GST-fusion proteins, the precipitates were separated by SDS/PAGE and subjected to Western blot analysis with mouse anti-BRAF35 polyclonal antibody. Amino acid residues 359–440 of KIF4 were found to be sufficient for precipitation of BRAF35, in accordance with the results from the β-gal assay (Figure 2C, lower panel).

The domain of BRAF35 interacting with KIF4 was also determined with the entire and internally deleted BRAF35 ORFs cloned in the pACT2 activation-domain vector. When the β-gal activity was measured, the region of amino acid residues 216–267 of BRAF35 was found to interact with KIF4 (Figure 3A). This region also contains an α-helical coiled coil. For further confirmation of this *in vitro* binding result, extracts from HeLa cells were incubated with two purified GST–KIF4-fusion proteins (GST–$KIF4_{320-464}$ and GST–$KIF4_{359-464}$) and were subsequently immunoprecipitated with anti-BRAF35 antibodies. When the immunoprecipitates were collected with Protein G–agarose and subjected to Western blot analysis with anti-GST antibody, GST–KIF4 proteins of expected sizes were detected (Figure 3B).

Taking the above results together, as depicted in Figure 3(C), we revealed that KIF4 interacted with BRAF35 *in vitro* and that the interaction between KIF4 and BRAF35 is presumably mediated by their respective α-helical coiled-coil domains.

KIF4 binds to BRAF35 *in vivo*

To verify that *in vitro* interaction between KIF4 and BRAF35 shown above occurs *in vivo*, the BRAF35-interacting domain of KIF4 and the KIF4-interacting domain of BRAF35 were cloned into the Myc-tagged and FLAG-tagged vectors respectively (Figure 4A). After these vectors were transiently co-expressed in HeLa cells, cell lysates were directly analysed with anti-FLAG or anti-Myc antibodies to check proper expression of both vectors (Figure 4B). When the same lysate was immunoprecipitated with anti-FLAG M2–agarose and Western blot analysis was performed with anti-Myc antibody, Myc-tagged KIF4 was detected, indicating *in vivo* interaction of the two proteins.

Since the *in vivo* interaction between KIF4 and BRAF35 was verified using products encoded by partial fragments of the two genes, it was necessary to examine the interaction between the two proteins encoded by full-length cDNAs of KIF4 and BRAF35. To do this, full-length cDNAs of KIF4 and BRAF35 were cloned into the Myc-tagged and FLAG-tagged vectors respectively. With proper expression of both vectors confirmed in the HeLa cell lysate, *in vivo* interaction experiments were carried out as above. When the same lysate was incubated with anti-FLAG M2–agarose and analysed with anti-Myc antibody, a KIF4 protein product of 140 kDa was detected (Figure 4C). Thus it was shown that KIF4 and BRAF35 interact directly *in vivo*.

To examine whether or not the complex containing both KIF4 and BRAF35 proteins can be further separated biochemically, we

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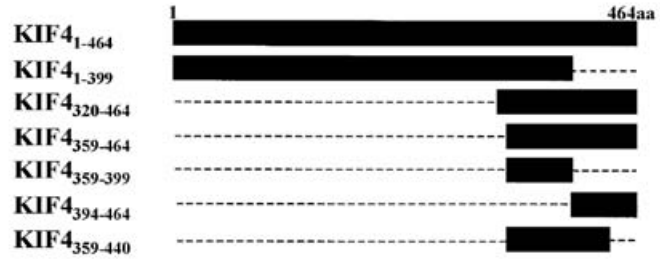

B

DNA binding vector(pGBT9)	Activation vector(pACT2)	β-Galactosidase activity (U)[a] (Mean ± S.D.)	Binding activity
pVA3	pTD1	117±2	+++
Vector	Vector	0	−
Vector	BRAF35	0	−
KIF4$_{1-464}$	BRAF35	5±1	+
KIF4$_{1-399}$	BRAF35	0	−
KIF4$_{320-464}$	BRAF35	2209±22	++++
KIF4$_{359-464}$	BRAF35	2235±34	++++
KIF4$_{359-399}$	BRAF35	0	−
KIF4$_{394-464}$	BRAF35	0	−
KIF4$_{359-440}$	BRAF35	934±16	++++

