A Monoclonal Antibody to a Mitotic Microtubule-associated Protein Blocks Mitotic Progression

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Abstract. A monoclonal antibody raised against mitotic spindles isolated from CHO cells ([CHO1], Sellitto, C., and R. Kuriyama. 1988. *J. Cell Biol.* 106:431–439) identifies an epitope that resides on polypeptides of 95 and 105 kD and is localized in the spindles of diverse organisms. The antigen is distributed throughout the spindle at metaphase but becomes concentrated in a progressively narrower zone on either side of the spindle midplane as anaphase progresses. Microinjection of CHO1, either as an ascites fluid or as purified IgM, results in mitotic inhibition in a stage-specific and dose-dependent manner. Parallel control injections with nonimmune IgMs do not yield significant mitotic inhibition. Immunofluorescence analysis of injected cells reveals that those which complete mitosis display normal localization of CHO1, whereas arrested cells show no specific localization of the CHO1 antigen within the spindle. Immunoelectron microscopic images of such arrested cells indicate aberrant microtubule organization. The CHO1 antigen in HeLa cell extracts copurifies with taxol-stabilized microtubules. Neither of the polypeptides bearing the antigen is extracted from microtubules by ATP or GTP, but both are ~60% extracted with 0.5 M NaCl. Sucrose gradient analysis reveals that the antigens sediment at ~11S. The CHO 1 antigen appears to be a novel mitotic MAP whose proper distribution within the spindle is required for mitosis. The properties of the antigen(s) suggest that the corresponding protein(s) are part of the mechanism that holds the antiparallel microtubules of the two interdigitating half spindles together during anaphase.

THE mitotic spindle is the structure responsible for the equipartition of chromosomes during mitosis. Although there are numerous descriptions of the mitotic events visible at the microscopic level, we know little about the molecular aspects of spindle function. The inherent complexity of the spindle, combined with its extreme lability, has made such analyses difficult. Efforts to identify spindle-specific molecules from a variety of cell types have demonstrated the complexity of the spindle, but have thus far told us little about the molecules essential for mitosis. To go beyond descriptive biochemistry one needs a functional assay whereby to gauge the importance of a given molecule for mitotic progression.

There are at present two methods to determine the role of a given protein during mitosis. One powerful approach involves the creation of mutations that alter the gene(s) encoding a protein of interest. Although this method allows one to examine the function of a protein with precision, the requisite mutant cells must be found, and the list of mitosis-blocking mutations is not very long at present (reviewed in McIntosh and Koonce, 1989). An alternative approach involves the microinjection of antibodies against putative mitotic proteins into dividing cells. The microinjection method is advantageous because the antigen(s) recognized by the antibody need not be well characterized at the initiation of the experiments. Furthermore, the effects of the antibodies can be monitored in real time. Microinjection has been used successfully to probe the mitotic functions of MAP 4 (Izant et al., 1983), calcium transport enzymes (Silver, 1986; Hafner and Petzelt, 1987), a 62-kD phosphoprotein in mitotic sea urchin spindles (Dinsmore and Sloboda, 1989) and the mammalian homologue of yeast CDC 2 (Riawol et al., 1989). In each case this method has provided useful information about the possible roles of the corresponding antigens during mitosis. In this paper we have used microinjection to probe the role in mitosis of an interesting but thus far poorly characterized antigen. Sellitto and Kuriyama (1988) described an mAb, CHO1, that was raised against spindles isolated from CHO cells. The antigen recognized by this antibody appears to be a component of interphase nuclei and centrosomes as well as the mitotic matrix that binds to interzonal microtubules during anaphase. It is confined to the region where interdigitating microtubules overlap near the spindle midplane. To obtain evidence about the mitotic functions of the CHO1 antigen we have microinjected CHO1 ascites fluid or purified CHO1 IgM into PtK_1 cells at various times in mitosis. These

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injections led to mitotic arrest in a dose-dependent and stage-specific manner, suggesting that the CHO1 antigen is important for normal mitotic progression. Biochemical analyses have indicated that the polypeptides recognized by CHO1 bind to microtubules in vitro in a nucleotide insensitive, salt sensitive manner. Collectively, these data suggest that the CHO1 antigen(s) are novel mitotic microtubule-associated proteins (MAPs)[1] required for mitosis in PtK_1 cells.

Materials and Methods

General Materials

Unless otherwise specified, all reagents were from Sigma Chemical Co. (St. Louis, MO). Taxol was the generous gift of Dr. Matthew Suffness (National Cancer Institute).

Preparation and Purification of Antibodies

An ascites fluid containing the CHO1 IgM (Sellitto and Kuriyama, 1988) was precipitated by the addition of 50% ammonium sulfate (pH 7.4), resuspended in half its original volume of PBS (136 mM NaCl, 80 mM Na_2HPO, 3 mM KCl, 2 mM $MgCl_2$, and 1 mM KPO_4) dialysed against PBS or injection buffer (140 mM K^+, 100 mM glutamic acid, 40 mM citric acid 1 mM $MgCl_2$, and 1 mM EGTA at pH 7.4), and either purified further or concentrated, using a microconcentrator (Centricon 30; Amicon, Danvers, MA) to one-tenth the original volume. Further purification was performed by loading 0.5 ml of ammonium sulfate-precipitated antibody onto a 21-ml (1.5 × 27 cm) Sephacryl S-300 gel filtration column (Pharmacia Fine Chemicals, Piscataway, NJ) and eluting with PBS at room temperature (Jehanli and Hough, 1981). 0.5-ml fractions were collected and analyzed electrophoretically followed by immunoblotting (Towbin et al., 1979) with goat antibodies to the μ chain of mouse IgM (Bio-Rad Laboratories, Richmond, VA) to locate the proteins of interest. Commercial mouse IgM (Calbiochem-Behring Corp., La Jolla, CA) was used on these blots as a standard. IgM- and non-IgM-containing fractions were pooled separately and concentrated with a Centricon 30 for subsequent microinjection. For some experiments the IgM was precipitated with 40% ammonium sulfate, chromatographed as before, then further purified by passage over a 700-μl protein G column (Pierce Chemical Co., Rockford, IL) that had been equilibrated with sodium phosphate buffer (145 mM NaCl, 7.5 mM NaH_2PO_4, and 2.5 mM $NaHPO_4$ at pH 7.2). The flow through from this column which contained purified CHO1 IgM was collected and concentrated to ~10 mg/ml as described above. In other experiments the ascites fluid from a mouse injected with a nonimmune, IgM-secreting hybridoma (gift of Bonnie Neighbors, University of Colorado) was processed and purified as above to serve as microinjection controls.

Microinjection

Antibody solutions were dialyzed against two changes of 300 vol of injection buffer (see above) then clarified by centrifugation for 30 min in a microfuge, and stored at 4°C until used. In most cases, FITC-conjugated dextran was added to the antibody solutions at a concentration of 1 mg/ml before dialysis. Microinjections were performed according to Saxton and McIntosh (1987). Based on earlier calculations (Zavortink et al., 1983) we estimate injections to be ~10% of the cell's volume. The protein concentrations of the injectates, as loaded into the needles, ranged from 1–50 mg/ml.

Cells to be microinjected were subcultured in Ham's F-12 medium supplemented with 10% FCS (Gibco Laboratories, Grand Island, NY) on etched glass coverslips (Bellco, Vineland, NJ). These coverslips facilitated subsequent identification of injected cells and their progeny. Coverslips containing cells at ~70% confluence were tacked down to 35-mm petri dishes with valap (vaseline, lanolin, and paraffin at 1:1:1) then covered with medium. Selected mitotic cells were microinjected with glass micropipettes made on a Brown-Flaming P-77 pipette puller (Sutter Instruments, San Francisco, CA) from capillaries of 1.0 mm o.d. and containing an internal filament to aid loading the needles (World Precision Instruments, New Haven, CT). Cells were injected at room temperature while being observed with a phase-contrast, water immersion objective (40×, n.a. 0.75; Carl Zeiss; Oberkochen, FRG). For most experiments between 30 and 40 cells per coverslip were injected over the course of 20 min.

