Characterization of a Minus End–directed Kinesin-like Motor Protein from Cultured Mammalian Cells

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Abstract. Using the CHO2 monoclonal antibody raised against CHO spindles (Sellitto, C., M. Kimble, and R. Kuriyama. 1992. *Cell Motil. Cytoskeleton.* 22:7–24) we identified a 66-kD protein located at the interphase centrosome and mitotic spindle. Isolated cDNAs for the antigen encode a 622-amino acid polypeptide. Sequence analysis revealed the presence of 340-amino acid residues in the COOH terminus, which is homologous to the motor domain conserved among other members of the kinesin superfamily. The protein is composed of a central α-helical portion with globular domains at both NH_2 and COOH termini, and the epitope to the monoclonal antibody resides in the central α-helical stalk. A series of deletion constructs were created for in vitro analysis of microtubule interactions. While the microtubule binding and bundling activities require both the presence of the COOH terminus and the α-helical domain, the NH_2-terminal half of the antigen lacked the ability to interact with microtubules. The full-length as well as deleted proteins consisting of the COOH-terminal motor and the central α-helical stalk supported microtubule gliding, with velocity ranging from 1.0 to 8.4 μm/minute. The speed of microtubule movement decreased with decreasing lengths of the central stalk attached to the COOH-terminal motor. The microtubules moved with their plus end leading, indicating that the antigen is a minus end–directed motor. The CHO2 sequence shows 86% identify to *HSET*, a gene located at the centromeric end of the human MHC region in chromosome 6 (Ando, A., Y. Y. Kikuti, H. Kawata, N. Okamoto, T. Imai, T. Eki, K. Yokoyama, E. Soeda, T. Ikemura, K. Abe, and H. Inoko. 1994. *Immunogenetics.* 39:194–200), indicating that HSET might represent a human homologue of the CHO2 antigen.

THE mitotic spindle is the structure responsible for equidistribution of the genetic material into each daughter cell. It is composed of microtubules plus a number of associated molecules. Microtubules are in a highly dynamic state, which accounts for the dynamic nature of the mitotic spindle during cell division. The dynamic properties of the relatively invariant microtubular structure are mediated by interaction with a variety of proteins, collectively referred to as microtubule-associated proteins.

Much evidence has recently been accumulated indicating that one class of microtubule-associated proteins, the microtubule motor proteins, are present in the mitotic spindle and play an important role during mitosis (for reviews see McIntosh and Pfarr, 1991; Sawin and Endow, 1993). Genetic analysis has allowed the identification of mitosis-specific kinesin-like molecules, such as cut7 (Hagan and Yanagida, 1990), bimC (Enos and Morris, 1990), KAR3 (Meluh and Rose, 1990), CIN8 (Hoyt et al., 1992), KIP1 (Roof et al., 1992; Hoyt et al., 1992) in fungi, and ncd (Endow et al., 1990; McDonald and Goldstein, 1990) and nod (Zhang et al., 1990) in *Drosophila.* Recent application of PCR technologies has made it possible to characterize additional kinesin-like proteins in a number of species (Roof et al., 1992; Aizawa et al., 1992; Mitsui et al., 1993; O'Connell et al., 1993; Heck et al., 1993; Kondo et al., 1994; Pesavento et al., 1994; Bernstein et al., 1994). Likewise, polyclonal antipeptide antibodies prepared to the conserved kinesin sequences have been widely used to identify additional kinesin-like molecules in sea urchin embryos (Cole et al., 1992), eukaryotic flagellar axonemes (Fox et al., 1994; Johnson et al., 1994), and cultured mammalian cells (Sawin et al., 1992; Wordemann and Mitchison, 1995). To identify molecular components of the mitotic spindle, mAb probes were prepared against isolated spindles (Sellitto et al., 1992) and chromosomes (Yen et al., 1991). Antigens correspond-

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ing to some antibodies have proven to be kinesin-like molecules, leading to identification of CHO1/MKLP1 (Nislow et al., 1992; Kuriyama et al., 1994) and CENP-E (Yen et al., 1992) located at the interzonal region of the spindle and kinetochores, respectively. These motors are believed to generate forces acting on different regions of the mitotic spindle.

Based on comparison of amino acid sequences, a variety of kinesin-like proteins are now classified into several subclasses (Goldstein, 1993; Goodson et al., 1994). One subclass, the KAR3 family, is of particular interest. It includes KAR3 (Meluh and Rose, 1990), ncd (Endow et al., 1990; McDonald and Goldstein, 1990), KLPA in *Aspergillus* (Roof et al., 1992), and KatA in *Arabidopsis* (Mitsui et al., 1993). They are unique in the sense that, unlike other members of the kinesin superfamily, the mechanochemical motor domain resides at the COOH terminus of the protein rather than the NH_2 terminus. In addition, ncd (McDonald et al., 1990; Walker et al., 1990) and KAR3 (Endow et al., 1994*b*) have been shown to move along microtubules towards the minus end, a direction opposite to that seen with kinesins and other kinesin-like proteins. Since chromosomes move towards opposite poles from plus to minus along kinetochore microtubules, members of the KAR3 subfamily could be involved in the mechanism of chromosome movement during mitosis.

Using the CHO2 mAb, raised against mitotic spindles isolated from CHO cells, we have identified a novel 66-kD spindle component (Sellitto et al., 1992). The antibody was originally screened as a specific probe for the centrosome in cultured mammalian cells. The antibody was also able to block the regrowth of microtubules onto isolated centrosomes, suggesting that the CHO2 antigen might be involved in nucleation of microtubules at centrosomes (Sellitto et al., 1992). To extend the study of this unique spindle component, we cloned the cDNA encoding the CHO2 antigen by screening an CHO expression library using the CHO2 antibody as a probe. Here we report isolation and characterization of the cDNA clones. Analysis of the nucleotide and deduced amino acid sequence showed that the COOH-terminal half of the protein contains a region of 340 amino acids that shares significant identity with the motor domain conserved among members of the kinesin superfamily. The NH_2-terminal half of the antigen, however, shows little homology to other kinesin-like proteins, suggesting that the CHO2 antigen is a novel COOH-terminal motor kinesin-like protein in cultured mammalian cells. The CHO2 motor domain can support the in vitro gliding of microtubules with their plus ends leading; therefore the antigen is, like other members of the KAR3 subclass, a minus end-directed microtubule motor.

Materials and Methods

Isolation and Sequence Analysis of cDNA Clones Encoding the CHO2 Antigen in CHO Cells

A commercially available CHO cell cDNA expression library cloned in λUni-Zap (Stratagene Inc., La Jolla, CA) was immunoscreened with the monoclonal CHO2 antibody (Sellitto et al., 1992) as described previously (Maekawa and Kuriyama, 1993). One positive clone (17a) was isolated, and its 1.7-kb insert was excised to use as a probe for further screening of the same library, yielding two more clones, 24a and 31b, with insert sizes of 2.0 and 0.9 kb, respectively. To extend the cDNA to include the start codon at the NH_2 terminus, the RACE protocol (Frohmann et al., 1988) was used with a commercially available 5'RACE system (GIBCO BRL, Gaithersburg, MD). The primer sequences used to synthesize cDNA were 5'TGCTCCTGGGCTTCTTTAAG3', which is complementary to nucleotide positions 361–381, and a nested CHO2-specific primer (5'AGCACTGATCCAACGGCAGT3') corresponding to nucleotide positions 175–185. 5'-CHO2 with a 450-bp insert was obtained. Two clones (5'-CHO2 and 24a) contain the entire coding sequence (1,869 bp) of the antigen, as well as 270 and 172 nucleotides of the 5'- and 3'-untranslated regions, respectively. The 5'-CHO2 PCR products were cloned using pCR™II (Invitrogen, San Diego, CA), and the DNA sequence of the three clones (17a, 24a, and 5'-CHO2) was analyzed as before (Maekawa and Kuriyama, 1993).

