Heterogeneity and microtubule interaction of the CHO1 antigen, a mitosis-specific kinesin-like protein

Analysis of subdomains expressed in insect sf9 cells

Ryoko Kuriyama, Sasa Dragas-Granoic, Takami Maekawa*, Alexei Vassilev, Alexey Khodjakov† and Hitoshi Kobayashi‡

Department of Cell Biology and Neuroanatomy, University of Minnesota, 4-135 Jackson Hall, 321 Church St SE, Minneapolis, Minnesota 55455, USA

*Present address: Hokkaido Red Cross Blood Research Center, Sapporo, Hokkaido, Japan
†Present address: Department of Histology and Cytology, Moscow State University, Moscow, Russia
‡Present address: Iwate Biotechnology Research Center, Kitakami, Japan

SUMMARY

The CHO1 antigen is a mitosis-specific kinesin-like motor located at the interzonal region of the spindle. The human cDNA coding for the antigen contains a domain with sequence similarity to the motor domain of kinesin-like protein (Nislow et al., Nature 359, 543, 1992). Here we cloned cDNAs encoding the CHO1 antigen by immunoscreening of a CHO Uni-Zap expression library, the same species in which the original monoclonal antibody was raised. cDNAs of CHO cells encode a 953 amino acid polypeptide with a calculated molecular mass of 109 kDa. The N-terminal 73% of the antigen was 87% identical to the human clone, whereas the remaining 27% of the coding region showed only 48% homology. Insect SF9 cells infected with baculovirus containing the full-length insert produced 105 and 95 kDa polypeptides, the same doublet identified as the original antigen in CHO cells. Truncated polypeptides corresponding to the N-terminal motor and C-terminal tail produced a 56 and 54 kDa polypeptide in SF9 cells, respectively. Full and N-terminal proteins co-sedimented with, and caused bundling of, brain microtubules in vitro, whereas the C-terminal polypeptide did not. Cells expressing the N terminus formed one or more cytoplasmic processes. Immunofluorescence as well as electron microscopic observations revealed the presence of thick bundles of microtubules, which were closely packed, forming a marginal ring just beneath the cell membrane and a core in the processes. The diffusion coefficient and sedimentation coefficient were determined for the native CHO1 antigen by gel filtration and sucrose density gradient centrifugation, respectively. The native molecular mass of overinduced protein in SF9 cells was calculated as 219 kDa, suggesting that the antigen exists as a dimer. Intrinsic CHO1 antigen in cultured mammalian cells forms a larger native complex (native molecular mass, 362 kDa), which may suggest the presence of additional molecule(s) associating with the CHO1 motor molecule.

Key words: mitosis, spindle, baculovirus, kinesin-like protein, mitotic motor

INTRODUCTION

To achieve faithful transmission of the genetic material from one cell to its daughters, cells assemble special devices called mitotic spindles during cell division. The highly coordinated mitotic events occur concomitantly with continuous changes in spindle structure. The major framework of the spindle is composed of microtubules that have been demonstrated to be in a highly dynamic state. Since the dynamic nature of microtubules is a key to the regulation of spindle morphogenesis and mitotic function (Kirschner and Mitchison, 1986), it is important to identify and characterize molecules controlling the dynamics of mitotic microtubules to understand the mechanism of mitosis and its regulation.

A growing body of evidence suggests that microtubule-based motor proteins are included in the spindle structure and play an essential role during mitosis (McIntosh and Pfarr, 1991; Gelfand and Scholey, 1992; Sawin and Endow, 1993). In vivo (Rieder et al., 1990; Gorbsky et al., 1987; Nicklas, 1989) and in vitro (Mitchison and Kirschner, 1985; Hyman and Mitchison, 1991; Hyman et al., 1992) observations of mitotic cells strongly suggested the presence of ATPase activity within the kinetochore. Localization of cytoplasmic dynein (Pfarr et al., 1990; Steuer et al., 1990), kinesin (Neighbors et al., 1988; Leslie et al., 1987) and kinesin-like proteins (Sawin et al., 1992a; Yen et al., 1992) was also shown by immunofluorescence microscopy at the kinetochore, pole and spindle fibers. Genetic analysis of yeast and Drosophila led to identification of mitosis-specific kinesin-like motor molecules, including cut7 (Hagan and Yanagida, 1990), BimC (Enos and Morris, ...
mitotic cells resulted in mitotic inhibition in a stage-specific manner. Microinjection of the purified CHO1 antibody into mammalian cells during mitosis, however, the antigen localizes throughout the spindle and centrosomes in interphase CHO cells. During mitosis, assembly of spindle structures in Xenopus egg extracts (Sawin et al., 1992b).

Using the CHO1 monoclonal antibody raised against mitotic spindles isolated from CHO cells, we have identified a novel 95/105 kDa spindle constituent (Sellitto and Kuriyama, 1988). The antibody was originally screened as a specific probe for kinesin-like proteins, including KLPA (O’Connell et al., 1993), KLP61F (Heck et al., 1993) and KIP2 (Roof et al., 1992). Mutations of some of these kinesin-like proteins led to deficiency in mitotic processes, and the importance of the kinesin-like motors for organization of functional spindles has been further implicated by in vivo analysis of antibody microinjection into living cells (Yen et al., 1991) and in vitro assembly of spindle structures in Xenopus egg extracts (Sawin et al., 1992b).