[a] 1 unit is defined as the amount which hydrolyses 1 μmol of o-nitrophenyl β-D-galactopyranoside to o-nitrophenol and D-galactose per min per cell

C

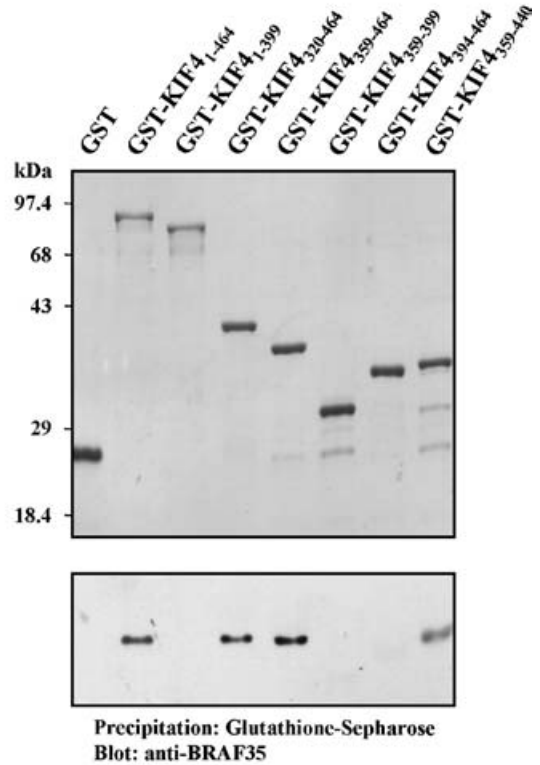

Figure 2 Determination of KIF4 domain interacting with BRAF35

(**A**) Schematic representation of the truncated KIF4 fragment. (**B**) Determination of KIF4 domain interacting with BRAF35 using the β-gal assay. GAL4 DNA-binding vectors containing the KIF4 were used to co-transform the yeast reporter strain SFY526 with the GAL4-activation vector containing BRAF35. β-Gal assays were performed three times independently from the yeast extract. (**C**) Determination of KIF4 domain interacting with BRAF35 using the GST pull-down assay. Upper panel: bacterially expressed and purified GST-fusion proteins of the truncated KIF4. Fusion proteins were expressed and purified as described in the Experimental section and were analysed following SDS/PAGE by Coomassie Blue staining. Lower panel: GST pull-down assay. Equal amounts of GST-truncated KIF4 fusion proteins bound to glutathione–Sepharose 4B beads were incubated with 2 mg of the HeLa cell extract for 4 h at 4 °C. After washing with the lysis buffer, proteins bound to beads were subjected to Western blot analysis using anti-BRAF35 antibody.

used size-exclusion chromatography. A total of 2 g of soluble nuclear protein in 400 μl of lysis buffer was loaded on a Superose column that was equilibrated with the same buffer. The running procedure was performed under the Bio-Logic System (Bio-Rad, Hercules, CA, U.S.A.) with a flow rate of 0.5 ml/min, and 50 fractions of 0.5 ml were collected. A volume of 10 μl of every other fraction was mixed with 2× sample buffer, boiled, and loaded on to a 10 % (w/v) polyacrylamide gel for Western blot analysis. Figure 5(A) shows that the majority of KIF4 protein was co-fractionated with BRAF35. The molecular mass of that fraction was determined to be approx. 540 kDa (Figure 5B).