RNA Blot Analysis

5–10 μg of mRNA, which was isolated and purified from CHO cells, was separated on a 1% agarose/formaldehyde gel, transferred to a nylon membrane, and probed with the 17a CHO2 cDNA clone labeled by random priming. The final wash of the nylon membrane was done at 65°C with a medium containing 0.2× SSC and 0.1% SDS.

Preparation of CHO2 Antigens Expressed in Bacteria and Sf9 Cells

Truncated polypeptides of the CHO2 antigen were overexpressed in bacteria as fusion proteins using pGEX expression vectors with a 26-kD leader sequence of glutathione *S*-transferase (Smith and Johnson, 1988). The 2.0- and 1.7-kb EcoRI-XhoI fragments of 24a and 17a, respectively, were isolated from the pBS vector and inserted into EcoRI and XhoI restriction sites of pGEX3UZ, which was generated by addition of an XhoI site to the pGEX3 vextor. Δ1 (1.3-kb EcoICRI-XhoI fragment), Δ2 (1.2-kb EspI-XhoI fragment), and Δ3 (1.0-kb NcoI-XhoI fragment) were derived from pGEX-17a, while Δ4 (0.86-kb BamHI-NcoI fragment) and Δ5 (0.67-kb EspI-NcoI fragment) were isolated from pVL1392-CHO2 (see below) and pBS-24a, respectively. They were cloned into pGEX1, 2, or 3 depending on the required reading frame. After incubation with 0.5–1.0 mM isopropyl β-D-thiogalactopyranoside for 5 h at 22–37°C, pelleted cells were washed twice with cold PBS and resuspended in 5 vol of either 100PEM (100 mM Pipes at pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$) or PBS plus 0.1 mg/ml PMSF. Cells were sonicated for 30 s to 1.5 min at 0°C, and then Triton X-100 was added at a final concentration 1% to the lysate, which was further cleared by centrifugation at 13,000 *g* for 10 min. 10 ml of the supernatant was incubated with 0.5 ml glutathione-conjugated agarose beads (Sigma Chemical Co., St. Louis, MO) at 4°C for 60 min with agitation. Beads were next washed three times with either 100-PEM or PBS and further incubated for 10 min with an equal volume of 10 mM glutathione. Eluates were dialyzed against 100PEM, aliquoted, and stored at −80°C until use. Recovery of the fusion proteins was monitored by 7.5% SDS-PAGE and immunoblot analysis.

For expression of the full coding sequence of CHO2 antigen, the clones of 5'-CHO2 and 24a were joined at the ApaI site (nucleotide position 118). Purified cDNA was subcloned into multicloning sites of pVL1392 and introduced into moth ovarian Sf9 cells by cotransfection with partially purified, deleted, linearized baculovirus DNA as described previously (Maekawa and Kuriyama, 1993; Kuriyama et al., 1994). Sf9 cells expressing the CHO2 antigen were washed with PBS, and supernatants were prepared in 100PEM (Vassilev et al., 1995).

Immunological Techniques

Antibody Preparation and Purification. Purified fusion proteins encoded by the 17a and 24a cDNAs were mixed with Freund's complete ajuvant and used to immunize rabbits by subcutaneous injections. Sera collected from the animals were affinity purified by binding to the protein immobilized on nitrocellulose blots (Maekawa and Kuriyama, 1993).

Immunofluorescence Staining. CHO cells grown on coverslips in Ham's F-10 medium plus 7.5% FCS were fixed in methanol for 5 min at −20°C. Mitotic spindles isolated from synchronized CHO cells (Kuriyama et al., 1984) were mounted on polylysine-coated coverslips and fixed with absolute methanol as above. After rehydration with PBS containing 0.05% Tween-20 (PBS-Tw20), coverslips were double stained with a purified rabbit polyclonal anti-17a fusion protein antibody and either a mouse monoclonal CHO2 antibody or a mouse monoclonal anti-chicken β-tubulin antibody (Amersham Corp., Arlington Heights, IL). Microscopic observation was made on a microscope (model BH2; Olympus Corp., Lake Success, NY) with eqifluorescence optics.

Immunoblot Analysis. Purified fusion proteins and isolated CHO spindles were boiled in SDS sample buffer for 5 min and run on 7.5% SDS-PAGE. Separated proteins were then transferred to nitrocellulose membranes and probed with monoclonal CHO2 and affinity-purified fusion protein antibodies as before (Maekawa and Kuriyama, 1993). Immunoreactive bands were visualized by alkaline phosphatase-conjugated secondary antibody (Hyclone Laboratories, Inc., Logan, UT) with NBT/BCIP as the chromogen.

Microtubule Binding and Bundling Assays

Purified fusion proteins in 100PEM were clarified by centrifugation at 13,000 *g* for 15 min at 4°C and mixed for 10 min at 0°C with a two-thirds volume of taxol-stabilized microtubule-associated protein-free brain microtubules prepared in a medium containing 100PEM, 1 mM GTP, and 20 μg/ml taxol (Maekawa and Kuriyama, 1993). Supernatants as well as pellets of microtubules plus associated proteins were separated by centrifugation, mixed with SDS sample buffer, and run on 7.5% SDS-PAGE. For assays of the microtubule bundling activity, the truncated polypeptides of the CHO2 antigen were mixed with polymerized microtubules and mounted on slide glass for observation by phase-contrast microscopy or Formvar-coated 200-mesh grids for whole-mount electron microscopy as described before (Kuriyama et al., 1994).

In Vitro Microtubule Motility Assays

Motor activity of full-length and truncated polypeptides of the CHO2 antigen was assayed according to the protocol described by Hyman (1991). Fluorescently labeled brain microtubules were prepared by polymerization of rhodamine-conjugated tubulin dimers (Hyman et al., 1991). Polarity-marked microtubules were made as described by Hyman (1991) with the following modifications. Equal volumes of rhodamine-labeled tubulin, cold tubulin, and glycerol buffer (33% glycerol, 160 mM MES at pH 6.8, 1 mM EGTA, 5 mM $MgCl_2$) were combined and incubated at 30°C for 30 min. After addition of 0.1 vol of 1 mM taxol, the mixture was allowed to sit for 10 min at 30°C and then incubated with 40 vol of 1:10 diluted rhodamine tubulin for an additional 30 min. Taxol was added to the polymerized microtubule fraction at a final concentration of 0.1 mM to stabilize the polarized microtubules.

For observation of microtubule movement, coverslips were first coated with 20 μl 2.5 mg/ml casein and washed with 100 μl HMDEK buffer, which contained 30 mM Hepes at pH 7.4, 5 mM $MgSO_4$, 1 mM DTT, 1 mM EGTA, and 25 mM KCl. 20 μl of Sf9 cell supernatants and/or purified bacterial fusion proteins in 100PEM were applied to the coverslip and allowed to adsorb for ~1 min. The absorption process of motor proteins onto the glass surfaces was repeated several times. After draining, the coverslip was perfused with 20 μl of fluorescently labeled microtubules in HMDEK buffer plus "anti-fade" (4.5 mg/ml glucose, 216 μg/ml glucose oxidase, 36 μg/ml catalase, 1% β-mercaptoethanol) (Harada et al., 1990), 5 mM MgATP, and 10 μM taxol. Unbound microtubules were washed out with 50–100 μl of the same solution without microtubules. For the assay of the full coding sequence of the antigen, the supernatant of Sf9 cells expressing the full-length CHO2 antigen was mixed with 1% NP-40 and clarified by centrifugation before mounting on coverslips. Microscopic observation and videotaping of fluorescence images were done as described by Harada et al. (1990). Velocities of microtubule movement were measured by a computer image processor (Avio Excel + NEC PC-9801RA; Nippon Avionics Co., Japan) (Harada et al., 1990).