Microinjection of the purified CHO1 antibody into mammalian mitotic cells resulted in mitotic inhibition in a stage-specific and dose-dependent manner, indicating that the CHO1 antigen is essential for mitotic progression (Nislow et al., 1990). To further extend the study of this unique spindle component, we have cloned the human gene by screening a HeLa expression library using CHO1 as a probe (Nislow et al., 1992). Analysis of the nucleotide and deduced amino acid sequence showed that the N-terminal half of the CHO1 antigen contains a region of 350 amino acids that has significant identity with the motor domain shared among kinesin-like proteins. The C-terminal half of the antigen, however, shows little homology to other kinesin-like proteins, suggesting that the CHO1 antigen is a novel member of the kinesin superfamily (Nislow et al., 1992).

Since the CHO1 antigen is a kinesin-like motor molecule specific to mitotic cells, it is also called mitotic kinesin-like protein-1, MKLP-1 (Nislow et al., 1992). cDNA encoding the CHO1 antigen was also cloned from a CHO expression library, the same species in which the original antibody was raised. Here we report sequence comparison of the protein between the two species. Full coding as well as truncated polypeptides were expressed in insect Sf9 cells using the baculovirus expression system. Overexpression of the motor domain resulted in characteristic reorganization of microtubule arrays in Sf9 cells. Molecular properties of induced CHO1 polypeptides were also compared with those of the intrinsic CHO1 antigen in cultured mammalian cells to gain more information regarding structure and function of this unique mitotic motor molecule.

MATERIALS AND METHODS

Isolation and sequence analysis of cDNA clones encoding the CHO1 antigen in CHO cells

A commercially available Chinese hamster ovary (CHO) cell cDNA expression library cloned in λUni-Zap (Stratagene, La Jolla, CA) was immunoscreened with the monoclonal CHO1 antibody (Sellitto and Kuriyama, 1988), according to the procedure described previously (Kuriyama et al., 1990; Maekawa and Kuriyama, 1993). Three out of four positive clones obtained cross-hybridized and showed identical overlapping regions (see Fig. 1A). The largest of these clones (CHO1-8a clone), which spans 3.2 kb, was chosen for further nucleotide sequence analysis. A set of nested deletions of both strands of the 8a clone in pBluescript KS+ (Stratagene, La Jolla, CA) was generated using an ExoIII/Mung bean deletion kit (Stratagene). Double-stranded DNA was then sequenced by the dideoxy chain termination method (Sanger et al., 1977), using the Sequenase kit (US Biochemical Corp., Cleveland, OH). The DNA sequence was assembled and analyzed with the IntelliGenetics Software Package (Maekawa and Kuriyama, 1993).

Preparation of CHO1 antigen expressed in Sf9 cells

Expression of full/truncated CHO1 antigens in insect Sf9 cells

The CHO1-8a clone was used to express the entire coding sequence of the CHO1 antigen, and the N- and C-terminal halves of the proteins were made by digesting the 8a clone at EcoRV site (nucleotide position 1,465; arrow in Fig. 1A). Purified cDNAs were subcloned into multi-cloning sites of pVL1392 or pVL1393 (PharMingen, San Diego, CA), and introduced into moth ovarian Sf9 cells by co-transfection with partially deleted linearized baculovirus DNA as described previously (Maekawa and Kuriyama, 1993). Viruses released by the transfected cells were collected and used for further amplification.

Preparation of CHO1 antigen from HeLa cells

HeLa cell lysates containing the intrinsic CHO1 antigen were prepared as described previously (Maekawa et al., 1991). Briefly, monolayer cultures of cells were treated with 0.1 µg/ml of nocodazole for 24 hours to prepare synchronous cells arrested at mitosis. After washing twice in cold PBS, cells were resuspended in 5-10 vols of 100PEM buffer (100 mM Pipes at pH 6.8, 1 mM EGTA, 1 mM MgCl₂) containing a mixture of protease inhibitors (20 µM leupeptin, 1% aprotinin and 0.1 mg/ml PMSF) at 0°C. After sonication, disrupted cells were fractionated into supernatants and pellets by centrifugation at 13,000 x g for 15 minutes. As shown in Results, a major part of the full-length is recovered in the pellet fraction. To solubilize the protein, CHO1 antigen-containing pellets were resuspended in 100PEM supplemented with 0.6 M NaCl and 5 mM MgATP. After 10 minutes of incubation on ice, the sample was centrifuged at 100,000 x g for 60 minutes in a Beckman TL-100 table-top centrifuge to recover supernatants, which were further dialyzed against an excess volume of 100PEM. Insoluble materials were separated by sedimentation, and the supernatant was used as the partially purified full-length CHO1 antigen.

Preparation of CHO1 antigen from HeLa cells

HeLa cell lysates containing the intrinsic CHO1 antigen were prepared as described previously (Maekawa et al., 1991). Briefly, monolayer cultures of cells were treated with 0.1 µg/ml of nocodazole for 24 hours to prepare synchronous cells arrested at mitosis. After washing twice in cold PBS, cells were resuspended in 5-10 vols of 100PEM plus protease inhibitors. Cells were disrupted by sonication for about 1 minute, supernatants were recovered after sedimentation at 15,000 x g for 15 minutes and stored at −80°C until use.