DISCUSSION

In the present study, we identified a protein molecule that interacts directly with KIF4. This protein was BRAF35, originally identified as a DNA-binding component of the BRCA2 multiprotein complex. Although numerous kinesins have been listed to date and many of them are found to be involved in transporting membrane organelles or mitotic apparatus, only several binding molecules have been identified by biochemical methods and YTHS (see [10,11]). Those include molecules that interact with conventional kinesin, such as kinectin, Jun kinase-interacting proteins, amyloid precursor protein, a vaccinia virus membrane protein, and molecules that interact with KIF17, KIF1C, KIF13A, KIF3, rabkinesin-6 and Eg5. Some of them are outer-membrane components of membrane organelles, and others are proposed scaffolding proteins that link the tail domain of kinesin to the transmembrane proteins of cargoes. It has been proposed that all those proteins bind to the cargo-binding domain of kinesin. Accordingly, our results demonstrating association of KIF4 with BRAF35 through the interaction of their respective α-helical coiled-coil domains is unique so far in mammals. A similar case has been reported from the Kar3 motor protein of *Saccharomyces cerevisiae* that is essential for nuclear fusion and has distinct roles during mitosis [12–14]. It has a predicted α-helical coiled-coil domain of approx. 300 amino acids at the central region. Kar3 interacts separately with two different proteins, Cik1p and Vik1p, which have been implicated in modulating Kar3 function in mitotic cells, possibly by targeting the motor to different sites within the cell [15,16]. They each also have a centrally located 300-amino-acid sequence predicted to form a α-helical coiled-coil. Especially, it has proven that Kar3 and Cik1 form a tight complex with a heterodimeric structure through their respective coiled-coil domains [17]. In HeLa cells during interphase, KIF4

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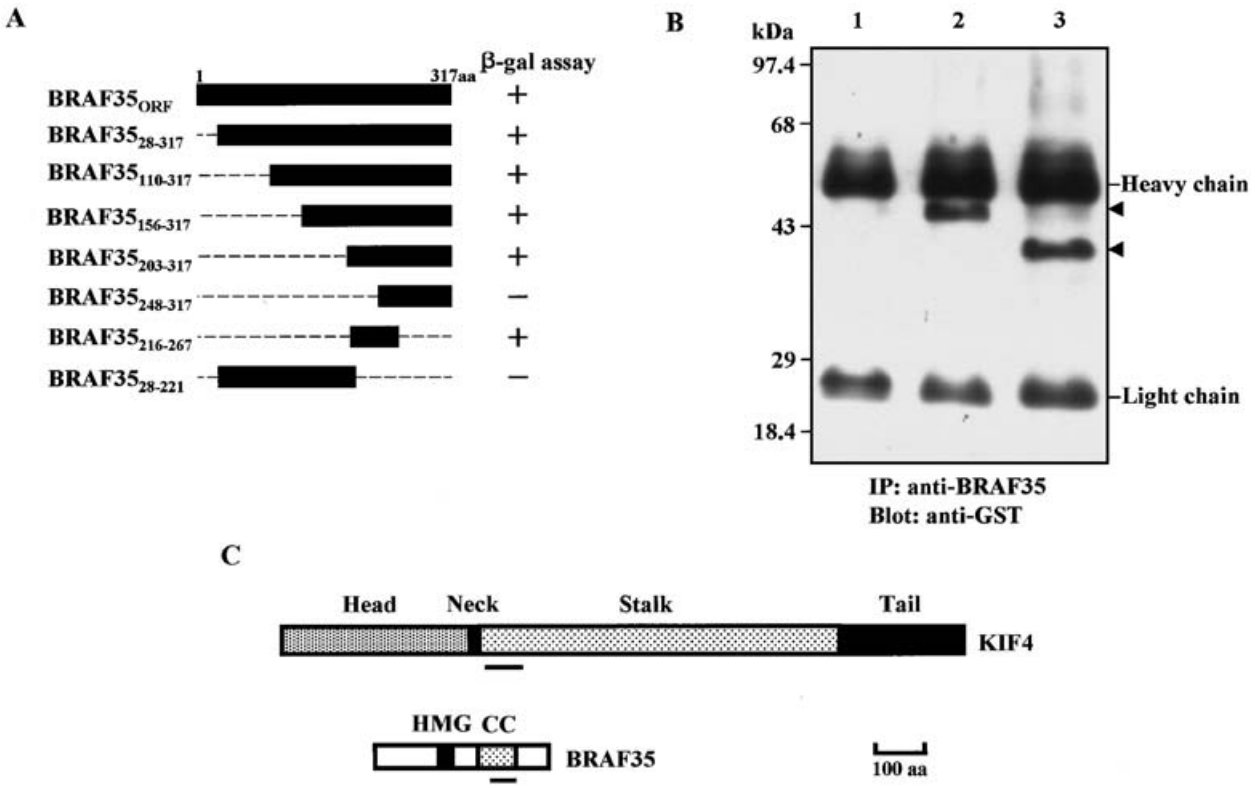