Results

Isolation and Sequence Analysis of DNA Encoding CHO2 Antigen

The 17a cDNA clone, isolated by immunoscreening of an expression library with the monoclonal CHO2 antibody, hybridizes to a single 2.3-kb transcript on Northern blot analysis (Fig. 1 *A*), a size which is appropriate to code for the CHO2 antigen, which has an apparent molecular mass of 66 kD. Analysis of the full-coding nucleotide sequence (accession number X83576 in EMBL data base) showed that the longest open reading frame predicts a protein of 622 amino acids in length with a calculated molecular mass of 69 kD. Fig. 1 *B* shows the predicted amino acid sequence of the

Figure 1. (*A*) Northern blot of mRNA prepared from exponentially growing CHO cells. The 1.7-kb fragment isolated from the 17a clone hybridized with a 2.3-kb transcript. (*B*) The predicted amino acid sequence of the CHO2 antigen. The consensus nucleotide-binding domain and the amino acid sequences conserved among members of the kinesin superfamily are marked by double and single underlines, respectively. The complete DNA sequence of the CHO2 antigen is available from EMBL/GenBank/DDBJ under accession number X83576.

CHO2 antigen. The prediction of the protein's secondary structure indicates that the CHO2 antigen is composed of three domains: an α-helical stalk extends from amino acid positions 85 to 258, which is flanked by globular domains located at both the NH_2 terminus (amino acids 1–84) and the COOH terminus (amino acids 259–622) (Fig. 5). The COOH-terminal globular domain possesses a conserved region of 340–amino acid residues corresponding to the putative motor domain. It contains the ATP-binding consensus sequences (GX_4GKT) at amino acid positions 360–367 (*double underline*), and several oligopeptide sequences, such as SSRSH and DLAGSE at amino acid positions 484–488 and 514–519 (*single underline*), respectively, which are conserved among different classes of kinesin-like molecules. In contrast, the NH_2-terminal 40% of the protein shows little homology to previously characterized kinesin-related molecules, suggesting that the CHO2 antigen is a novel member of the kinesin superfamily. The isoelectric point of the CHO2 antigen was calculated as 8.6. While the COOH-terminal globular domain has pI = 8.6, the NH_2-terminal globular domain is highly basic (pI = 12.2), and the central α-helical region is highly acidic (pI = 4.6). The NH_2-terminal globular domain is also rich in proline residues, which constitute up to 11.9% of the total amino acids.

Immunofluorescence Localization of the CHO2 Antigen Probed by the Monoclonal and Polyclonal Antibodies

Bacterially expressed fusion proteins were generated from clones 24a and 17a to prepare specific polyclonal antibodies. Lanes *1* and *2* in Fig. 2 illustrate polypeptide species included in fusion protein fractions affinity purified through glutathione beads. Both truncated CHO2 polypeptides with a leader sequence of 26-kD glutathione *S*-transferase were susceptible to protease digestion, resulting in production of degraded smaller molecular weight polypeptide bands on SDS-PAGE. The amounts of such degraded products can be reduced to a certain extent, if induction temperatures are reduced (for example, Column *24a* in Fig. 6). We could not prepare pure fractions of intact 17a and 24a fusion proteins

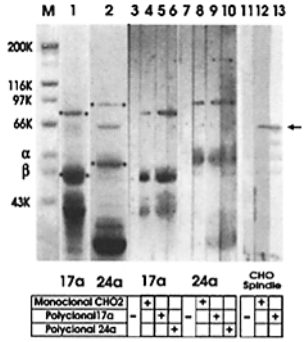


Figure 2. Preparation of polyclonal anti-17a and 24a fusion protein antibodies. (Lane *M*) Molecular weight markers containing myosin (200 kD), β-galactosidase (116 kD), phosphorylase B (97 kD), BAS (66 kD), and ovalbumin (43 kD). α and β indicate the position of brain α- and β-tubulin, respectively. (Lanes *1* and *2*) Affinity-purified 17a and 24a fusion proteins detected by 7.5% PAGE stained with Coomassie blue. In addition to the intact 84- and 92-kD polypeptides for clones 17a and 24a (*upper asterisks*), there are degraded polypeptide species included in the fraction. Some bands (*lower asterisks*) are immunoreactive to both monoclonal and polyclonal CHO2 antibodies. (Lanes *3–10*) immunoblot analysis of 17a (lanes *3–6*) and 24a (lanes *7–10*) fusion protein–containing nitrocellulose strips probed with secondary antibody alone (lanes *3* and *7*), monoclonal CHO2 (lanes *4* and *8*), polyclonal 17a (lanes *5* and *9*), and polyclonal 24a (lanes *6* and *10*) antibodies. (Lanes *11–13*) Immunoblot analysis of mitotic spindles isolated from CHO cells probed with secondary antibody alone (lane *11*), monoclonal CHO2 (lane *12*), and polyclonal 17a fusion protein (lane *13*) antibodies. Both the monoclonal and polyclonal CHO2 antibodies recognize a 66-kD polypeptide species (*arrow*).

with apparent molecular masses of 84 and 92 kD, respectively. Immunoblot analysis showed the positive cross-reactivity of these polypeptides to the original monoclonal antibody (lanes *4* and *8*). The same bands were labeled by the polyclonal antibodies raised against 17a (lanes *5* and *9*) and 24a (lanes *6* and *10*) fusion proteins. The original CHO2 monoclonal antibody was prepared against isolated mitotic spindles, in which a major 66-kD polypeptide species (*arrow* in Fig. 2) reactive to the antibody is included (lane *12*). A polypeptide of the same apparent molecular mass is also recognized by both polyclonal anti-17a (lane *13*) and 24a (data not shown) fusion protein antibodies, indicating that the isolated clones indeed encode the authentic CHO2 antigen molecule. Both the monoclonal and polyclonal antibodies were also noted to occasionally cross-react with 240- and 116-kD polypeptides, besides the 66-kD band. Since these higher molecular mass bands were detected only sporadically on nitrocellulose strips containing highly concentrated spindle proteins, no further attempts have been made to characterize them.