After a series of injections, the coverslip was scanned and any injected cells that appeared to be injured were discounted from further analysis. Cells were placed in fresh medium in a 60-mm petri dish and placed in a 37°C incubator for 3 h. After this recovery period, injected cells were examined to determine their mitotic stage. Cells that had divided successfully were identified as pairs of fluorescent cells, whereas cells that had failed to divide were seen as individual fluorescent cells. These arrested cells were examined microscopically to determine the stage at which they had arrested. The microinjection experiments were performed under double-blind conditions. For each series of injections, two antibody solutions were prepared, a control IgM and CHO1 IgM. (The commercially prepared mouse IgM used as a control did not stain any mitotic structures in our immunofluorescent preparations.) Aliquots of these samples were then coded so that the individual performing the injections and subsequently scoring the cells did not know the identity of the samples.

Immunofluorescence Microscopy

Both PtK_1 and HeLa cells were grown to ~70% confluence on 12-mm circular coverslips as described above, then fixed directly in cold methanol, as described by Neighbors et al. (1988), or lysed for 1 min in PMEG buffer (100 mM Pipes, 5 mM $MgSO_4$, 5 mM EGTA, 0.5 mM EDTA, and 0.9 M glycerol at pH 6.9) containing 1.0% Triton X-100 and 1 μM taxol, then fixed. CHO1 ascites or purified CHO1 IgM was typically used as a primary antibody at a 1:500 dilution in PBS for 2 h followed by three washes in PBS and a 1 h incubation with a Texas Red–conjugated goat anti-mouse secondary antibody (Fisher Scientific Co., Springfield, NJ) diluted 1:200 in PBS for 1 h. For double label experiments, cells labelled as described were washed for 40 min in PBS, then incubated for 2 h with a mouse monoclonal antitubulin (Scholey et al., 1984) followed by an FITC-conjugated goat anti-mouse secondary antibody (U.S. Biochemical Corp., Cleveland, OH) diluted 1:100 in PBS for 1 h. Though this procedure used two primary antibodies from mouse, no cross-reactivity of the second goat antibody with the first primary antibody was observed. Control experiments in which the antitubulin incubation was omitted confirmed that the FITC-conjugated goat anti-mouse did not stain the CHO1/Texas Red–labeled cells. In some experiments DAPI (4',6-diamidino-2-phenylindole dihydrochloride) was added to the final PBS wash (at 1 μg/ml) to stain the chromosomes. Cells injected with either CHO1 or control antibodies were processed as above, except that the first antibody incubation step was omitted. Coverslips were mounted in Gelvatol (Monsanto, Indian Orchard, MA) with 5% *n*-propyl gallate and observed on a Zeiss universal microscope equipped with both phase and epi-fluorescence optics, using a Zeiss phase contrast oil immersion lens (100×, n.a. 1.25). Images were recorded on T-Max 3200 film (Eastman Kodak Co., Rochester, NY) developed at 29°C according to the manufacturer's instructions.

Immunoelectron Microscopy

PtK_1 cells were grown as described above on glow discharged, Formvar and carbon-coated gold finder grids (Fullam, Latham, NY). Mitotic cells were injected with CHO1 IgM as described, lysed for 1 min at room temp with 0.2% Triton X-100 in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, and 2 mM $MgSO_4$) then fixed with 2.0% paraformaldehyde and 0.1% glutaraldehyde in PHEM for 20 min at room temperature. Fixed cells were treated with 10 mg/ml sodium borohydride in 50% ethanol and 50% PBS then returned to PBS. Fixed cells were incubated with antitubulin for 2 h at 37°C followed by goat anti-mouse antibodies conjugated to 15 nm colloidal gold (Janssen Life Sciences, Olen, Belgium) for 3 h at room temperature. Labeled cells were dehydrated in a graded ethanol series, transferred to dry acetone, and then critical point dried from liquid CO_2 in a CPD 020 (Balzers, Hudson, NH). Dried specimens were viewed in a JEOL JEM-1000 electron microscope at 1 MeV.

Preparation of CHO1 Antigen from HeLa Cells

HeLa cells were grown in suspension culture and harvested by centrifugation and separated into nuclear and cytoplasmic fractions according to the method of Arias and Dynan (1989). Briefly, cells were lysed in a hypotonic buffer (10 mM Tris-HCl, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$ and 1 mM DTT) and centrifuged at 3000 rpm in a rotor (model JS 13.1; Beckman In-

1. *Abbreviations used in this paper*: MAP, microtubule-associated protein; PHEM, 60 μM Pipes, 25 μM Hepes, 10 mM EGTA, and 2 mM $MgSO_4$; PMEG, 100 mM Pipes, 5 mM $MgSO_4$, 5 mM EGTA, 0.5 mM EDTA, 0.9 M glycerol, pH 6.9.

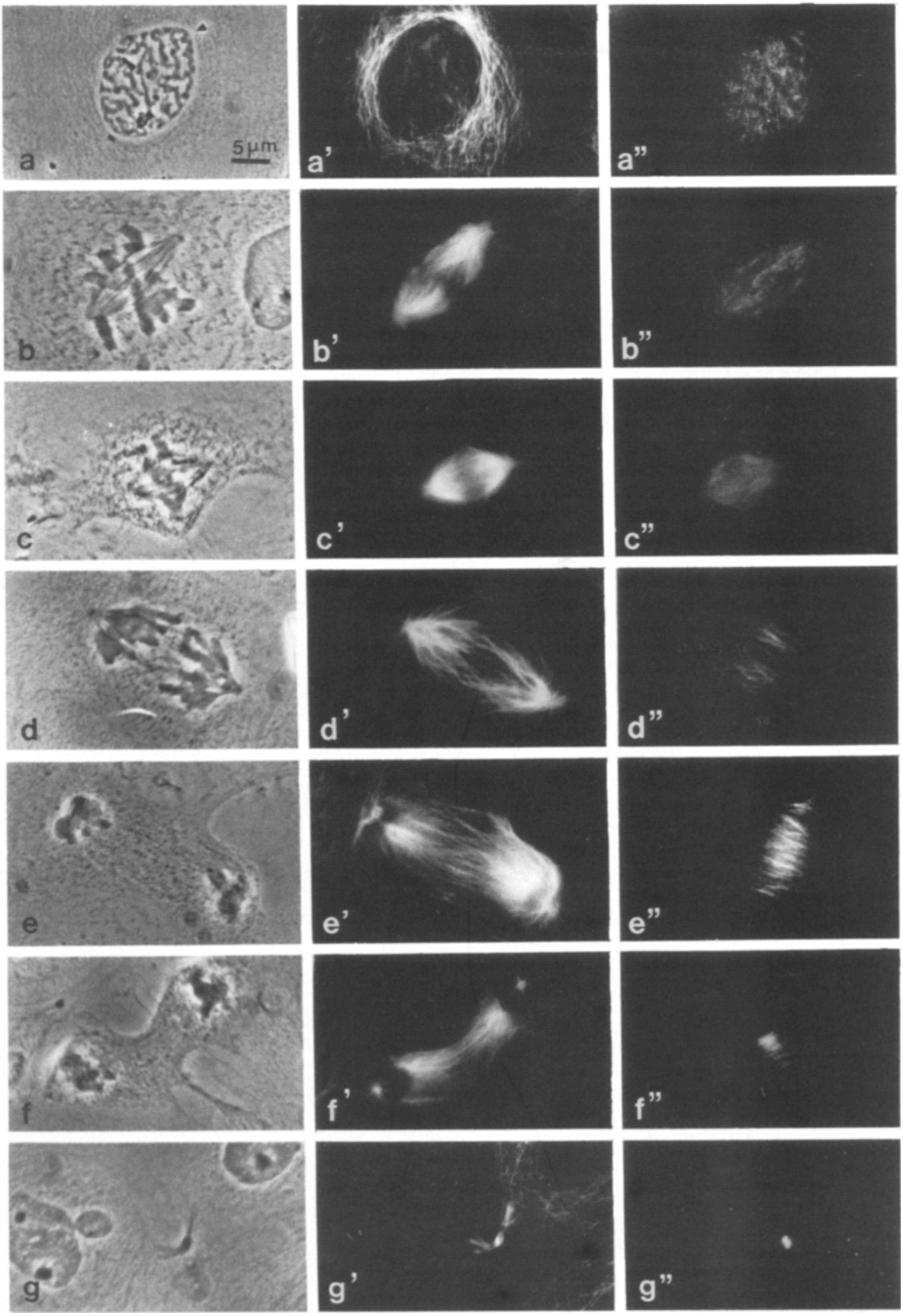


Figure 1. Immunolocalization of the CHO1 antigen at different stages of mitosis in PtK_1 cells. Cells were lysed and prepared as described in Materials and Methods. The same cell for each stage is seen in phase contrast (*a–g*), after staining with anti-tubulin (*a'–g'*) and CHO1 (*a"–g"*). Bar, 5 μm in all micrographs.