Analysis of physical parameters of the native CHO1 protein complex

To determine the native size of CHO1 antigen, the intrinsic HeLa CHO1 antigen as well as recombinant proteins of full and truncated N- and C-terminal CHO1 antigen in the cell supernatant were subjected to FPLC on a Superdex 200 HR 10/30 column (Pharmacia LKB Biotechnology, Piscataway, NJ). The column was run according to the manufaacture’s protocol with 100PEM as the elution buffer. For determination of the sedimentation coefficient (s20,w = 10^{-13} seconds), 5% to 15% or 5% to 20% sucrose density gradients in 100PEM were made in Beckman 14 mm x 89 mm centrifuge tubes. Samples of 0.5 ml of cell supernatants were loaded, and the gradients were cen-
trifuged at 200,000 g for 12 (for 5% to 20% gradient) or 18 hours (for 5% to 15% gradient) at 4°C in a Beckman SW41Ti rotor. Fractions of 0.5 ml were collected and analyzed on Coomassie-stained SDS-PAGE and immunoblots. The markers used for calculation of the apparent molecular mass, diffusion coefficient ($D_{20,w}$ × 10^{-7} cm^2 s^{-1}) and sedimentation coefficient are thyroglobulin (669 kDa, $D_{20,w} = 2.6$, $s_{20,w} = 19.4$), ferritin (440 kDa, $D_{20,w} = 3.6$, $s_{20,w} = 17.6$), catalase (232 kDa, $D_{20,w} = 4.1$, $s_{20,w} = 11.3$), aldolase (158 kDa, $D_{20,w} = 4.6$, $s_{20,w} = 7.4$), brain $\alpha$-heterodimer (100 kDa), BSA (66 kDa, $D_{20,w} = 6.1$, $s_{20,w} = 4.4$) and ovalbumin (43 kDa, $s_{20,w} = 3.6$). The native molecular mass, Stokes' radius and axial ratio were calculated as described by Bloom et al. (1988).

**Assays of microtubule interaction with the CHO1 antigen**

Supernatants of Sf9 cells expressing either the full-length, or the N- or C-terminal half of the protein were first dialyzed against excess volume of 100PEM. After clarifying by centrifugation at 13,000 g for 15 minutes at 4°C, the samples were mixed with taxol-stabilized MAP-free brain microtubules for 10 minutes at 0°C in a buffer containing 100PEM plus 0.5 mM GTP and 10 µg/ml taxol (Maekawa et al., 1991; 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-polyacrylamide gels. For assay of the microtubule bundling activity, a sample of the CHO1 antigen-containing fraction and stabilized brain microtubule mixtures was mounted on a slide for observation by phase-contrast microscopy or Formvar-coated 200-mesh grids for whole-mount electron microscopy as described previously (Kuriyama and Borisy, 1981).

**Immunological techniques**

**Antibody preparation and purification**

The recombinant CHO1 antigen with an apparent molecular mass of 95 kDa was purified by excising from gels. The gel bands were mixed with Freund's complete adjuvant, 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**

Immunofluorescence staining of CHO and Sf9 cells, and isolated CHO mitotic spindles was performed as before (Sellitto and Kuriyama, 1988; Maekawa and Kuriyama, 1993). Briefly, CHO cells grown on coverslips in F-10 medium plus 7.5% fetal calf serum (FCS) were fixed with 4% paraformaldehyde in 100PEM for 30 minutes at room temperature, rinsed in PBS, then postfixed in 1% OsO$_4$. After washing with distilled water, the samples were next dehydrated through an ethanol series, infiltrated and embedded in Epon-Araldite plastic. Cells with cytoplasmic processes were marked with a diamond scribe. Ultrathin sections were cut with LKB Nova Ultramicrotome and stained with uranyl acetate and lead citrate. The specimen were examined in a JEOL 100CX electron microscope.

**RESULTS**

Sequence analysis of the CHO1 antigen in CHO cells; comparison with the human gene

By screening a λUni-Zap expression library using CHO1 as a probe, we isolated three immunopositive clones out of 10$^6$ plaques screened. They shared common restriction maps, and analysis of their partial sequence showed them to be identical in the overlapping regions (Fig. 1A). The longest clone (8a), which contains 3,248 nucleotides, overlapped with the other two DNA fragments (12a and 14a clones) at the C terminus, implying that the epitope to the CHO1 antibody resides on the C-terminal half of the protein. The nucleotide sequence of the 8a clone is shown in Fig. 1B. The clone contains the entire coding sequence (2,859 base pairs) of the CHO1 antigen along with 379 nucleotides of 3'-untranslated region in which a potential polyadenylation signal and a long poly(A)$^+$ tract are included. This open reading frame predicts a protein of 953 amino acids in length with a calculated molecular mass of 109 kDa, which is in good agreement with 105/95 kDa of the CHO1 antigen determined from SDS-PAGE (Sellitto and Kuriyama, 1988). The isoelectric point of the CHO1 antigen was calculated as 8.4; the C-terminal half of the protein is much more basic (pI = 9.8) than the N terminus (pI = 6.9). Particularly basic regions are included in the N terminus at amino acid positions 600-625 (pI = 11.6), 650-675 (pI = 10.3), 800-825 (pI = 12.3), 900-910 (pI = 12.8) and 940-953 (pI = 10.2).

Fig. 1B also compares the amino acid sequence of the CHO1 clone with that of HeLa cells (Nislow et al., 1992). As reported before, the CHO1 antigen possesses a conserved region of 350 amino acid residues corresponding to the putative motor domain at the N terminus (Vale and Goldstein, 1990; Goldstein, 1993) (Fig. 1C). It contains sequences similar to the consensus sequence proposed for ATP binding proteins at position 1 (pI = 9.8) than the N terminus (pI = 6.9). Particularly basic regions are included in the N terminus at amino acid positions 600-625 (pI = 11.6), 650-675 (pI = 10.3), 800-825 (pI = 12.3), 900-910 (pI = 12.8) and 940-953 (pI = 10.2).