Figure 3 Determination of BRAF35 domain interacting with KIF4

(**A**) Determination of KIF4 domain interacting with BRAF35 using β-gal assays. GAL4-activation vectors (pACT2) containing the truncated BRAF35 fragments were co-transformed into the yeast reporter strain SFY526 with GAL4 DNA-binding vector (pGBT9) containing $KIF4_{1-464}$. β-Gal assays were performed from colonies of co-transformants. (**B**) *In vitro* binding of BRAF35 with KIF4 using two GST–KIF4 fusion proteins (GST–$KIF4_{320-464}$ and GST–$KIF4_{359-464}$). After incubating 2 mg of HeLa cell extracts with GST, GST–$KIF4_{320-464}$ or GST–$KIF4_{359-464}$, the protein complexes were precipitated with 10 μl of Protein G–agarose conjugated with anti-BRAF35 antibody. Precipitated proteins were subjected to Western blot analysis using anti-GST antibody. (**C**) Schematic diagram of interacting domains of KIF4 and BRAF35. The thick lines under the bars represent interacting domains determined from Figures 2(A) and 3(A). The coiled-coil domains of KIF4 are not specified, since they are scattered all over the stalk region with short intervals. The position of the coiled-coil domain is based on amino-acid-sequence analysis using the MultiCoil score program provided by the website http://multicoil.lcs.mit.edu/cgi-bin/multicoil. CC, coiled coil.

is partitioned into the cytoplasm (30%) and the nuclear matrix (70%). In mitotic cells, it is associated with the chromosomes during the entire process [4]. Meanwhile, BRAF35 is predominantly associated with the nuclear matrix during the interphase and is localized to the metaphase chromosomes, but dissociates from the early anaphase chromosomes [6,7]. It is highly probable that appropriate subcellular localization of both proteins may be important for performing their functions, and that such localization may be directed by associated protein, as in the case of Kar3 and Cik1. At present, it is not clear at which subcellular locus the interaction between KIF4 and BRAF35 occurs. Whether or not the function(s) of KIF4, even though not known, is modulated by BRAF35, or vice versa, remains to be elucidated.

BRAF35, initially identified as a component of a large BRCA2-containing protein complex, contains a non-specific DNA-binding HMG domain and a KLCC domain [6]. Inhibition of BRAF35 by antibody microinjection causes a G_2 cell-cycle delay, suggesting a role in the progression through mitosis in association with BRCA2. Recent biochemical analysis revealed that BRAF35 is also present in a smaller complex devoid of BRCA2 [18]. It is a six-subunit complex with the capacity to deacetylate histones, contains polypeptide reminiscent of the chromatin-remodelling complexes SWI–SNF and NuRD (nucleosome remodelling and deacetylating), and mediates repression of neuron-specific genes by recruiting a neuronal repressor. BRAF35, also known as SMARCE1r, is closely related to BAF57/SMARCE1 that is associated with BRG1, a human homologue of SWI2–SNF2 [19]. Since the BRG1 complex is involved in ATP-dependent nucleosome disruption for activation of gene expression [20–22], its high similarity to BAF57/SMARCE1 suggests that BRAF35 may be involved in the regulation of gene expression through remodelling of chromatin structure. Thus it is likely that BRAF35 is a multi-functional protein, regulation of which may be directly or indirectly controlled by associated proteins, one of which may be KIF4.

Dimeric kinesin members contain the coiled-coil neck region of approx. 50 residues, immediately adjacent to the globular motor domain, that contributes to dimerization and processive movement along a microtubule [23]. A rather complicated kinesin complex is heterotrimeric KIF3 that is composed of a KIF3A–KIF3B heterodimer and KAP3 (kinesin superfamily-associated protein 3), thus forming a heterotrimeric complex (KIF3A–KIF3B–KAP3) (see [24] and references therein). KAP3 is thought to bind to the C-terminal cargo-binding tail domain of the KIF3 heterodimer and regulate the cargo binding. Thus the tertiary structure of the KIF4–BRAF35 complex is very informative in understanding their functions. Sequences of interacting domains of KIF4 and BRAF35 are characterized by a seven-residue periodicity (heptad repeat) (Figure 6), suggesting that both domains are left-handed coiled coils. BRAF35 contains a single