struments, Palo Alto, CA) to separate nuclei and cytoplasm. Clarified cytoplasmic extracts were prepared by centrifugation in PMEG buffer, pH 6.9 containing soybean trypsin inhibitor, aprotinin, leupeptin, pepstatin, and benzamidine at 1 μg/ml at 140,000 *g* at 4°C for 45 min. Nuclear fractions were prepared according to Arias and Dynan (1989) by extracting nuclei with 50 mM Tris-HCl, pH 7.5, 20% glycerol, 10% sucrose, 0.42 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 2 mM DTT for 30 min at 4°C then centrifuging at 21,000 rpm in a rotor (model SW27; Beckman Instruments) for 1.5 h at 4°C. Ammonium sulfate (33%) was then added to the supernatant from this spin. This mixture was stirred for 1 h at 4°C then centrifuged at 15,000 rpm in a rotor (model SW27; Beckman Instruments) for 15 min at 4°C. The resulting pellet was resuspended in 50 mM Tris-HCl, pH 7.9, 1 mM EDTA, 20% glycerol, 12.5 mM $MgCl_2$, and 0.1 M KCl containing the same cocktail of protease inhibitors used for the cytoplasmic extract. The resulting nuclear extract was then clarified in a microfuge. Clarified cytoplasmic and nuclear extracts were supplemented with 1 mM GTP and 10 μM taxol, then incubated at 37°C for 5 min, followed by the addition of 1 mg/ml taxol-stabilized microtubules (prepared from phosphocellulose purified tubulin [Williams and Detrich, 1979]) and incubated at 37°C for an additional 40 min. The microtubules and their associated proteins were pelleted through a 15% sucrose cushion for 1 h at 25,000 *g* at 30°C. The pellet of microtubules plus associated proteins (MTP) was washed in PMEG containing 10 μM taxol, then extracted simultaneously with 5 mM MgATP and 5 mM MgGTP (in PMEG containing 10 μM taxol) for 30 min at 25°C. After extraction, the pellet and supernatant fractions were separated by centrifugation at 40,000 *g* for 30 min at 30°C. This pellet was then resuspended in PMEG plus taxol and extracted sequentially with 0.5 M NaCl then 1.0 M NaCl in PMEG containing 10 μM taxol. To control for proteins that might sediment nonspecifically with microtubules, clarified cytoplasmic extracts were incubated and processed as above except both taxol and GTP were omitted and 0.1 μg/ml nocodazole was added to prevent the polymerization of microtubules.

Sucrose Density Gradient Centrifugation

200-μl samples of high speed supernatant prepared from HeLa cytoplasm were loaded onto 4.2 ml 5–20% sucrose gradients (prepared in PMEG), then centrifuged at 300,000 *g* for 6.5 h at 4°C in an SW60 rotor (Beckman Instruments). For each run, two gradients were loaded with 1 mg/ml of the following standards: cytochrome *c* (2.1S), catalase (11.1S), and thyroglobulin (19.2S). Each gradient was collected into 14 fractions of 300 μl each, adjusted to 1 mM GTP, 10 μm taxol, and 1 mg/ml taxol-stabilized microtubules. These fractions were incubated for 30 min at room temperature, then centrifuged in an airfuge (Beckman Instruments) at 100,000 *g* for 15 min at 22°C. Supernatants from these preparations were removed and the pellets were resuspended in one-third the original volume of PMEG before analysis via SDS-PAGE and immunoblotting.

Other Biochemical Procedures

Whole PtK_1 cell extracts were prepared by rinsing adherent cells with PMEG at 4°C followed by removal of the cells by scraping with a rubber policeman. PtK_1 cells were then rinsed twice in PMEG, resuspended in 2 vol of PMEG containing the same cocktail of protease inhibitors used for HeLa cells, disrupted by sonication and boiled in gel sample buffer.

Protein concentrations were measured according to the method of Bradford (1976) using bovine IgG as a standard. SDS-PAGE was performed on 7.5% acrylamide slab gels (0.75 mm thick) in a Mini Slab apparatus (Idea Scientific, Corvallis, OR) using a modification of the buffer system of Laemmli (1970). The bis/acrylamide ratio was reduced to one-one hundredth to allow better transfer of proteins to nitrocellulose. Gels were stained with Coomassie blue and/or silver nitrate. For immunoblots, proteins were transferred to 0.22-μm nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) according to Towbin et al. (1979) for 1.75 h at 12 V in a Genie Mini Blot apparatus (Idea Scientific). Blots were probed sequentially with antibodies for 2 h each at 37°C, then visualized via the immunoperoxidase reaction (Bio-Rad Laboratories, Richmond, CA).

Results

Localization of CHO1 in PtK Cells

The mAb designated CHO1 recognizes an epitope within the mitotic spindle and midbody of CHO cells (Sellitto and Kuriyama, 1988). This cell type is not convenient for microinjection during mitosis because the cells are small, and they round up during prophase. PtK_1 cells, however, are larger and remain relatively flat during mitosis, making them suitable for microinjection. To determine the normal distribution of CHO1 in mitotic PtK_1 cells, we processed these cells for double-label immunofluorescence, using CHO1 and an antitubulin mAb (Fig. 1). Both lysed and unlysed cells displayed similar localization patterns, but the lysed preparations afforded a clearer immunofluorescent signal, so we will focus our descriptions on them. In prophase cells, CHO1 stains fibers and punctate spots within the nucleus, but there is no detectable cytoplasmic staining (Fig. 1, *a–a″*). By prometaphase a dim, speckled staining by CHO1 is visible along the spindle microtubules (Fig. 1, *b–b″*). This staining appears more uniform and intense by metaphase (Fig. 1, *c–c″*). At the onset of anaphase, CHO1 staining becomes localized to a discrete band of fibers that run between the separating chromosomes (Fig. 1, *d–d″*). By late anaphase, staining intensity increases and is more sharply localized to the region near the middle of the spindle where interpolar microtubules interdigitate (Fig. 1, *e–e″*). During telophase, staining is seen only within the cytoplasmic bridge that separates the two daughter cells. At this stage CHO1 staining is brightest where interzonal microtubules remain interdigitated (Fig. 1, *f–f″*). By the completion of cytokinesis staining is confined to the midbody (Fig. 1, *g–g″*). From these images it appears that the CHO1 staining pattern is similar in both PtK_1 and CHO cells.

Microinjection of CHO1

We have examined the effect of microinjecting CHO1 into mitotic PtK_1 cells. Ascites fluid containing either CHO1 or a nonimmune IgM at various concentrations ranging between 1 and 50 mg/ml was injected into PtK_1 cells at different stages of mitosis (all concentrations cited refer to the total protein concentration of a given sample within the needle before microinjection). Microinjection of mitotic cells with ~10% of their volume containing 12.5 mg/ml of CHO1 ascites fluid led to mitotic arrest in a stage-specific manner. When cells were injected during prophase, prometaphase or metaphase, 70–75% became arrested in a metaphase-like configuration (Table I). Higher concentrations of ascites fluid proteins (up to 50 mg/ml) had no greater inhibitory

Table I. The Effects of Microinjection of an Ascites Fluid Containing CHO1 and a Nonimmune Ascites Fluid into Mitotic PtK_1 Cells

Sample*		Mitotic arrest (*n*=)	
		Before AO‡	After AO§
		%	
CHO1	*(12.5 mg/ml)*	73(128)	22(85)
CHO1	*(8.0 mg/ml)*	44(36)	ND
CHO1	*(5.5 mg/ml)*	30(28)	ND
CHO1	*(4.0 mg/ml)*	21(30)	ND
Control	*(12.5 mg/ml)*	14(113)	13(50)

* For each sample the protein concentration listed refers to the concentration within the needle before microinjection.
‡ Before AO, cells were injected before the onset of anaphase.
§ After AO, cells were injected after the onset of anaphase.

