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SHORT ORIGINAL COMMUNICATION

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Immunohistochemistry of a cytoplasmic dynein (MAP 1C)-like molecule in rodent and human brain tissue: an example of molecular mimicry between cytoplasmic dynein and influenza A virus

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Abstract Immunohistochemistry with an antibody to influenza A/Aichi/2/68 (H3N2) virus was performed using normal mouse, rat and human brain tissues. Dot-like or filamentous structures in the neuronal cytoplasm were clearly stained. Axons were also stained, but weakly. Lewy bodies in Parkinson's disease substantia nigra were also positive. Immunoscreening of the antibody using mouse brain cDNA revealed that this antibody recognized the heavy chain of cytoplasmic dynein. Immunoblot analysis also showed that the reactive molecule was the same size as cytoplasmic dynein (microtubule-associated protein 1C). This is an example of molecular mimicry between cytoplasmic dynein and influenza A virus, and the antibody appears to be useful for the localization on cytoplasmic dynein in the central nervous system.

Key words Cytoplasmic dynein · Influenza A virus · A/Aichi/2/68 · Molecular mimicry · Immunohistochemistry

Introduction

An antibody raised against a viral determinant sometimes reacts with the host cells. Of 635 antiviral monoclonal antibodies, 21 gave positive reactions with one or more tissues [8]. It has been suggested that molecular mimicry between an infectious agent and normal tissue components is a relatively common phenomenon. Here we present one example of cross-reactivity of an antiviral antibody. We used a polyclonal anti-influenza A/Aichi/2/68 (H3N2) virus antibody and found that it recognized cytoplasmic dynein in rodent and human brain tissues. Immunohistochemical data on cytoplasmic dynein in human brain tissue have not been reported. Our present results showed a clear localization of the dynein-like molecule in neuronal cytoplasm in all brains examined, and in Lewy bodies (LBs) in parkinsonian brain.

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Materials and methods

Immunohistochemistry

The polyclonal rabbit antibody to influenza A/Aichi/2/68 was raised against purified virions. For immunohistochemistry for rodent brain tissues, ten male C3H/HeN mice weighing 20–30 g and six male Fisher rats weighing 200–225 g were used. Animals were anesthetized with sodium pentobarbital and perfused through the heart, first with 10 m*M* phosphate-buffered saline (pH 7.4; PBS), and then with a fixative consisting of 4% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.4). The brains were quickly removed and left in the same fixative for 48 h at 4° C. The brains of two other C3H/HeN mice inoculated intracerebrally with 5×10^3 pfu of the influenza A/Aichi/2/68 virus were also examined.

For immunohistochemistry on human brain tissue, we used five non-neurological (age range 61–85) and four Parkinson's disease (age range 61–81) cases. Brains in all cases were obained 2–12 h after death. Small blocks of the midbrain were dissected and fixed for 48 h at 4° C in phosphate-buffered 4% paraformaldehyde. All brain samples were transferred to 0.1 *M* PBS containing 15% sucrose and 0.1% sodium azide, where they were maintained at 4° C until used. Sections were cut on a cryostat at 20 μm thickness, and stored at 4° C in the same solution until immunohistochemical staining was carried out.

The sections were treated with the primary antibody for 48 h in the cold. They were then treated for 2 h at room temperature with biotinylated secondary antibody (Vector), followed by incubation with the avidin-biotinylated horseradish peroxidase (HRP) complex (Vector). Washing between steps was done with PBS containing 0.3% Triton X-100 (PBST). Peroxidase labeling was visualized by incubating with a solution containing 0.001% 3.3′-diaminobenzidine (DAB), 0.6% nickel ammonium sulfate, 0.05% imidazole and 0.0003% H_2O_2. When a dark purple product formed, the reaction was terminated. Sections were washed,

mounted on glass slides, dehydrated with graded alcohols and coverslipped with Entellan.