**Immunoblot analysis**

Proteins in whole cell lysate, supernatant and pellet fractions as well as isolated CHO spindles were separated in 7.5% SDS-polyacrylamide gels, transferred to nitrocellulose membranes as before (Maekawa et al., 1991), then probed with monoclonal CHO1, polyclonal 95 kDa CHO1 antibody, and affinity-purified polyclonal antibodies raised against 10 amino acid residues (LNLVDLAGSE) that are conserved among kinesin-like molecules (generous gift from Dr Linda Wordeman) (Sawin et al., 1992a). Immunoreactive bands were visualized by alkaline phosphatase-conjugated secondary antibodies (HyClone, Logan, UT) with NBT/BCIP as the chromogen (Maekawa et al., 1991).
Fig. 1. Schematic diagram of cDNA clones and the primary sequence of the CHO1 antigen in CHO cells. (A) Map of the cDNA clones coding for the CHO1 antigen. The open and filled triangles indicate the position of the start and stop codons, respectively. The numbers underneath at the end of each clone represent nucleotide numbers encoded by the clones. An arrow shows the position (nucleotide number: 1465) where the CHO1-8a clone was cut with EcoRV to prepare N-terminal motor and C-terminal tail domains for expression in Sf9 cells. (B) Complete DNA sequence of the CHO1 antigen in CHO cells. The deduced amino acid sequence indicated by the single letter code below the DNA sequence was also compared with that of HeLa clone (Nislow et al., 1992). Consensus nucleotide binding domain and the amino acid sequences conserved among the members of kinesin superfamily are underlined. Double-underlining shows the position of a potential polyadenylation signal. (C) Sequence comparison between CHO and HeLa clones. Stippled areas indicate the regions with high amino acid sequence similarity. An arrowhead shows the position where the CHO1 clone was cut to prepare N- and C-terminal domains.
CHO1 antigen is a novel member of the kinesin superfamily. The N-terminal 73% of the CHO1 antigen is 87% identical to the human clone (Fig. 1C). In addition, a region at amino acid position 797 to 938 in the CHO clone corresponded well to the HeLa sequence between 698 and 839 (87% homology); that is, there is a shift of nearly 300 nucleotides in the aligned sequence. In contrast, the region from amino acid 692 to 796, showed no similarity to the HeLa sequence. Thus the remaining 27% of the coding sequence in the C-terminal domain of the CHO clone showed only 48% homology to the human clone. Since the sequence of two other clones (CHO1-12a and CHO1-14a) are identical to the CHO1-8a clone in the C-terminal region (Fig. 1A), it suggests that either the CHO1 antigens are heterogeneous among different species, or different forms of CHO1 antigen molecules may exist in a single species, or both.

**In vivo expression of full and truncated polypeptides of the CHO1 antigen in insect Sf9 cells**

The CHO1 antigen was originally identified as a spindle component localized at the equatorial region of the anaphase mitotic spindle. It possesses a plus-end-directed motor activity in vitro (Nislow et al., 1992) and is essential for mitotic progression (Nislow et al., 1990). Towards the goal of analyzing the regulatory mechanism of the CHO1 function during mitosis, we have expressed subregions of the antigen in insect ovarian Sf9 cells using the baculovirus expression system. The CHO1 antigen was split in the middle of the protein between nucleotide positions 1465 and 1466, indicated by an arrow in Fig. 1A. The cDNA fragments coding for the full-length as well as N- and C-terminal halves of the protein were inserted into the genome of the *Autographa californica* nuclear polyhedrosis virus at the polyhedrin gene locus by homologous recombination. SDS-PAGE analyses revealed the presence of major 105 and 95 kDa polypeptides in Sf9 cells expressing the full-length CHO1 antigen (Fig. 2, lanes 3 and 3’ in CBB column), the same as the doublet identified as the CHO1 antigen in CHO cells (Sellitto and Kuriyama, 1988; lane m in Spindle column). Amounts of the upper 105 kDa polypeptide vary among different preparations as is shown in lanes 3 and 3’. It was also noted that, as the antigen was further purified, the upper band of the expressed full-length CHO1 antigen eventually disappeared (data not shown). This indicates that the lower 95 kDa band could be a degraded product of the 105 kDa molecule. Expression of clones corresponding to N- and C-terminal halves of the protein resulted in production of 56 and 54 kDa polypeptides, respectively (lanes 2 and 4 in CBB column). The epitope for the CHO1 monoclonal antibody resides at the C terminus (Fig. 1A); thus the original monoclonal antibody recognized 95/105 kDa full-length and 54 kDa C-terminal fragment (Fig. 2 lanes 3m and 4m in IB column), but not the 56 kDa N-terminal half of the protein (data not shown). In contrast, affinity-purified antibodies raised against a decapeptide corresponding to a conserved region from the kinesin motor domain (LDVLAGSE: amino acid positions 335 to 342) (Sawin et al., 1992) reacted with the amino-terminal (lane 2L in IB column) and full-length CHO1 antigen, but not the C-terminal fragment (data not shown).