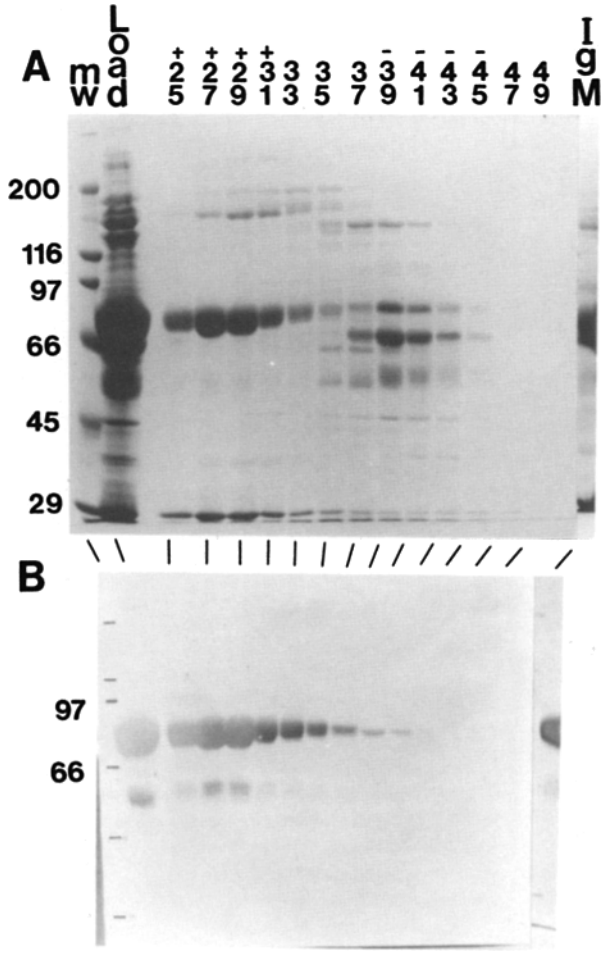


Figure 2. Purification of the CHO1 IgM via gel filtration chromatography. Column fractions were electrophoresed on a 7.5% polyacrylamide gel and stained with Coomassie blue (*A*) or blotted to nitrocellulose and probed with goat anti-mouse IgM (*B*). (*MW*) Molecular weight markers in kilodaltons. (*Load*) Ammonium sulfate precipitated CHO1 ascites fluid that was loaded onto the column. (*25–49*) Column fractions. (*IgM*) Commercial mouse IgM. Fractions 25–31 (+) and 39–45 (−) were pooled, concentrated, and denoted "*IgM*" and "non IgM," respectively.

affect, but at lower concentrations (<8 mg/ml) mitotic inhibition was proportionally less (Table I). Significantly less inhibition was also observed when cells were injected after the onset of anaphase (20–30%). Injection of control ascites at 12.5 mg/ml resulted in 10–15% inhibition, regardless of the time of injection.

To control for the possibility that CHO1-injected cells were simply delayed in completing mitosis, we observed several injected cells 6–7 h after injection. None of the arrested cells divided. By that time they appeared to lose viability, as determined by their appearance in the phase contrast microscope and their permeability to trypan blue (data not shown).

To determine whether the mitotic inhibition observed in injected cells was really due to the action of CHO1 (as opposed to an unidentified component of the ascites fluid) we used gel filtration chromatography to enrich the preparation for IgM. SDS-PAGE and immunoblot analysis of the gel filtration column fractions with anti-mouse IgM antibodies revealed that these immunoglobulins eluted immediately after the void volume with few contaminating proteins (compared with commercially purified mouse IgM) (Fig. 2).

To determine if the chromatography had damaged the ability of CHO1 to recognize its antigen, we tested the avidity of this partially purified IgM relative to the original ascites by serially diluting both preparations (at comparable protein concentrations) until no signal was detected via indirect immunofluorescence of fixed PtK_1 cells. Assuming that the IgM constituted approximately half of the ascites fluid proteins (see Fig. 2), we estimate that the chromatographed IgM was ~75% as avid as its counterpart in the original ascites fluid.

Column fractions containing either the isolated IgM or non-IgM proteins were pooled separately, concentrated via ultrafiltration and dialysed as described in the Materials and Methods. Microinjection of purified IgM into mitotic cells resulted in a pattern of stage-specific mitotic arrest similar to that obtained with whole ascites, although the inhibition was significant at lower protein concentrations (compared with the whole ascites fluid) (Fig. 3). Injection of CHO1 IgM at ≥8 mg/ml into prophase, prometaphase or metaphase cells resulted in a metaphase-like arrest in 80–85% of the cases. As with the ascites fluid, injections following anaphase onset had little effect. Control injections with a purified nonimmune IgM monoclonal antibody at equivalent concentrations led to 9–16% arrest, regardless of the stage injected. The control cells that did not divide usually ceased mitotic progression in the stage at which they were injected, in contrast to the metaphase-like block observed with CHO1 injections.

To determine the response of PtK_1 cells to different doses of purified CHO1, we injected a dilution series of the antibody during prometaphase, then scored these cells for completion of mitosis 3 h later (Fig. 4). Inhibition of mitotic progression decreased from 85% at 8 mg/ml to 24% inhibi-

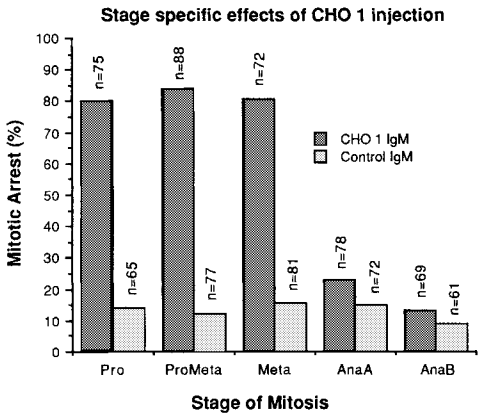


Figure 3. Effect of microinjection of CHO1 IgM or a control IgM on mitotic progression in PtK_1 cells. Cells were injected at different stages (*Pro*, prophase; *ProMeta*, prometaphase; *Meta*, metaphase; *AnaA*, anaphase A; and *AnaB*, Anaphase B) with ~10% of their volume of IgM at 8 mg/ml (protein concentration in needle). Injected cells were observed and scored 3 h after injection. The number of cells injected at each stage is indicated (n=).

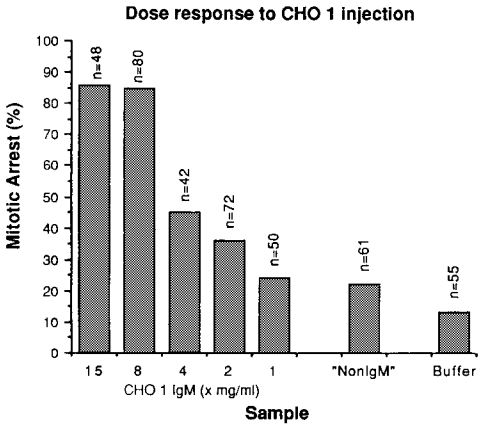


Figure 4. Dose response of mitotic PtK_1 cells to microinjection of CHO1. Cells were injected before the onset of anaphase with a dilution series of CHO1 IgM (1–15 mg/ml), with non IgM at 8 mg/ml (see Fig. 2) or with injection buffer alone. The number of cells injected with each sample is indicated (n=).

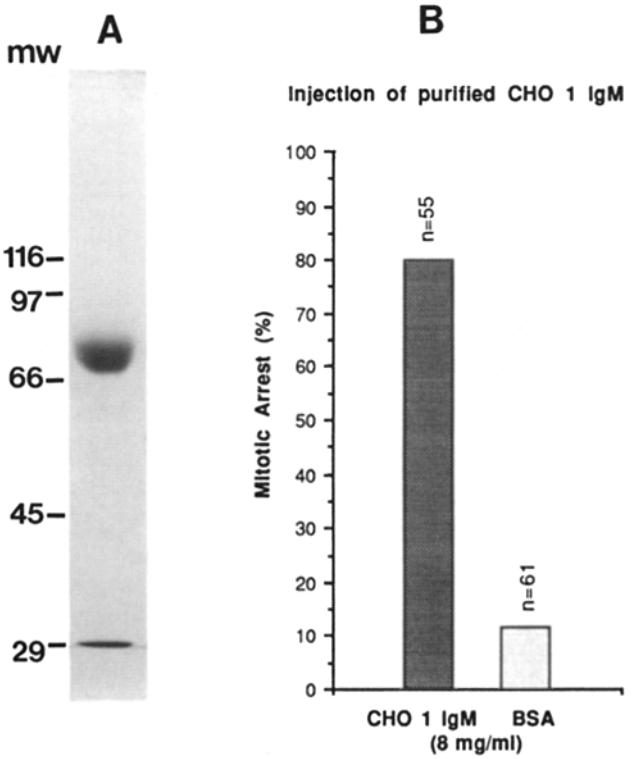


Figure 5. Effect of microinjection of highly purified CHO1 IgM into mitotic PtK_1 cells. (*A*) Coomassie blue–stained gel of the purified CHO1 IgM. Note that the predominant polypeptides migrate at ~70 and ~29 kD, corresponding to the IgM heavy and light chains, respectively. (*B*) Histogram showing the response of PtK_1 cells injected before anaphase onset with 8 mg/ml of either CHO1 IgM (*dark bar*) or BSA (*light bar*). Injected cells were observed and scored 3 h after injection. The number of cells injected with each sample is indicated (n=).