Western blotting

Immunoblot analysis was done on samples of rodent brain stem and on midbrain tissue from a non-neurological human case. The tissues were homogenized in buffer [5 volumes of 20 m*M* TRIS-HCl (pH 7.4), 1 m*M* EGTA, 1 m*M* EDTA, 10 μ*M* leupeptin, 1 μ*M* pepstain and 0.3 μ*M* aprotinin]. The homogenate was centrifuged at 15,000 rpm for 30 min at 4° C, and the supernatant was collected as a crude cytosolic fraction. A portion of this fraction containing 50 μg of protein was electrophoresed on sodium dodecyl sulfate/polyacrylamide gel (15% polyacrylamide gel; non-reducing condition) and then transferred to a nitrocellulose membrane [25 m*M* TRIS-glycine buffer (pH 8.3), containing 20% methanol]. The membrane was pretreated with 5% skim milk powder in 25 m*M* TRIS (pH 7.4) containing 150 m*M* NaCl (TBS), and then incubated with an anti-Aichi antibody (1 : 10,000) in 2% skim milk in TBS, for 18 h at 4° C. The membrane was extensively washed in TBS + 0.1% Tween 20 (TBST), and the bound antibody labelled with alkaline phosphatase-conjugated anti-rabbit antibody for 2 h at room temperature (BRL; 1 : 5,000) in TBST containing 1% skim milk. Following further washing, the membrane was developed in alkaline phosphatase substrate buffer [0.33 mg/ml nitroblue tetrazolium (BRL), 0.44 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BRL), 0.1 *M* NaCl and 50 m*M* $MgCl_2$ in 0.1 *M* TRIS-HCl (pH 9.5)].

Immunoscreening

Immunoscreening of the antibody using a mouse brain cDNA library was done as follows. λgt11 cDNA clones (Clonetech) were plated on *E. coli* Y 1090 and incubated at 42° C for 4 h. Isopropyl-thio-β-D-galactoside (IPTG)-saturated nitrocellulose filters were overlayed to induce expression of LacZ-cDNA fusions and incubated at 37° C for 4 h. The membranes were washed several times in TBST and then incubated in TBST with 10% skim milk for 1 h. Then the membranes were incubated with the primary antibody (1 : 10,000 dilution) for 25 h. After washing several times with TBST, they were incubated with biotinylated anti-rabbit IgG (1 : 1,000 dilution) for 2 h, followed by incubation in the avidin-biotinylated HRP complex (Vector). Peroxidase labeling was visualized by incubating with a solution containing 0.001% DAB, 0.6% nickel ammonium sulfate, 0.05% imidazole and 0.0003% H_2O_2. After picking up positive colonies, the phage DNA was extracted and a cDNA insert (about 800 bp by agarose gel electropheresis) was obtained. It was subcloned into plasmid vector pbluescript SK(+) and the cDNA inserts were sequenced by the dyedeoxy cycle sequencing method.

Results

In immunoblot analysis, three clear bands of approximately 400, 150 and 75 kDa were detected in the cytosolic fraction of rodent brain stem by the anti-influenza A/Aichi/2/68 antibody (Fig. 1). In the human brain tissue, the two higher molecular weight bands were seen (Fig. 1).

Immunohistochemically, the anti-Aichi antibody stained dot-like or filamentous structures in the neuronal cytoplasm in all brains (Fig. 2 A–H). Only some cortical neurons showed such immunolabeling (Fig. 2 A), while stronger staining was observed in most neurons in the diencephalon and brain stem. This was especially true of structures located in the midline, such as the hypothalamus (Fig. 2 B), dorsal raphe nucleus (Fig. 2 C), etc.. Individual axons were sometimes weakly stained (Fig. 2 D), but axonal bundles were clearly visualized (Fig. 2 G). In the mice given intracerebral inoculations of influenza A/Aichi/2/68 virus there was capillary staining, which may indicate hematogenous invasion of the viruses, as well as the usual neuronal staining (Fig. 2 H).

In the human brain stem, neurons of the substantia nigra (SN) zona compacta (Fig. 3 A, B), oculomotor nucleus and red nucleus all showed immunolabeling, but positive staining was not seen in glial cells. In the SN of Parkinson's disease cases, LBs (Fig. 3 C) and dendritic spheroid bodies (Fig. 3 D) [10] were positively stained by this antibody. LBs were stained either laminally (Fig. 3 C) or diffusely. In the neurons containing LBs, filamentous structures were rarely seen in the cytoplasm, although there were positive dot-like structures.