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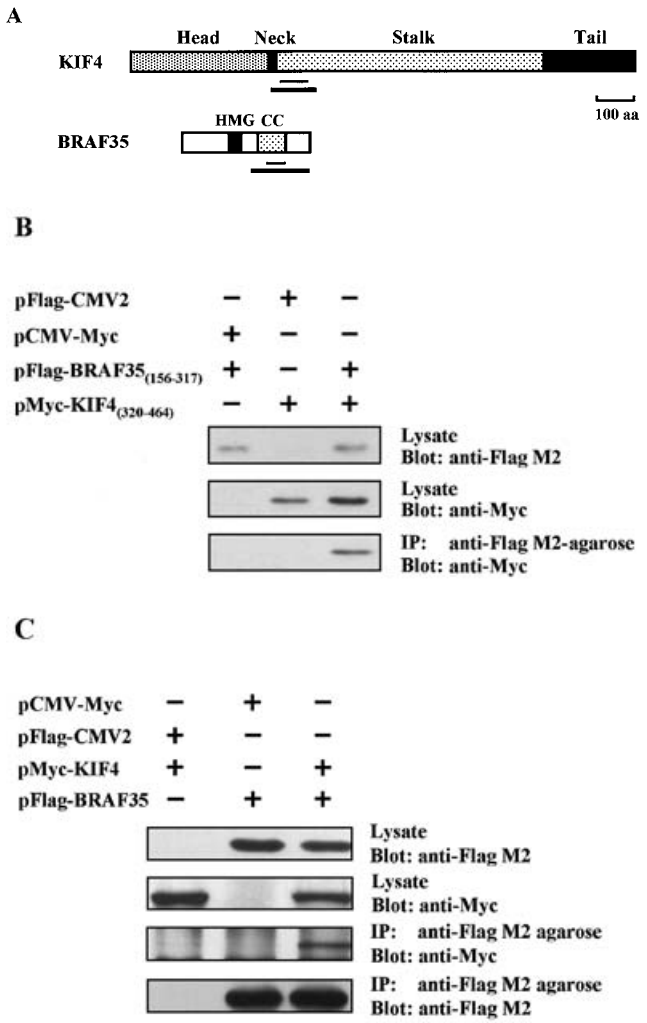

Figure 4 ***In vivo*** **interaction between BRAF35 and KIF4**

pCMV-Myc-KIF4 (5 μg) was transiently co-transfected into HeLa cells with 5 μg of FLAG-tagged BRAF35 or empty vector. At 48 h after transfection, whole-cell extracts were precipitated with anti-FLAG M2–agarose. The precipitates were subjected to Western blot analysis with anti-Myc antibody or anti-FLAG M2 antibody. (**A**) Schematic diagram of the regions used in this experiment [thick lines under the bars for (**B**) and bars for (**C**)]. Thin lines represent interacting domains of two proteins as identified in Figure 3. CC, coiled coil. (**B**) Interaction between protein products encoded by partial cDNA fragments of KIF4 and BRAF35. (**C**) Interaction between protein products encoded by full-length cDNAs of KIF4 and BRAF35.