The lower molecular mass 95 kDa of the full-length protein was excised from gels and injected into rabbits as an immunogen (Fig. 2, lane 3p in IB column). Polyclonal antibodies affinity-purified from such immune sera reacted with the same antigen in isolated CHO mitotic spindles (Fig. 2, lane p in Spindle column, arrow) as the original monoclonal antibody. They also recognized truncated N- as well as C-terminal proteins (Fig. 2, lanes 3p and 4p in 1B column). Fig. 3 shows whole CHO cells (A) and isolated spindles and midbodies (B) at different mitotic stages double-stained with affinity-purified polyclonal anti-kinesin oligopeptide antibody (LAGSE) (lane 2L); Spindle, mitotic spindles isolated from CHO cells were run on 7.5% gel and stained with Coomassie Blue (lane CBB). Blotted nitrocellulose filters were stained with monoclonal (lane m) and affinity-purified polyclonal (lane p) CHO1 antibodies. Bars indicate the position of molecular mass markers; myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (93 kDa), BSA (66 kDa) and ovalbumin (45 kDa).
polyclonal CHO1 antibodies (A,a' to d' and B,b) and either tubulin (A,a'' to d'') or the original monoclonal CHO1 antibody (B,c). The polyclonal antibody labeled interphase centrosomes (arrowheads in A,a') and interzonal region of mitotic spindle structures (A,c' and B,b); those are the structures typically revealed by the original monoclonal antibody (Sellitto and Kuriyama, 1988; Fig. 3B,c). Although the spindle pole was not stained by the monoclonal antibody at all, faint staining of...
spindle poles by the polyclonal antibody was sometimes noted (indicated by arrows in A,b′). Antibodies raised against HeLa cell fusion protein were also able to label spindle poles (Nislow et al., 1992). These immunofluorescence staining and western blotting results indicate that the isolated clones indeed encode the authentic CHO1 antigen molecule.

**Molecular properties of the CHO1 antigen**

In order to examine the CHO1 antigen in more detail, we analyzed the molecular properties of the polypeptides corresponding to different regions of the CHO1 antigen and compared them with those of intrinsic CHO1 antigen prepared from cultured mammalian cells.

**Full-length and truncated polypeptides expressed in Sf9 cells**

Overexpressed N-terminal fragment was recovered in the supernatant fraction, whereas the major part of the full-length as well as C-terminal half of the protein came to the pellet after centrifugation at 13,000 g for 15 minutes (Table 1). To determine the physical properties of the CHO1 antigen, proteins in the supernatant were analyzed by gel filtration and sucrose density gradient centrifugation. As summarized in Table 1, FPLC analysis on a Superdex 200 column showed that the full-length, N- and C-terminal CHO1 antigen in supernatants eluted in a peak corresponding to a molecular mass of 440, 100 and 200 kDa, respectively. Sucrose density gradient centrifugation allowed us to calculate the sedimentation coefficient ($s_{20,w}$) as 8.0, 4.6 and 5.5 S, respectively. Based on the value of $s_{20,w}$ and the diffusion coefficient ($D_{20,w} \times 10^{-7}$ cm$^2$ s$^{-1}$) estimated from elution profiles of Superdex 200 columns, we calculated the native molecular mass of full-length, N- and C-terminal CHO1 antigens as 219, 74 and 114 kDa, respectively (Table 1). These results indicate that the overinduced CHO1 antigen in Sf9 cells forms a dimer complex. The protein is expected to be asymmetric with an axial ratio of approximately 17.

**Comparison of the CHO1 antigen between Sf9 and HeLa cells**

To compare the size of native protein in Sf9 cells with that of intrinsic CHO1 antigen, we prepared cytosolic fractions from cultured mammalian cells and analyzed by both FPLC and sucrose density gradient centrifugation. HeLa cells were employed for these analyses because: (1) large quantities of these cells were available; (2) immunofluorescence/immunoblotting pattern of the antigen is identical to that in CHO cells (Nislow et al., 1990); and (3) despite the fact that there is ~300 nucleotide shift in the C terminus, the HeLa CHO1 antigen shows high homology to the protein in CHO cells.

The CHO1 antigen is localized at both the centrosome and nucleus in interphase cells, and the antigen is recovered in nuclear as well as cytosolic fractions (Nislow et al., 1990) (Table 1). When the soluble fraction was applied to the FPLC Superdex 200 sizing column, the protein fractionated into a single peak with an apparent molecular mass of ~800 kDa. Sucrose density gradient centrifugation provided evidence that the intrinsic CHO1 antigen possesses a sedimentation value of 10.3, which is in good agreement with the value of 11 S previously reported by Nislow et al. (1990) in HeLa cells. The intrinsic CHO1 antigen has the native molecular mass of 362 kDa with approximate radial axis of 18.3. The protein in HeLa cells is apparently larger than that in Sf9 cells, indicating the possibility that additional molecule(s), such as the light chains associated with the conventional kinesin motor (Bloom et al., 1988; Kuznetsov et al., 1988), is (are) included in the CHO1 antigen fraction.