In the immunoscreening using a mouse brain cDNA library, we obtained a fragment of approximately 800 bp as shown by agarose gel electropheresis. The nucleotide sequence of this insert is given in Fig. 4. It consisted of 867 bp, and had high homology to the heavy chain of rat cytoplasmic dynein, as reported by Mikami et al. [7]. A search of the EMBL/GenBank data base revealed that this fragment had 80.6% homology with cytoplasmic dynein, and that the nucleotide sequence 12 571–13 310 in the heavy chain of cytoplasmic dynein had 94.4% similarity with this fragment.

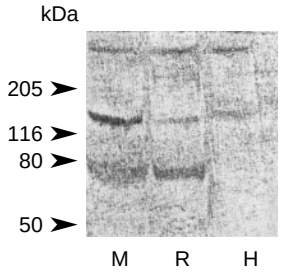

Fig. 1 Immunoblot analysis using brain tissues and the anti-influenza A/Aichi/2/68 antibody. Three major bands of approximately 400, 150 and 75 kDa were seen in the cytosolic fraction from the brains of mouse *(M)* or rat *(R)* and two of these bands in that fraction of human midbrain *(H)*

Fig. 2 Immunostaining with the antibody to influenza A/Aichi/2/68 in the brains of mouse (**A–D, F, G, H**) and rat (**E**), counterstained with neutral red except for **G**. **A** Positive immunolabeling was seen in the cytoplasm and processes of cortical neurons. × 160. **B** In the hypothalamus (paraventricular nucleus), almost all the neurons were positively stained. × 160. **C** In the dorsal raphe nucleus, clear staining was seen. × 160. **D** High magnification of the neurons of **C**. Filamentous or a few dot-like structures were seen. Weakly stained axons were also seen. × 512. **E** In the locus ceruleus, neuronal cells and processes were clearly stained. × 320. **F** Neurons of the oculomotor nucleus had the same staining pattern as those in the locus ceruleus (× 640). **G** Axonal bundles, such as the oculomotor nerve *(arrows)* in the region of the substantia nigra zona reticulata, were positive. × 160. **H** In mice inoculated intracerebrally with influenza A/Aichi/2/68 there was capillary staining as well as neuronal staining. (× 160) ▶