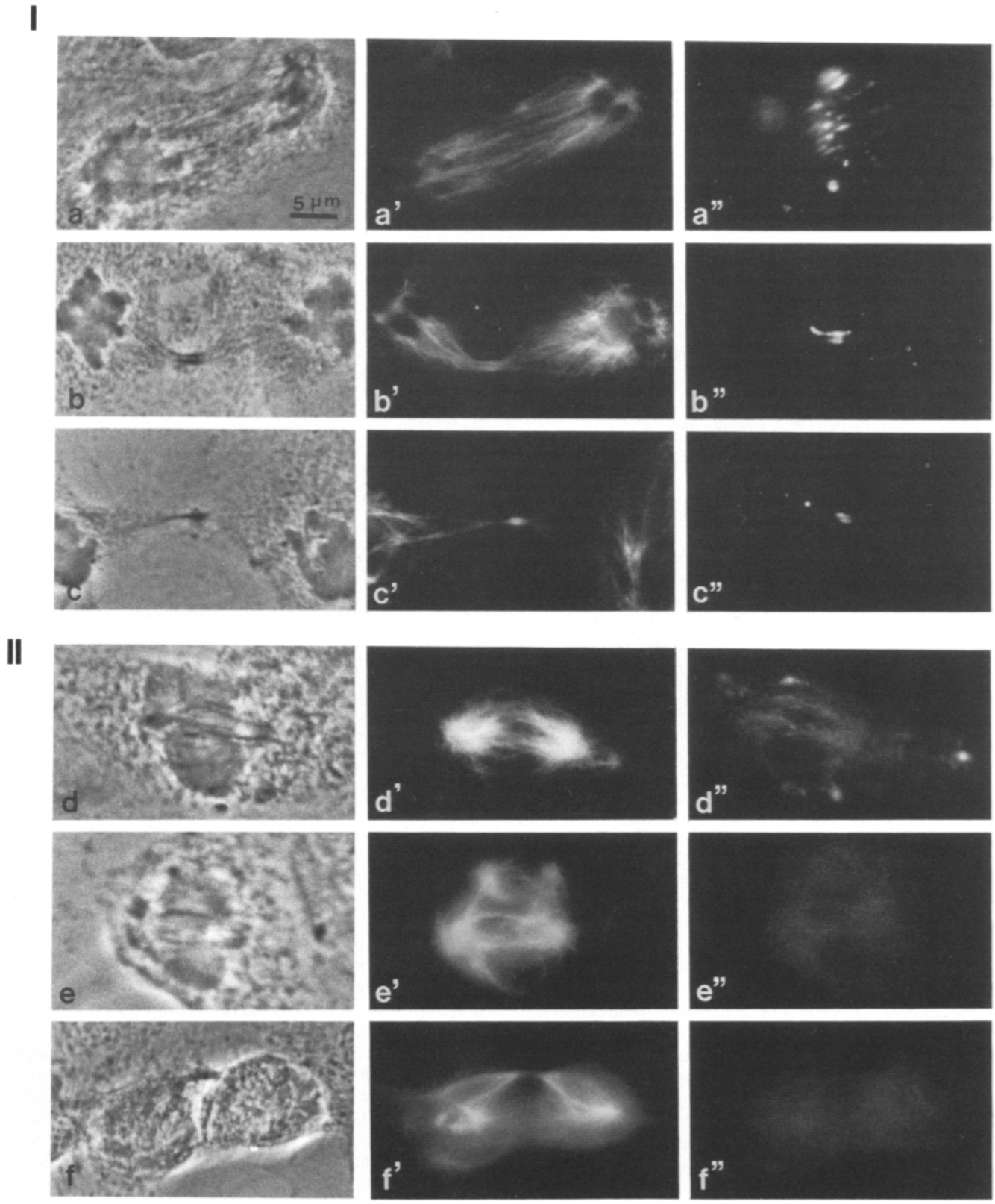
tion at 1 mg/ml. For these experiments, two different control solutions were prepared. First we concentrated column fractions containing primarily non-IgM proteins to 8 mg/ml and injected this sample into PtK_1 cells during prometaphase. Approximately 20–23% of these cells became arrested at metaphase (Fig. 4). These numbers are strikingly similar to those obtained with injection of 1 mg/ml of CHO1 IgM and probably reflect a contamination of the non-IgM column fractions with a small quantity of CHO1 IgM (see Fig. 2). To circumvent this contamination, we constructed a second control in which prometaphase cells were injected with commercially purified mouse IgM at 8 mg/ml. In these experiments only 8–10% of the injected cells failed to complete mitosis (data not shown).

As a third control, we attempted to remove all contaminating IgG and further purify CHO1 IgM. CHO1 does not precipitate in low ionic strength buffers as described by Kiehart et al. (1986). However, a second chromatographic step (as described in Materials and Methods) resulted in nearly homogeneous IgM which was devoid of IgG (Fig. 5 *a*). This preparation and control BSA were injected into mitotic PtK_1 cells before anaphase onset using a double blind experimental design (Fig. 5 *b*). The degrees of mitotic arrest achieved with this material was similar to that seen with the singly chromatographed CHO1 IgM (compare Figs. 5 and 3). Based on these several controls we are confident that the metaphase-like arrest observed in cells injected with CHO1 is due to the action of the antibody.

To obtain a more detailed view of the effect of microinjected CHO1 on mitotic progression, injected cells were processed for indirect immunofluorescent staining to visualize either the injected antibody or the spindle and its chromosomes. Cells injected at prometaphase with 2 mg/ml CHO1 IgM (a concentration that did not normally inhibit mitosis) were fixed at various times after injection and reacted with secondary antibody conjugated to Texas Red to localize the bound CHO1 (Fig. 6, *a* and *b*). In all cases the staining pattern of these injected cells was similar to that observed in uninjected cells stained directly with CHO1. Cells injected during anaphase with 8 mg/ml of CHO1 IgM (another treatment that normally has no effect on mitotic progression) also showed the normal localization of CHO1 (Fig. 6 *c*). These results demonstrate that CHO1 injected into the cytoplasm can recognize its native antigen in a normal manner. Both spindle and chromosome morphology appeared normal in those cells that were capable of dividing.

Next we focused on cells that were injected before anaphase onset with 8 mg/ml of CHO1 IgM and became arrested at metaphase. Cells injected before nuclear envelope breakdown proceeded to a metaphase-like state on schedule with control cells but did not develop further. The chromosomes in these arrested cells remained condensed near the spindle equator (Fig. 6, *d* and *e*). Staining these cells with antitubu-

Figure 6. Immunofluorescent analysis of injected cells. The same cells are seen in phase contrast (*a–f*), stained with fluorescein labeled anti-tubulin (*a′–f′*) or stained with a Texas Red–labeled goat anti-mouse secondary antibody to visualize injected IgMs (*a″–f‴*). Cells were lysed and fixed as in Fig. 1. Top (*I*) illustrates the effects of microinjection of nonrestrictive doses of CHO1 IgM. In all cases the



injected antibody localizes normally to its antigen in the cell. (*a–a″*) Cell injected at prometaphase with 2 mg/ml CHO1 then fixed 1 h later. (*b–b″*) Cell injected as in *a* but fixed 3 h later. (*c–c″*) Cell injected during anaphase A with 8 mg/ml CHO1 then fixed 3 h later. Bottom (*II*) illustrates the effects of restrictive doses of CHO1 IgM. (*d–d″*) Cell injected during prometaphase with 8 mg/ml CHO1 IgM then fixed 3 h later. This cell has become arrested at metaphase. (*e–e″*) Cell injected as in *d* and fixed 3 h later. Note the formation of supernumerary asters (*e'*). (*f–f″*) Cell injected during prometaphase with 8 mg/ml control mouse IgM and then fixed 3 h later. Note that the extent of delocalization of CHO1 from the spindle varies widely from *d″* (extreme delocalization) to *e″* (moderate delocalization). Bar, 5 μm in all micrographs.