Despite the fact that no significant difference has been noted between the monoclonal and polyclonal antibodies on immunoblot analysis, there was a certain degree of difference in the pattern of immunofluorescence staining (Fig. 3). The mAb labels interphase centrosomes as bright fluorescent aggregates (A_2–D_2), whereas one or two distinct dots next to each nucleus were revealed by the polyclonal probes (A_3–D_3). The mAb staining is restricted exclusively to the centrosomal structure in interphase cells (A_2–D_2). In contrast, the polyclonal antibodies cross-reacted with nuclei, besides centrosomes, with varying degrees of intensity (B_3–D_3). The intensity of the nuclear staining with the polyclonal antibodies was found to be cell cycle dependent, with the most intense staining of nuclei occurring just before mitosis (data shown). Micrographs in Fig. 3, *E* and *G*, correspond to mitotic spindles in whole cells (*E*) and the isolated spindle fraction (*F* and *G*) double stained with either the monoclonal (E_2 and F_2) and polyclonal (E_2 and F_3) CHO2 antibodies, or anti-tubulin (G_2) and polyclonal CHO2 (G_3) antibodies. The monoclonal probe revealed the presence of the CHO2 antigen concentrated at the spindle poles (E_2, F_2), whereas intense spindle fiber staining became apparent with the polyclonal antibodies (E_3, F_3). Although the polyclonal antibodies showed the presence of the CHO2 antigen in almost the entire spindle region (G_3), the spindle fiber staining did not entirely overlap with that of tubulin (G_2). This difference, which is particularly striking around the midzonal region of the spindle, may reflect the difference in availability of the epitope to the two kinds of antibodies. The monoclonal CHO2 antibody labels the centrosome primarily in CHO cells. In contrast, the polyclonal antibodies cross-react with the antigen present in the centrosome, nucleus, and spindle poles/fibers in sea urchin embryos as well as in other mammalian cells, such as HeLa, PtK_1, 3T3, MDCK, and gerbil fibroma, indicating that the CHO2 antigen is a ubiquitous component of the mitotic spindle/centrosome.

In Vitro Interaction of Microtubules with Truncated CHO2 Polypeptides

Preparation of Truncated COOH- and NH_2-terminal Polypeptides. To identify subdomains of the CHO2 antigen necessary for interaction with microtubules in vitro, we have prepared a series of deletion constructs (Fig. 4). Full-length protein was expressed in insect ovarian Sf9 cells using the baculovirus expression system, while the truncated polypeptides were prepared in bacteria as fusion proteins with the 26-kD glutathione *S*-transferase leader sequence. 24a encodes nearly the full-length protein, in which the first 11-amino acid residues are missing. 17a, Δ1, and Δ2 contain the conserved mechanochemical motor domain at the COOH terminus with differing lengths of central α-helical stalk, whereas Δ3 encodes the COOH-terminal globular region only. Two deletion constructs, Δ4 and Δ5, encode the NH_2-terminal globular domain plus different lengths of the α-helical stalk. Substantial parts of the proteins induced in bacteria were recovered in the supernatant, and individual truncated polypeptides were purified using glutathione-conjugated beads (Fig. 2, lanes *1* and *2*; Fig. 5, *CBB*). Immunoblot analysis with the monoclonal and polyclonal fusion protein antibodies shows that while all molecular species of truncated polypeptides were recognized by the polyclonal antibodies, the monoclonal CHO2 antibody failed to react with the proteins encoded by the clones Δ1, Δ2, and Δ3. Since the protein encoded by clone 17a was stained with the monoclonal CHO2 antibody (Fig. 2, lane *4*), the epitope for the mAb resides in the α-helical central portion between amino acid positions 110–179.

Microtubule Binding and Bundling Activities. The ability of the truncated CHO2 polypeptides to bind to microtubules was measured by microtubule cosedimentation experiments (Fig. 6). Columns *24a* to Δ2 show Coomassie-stained gel patterns of the proteins derived from clones 24a, 17a, Δ1, and Δ2, respectively. Since the Δ3, Δ4, and Δ5 fusion proteins comigrate with either brain α- (Δ3 and Δ4) or

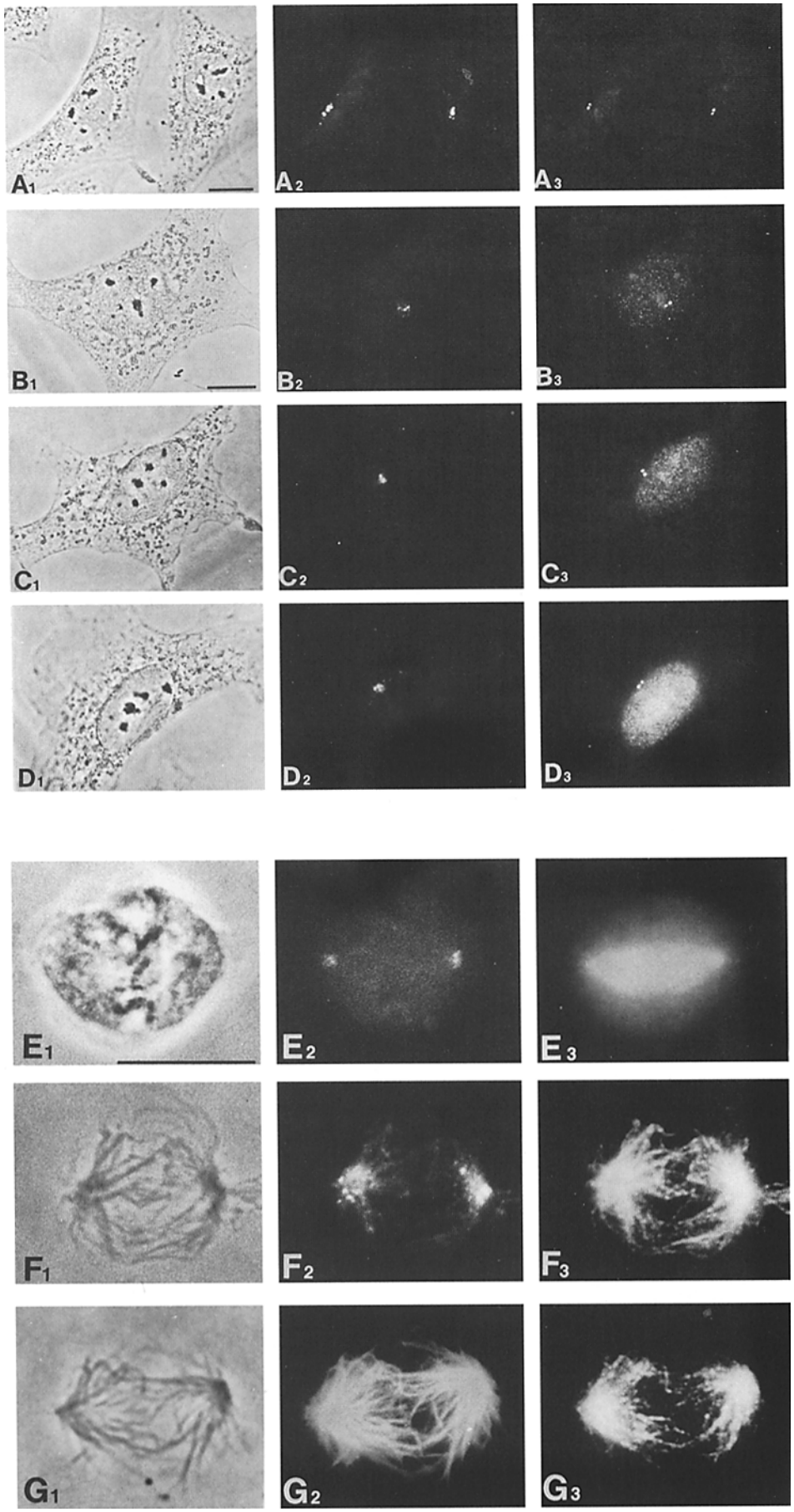
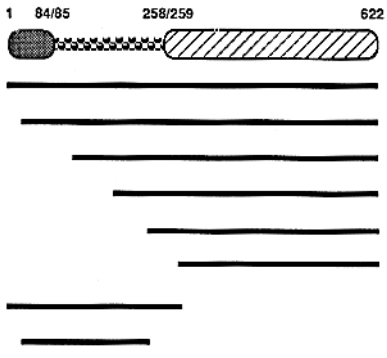


Figure 3. Localization of the CHO2 antigen in whole CHO cells (*A–E*) and isolated mitotic spindles (*F–G*). The same cells/spindles are seen by phase-contrast (A_1–G_1) and fluorescence microscopy after double staining with either monoclonal CHO2 (A_2–F_2) and affinity-purified polyclonal anti-17a fusion protein (A_3–F_3) antibodies, or antitubulin (G_2) and polyclonal 17a fusion protein (G_3) antibodies. While the mAb labels centrosomal aggregates, the polyclonal antibody reveals only one or two dots next to each nucleus in interphase cells. Nuclei as well as spindle fibers are also stained by the polyclonal antibody. Bar, 10 μm.