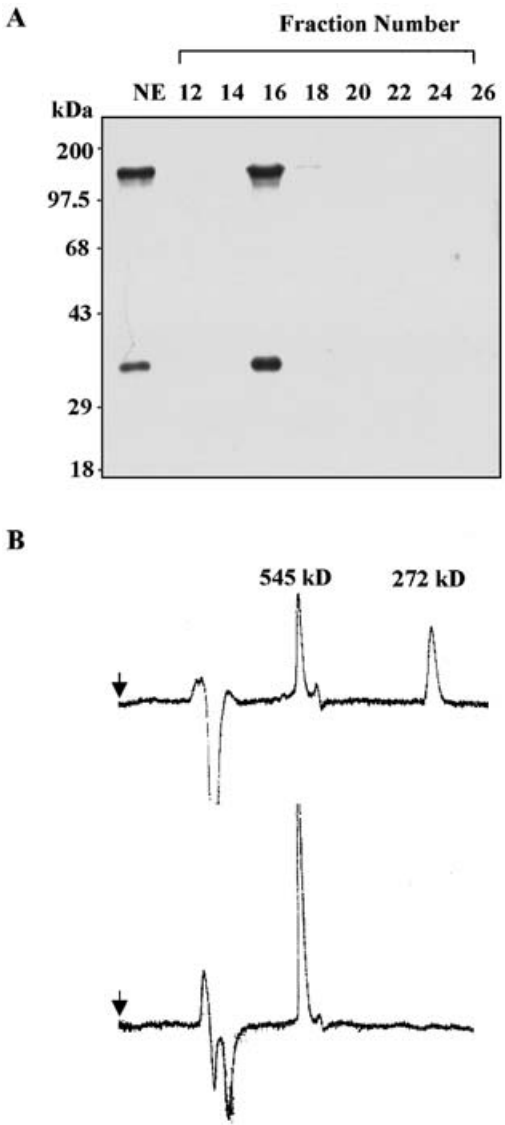

Figure 5 **Co-fractionation of KIF4-BRAF35 complex**

(**A**) Identification of a fraction containing the KIF4–BRAF35 complex. A total of 2 g of the HeLa cell nuclear extract was fractionated by gel filtration with FPLC (fast-performance column chromatography). Aliquots (10 μl) of designated fractions were subjected to Western blot analysis using anti-KIF4 and anti-BRAF35 antibodies. (**B**) Determination of the molecular mass of the KIF4–BRAF35 complex. A portion of fraction number 16 was run on an FPLC column, calibrated with hexamer (545 kDa) and trimer (272 kDa) of *Canavalia ensiformis* urease (upper panel). The KIF4–BRAF35 complex nearly coincides with urease hexamer (lower panel). Arrows indicate the first fraction collected.

coiled coil of approx. 35 residues, whereas KIF4 possesses two short coils on its sides and one of approx. 35 residues in the middle. There are several types of multimerization of coiled-coil-containing proteins [25]. A SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) complex comprising a heterotetrameric coiled-coil is characterized by the highly conserved glutamine or arginine residue at a **d** position of heptad [25,26]. Since neither KIF4 nor BRAF35 contains such residues, it is unlikely that the KIF4–BRAF35 complex is a heterotetrameric structure. KIF4 has at least two distinct coiled-coil domains identified as being involved in protein–protein interactions, one in the neck for dimerization and the other for interaction with BRAF35. Accordingly, the stoichiometry of KIF4 and BRAF35 is presumed to be 2:1. However, the possibility that the interaction of KIF4 with BRAF35 inhibits the formation of

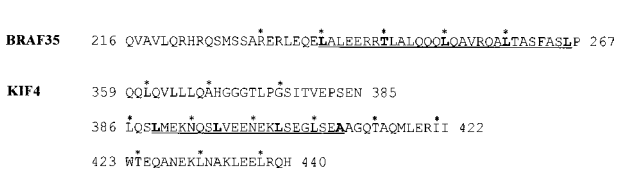

Figure 6 **Coiled coils of KIF4 and BRAF35 are required for interaction**

The interacting region shown here is based on the results shown in Figure 3. Asterisks (*) indicate **a** positions of heptad. Leucine-zipper-like sequences are underlined, with leucine residues in bold.

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a homodimer of KIF4 cannot be excluded. Leucine-zipper-like sequences can be noted in both proteins (Figure 6), suggesting that the interaction between them may form a zipper. Considering these presumptions, the complex of 540 kDa containing KIF4 and BRAF35 is large enough to occupy other proteins that may also be important for proper function(s) of KIF4 and BRAF35.

At the beginning of the present study, considering that most of the region used as bait derived from the complete motor domain responsible for binding to microtubules, we expected tubulin to be a prominent candidate molecule that binds to KIF4. KIF4 itself also was expected, since it has been thought that KIF4 is present inside the cell as a homodimer. However, no clone containing inserts of tubulin or the coiled-coil domain of KIF4 was obtained, in contrast with repeatedly isolated BRAF35, suggesting that the experimental condition for screening with YTHS was optimal for detection of BRAF35, but not for tubulin and KIF4 itself, or that KIF4 may indirectly bind to microtubules, or that an additional domain of KIF4 may be required for dimerization.

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