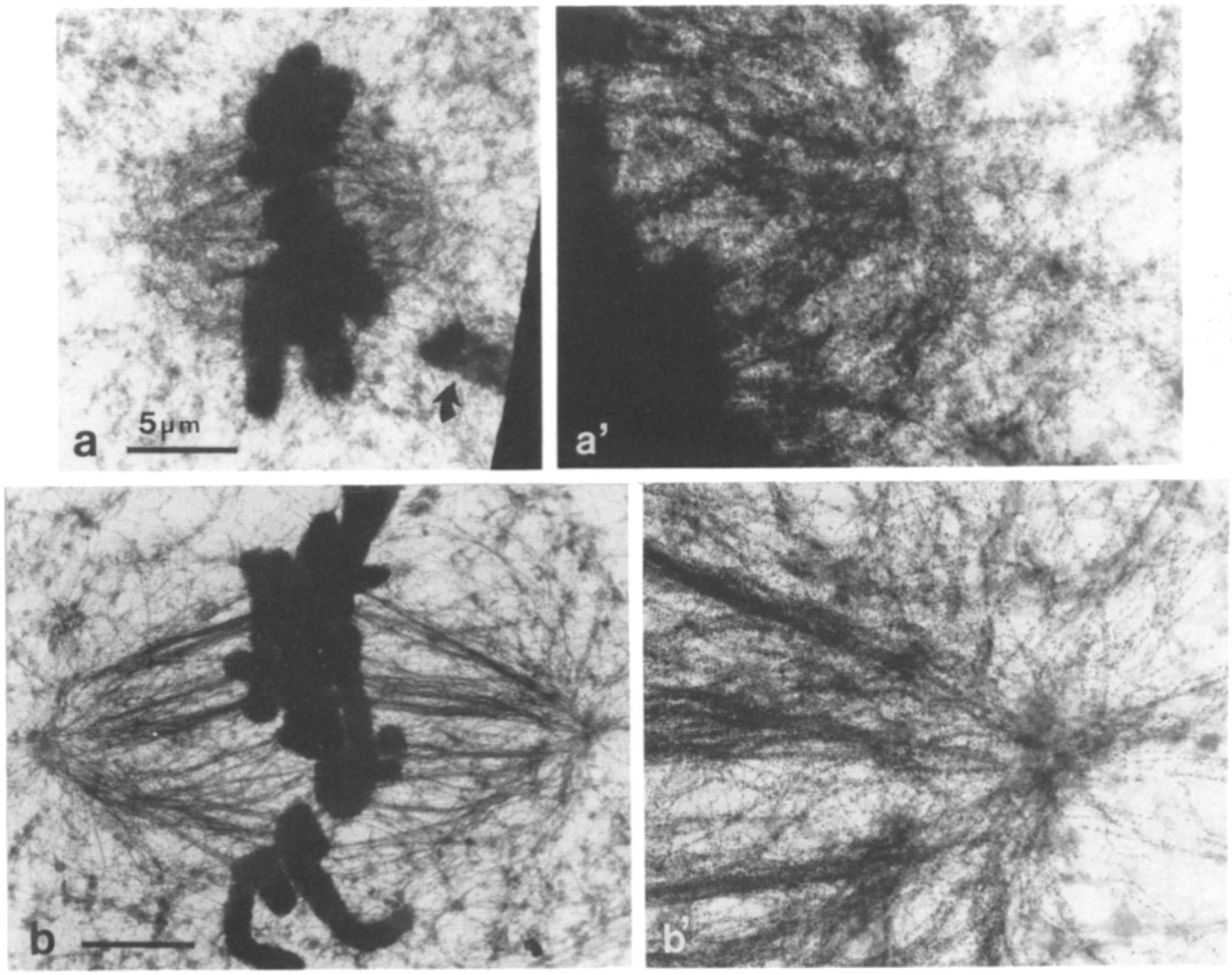


Figure 7. Whole mount immunoelectron microscopic analysis of a CHO1-arrested cell. Cells were prepared as described in Materials and Methods and stained with antitubulin and 10 nm colloidal gold–labeled secondary antibodies to enhance the contrast of the microtubules. (*a*) Low-magnification view of a representative cell injected with 8 mg/ml CHO1 IgM during prometaphase and fixed 3 h later. Note the short, stubby appearance of the spindle and the lack of focused spindle poles in this cell. (*a'*) Higher magnification view of the spindle pole of *a*. (*b*) Low-magnification view of an uninjected metaphase cell that was on the same grid as the cell in *a* shown for comparison. (*b'*) Higher magnification view of the pole in (*b*). Bar, 5 μm in all micrographs.

lin antibodies showed that their spindles look reasonably normal at the light microscopic level, although 30–40% of these cells contain one to three additional spindle poles (Fig. 6 *e*). Visualization of CHO1 in the same cells revealed that the injected antibody was diffusely distributed throughout the cytoplasm with spindle staining barely detectable above background, in either lysed (Fig. 6) or unlysed preparations (data not shown). This observation is distinct from the spindle staining seen in fixed, uninjected metaphase cells treated directly with CHO1 (compare with Fig. 1, *b''* and *c''*). The dispersal of CHO1 antigen in cells arrested by CHO1 injection suggests that the mechanism of mitotic arrest may be related to the lack of proper localization of the CHO1 antigen in the spindle (see Discussion).

Spindle morphology in injected cells was studied at higher resolution by whole-mount immunoelectron microscopy, using antitubulin and colloidal gold–labeled secondary antibodies. Seven cells that were injected with 8 mg/ml CHO1 IgM at prometaphase and became arrested at metaphase were examined ultrastructurally. The overall morphology of these cells resembled similarly treated cells observed with phase microscopy, yet closer analysis of the microtubule structure of these cells revealed several features not detected in the immunofluorescent preparations. The cells examined appeared to have arrested before achieving a true metaphase configuration; often one or more chromosomes or chromosome fragments were several micrometers from the spindle equator (Fig. 7 *a*, *arrow*). The microtubules in each half spindle were not organized into discrete spindle fibers, nor did they focus at the poles, as they do in uninjected cells at metaphase (Fig. 7 *b*). Instead these microtubules emanate diffusely from the polar region. Organized centrosomes were not readily discernible in these cells. The ultrastructural data suggest that the microtubule organization of injected cells compromised, particularly near the poles.

Biochemical Characteristics of the CHO1 Antigen

The microinjection experiments suggest that the CHO1 antigen might be an important mitotic MAP. To investigate this