Constructs		MT-INTERACTION		
		Binding	*Bundling*	*Gliding*
CHO2	(1-622)	ND	ND	+
24a	(12-622)	+	+	+
17a	(110-622)	+	+	+
Δ 1	(180-622)	+	+	+
Δ 2	(235-622)	+	+	-
Δ 3	(286-622)	+/-	-	-
Δ 4	(1-285)	+	-	ND
Δ 5	(12-234)	+/-	-	ND

Figure 4. Deletion constructs of the CHO2 antigen created for in vitro analysis of microtubule interactions. The antigen is composed of three domains: the NH_2-terminal globular (amino acid positions 1–84), central α-helical stalk (85–258) and COOH-terminal globular (259–622) regions. The results of microtubule binding, bundling, and gliding assays are shown at the right.

β- (Δ5) tubulin on SDS-PAGE, the supernatant and pellet fractions were probed with the polyclonal (Δ3) and monoclonal (Δ4 and Δ5) antibodies to detect the fusion proteins (Δ3 to Δ5). After incubation with (lanes *1* and *2*) or without (lanes *3* and *4*) brain microtubules for 10 min on ice, protein species included in supernatants (lanes *1* and *3*) and microtubule pellets (lanes *2* and *4*) were analyzed. Almost all 24a, and substantial amounts of 17a, Δ1, Δ2, and Δ4 proteins were recovered in the pellet fraction after incubation with brain microtubules. Copurification of these polypeptides with microtubules was partially sensitive to ATP and high salt. In contrast, much lower amounts of Δ3 and Δ5 proteins were pelleted with microtubules.

During the 10-min incubation on ice in the presence of 0.1–1 mM GTP, microtubules mixed with 24a, 17a, Δ1, and Δ2 became cross-linked to form large bundles that were easily detected by phase-contrast microscopy. Bundle formation was also evident on coverslips, which were monitored for microtubule gliding activity of the truncated CHO2 polypeptides in vitro (see below). Whole-mount electron microscopy revealed the presence of bundled microtubules, typically composed of several to ~20 microtubules, and the individual microtubules are closely associated side by side along their entire length (data not shown). The overall appearance of bundled microtubules was quite similar to those cross-linked by the CHO1 antigen, another kinesin-like protein with an NH_2-terminal motor domain (Kuriyama et al., 1994). However, the Δ3 clone, which encodes only the COOH-terminal globular domain, and clones Δ4 and Δ5, which code for the NH_2-terminal tail domain with or without the central α-helical stalk, failed to bundle microtubules in vitro as determined by both light and electron microscopic analysis (Fig. 4). These results indicate that the microtubule

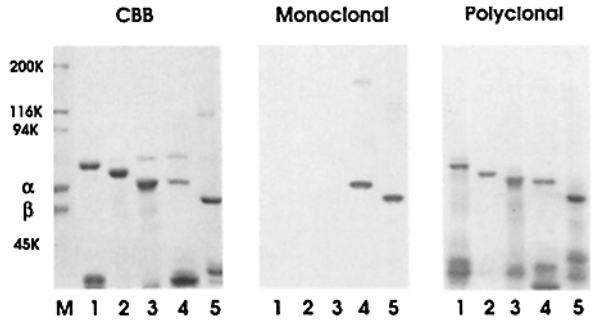


Figure 5. Purification of truncated polypeptides of the CHO2 antigen. (Lane *M*) Molecular mass markers containing myosin (200 kD), β-galactosidase (116 kD), phosphorylase B (94 kD), brain α- and β-tubulin, and ovalbumin (45 kD). (Lanes *1–5*) Affinity-purified fusion proteins derived from the deletion constructs of Δ1 to Δ5, respectively. *CBB*, 7.5% polyacrylamide gel stained with Coomassie blue. *Monoclonal*, immunoblot analysis of the nitrocellulose membrane probed with the monoclonal CHO2 antibody. *Polyclonal*, immunoblot analysis of the nitrocellulose membrane probed with affinity-purified polyclonal anti-17a fusion protein antibody. The monoclonal CHO2 antibody recognizes the NH_2-terminal tail-containing Δ and Δ5, but not Δ1, Δ2, and Δ3, indicating that the epitope to the mAb resides in the central α-helical stalk.

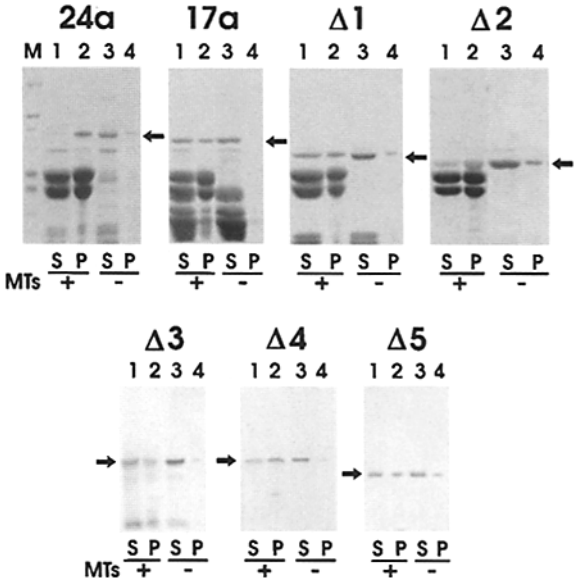


Figure 6. Cosedimentation of taxol-stabilized brain microtubules with affinity-purified bacterially expressed fusion proteins. (Columns *24a*, *17a*, *Δ1*, *Δ2*) 7.5% polyacrylamide gel stained with Coomassie blue. (Columns *Δ3*, *Δ4*, *Δ5*) Immunoblot analysis. (Lanes *1* and *3*) Supernatant; (lanes *2* and *4*) pellet after centrifugation at 13,000 *g* for 15 min. Each fusion protein, in 100PEM, was incubated for 10 min at 0° in the presence (lanes *1* and *2*) or absence (lanes *3* and *4*) of a two-thirds volume of taxol-stabilized brain microtubules prepared in 100PEM, 1 mM GTP, and 20 μg/ml taxol. Arrows indicate the position of fusion protein bands.