**Table 1. Physical properties of the CHO1 antigen in Sf9 and HeLa cells**

<table>
<thead>
<tr>
<th>Cell fractionation</th>
<th>App. mol. mass (kDa)</th>
<th>$D_{20,w}$</th>
<th>$s_{20,w}$</th>
<th>Stokes' radius</th>
<th>Axial ratio</th>
<th>Native mol. mass (kDa)</th>
<th>Oligomerization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sf9 cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full-length</td>
<td>Insoluble</td>
<td>95/105</td>
<td>440</td>
<td>3.2</td>
<td>8.0</td>
<td>6.9</td>
<td>16.6</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Soluble</td>
<td>56</td>
<td>100</td>
<td>5.5</td>
<td>4.6</td>
<td>3.9</td>
<td>7.1</td>
</tr>
<tr>
<td>C-terminal</td>
<td>Insoluble</td>
<td>54</td>
<td>200</td>
<td>4.2</td>
<td>5.5</td>
<td>4.9</td>
<td>6.3</td>
</tr>
<tr>
<td>HeLa cells</td>
<td>CHO1 antigen</td>
<td>100/115*</td>
<td>800</td>
<td>2.5</td>
<td>10.3</td>
<td>8.7</td>
<td>18.3</td>
</tr>
</tbody>
</table>

* Nislow et al. (1990).
pellet fraction, even in the absence of microtubules (lanes 6 and 8), the majority of full as well as N-terminal polypeptides specifically co-sedimented with the taxol-stabilized microtubules (lanes 4 in column F and N). In contrast, the polypeptide corresponding to the C-terminal half of the CHO1 antigen stayed in the supernatant, as in the control without microtubules (lanes 4 and 8 in column C), indicating that the antigen associates with microtubules through the globular motor domain at the N terminus. When the full-length and/or the N-terminal half of the protein was incubated with brain microtubules in the presence of 5 mM MgATP, the amount of immunoreactive polypeptides that co-sedimented with microtubules decreased to some extent (lanes 2 in columns F and N). These results suggest that the N-terminal motor domain contains both nucleotide-dependent and -independent microtubule binding sites. Interaction of the full-length, C- and N-terminal fragments with microtubules is summarized in Table 2.

Reorganization of microtubule arrays in Sf9 cells by expression of N-terminal motor domain of the CHO1 antigen

During the course of immunofluorescence screening of Sf9 cells, we noticed reorganization of cytoplasmic microtubules in cells overexpressing the motor domain of the CHO1 antigen (Fig. 6). While only diffuse tubulin staining was seen in non-infected control cells (data not shown), Sf9 cells overexpressing the N-terminal fragment contain bundled microtubules running in different directions (a'). The majority of the cells, however, included only a very thick microtubule ring just beneath the cell membrane (b). Since these kinds of microtubule arrays were never detected in control cells, it is concluded that the motor domain of CHO1 antigen modified microtubule organization inside the Sf9 cells. Fig. 7 shows electron micrographs of cells expressing the N-terminal motor domain, confirming the presence of thick bundles of microtubules, running in different directions (a) or forming a ring just beneath the cell membrane (b). A higher magnification of a microtubule ring is seen in d; although the bundle is packed too tightly to distinguish individual tubular structures, there is an area where microtubules are seen spaying off the bundle (d). In cross-

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**Table 2. Interaction of the CHO1 antigen with microtubules**

<table>
<thead>
<tr>
<th>In vitro MT-binding</th>
<th>In vitro MT-bundling</th>
<th>Process in Sf9 cells</th>
</tr>
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<tbody>
<tr>
<td><strong>Sf9 cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full-length</td>
<td>+</td>
<td>MT-binding</td>
</tr>
<tr>
<td>N-terminal</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C-terminal</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td><strong>HeLa cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHO1 antigen</td>
<td>+*</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined; NA, not applicable.

*Nislow et al. (1990).*
sectional views, it is seen that the bundles consist of 10-20 microtubules embedded in a kind of electron-dense material (c).

Although the microtubule ring generally had a nice rounded shape during the early stage of virus infection (Fig. 6b), at later stages it started to bend or to have an uneven surface. Subsequently changes in cell shape were observed (Fig. 6c-c’). Once microtubule bundles find a site where they can extend, the rings start unwinding to elongate (Fig. 6d), ultimately other bundles of microtubules appeared to join this outlet (Fig. 6e), resulting in the formation of long cytoplasmic processes (Fig. 6f-f’). The number of cytoplasmic extensions per cell ranged from one to many (Fig. 6g-i). No tubulin/microtubules appear to remain in the body of cells with long processes; as a result we detected very low background of tubulin fluorescence in the cell body. Electron microscopic analyses of thin-sectioned processes show bundles of microtubules present in the center, which appears to provide a structural core for the cytoplasmic extensions (Fig. 8a). In cross-sectional view, a group of microtubules are bundled together at the center of the process (Fig. 8b). Individual microtubules are packed tightly and linked to adjacent microtubules by bridge-like structures (arrows in Fig. 8c), which might represent the N-terminal motor domain of the antigen.

In contrast to the cells overproducing the N-terminal motor domain, the SF9 cells that expressed the full or C-terminal tail did not show any prominent morphological alterations (Table 2). It is, however, sometimes possible to detect microtubule bundles in cells expressing the full-length molecule, by immunofluorescence microscopy (data not shown). Such bundles of microtubules run in different directions inside the cells, and their fluorescence was far less intense than those in motor domain-expressing cells. Fig. 8d is a thin-section electron micrograph of a cell expressing the full-length CHO1 antigen. Although there are parallel arrays of microtubules (arrows in Fig. 8d), individual microtubules are not packed as tightly as in

Fig. 6. Immunofluorescence staining of SF9 cells with anti-tubulin antibody. The same cells are seen by phase-contrast (a,c,f) and immunofluorescence (a’,c’,f’) microscopy. Cells expressing the N-terminal motor domain of the antigen form either microtubule bundles running in different directions (a’), or thick microtubule rings just beneath the cell membrane (b,c’), and eventually produce a microtubule extension and cytoplasmic process (f,f’). Multiple extensions are also formed (g,h,i). Bars, 10 µm.
N-terminal motor-expressing cells. On very rare occasions, we found examples in which Sf9 cells expressing the full-length CHO1 antigen formed cytoplasmic extensions. As in the cellular processes induced in motor domain-expressing cells, these extensions appeared to contain a core of microtubules; however, the spacing between individual microtubules is much wider than that found in N-fragment expressing cells (Fig. 8e).