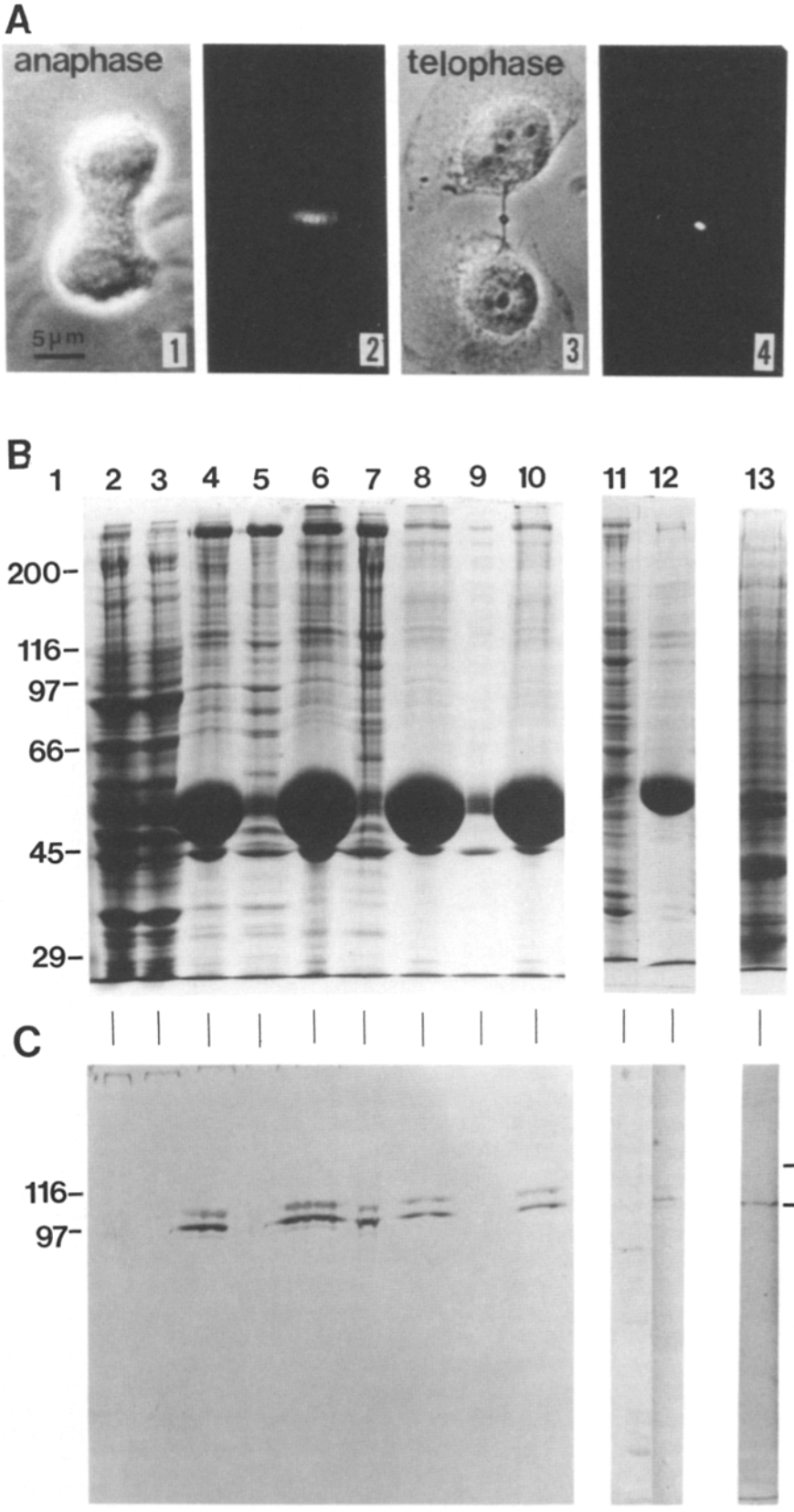


Figure 8. Analysis of the CHO1 antigen(s) in HeLa cells. Samples were prepared as described in Materials and Methods. (*A*) Immunolocalization of the CHO1 antigen in mitotic HeLa cells. The same cell is seen in phase contrast (*1* and *3*) and stained with CHO1 (*2* and *4*). Bar, 5 μm in all micrographs. (*B*) 7.5% polyacrylamide gel stained with Coomassie blue. (*C*) Corresponding immunoblot probed with CHO1 IgM. Lanes correspond to: (*1*) molecular weight markers (in kilodaltons); (*2*) high-speed supernatant prepared from HeLa cytoplasm; (*3*) supernatant after addition and centrifugation of taxol microtubules; (*4*) pellet of taxol microtubules and MAPs (MTP); (*5*) supernatant after extraction of MTP with 5 mM MgATP plus 5 mM MgGTP; (*6*) MTP pellet after MgATP and MgGTP extraction; (*7*) supernatant after extraction of MTP with 0.5 M NaCl; (*8*) MTP pellet after 0.5 M NaCl extraction; (*9*) supernatant after extraction of MTP with 1.0 M NaCl; (*10*) MTP pellet after 1.0 M NaCl extraction. (*11*) high-speed supernatant prepared from HeLa cell nuclei; (*12*) pellet of taxol microtubules and nuclear MAPs; and (*13*) whole, sonicated PtK cells. Lanes *2*, *3*, *11*, and *13* represent equal loadings of samples of a similar protein concentration. Lanes *4–10* and *12* represent a thirty-fold reduction in sample volume.

possibility further we initiated a biochemical analysis of the protein(s) recognized by CHO1. It has been shown that CHO1 binds to polypeptides of ~95 and ~105 kD on immunoblots of isolated CHO spindles (Sellitto and Kuriyama, 1988). These polypeptides appeared to be minor components of these preparations, because corresponding gel bands could not be identified in Coomassie-stained gels of these preparations. As an alternative approach to characterizing

the CHO1 antigen, we used microtubule affinity methods on HeLa cell extracts. We separated HeLa cells into nuclear and cytoplasmic components (see Materials and Methods), prepared a high-speed supernatant (HSS) from the cytoplasmic fraction, and then prepared a crude microtubule plus MAPs (MTP) pellet by supplementing it with preformed, taxol-stabilized microtubules, followed by centrifugation. HeLa cells were chosen for this analysis because large quantities of these cells were available to us and the immunofluorescent staining pattern of CHO, PtK_1, and HeLa cells is indistinguishable (Fig. 8, *a* and Sellitto and Kuriyama, 1988).

Blots of the HeLa fractions probed with purified CHO1 antibody revealed the presence of two polypeptides of molecular weights similar to those described for CHO cells (~100 and ~115 kD). Faint antibody staining on blots was detected in the HSS (Fig. 8 *c*, lane *2*). The MTP fraction was greatly enriched for these antigens (over HSS), indicating that the CHO1 antigens bind to microtubules in vitro (Fig. 8 *c*, lane *4*). When the HSS was incubated with 0.1 μg/ml nocodazole to prevent microtubule assembly, the CHO1 antigens did not pellet in a comparable centrifugation (see Materials and Methods, data not shown). To examine the microtubule binding characteristics of the cytoplasmic antigen we extracted the cytoplasmic taxol-stabilized MTP with 5 mM MgATP and 5 mM MgGTP (Fig. 8, lanes *5* and *6*) and then with two concentrations of NaCl (Fig. 8, lanes *7* and *8*). Both polypeptides remained associated with the microtubules after extraction with nucleotides (Fig. 8, lanes *5* and *6*), but 0.5 M NaCl removed between one-half and two-thirds of both polypeptides from the microtubule pellet (Fig. 8, lanes *7* and *8*). Subsequent extractions with 1.0 M NaCl did not extract any more of the antigen from the microtubule fraction (Fig. 8, lanes *9* and *10*). These results indicate that the polypeptides recognized by CHO1 bind to exogenous bovine brain microtubules in an ATP/GTP insensitive, but partially salt-sensitive manner. The identification on Coomassie-stained gels of the polypeptides responsible for the observed immunoreaction with CHO1 was hampered by the presence of numerous bands in the relevant region of the gel.

We also asked whether the nuclear staining by CHO1, observed in immunofluorescent preparations, was due to the same antigen as that recognized by CHO1 in the cytoplasm. HeLa nuclei and nuclear proteins, prepared according to Arias and Dynan (1989), were fractionated into nuclear HSS and MTP as above, then analyzed on immunoblots with CHO1 (Fig. 8, lanes *11* and *12*). The CHO1 antigen in interphase nuclei comprise a microtubule binding protein of about the same R_f as at that seen in the cytoplasm. This observation corroborates the immunofluorescent images of interphase and prophase cells and further suggests that the nuclear form of CHO1 antigen retains its microtubule binding properties.

To control for the use of two different cell types in these experiments, PtK_1 cells for microinjection and immunofluorescence and HeLa cells for the biochemical analysis, we probed an immunoblot of whole PtK_1 cells with CHO1 IgM. This experiment revealed the presence of an immunoreactive band of ~100 kD and the absence of any other cross reacting polypeptides in PtK_1 cells (Fig. 8, lane *13*).

The sedimentation velocity of the CHO1 cytoplasmic antigen from HeLa cells was determined for comparison with those of other known MAPs (reviewed in Olmstead, 1986). HeLa cell cytoplasmic HSS was fractionated on a 5–20% sucrose gradient. Each fraction from the gradient was supplemented with preformed microtubules, centrifuged, and the resulting pellets were analyzed electrophoretically. Immunoblots of these fractions reveal that the CHO1 antigen(s) in HeLa cells sediment at 11S (data not shown).

Discussion

We have demonstrated that the epitope recognized by CHO1 is associated with the spindle microtubules of PtK_1 cells in stage-specific patterns that resemble those observed previously in CHO cells (Sellitto and Kuriyama, 1988). Microinjection of sufficient quantities of CHO1 antibody into mitotic PtK_1 cells inhibits mitotic progression, supporting the idea that the CHO1 antigen is an essential mitotic protein. The exact mechanism whereby the antibody arrests mitosis is, however, unclear. Examination of injected and arrested cells reveals that CHO1 blocks mitotic progression just before full metaphase; one or two chromosomes are often some distance from the spindle equator, suggesting that chromosome congression was partially impaired. The spindle microtubules of injected cells were also disorganized, failing to bundle into discrete kinetochore fibers and to focus at the poles; rather they terminated some distance from the centrosome and formed broad poles and a "stubby" spindle.