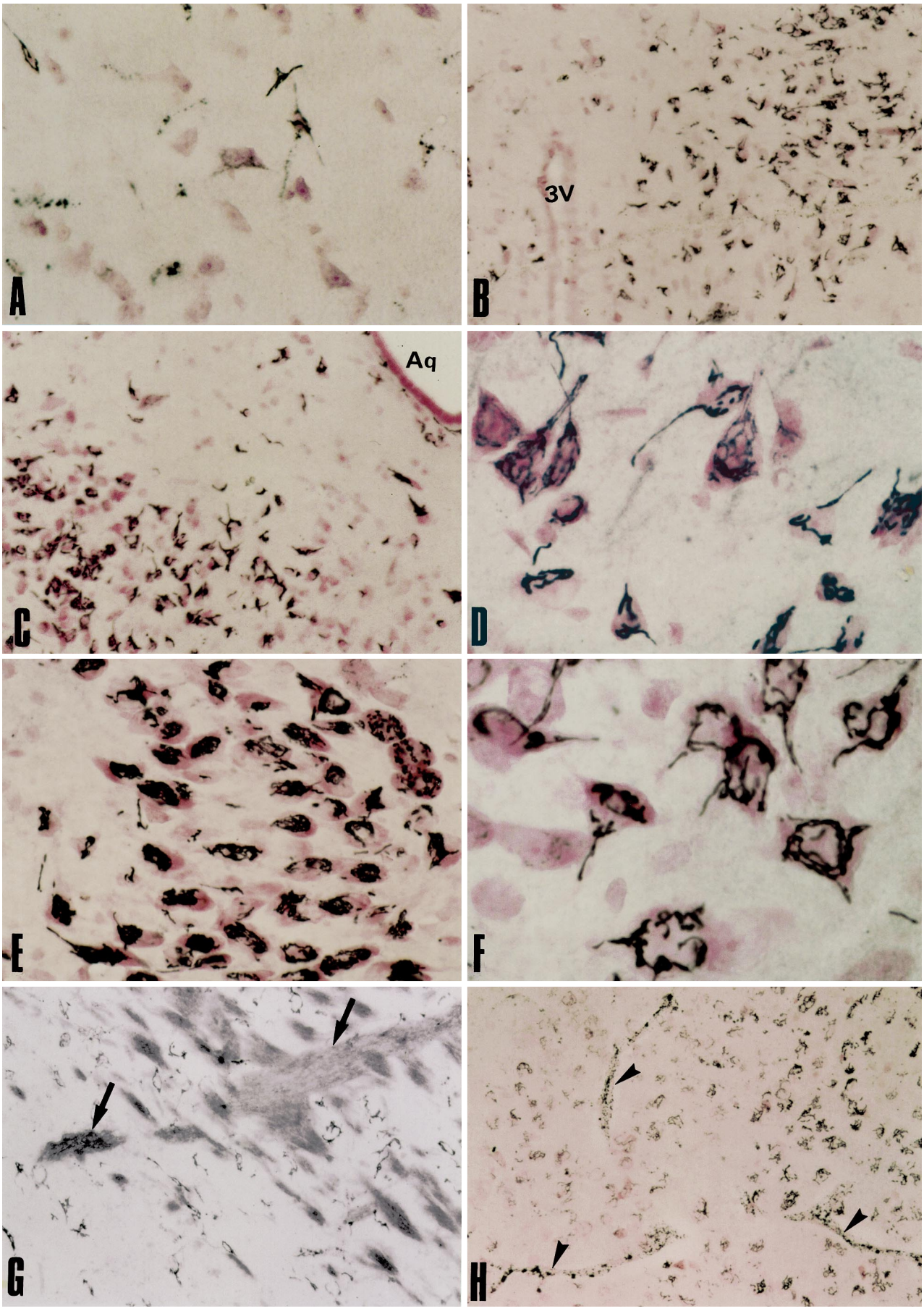

Fig. 2 A–H (for legend see p. 307)