**DISCUSSION**

The gene encoding a kinesin-like motor protein, the CHO1 antigen, which is also called mitosis-specific kinesin-like protein-1 (MKLP-1) in HeLa cells (Nislow et al., 1992), was cloned and sequenced in CHO cells. Comparison of the deduced amino acid sequence of the protein between Chinese hamster and human cells showed over 87% homology in the N-terminal half of the protein (amino acid position: 1 to 691 in total 953 amino acid residues). Although over half of the remaining C-terminal sequence in CHO cells (amino acid position: 797 to 938) is well-aligned with the human sequence between 698 and 839 with 87% homology, the remaining sequence between 692 and 796 does not match any regions of the HeLa clone (Fig. 1C). It is not clear why the homologous
region in the C-terminal domain is shifted between the two species; this may, however, represent heterogeneity of the CHO1 antigen.

There are good reasons to believe that the CHO1 antigen plays an essential role during mitosis. Microinjection of the CHO1 antibody into living cells results in mitotic inhibition in a stage-specific and dose-dependent manner (Nislow et al., 1990). The protein displays a unique subcellular localization in dividing cells, shifting from diffuse spindle staining at metaphase to an increasingly narrow band of microtubules within the interzone during anaphase (Sellitto and Kuriyama, 1988; Kuriyama and Nislow, 1992). Immunofluorescence analysis of injected cells revealed that those that complete mitosis display normal localization of the CHO1 antigen, whereas arrested cells show no specific localization within the spindle (Nislow et al., 1990). These results suggest the importance of translocation of the protein from throughout the spindle to the midplane for its function in mitotic cells.

**Fig. 8.** Thin-section electron micrographs of Sf9 cells expressing full-length (d,e) and N-terminal motor domain (a to c) of the CHO1 antigen. Longitudinal (a) as well as cross-sectional (b) view of the cytoplasmic process formed in N-terminal fragment-expressing cells show the presence of highly packed microtubule bundles. Area outlined in b is shown in c at higher magnification. Small arrows in e indicate the cross-bridge structure formed between neighboring microtubules. Full sequence-expressing Sf9 cells contain microtubule bundles in both the cell body (arrows in d) and process (e), but spacing between neighboring microtubules was wider than in N-terminal fragment-expressing cells. Bars, 0.1 μm (a-c,e), and 5 μm (d).
CHO1 antigen is a plus-end-directed motor molecule (Nislow et al., 1992), thus it may be possible for it to move along the length of spindle microtubules toward the plus end at the spindle midzone. It is suggested that the normal size of the bipolar spindle is maintained by the balance of forces acting on the spindle structure. Motors located in the metaphase kinctochore exerting force toward the spindle poles pull the spindle poles toward each other. In order to prevent the spindle from shrinking, therefore, this force must be opposed by one or more forces that keep the spindle poles apart. The CHO1 antigen could be a candidate for producing such force by interaction between microtubules of opposite polarities in the center of the spindle. In support of this hypothesis, we have already observed that short and stubby spindle structures are formed in dividing cells microinjected with the CHO1 antibody (Nislow et al., 1990). Alternatively, since the CHO1 antigen is present in the interzonal region of the spindle where antiparallel microtubules overlap, the motor may be involved in the generation of force necessary for sliding of the pole-to-pole microtubules during anaphase B.

On the basis of comparison of amino acid sequences, a number of kinesin-like proteins are now classified into several subclasses, including the bimC family and the KAR3 family (Goldstein, 1993). The human gene corresponding to the CHO1 antigen is an apparent ‘orphan’; that is, it cannot be placed into any of the previously identified families. Although functional specificity of the kinesin-like motors appears to be regulated by the tail domain of the molecule (Vale and Goldstein, 1990), there is a certain degree of functional redundancy among different types of kinesin-like proteins despite the fact that the primary amino acid sequences of the tail regions are apparently not well conserved (Roof et al., 1992; Hoyt et al., 1992; Saunders and Hoyt, 1992; O’Connell et al., 1993). Localization of the CHO1 antigen at the midzone and pole in the spindle structure resembles that of cut7, a kinesin-like protein in Schizosaccharomyces pombe (Hagan and Yanagida, 1992). It is thus of interest to determine whether the CHO1 antigen is the homolog to cut7 and whether they are able to complement each other functionally during the process of spindle formation and maintenance.