A comparison of the morphology of cells injected with CHO1 IgM or control IgM suggests several interpretations of the effects of the antibody injections. First, the antibody might be cross-linking adjacent microtubules within the spindle (directly or indirectly), effectively inhibiting further microtubule movements. We think this possibility unlikely, considering that cells injected during prophase proceed through most of prometaphase. Second, injected CHO1 might block the binding of the antigens to the spindle or remove them from the spindle during prometaphase and metaphase. Removal of these antigens from the spindle before anaphase onset may prevent the establishment of a normal metaphase configuration, causing the cells to arrest prior to this stage. Injection of CHO1 appears to delocalize the antigen from the spindle, resulting in a higher concentration of antigen within the cytoplasm (Fig. 6, *d* and *e*). Because the CHO1 antigen is localized in normal cells where it may serve to connect antiparallel spindle microtubules, the inactivation of the antigen by antibody injection might destabilize the spindle microtubules. Also, the formation of a stable interzone, which normally occurs during anaphase, might be impaired in the injected cells.

Considering the staining pattern of CHO1 in cells fixed during anaphase, we were surprised that injection of CHO1 had little effect on mitotic progression once anaphase commenced. From this we infer that once the CHO1 antigen has reached its target within the interzone during anaphase, it is protected from the perturbing effects of the antibody. This protection could arise if the association of CHO1 antigen with the interzonal microtubules and/or spindle matrix alters the antigen in such a way to make it less susceptible to removal by the antibody. The lack of an anaphase effect might also reflect either steric hindrance of the antibody by the closely aligned microtubules within the interzone or an insufficient amount of time for the antibody to have an effect before the CHO1 antigen accomplishes its task. Further, it

is possible that the CHO1 antigen may have already served its mitotic function by the onset of anaphase.

An alternative mechanism whereby CHO1 might inhibit mitotic progression is suggested by the observation that cells arrested by antibody injection persist in a metaphase-like state for 6–7 h until they lose viability. This persistence is not observed in cells blocked with various microtubule poisons. Such drugged cells usually resume an interphase configuration within 2–3 h of treatment (Zieve et al., 1980). That CHO1 arrested cells remain in mitosis for extended periods may mean that the corresponding antigens play a role in cell cycle regulation. The localization of the CHO1 antigen is similar in some ways to the distribution of a few molecules known to be important for mitotic control, such as the antigens recognized by MPM-2 (Vandre et al., 1984) and the inner centromere (INCENP) antigens (Cooke et al., 1987).

We have been puzzled by the high concentration of CHO1 antibody required to inhibit mitotic progression. There are several possible explanations for the need of such a large quantity of antibody. First, there could be a large pool of CHO1 antigen in the cytoplasm. This is unlikely given the low levels of background staining observed with CHO1 even in unlysed preparations. Second, the avidity of the antibody might be low. Again this does not appear to be the case since CHO1 staining is detected on fixed cells at 1:5,000 dilution of the ascites fluid. Third, the CHO1 antigen might comprise an enzyme. Accordingly, it might be necessary to inactivate all of the antigen to block mitosis. Fourth, the PtK_1 cytoplasm might be degrading the injected antibody. While we have no direct evidence on the half-life of the injected antibody, fluorescent IgGs injected into fibroblasts persist as diffuse fluorescence for several hours, suggesting that they remain soluble and probably retain their ability to bind antigen (Stacey and Allfrey, 1977). Finally, the accessibility of the injected antibody to its antigen in the cytoplasm might be impaired. This is plausible given the extreme density of the cytoplasm near the spindle. Indeed, Fuchtbauer et al. (1985) have shown that even high concentrations of high-affinity antitubulin antibodies that cause disintegration of cytoplasmic microtubules do not affect spindle microtubules. Additionally, these authors observed the disintegration of cytoplasmic microtubules in lysed cell models at concentrations significantly lower than those required for similar effects in vivo. This observation supports the idea that cytoplasm is deleterious to the activity of some antibodies.

The CHO1 antigen appears to be a MAP. This assertion is based on the observation that the antigen binds to preformed, taxol-stabilized microtubules in vitro. Unlike many other previously described MAPs (Olmsted, 1986) the CHO1 antigen(s) does not bind to all cytoplasmic microtubules, rather it appears to colocalize only with interdigitating, antiparallel microtubules during mitosis. Given its localization, its molecular weight, its microtubule binding properties, and its sedimentation velocity, we suppose that the CHO1 antigen(s) make up a novel MAP. Several other proteins do, however, display similar immunolocalization patterns on interzonal microtubules during anaphase and telophase. Among these are epitopes identified using particular autoimmune sera, i.e., INCENPs (Cooke et al., 1987); a 38-kD antigen (Kingwell et al., 1987); a 200-kD antigen (Lyderson and Pettijohn, 1980); the tektins (Steffen and Linck, 1989); mitosis-specific phosphoproteins (Vandre et al., 1984); calpain II (Schollmeyer, 1988); a 112-kD protein from grasshopper (Bastmeyer and Russell, 1987); a 205-kD thiophosphorylated protein in diatoms (Wordeman et al., 1989); and a 225-kD phosphoprotein in sea urchin spindles (Kuriyama, 1989). Additionally, Mullins and McIntosh (1982) observed a 115-kD doublet on Coomassie stained gels of isolated midbodies. CHO1 antigen(s) are, however, unique in the localization patterns they display through both interphase and mitosis.

The CHO1 antigen is distributed among a subset of spindle microtubules. During mitosis the CHO1 staining pattern changes markedly, shifting from diffuse spindle staining at metaphase to an increasingly narrow band of microtubules within the interzone during anaphase. The strong inference from the anaphase images is that the CHO1 antigen localizes specifically to regions where antiparallel microtubules from opposite poles interdigitate. If this is the case throughout mitosis, our data suggest that during prometaphase and metaphase some microtubules overlap almost all the way to the opposite pole. We were unable to test this assertion by immunoelectron microscopy because the CHO1 epitope is extremely sensitive to fixation with aldehydes. Several studies have, however, addressed this question. By reconstructing spindles from serial sections prepared for EM (McDonald, 1989) or by lysing cells with exogenous polarity markers (Euteneur et al., 1982; Haimo and Telzer, 1981; Heidemann and McIntosh, 1980), microtubules in prometaphase and metaphase cells have been shown to extend far into the opposite half spindle. Although both these methods share several potential artifacts, the data suggest that before anaphase, some spindle microtubules extend very nearly to the pole opposite their site of initiation.

Our immunofluorescence observations show that CHO1 stains the nuclei of approximately one-fourth to one-third of interphase PtK_1 cells. We have tested to see whether this staining was attributable to the same antigen as that seen in the spindle by using microtubule-affinity methods on nuclear extracts. Indeed, it appears that before nuclear envelope breakdown the CHO1 antigen is located in the nucleus. These observations suggest a scenario whereby CHO1 antigen is sequestered within the nucleus prior to mitosis (possibly to prevent its binding to cytoplasmic microtubules); then as the nuclear envelope disperses during the transition to prometaphase, CHO1 antigen is released into the cytoplasm to participate in the assembly and/or stabilization of the spindle.

Once mitosis is underway, it is clear that the CHO1 antigen moves relative to the surface of spindle microtubules. It might therefore be a component of a microtubule-dependent motor complex, perhaps involved in anaphase B (Cande et al., 1989). Unlike previously characterized microtubule-based motor molecules, however, the CHO1 antigen is not extracted from microtubules with MgATP or MgGTP. This property may result simply from the use of inappropriate buffer conditions or from an adventitious blocking of the relevant active sites on this antigen by interaction with another spindle component. In support of the latter idea, a previous report demonstrated that the CHO1 antigen is tightly associated with the spindle matrix, an amorphous, electron-dense material that begins to appear within the spindle during metaphase and that becomes concentrated in the interzone and midbody during anaphase and telophase

(Sellitto and Kuriyama, 1988). Extraction of isolated CHO midbodies with the detergent Sarkosyl revealed that the CHO1 staining persisted, even after most of the midbody microtubules had been solubilized. Thus, in addition to binding to spindle microtubules, it is likely that the CHO1 antigen interacts with other, as yet undefined spindle components. We plan to identify the other proteins that associate with the CHO1 antigen(s) in an effort to elucidate the role of these proteins in mitosis.

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