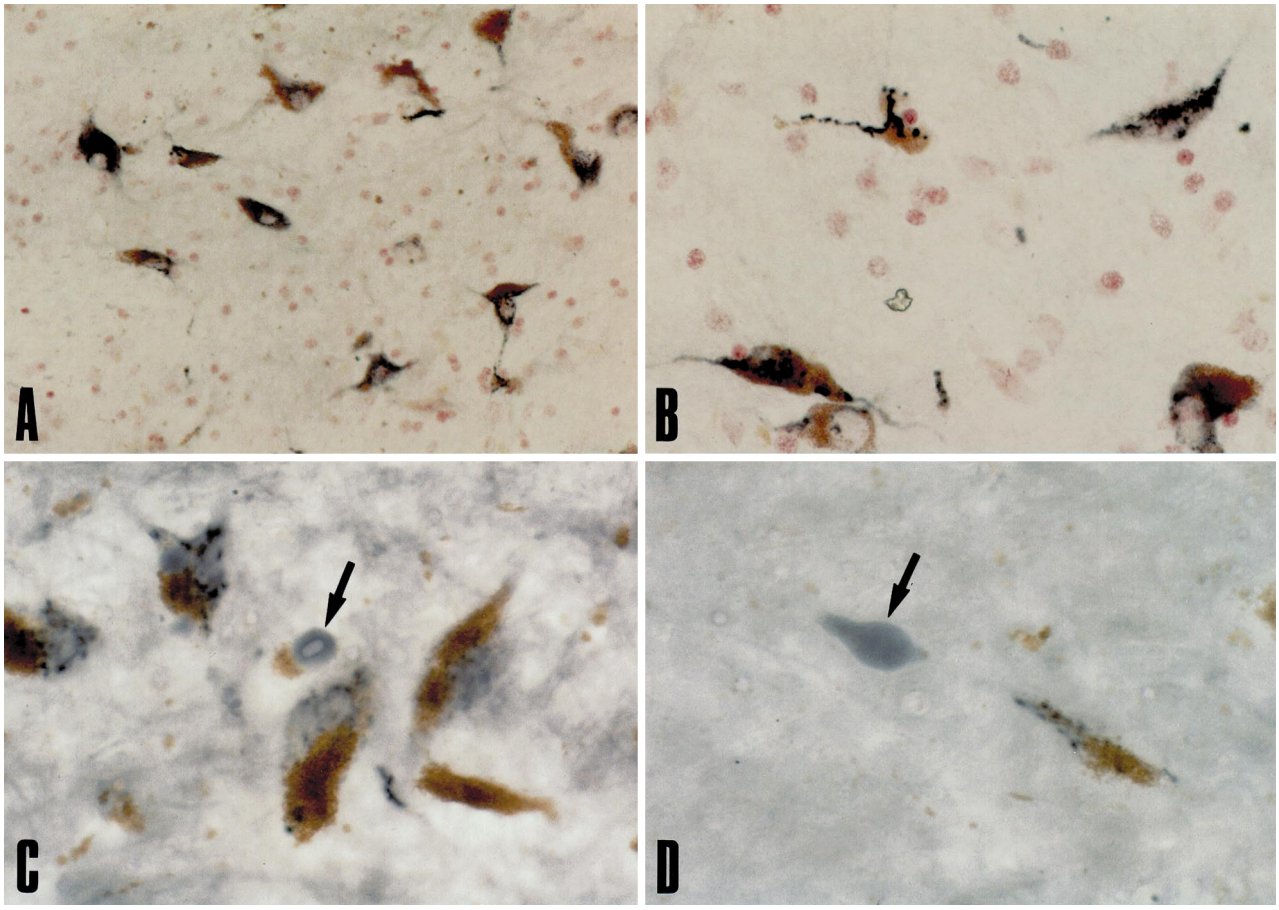

Fig. 3 Immunohistochemistry with the antibody to influenza A/Aichi/2/68 in non-neurological (**A, B**) and Parkinson's disease (**C, D**) midbrain. **A** × 160, **B** × 320. Normal nigral neurons had filamentous or dot-like staining. **C** A Lewy body *(arrow)* was positively stained in parkinsonian substantia nigra, while only dot-like staining was seen in the remaining nigral neurons. × 320. **D** A dentritic spheroid body *(arrow)* was positively stained. In a remaining neuron, dot-like staining was seen. × 320

Discussion

The cDNA fragment obtained by immunoscreening corresponded closely with the C-terminal third of cytoplasmic dynein. This region is a conserved portion and has approximately 50% homology to the hemagglutinin of influenza A/Aichi/2/68 (GenBank) [9]. Immunoblot analysis showed that this antibody reacted with a molecule of size reported for cytoplasmic dynein by Yoshida et al. [11, 12]. Therefore, we suggest that the antibody produced against Aichi/2/68 proteins cross-reacted with cytoplasmic dynein. However, the reason why glial cells were not stained by this antibody is still unknown.

Using this antibody, we could show very clear localization of dynein in the cytoplasm of various neurons. Cytoplasmic dynein, one of the motor proteins with ATPase, plays a part in the microtubule-dependent transport of organelles within cells. This includes retrograde axonal transport, as well as the centripetal transport of endosomes, lysosomes, the elements of Golgi apparatus and chromosomes [1, 3, 5, 6]. Four immunohistochemical studies [2, 4, 6, 12] have reported co-localization of cytoplasmic dynein with microtubules, lysosomes and membranous organelles in axons of peripheral nerve fibers. Our immunohistochemical finding of positive dot-like or filamentous structures in the neuronal cytoplasm may reflect the association of this molecule with microtubules and/or lysosomes. The absence of filamentous staining in parkinsonian SN may indicate damaged microtubules.

The preferential staining in rodent diencephalon and brain stem with the antibody is interesting. Localization of dynein molecules in these areas may show that these neurons require relatively active traffic of organelles for their survival and/or functioning.

This antibody may be useful for observing pathological abnormalities in dynein-containing structures.