Like conventional kinesin as well as many other members of the kinesin superfAMILY, the motor domain of the antigen is located in 350 amino acid residues at the N terminus. Expression of different domains in SF9 cells has revealed several characteristic features. When SF9 cells overproducing the CHO1 antigen were extracted in a detergent-containing buffer, it was found that the N-terminal half of the protein was recovered in the supernatant. However, major amounts of full-length as well as C-terminal polypeptides sedimented in the pellets. These results may suggest that the C-terminal domain could be sticky and has a tendency to form aggregates, which is also suggested by the fact that some full-length as well as C-terminal proteins expressed in SF9 cells eluted in the void volume on a sizing column (data not shown). Alternatively, sedimentation into the cell pellet fraction may be due to the presence of a nuclear localization signal (NLS) in the C terminus, since the CHO1 antigen is known to be localized inside the nucleus in interphase cells (Sellitto and Kuriyama, 1988); thus part of the molecule is recovered in the nuclear fraction (Nislow et al., 1990).

The predicted secondary structure of the CHO1 antigen is composed of two globular heads at N and C termini connected by a central stalk portion. The expressed full-length as well as N- and C-terminal polypeptides appear to exist as a dimer, which could be due to the presence of an α-helical coiled-coil central stalk. While the full-length as well as N-terminal half of the protein can bind to and bundle microtubules, the C terminus failed to interact with microtubules. The very basic carboxy domains of CHO1 apparently are not involved in forming electrostatic interactions with the acidic regions of tubulin. Dimer formation of the full-length and N-terminal polypeptides allows the globular heads with microtubule-binding capacity to extend outward, which might allow the protein to cross-link microtubules. The CHO1 antigen is located in the interzonal region of the spindle where antiparallel microtubules originating from opposite poles overlap. Since microtubules in the midzone region/midbody are packed closely together, it would be of interest to compare the length of the molecular complex of CHO1 antigen and the spacing between neighboring microtubules in the midbody.

Intrinsic CHO1 antigen in HeLa cells always forms a larger molecular complex (native molecular mass = 362 kDa) than the protein expressed in SF9 cells (native molecular mass = 219 kDa). One possible explanation is that, like the conventional kinesin protein, the CHO1 motor could be associated with additional molecule(s), such as kinesin light chains (Bloom et al., 1988; Kuznetsov et al., 1988). Suppose each 110 kDa CHO1 motor in HeLa cells binds to a second molecule in an equimolar ratio to form a 362 kDa dimer complex, the molecular mass of the associated protein of the CHO1 antigen would be calculated as 72 kDa, which is quite close to the molecular mass of kinesin light chain (62-84 kDa) identified in several different species (Bloom et al., 1988; Kuznetsov et al., 1988; Johnson et al., 1990).

As they overproduce the motor domain of the kinesin-like protein, rounded SF9 cells alter their shape to extend long cytoplasmic processes. Nerve-specific MAPs, such as tau (Knops et al., 1991; Chen et al., 1992) and MAP2 (Chen et al., 1992; LeClerc et al., 1993), have also been shown to produce long cytoplasmic processes in infected SF9 cells. The appearance of such extensions is similar to the neuronal outgrowth. Moreover, both the processes are supported by a core of microtubule bundles, and microtubules formed in SF9 cells extensions are organized in a pattern similar to that in neuronal processes, from the point of polarity (Baas et al., 1991; Chen et al., 1992) and MAP2 (Chen et al., 1992; LeClerc et al., 1993), have also been shown to produce long cytoplasmic processes in infected SF9 cells. The appearance of such extensions is similar to the neuronal outgrowth. Moreover, both the processes are supported by a core of microtubule bundles, and microtubules formed in SF9 cells extensions are organized in a pattern similar to that in neuronal processes, from the point of polarity (Baas et al., 1991; Chen et al., 1992) and spacing between microtubules (Chen et al., 1992). Cytoplasmic extensions in SF9 cells might therefore be considered as a model system for the study of neuronal process formation. However, processes in SF9 cells have organelar distribution patterns similar to those in the cell body (Knops et al., 1991; Baas et al., 1991), which is in sharp contrast to the axonal process. Extensions in SF9 cells could also be induced by expression of the catalytic subunit of cAMP-dependent protein kinase (Cheley et al., 1992). Nuclear polyhedrosis viral polypeptide p10 (Kuzio et al., 1984) becomes phosphorylated by the action of overinduced kinase enzyme, which in turn causes p10 to associate with microtubules and cells to produce processes. These results clearly indicate that formation of cellular outgrowth results from modification of microtubule architecture, thus it is not restricted to cells infected with recombinant baculovirus expressing neuron-specific MAPs.

We are puzzled by the fact that cells expressing the full-
length protein form neither cytoplasmic processes nor prominent microtubule bundles. The major part of the full-length protein expressed in Sf9 cells was found in the pellet after centrifugation at 13,000 g for 15 minutes. Therefore the amount of protein remaining in the cytosol may be insufficient to induce microtubule reorganization. Both the full-length and N-terminal domain contain ATP-dependent and -independent microtubule binding sites, and can cross-link microtubules in vitro. Since a major difference between cells overexpressing the full-length and N-terminal CHO1 antigen is the presence or absence of highly packed bundles of microtubule, it is reasonable to speculate that Sf9 cells must induce densely packed microtubule bundles, to a certain extent, in order to extend cellular processes. In fact spacing between microtubules in a bundle present inside the cell body was much wider than that in the process (Fig. 8d and e). It is likely that microtubule interactions with the intact CHO1 antigen may be blocked by some factor(s) through association with the C-terminal tail domain of the antigen. In natural conditions, such inhibition could be prevented by the presence of additional specific molecules, such as light chain component(s), to form a stoichiometric complex with the heavy chain of CHO1 kinesin-like molecule.

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REFERENCES

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