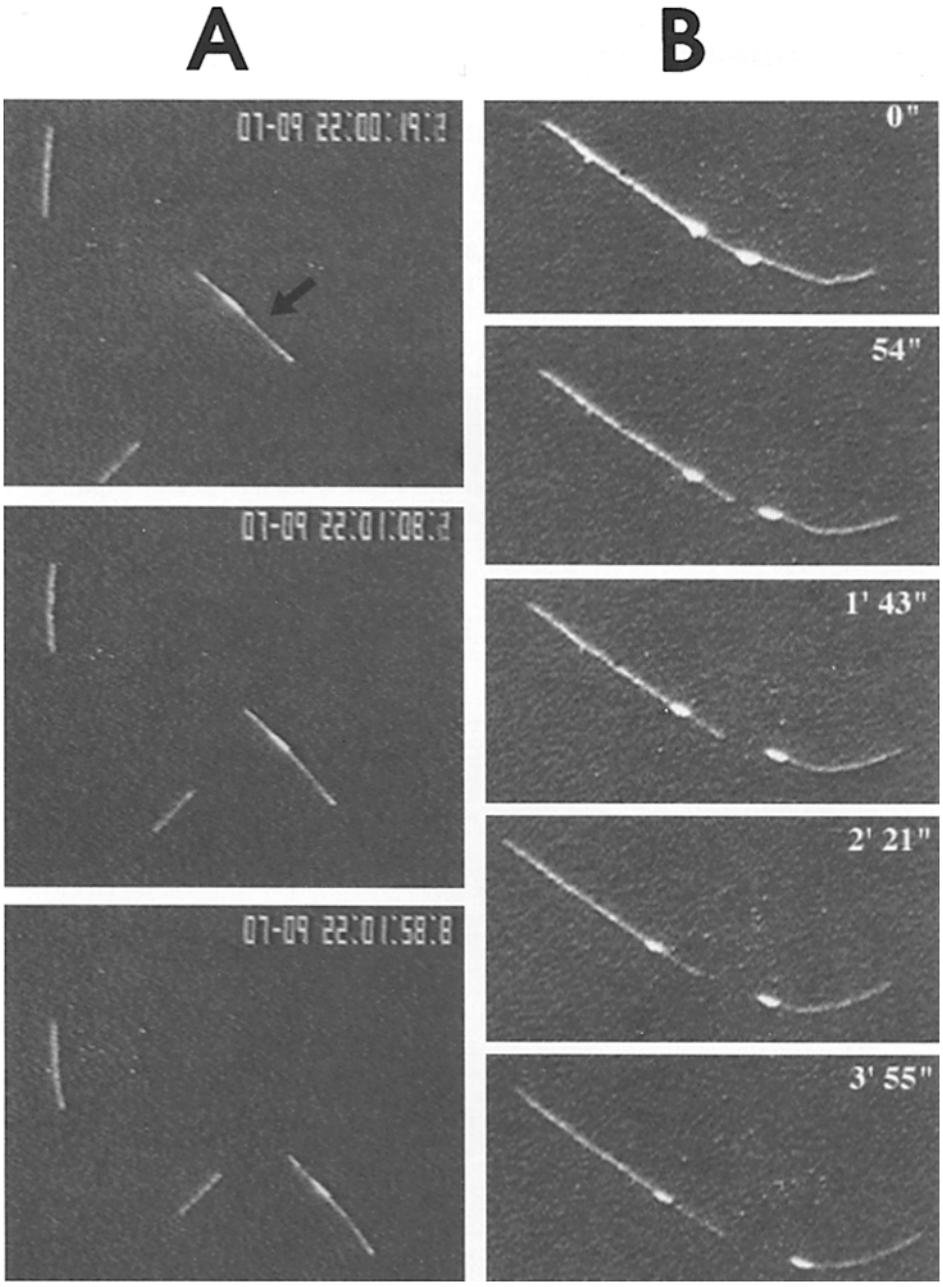


Figure 7. In vitro motility of fluorescently labeled brain microtubules in the presence of affinity-purified 17a fusion protein (*A*) and full-coding CHO2 sequence included in the Sf9 cell supernatant (*B*). Video frames from the times designated in each frame were photographed. Asymmetrically labeled microtubules, one in *A* (*arrow*) and two in *B*, move with the brightly marked minus end lagging, indicating that the CHO2 antigen is a minus end–directed motor.

binding and bundling activities require the presence of both the COOH terminus and the α-helical domains.

Microtubule Gliding Activity. In vitro motor activity of the CHO2 antigen was examined by monitoring microtubule gliding on coverslips coated with various domains of the antigen by fluorescence video microscopy (Fig. 7). Although lysates of the Sf9 cell expressing the full-length CHO2 antigen contained a visible 66-kD protein band on Coomassie-stained SDS gel, the major part of the protein pelleted after centrifugation of extracts at 13,000 *g* for 15 min. The supernatant was brought to 1% NP-40, centrifuged, and the recovered supernatant was applied to a coverslip. When fluorescently labeled taxol-stabilized microtubules were added with 5 mM MgATP, the microtubules attached to and moved over the glass surface (Fig. 7 *B*). The velocity ranged between 1.1 and 5.2 μm/min (n = 30) among different microtubules on the coverslip, different coverslip preparations, and different protein sample preparations. Microtubule movement did not occur in the absence of MgATP. When the CHO2 antigen-containing supernatant was not mixed with NP-40 before centrifugation, microtubules did not bind. Microtubules also failed to bind to coverslips coated with 13,000-*g* supernatants prepared from noninfected Sf9 cells, or cells expressing a nonmotor protein (*Chlamydomonas* γ-tubulin; Vassilev et al., 1995).

Microtubule movement was also observed with various bacterially expressed fusion proteins (Fig. 4). The fusion proteins encoded by 24a, 17a (Fig. 7 *A*) and Δ1 supported microtubule movement with mean velocities of 3.2 $\pm$ 1.1 (1.0–5.0 μm/min; n = 67), 5.2 $\pm$ 1.6 (2.2–8.4 μm/min; n =

41), and 1.7 ± 0.5 μm/min (0.5–2.6 μm/min; n = 57), respectively. In contrast, although microtubules bound to coverslips coated with fusion protein Δ2, but they did not exhibit any movement. Finally, microtubules failed to bind to coverslips coated with fusion protein Δ3. To determine the directionality of microtubule movement, microtubules polarity-marked by asymmetrical fluorescent labeling were prepared according to the procedures developed by Hyman et al. (1991) (Fig. 7 *B*; Fig. 7 *A*, arrow), then applied to the cover glass chamber precoated with various CHO2 polypeptides. The polarity-marked microtubules moved with their plus ends leading, indicating that the CHO2 antigen is a minus end-directed motor molecule.

Sequence Comparison of the CHO2 Antigen With Other Members in the KAR3 Subclass

Since the CHO2 antigen is a minus end-directed motor with the motor domain at the COOH terminus, its amino acid sequence was compared with that of other members of the KAR3 subclass in the kinesin superfamily (Fig. 8). The CHO2 antigen showed the highest degree (38%) of identity to KatA in *Arabidopsis* than any other COOH-terminal motor proteins in animal and fungal cells (ncd, 32%; KLPA, 34%; KAR3, 31%). The highly conserved motor domain of the CHO2 antigen (amino acid positions 243–622) shares 47% identity to KatA and KLPA, but to a lesser extent to ncd (43%) and KAR3 (44%). The NH_2-terminal third of the CHO2 protein (amino acid positions 1–242) shows a limited degree of identity to ncd (15%), KLPA (15%), and KAR3 (11%). In contrast, KatA has a relatively high degree of identity (24%) to CHO2 antigen in this NH_2-terminal region. These results indicate that the CHO2 antigen is a novel member of the COOH-terminal motor kinesin-like protein subfamily. In addition to the several consensus sequences in the motor domain that are conserved among all kine-