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cDNA CATGCTC CCGGCCGCCA TGGCCGCGGG ATTAGCCCGT CAGTATCGGC GGAATTCCGG
Dynein TGCCCGT GAACCTGCTC CGTGCAGGTC GGATCTTTGT GTTTGAGCCG CCACCTGGCG

TGAAAGCCAA CATGCTGAGG ACATTTAGCA GCATCCCTGT CTCTAGGATA TGCAAGTCTC
12571 TGAAGGCCAA CATGCTGAGG ACCTTCAGCA GCATCCCCGT CTCCAGGATG TGCAAGTCTC

CAAATGAGCG CGCCCGCTTG TATTTCCTGC TGGCTTGGTT CCATGCCATC ATCCAAGAGC
12631 CAAATGAGCG AGCCCGCTTG TACTTCCTGC TGGCCTGGTT CCATGCTGTC ATCCAAGAGC

GCCTGCGCTA TGCTCCACTG GGGTGGTCAA AGAAGTACGA ATTTGGAGAA TCTGACCTTC
12691 GCCTGCGCTA TGCTCCACTC GGGTGGTCCA AGAAATACGA ATTTGGAGAA TCTGACCTTC

GATCAGCTTG TGACACGGTG GACACGTGGC TGGACGACAC AGCCAAGGGC CGGCAGAACA
12751 GATCGGCTTG TGACACGGTG GACACATGGT TGGATGACAC AGCCAAGGGC CGACAGAACA

TCTCACCGGA TAAGATCCCA TGGTCTGCCC TGAAGACCCT GATGGCGCAG TCCATCTATG
12811 TCTCACCAGA TAAGATCCCA TGGTCTGCCC TGAAGACATT GATGGCCCAG TCCATCTATG

GTGGGCGGGT GGACAATGAG TTCGACCAGC GTCTGCTCAA CACCTTCCTG GAACGGCTGT
12871 GTGGGCGGGT GGACAATGAG TTTGACCAGC GTCTGCTCAA TACCTTCCTG GAGCGCCTGT

TCACCACCCG CAGCTTCGAC AGTGAATTCA AGCTGGCGTG CAAGGTTGAC GGGCACAAGG
12931 TTACCACCCG GAGTTTCGAT AGTGAATTCA AGCTGGCGTG CAAGGTCGAT GGGCACAAGG

ACATCCAGAT GCCTGATGGT ATCAGGCGAG AGGAGTTTGT GCAGTGGGTG GAGCTGCTGC
12991 ACATTCAGAT GCCTGACGGT ATCAGGCGAG AGGAGTTTGT GCAGTGGGTG GAGCTGCTGC

CTGACGCCCA GACACCCTCA TGGCTGGGCC TCCCCAACAA TGCTGAAAGG GTCCTGCTTA
13051 CTGACGCCCA GACACCCTCA TGGCTGGGCC TCCCCAACAA TGCTGAAAGG GTCCTGCTTA

CCACCCAAGG CGTGGACATG ATCAGCAAGA TGCTGAAGAT GCAGATGCTG GAGGACGAGG
13111 CCACCCAAGG CGTGGACATG ATCAGCAAGA TGCTGAAGAT GCAGATGCTG GAGGACGAGG

ATGACCTGGC CTACGCAGAG ACGGAGAAGA AGGCAAGGAC GGACTCCACC TCCGATGGCC
13171 ACGACCTGGC CTACGCAGAG ACGGAGAAGA AGACAAGGAC GGACTCCACC TCAGACGGCC

GTCCAGCCTG GATGAGGACA CTGCACACCA CGGCGTCCAA CTGGCTGCAC CTCATCCCAC
13231 GTCCAGCCTG GATGAGAACA CTGCACACGA CGGCCTCAAA CTGGCTGCAC CTCATCCCAC

AGACACTGAG CCCCCTGAAG CCCGGAATTCC AGCTGAGCG CCGGTCGCAA TCACTAGTGC
13291 AGACACTGAG CCCACTGAAG CGCACGGTGG AGAACATCAA GGACCCTTTG TTTAGGTTCT

GGCCGCCTGC AGGTCGACCA TATGGGAGAG
13361 TCGAGAGAGA GGTGAAGATG GGGGCCAAGT

Fig. 4 Comparison of nucleotide sequence in cDNA obtained by immunoscreening with that in dynein. Different nucleotides between the sequences of the cDNA and rat dynein dynein were underlined

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