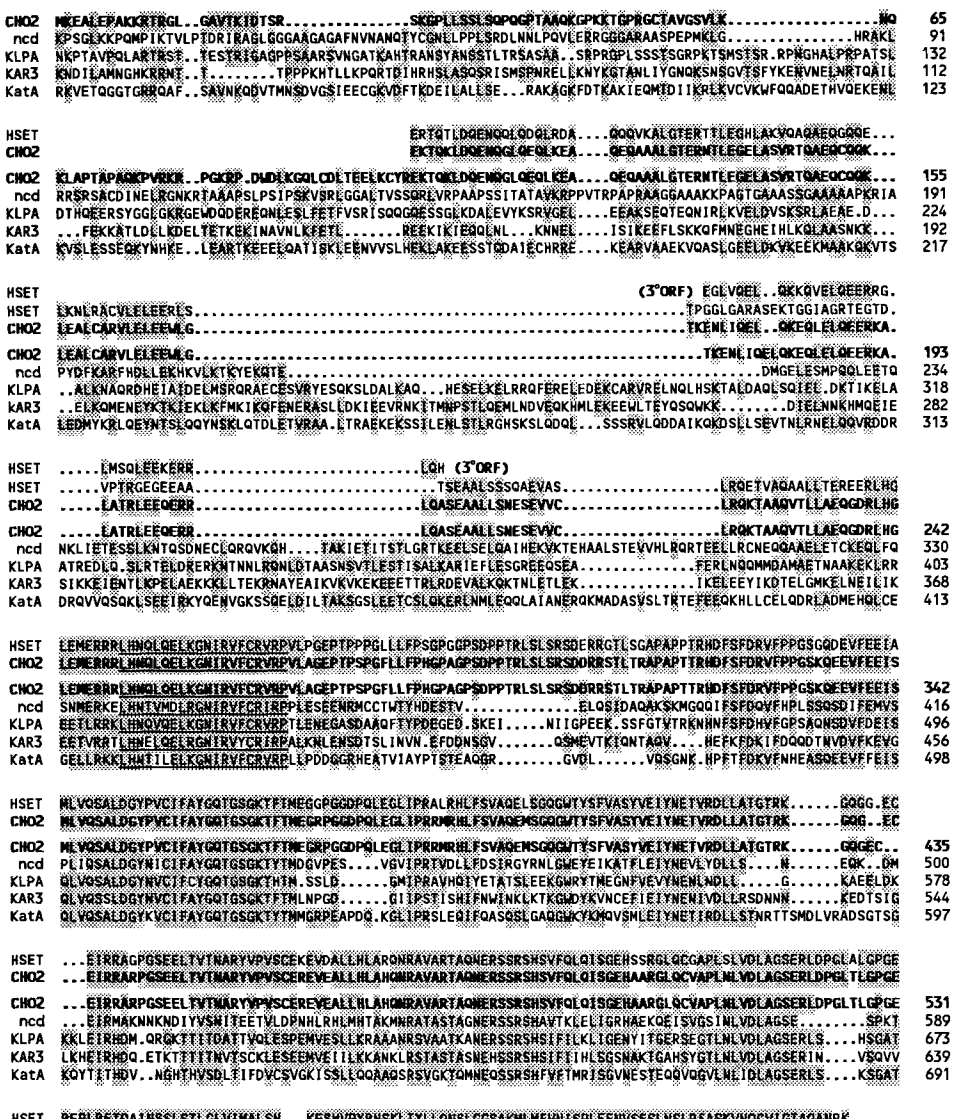


Figure 8. Comparison of the amino acid sequence of the CHO2 antigen with other COOH-terminal motors, including *Drosophila* ncd (Endow et al., 1990; McDonald and Goldstein, 1990), *Aspergillus* KLPA (O'Connell et al., 1993), *Saccharomyces* KAR3 (Meluh and Rose, 1990) and *Arabidopsis* KatA (Mitsui et al., 1993). The antigen's sequence is also compared with HSET, a gene product located at the centromeric end of the human major histocompatibility complex region (Ando et al., 1994). A nucleotide stretch shared among all members of the KAR3 subclass is underlined.

sins/kinesin-like proteins, there is an extra nucleotide stretch shared among all members of the KAR3 subclass. It is located at the border region between the NH_2-terminal globular domain and the central stalk of the CHO2 antigen (amino acid residues 250–269) (Fig. 8, *underline*). It should be pointed out that this consensus sequence is not found in conventional kinesin or kinesin-like proteins with an NH_2-terminal motor domain.

Besides a number of kinesin-related proteins, the computer search of the DNA sequence data base identified a human gene called *HSET* that has significant sequence identity with the CHO2 antigen (Ando et al., 1994). *HSET* was originally identified as a gene located at the centromeric side of the class II gene region of the human major histocompatibility complex (Ando et al., 1994). Although *HSET* analysis appears to be incomplete, alignment of the amino acid sequence showed striking similarity between the CHO and human clones (shown in the first two rows of Fig. 8). Two thirds of the CHO2 antigen (amino acids positions 243–622) is 92% identical to the human clone. In contrast, a region at amino acid positions 105–242 of the antigen shows only 49% homology to the *HSET* sequence. However, it was noted that the sequence difference in the region between amino acid positions 174–206 could be explained by a frame shift, as shown in the line designated 3°ORF, resulting in an overall 67% identity in this region. Assuming that the sequence labeled 3°ORF is correct, the CHO2 antigen and the HSET share an overall identity of 86%, suggesting that HSET is the human homologue of the CHO2 antigen.

Discussion

The CHO2 antigen, which is associated with the interphase centrosome and mitotic spindle, is a kinesin-like molecule with the motor domain located at the COOH terminus. Based on sequence comparison, members of the kinesin superfamily are now classified into several subclasses (Goldstein, 1993; Goodson et al., 1994). Since the CHO2 antigen is a minus end–directed COOH-terminal motor, it is reasonable to classify it into the KAR3 subclass, which includes KAR3 (Meluh and Rose, 1990), KLPA (O'Connell et al., 1993), ncd (Endow et al., 1990; McDonald and Goldstein, 1990), and KatA (Mitsui et al., 1993). The CHO2 antigen from CHO cells shows a higher degree of identity to KatA in *Arabidopsis* than to the other members of the KAR3 subclass (Fig. 8). This suggests that, besides the CHO2 antigen, CHO cells could contain another COOH-terminal kinesin-like motor molecule(s) that is more homologous to animal COOH-terminal kinesin motors than to *Arabidopsis* KatA.

The CHO2 clone encodes a protein with a calculated molecular mass of 69 kD, which is the smallest kinesin-like protein identified thus far (Goldstein, 1993). Although the monoclonal and polyclonal antibodies recognize the polypeptides on immunoblots in an identical manner, there is a distinct difference in the immunofluorescence staining patterns of the two kinds of antibodies (Fig. 3). While the spindle fibers and interphase nuclei are stained with the polyclonal antibodies, the monoclonal CHO2 antibody failed to reveal any of these structures. The epitope to the mAb, which resides in the central α-helical stalk portion (amino acid positions 110–179), may be masked within the structure of nuclei and spindle fibers. We are also puzzled by the fact that the large pericentriolar aggregates of the centrosome are stained with the monoclonal, but not polyclonal, antibodies. Since the polyclonal antibodies were raised against 24a and 17a fusion proteins lacking NH_2-terminal 11- and 109–amino acid residues, respectively, the NH_2-terminal domain might be crucial in subcellular localization of the CHO2 antigen at the pericentriolar region. The centrosomal matrix is known to include different kinds of intermediate filament-like components (Kimble and Kuriyama, 1992). Thus, the possibility exists that the monoclonal CHO2 antibody immunofluorescently recognizes other centrosomal molecules with α-helical coiled-coil regions that share the common epitope with the CHO2 antigen.

Structural organization of the CHO2 antigen resembles that of *ncd*, which is composed of an NH_2-terminal tail, α-helical central stalk portion, and the COOH-terminal motor domain (Endow et al., 1990; McDonald and Goldstein, 1990). The NH_2-terminal globular domains of both proteins are extremely basic (pI = 12.2 for both the CHO2 antigen and ncd) and high in proline content (11.9% in CHO2 antigen and 10.4% in ncd). The COOH-terminal motor domain in the CHO2 antigen possesses the capacity to cross-link microtubules, which may mean that the COOH terminus contains additional microtubule-binding regions besides the site(s) associated with the mechanochemical motor activity. A striking difference between two molecules is the interaction of the NH_2 terminus of the proteins with microtubules in vitro. Chandra et al. (1993) reported that the NH_2-terminal tail of ncd was insoluble, and the proteins must be purified with buffers containing urea and/or guanidine HCl. Nevertheless, such purified proteins were able to bind to microtubules and caused extensive microtubule bundling. Although the NH_2 terminus of the CHO2 antigen is as basic as the ncd NH_2 terminus, the truncated NH_2-terminal tail derived from clone $\Delta 2$ failed to bind to and bundle microtubules. Apparently, the charge effect is not sufficient to cause binding of the protein to microtubules. This may be due to the difference in size of the NH_2-terminal globular domain included in the CHO2 antigen (84 amino acid residues) and ncd (199 amino acid residues). Since the nonmotor globular domains are believed to be important for defining the functional specificity of individual kinesins and kinesin-like proteins (Vale and Goldstein, 1990), such differences in the NH_2-terminal domains and microtubule interaction between the CHO2 antigen and ncd may reflect differences in their biological functions.

The CHO2 antigen moves microtubules with the plus end leading, showing that, like all COOH-terminal motors analyzed so far, the antigen possesses a minus end–directed motor activity. Although there are slight variations among different CHO2 constructs, velocities of microtubule movement ranged between 1.0 and 8.4 μm/min, which is slower than ncd (15 μm/min measured by McDonald et al., 1990; 4–10 μm/min analyzed by Walker et al., 1990, and Chandra et al., 1993), but faster than KAR3 (1–2 μm/min) (Endow et al., 1994*b*). Supernatant lysates of Sf9 cells expressing the antigen contain a limited amount of the full coding CHO2 antigen. In addition, fusion proteins purified from bacterial extracts, especially those derived from clones 24a and 17a, include a relatively large amount of degraded polypeptide species (Fig. 2). The somewhat slower in vitro motility of microtubules may thus be a result of interference from con-

taminating degradation products. On the other hand, the difference in velocity may reflect a real difference in motile activity between various COOH-terminal kinesin-like molecules. There are also significant differences in velocities among the three different CHO2 fusion proteins, with 17a having the highest velocity and Δ1 the lowest. However, it is premature to speculate on the structural basis for this difference.

The COOH-terminal motors, KAR3 and ncd, were originally identified as essential molecules for yeast nuclear fusion and *Drosophila* meiosis. Endow et al. (1994*a*) have recently created null and deletion mutants of ncd to demonstrate that the protein is important in maintenance of structural as well as functional integrity of the spindle poles in meiosis and early mitosis. The CHO2 antigen, on the other hand, was found using an antibody probe specific to mammalian centrosomes (Sellitto et al., 1992), and the functional property of the protein has not yet been established. Based on subcellular localization of the antigen in dividing cells, it is possible that, like other COOH-terminal motor molecules, the CHO2 antigen is also involved in the mechanism of mitosis and its regulation in mammalian cells. The antigen appears to associate with the length of spindle fibers. If the protein is immobilized in the spindle matrix, the CHO2 would push the microtubules toward the plus end, resulting in the two spindle poles moving close together. The force produced by CHO2 is likely balanced by opposing forces generated by other motors present in the spindle in order to maintain the normal size of bipolar spindle structures. Alternatively, the CHO2 antigen may move microtubules toward the kinetochore, where kinetochore microtubules are depolymerized, thereby functioning in the movement of chromosomes to the poles during anaphase A.

If the protein serves an essential function, it is likely that the cells have developed back-up mechanisms to protect the cell from loss of CHO2 function. As a matter of fact, it is now well established that a certain degree of functional redundancy is seen among different types of mitosis-specific kinesin-like proteins (Roof et al., 1992; Hoyt et al., 1992; Saunders and Hoyt, 1992). As for the COOH-terminal motors, O'Connell et al. (1993) have provided evidence that *klpA* in *Aspergillus* can complement a null mutation in *kar3*, although chromosomal deletion of *klpA* resulted in no observable mutant phenotype. Moreover, deletion of *klpA* can suppress the temperature-sensitive phenotype of a mutation in the NH_2-terminal motor gene, *bimC*. These results clearly indicate that there is a functional interaction of the COOH-terminal motor with other mitosis-specific COOH- and NH_2-terminal kinesin-like proteins (O'Connell et al., 1993). Thus the CHO2 antigen likely interacts with other motor molecules in mammalian mitotic cells. Further identification of other COOH-terminal motor(s) and/or molecules interacting with the CHO2 antigen in the spindle structure, such as Cik1 for KAR3 in *Saccharomyces* (Page et al., 1994), will be important for our understanding of the mechanism of mitosis and its regulation.

The CHO2 antigen was originally identified as a centrosomal component in CHO cells (Sellitto et al., 1992). Using another centrosomal antibody, we have also identified a novel NH_2-terminal kinesin-like motor (CHO1 antigen) in CHO cells (Sellitto and Kuriyama, 1988; Kuriyama and Nislow, 1992). Like the CHO2 antigen, the CHO1 motor is composed of a central α-helical stalk plus globular domains at both NH_2 and COOH termini (Nislow et al., 1992; Kuriyama et al., 1994). Both CHO1 and CHO2 antibodies cross-react with the central stalk, suggesting that the α-helical coiled-coil region must display a strong antigenicity. Since two out of seven mAbs that we raised against mammalian centrosomes (Sellitto et al., 1992) turned out to be mitosis-specific kinesin-like motors, it is likely that more kinesin-like proteins present in the spindle structure, as suggested previously (Gelfand and Scholey, 1992; Cole et al., 1992; Sawin et al., 1992). It is noteworthy that both antigens are localized at centrosomes and in nuclei in interphase cells. A question arises whether these kinesin-like motors are functioning in interphase centrosomes and nuclei. The CHO1 antigen has been shown to be a plus end motor (Nislow et al., 1992); it is, therefore, unlikely that the motor is translocated along the length of astral microtubules to accumulate around the interphase centrosome. The nuclear motors could be precursors for the centrosomal component, which is sequestered inside nuclei before mitosis. Alternatively, the CHO1 and CHO2 antigens might play important roles in nuclear functions during interphase and become associated with spindle fibers in mitotic cells to ensure equal segregation to each daughter cell. A somewhat surprising result obtained by our computer search was that the CHO2 antigen shows striking similarity to the product of the *HSET* gene, which is located in the major histocompatibility complex region of human chromosome 6 (Ando et al., 1994). The high degree of identity (86%) suggests that HSET may be a human homologue of the CHO2 antigen. Since HSET is highly expressed in lymphocytes, macrophages, and spleen, as well as testis/ovaries in both humans and mice (Yeom et al., 1992; Ando et al., 1994), the CHO2 antigen may be involved in the process of immune responses and/or during early stages of mammalian embryogenesis. Kinesin and kinesin-like motor molecules may participate in a wide range of biological activities.

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