

# Centrophilin: A Novel Mitotic Spindle Protein Involved in Microtubule Nucleation

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**Abstract.** A novel protein has been identified which may serve a key function in nucleating spindle microtubule growth in mitosis. This protein, called centrophilin, is sequentially relocated from the centrosomes to the centrosomes to the midbody in a manner dependent on the mitotic phase. Centrophilin was initially detected by immunofluorescence with a monoclonal, primate-specific antibody (2D3) raised against kinetochore-enriched chromosome extract from HeLa cells (Valdivia, M. M., and B. R. Brinkley, 1985. *J. Cell Biol.* 101:1124–1134). Centrophilin forms prominent crescents at the poles of the metaphase spindle, gradually diminishes during anaphase, and bands the equatorial ends of midbody microtubules in telophase.

The formation and breakdown of the spindle and midbody correlates in time and space with the aggregation and disaggregation of centrophilin foci. Immunogold EM reveals that centrophilin is a major component of pericentriolar material in metaphase. During recovery from microtubule inhibition, centrophilin foci act as nucleation sites for the assembly of spindle tubules. The 2D3 probe recognizes two high molecular mass polypeptides, 180 and 210 kD, on immunoblots of whole HeLa cell extract. Taken together, these data and the available literature on microtubule dynamics point inevitably to a singular model for control of spindle tubule turnover.

**P**ROGRESSION of cells into mitosis is characterized by the disassembly of the cytoplasmic microtubule complex (CMTC)<sup>1</sup> and the concomitant assembly of microtubules of the mitotic spindle. The dissolution of the spindle is similarly synchronous with the formation of the midbody. When division is complete, midbody microtubules depolymerize, and a new CMTC is formed in each daughter cell (Brinkley et al., 1975; Brinkley, 1985). The organization of microtubule arrays *in vivo* appears to be regulated by discrete foci known as microtubule organizing centers (MTOCs) (see Brinkley, 1985). These foci either nucleate or capture microtubules.

The centrosome, the cell's major MTOC, is usually located near the cell center and serves as the nucleation site for the interphase CMTC. Other MTOCs in mammalian cells include kinetochores and midbodies. As cells progress from the G2 phase of the cell cycle into mitosis, the centrosome splits into two identical components (diplosomes) with each functioning to nucleate opposing halves of the microtubule array making up the mitotic apparatus (see McIntosh, 1983). At the same time, there is a prominent increase in protein kinase activity at the centrosome (Verde et al., 1990; Bailly et al., 1989), and a corresponding rise in its capacity to nucleate microtubules (Telzer and Rosenbaum, 1979;

McIntosh et al., 1975). These changes in centrosomal activity are accompanied by an increase in the abundance of electron-dense pericentriolar material (Rieder and Borisy, 1982). Aggregation of electron-dense material is also seen during the formation of the midbody in telophase. This material has been shown to nucleate microtubule growth *in vitro* (Gould and Borisy, 1977). Such major changes in structure and activity suggest that MTOCs and associated proteins play an important role in regulating the assembly and distribution of microtubules during the cell cycle.

Despite an extensive body of literature, the phenomenon of microtubule nucleation in the living cell remains nebulous. Neither is it clear how the shortening of kinetochore microtubules is orchestrated with both the elongation of inter-polar microtubules and the formation of the midbody. The mechanism by which MTOCs intervene in microtubule dynamics awaits the clarification of their molecular composition.

Antibodies have provided a seminal approach to defining the molecular composition of centrosomes and related MTOCs. The availability of human and rabbit autoantibodies directed against the centrosome (Gosti-Testu et al., 1986; Sager et al., 1986; Calarco-Gillam et al., 1983; Connolly and Kalnins, 1978; Brenner et al., 1981) has indicated that common antigenic sites exist among cells of varied origins including those of higher plants (Clayton et al., 1989). A potentially unique type of microtubule-associated protein

1. *Abbreviations used in this paper:* CMTC, cytoplasmic microtubule complex; MTOC, microtubule organizing center; MAP, microtubule-associated protein.

(MAP) at the centrosome was detected by antibodies raised against brain MAP 1A (Sato et al., 1983). This MAP may lower the critical concentration of tubulin required for microtubule assembly, thus, allowing for favored nucleation at the centrosome. Antibodies that are respectively specific for kinesin (Neighbors et al., 1988) and dynein (Pfarr et al., 1990) also recognize mammalian centrosomes. Vandres et al. (1986) initially identified a family of phosphoproteins associated with the centrosome, centromeres, and midbody utilizing an mAb, MPM-2. A 225-kD centrosomal phosphoprotein detected with an mAb, CHO3, appears crucial to the shift from mitosis to interphase (Kuriyama and Sellitto, 1989). The cdc2 protein kinase has been localized in the pericentriolar region using the monoclonal probe, CTR453 (Bailey et al., 1989). Anticalmodulin immunofluorescence reveals a polar pattern (Welsh et al., 1978) and a 165-kD centrosomal protein in mammals aggregates in the presence of  $\text{Ca}^{2+}$ , as revealed with anticentrin antibody (Salisbury et al., 1986). Centrosomal proteins with molecular masses of 43 kD (Rao et al., 1989), 64 kD (Petzelt, 1979), 110–115 kD (Sager et al., 1986), and a series of proteins characterized by molecular masses of 60–65, 130, and 180–250 kD (Gosti-Testu et al., 1986, 1987) have also been discovered using immunological probes. Although strong similarities exist, these probes recognize antigens with different immunofluorescent and/or electrophoretic patterns from that recognized by a new spindle probe to be described here.

In this report, we describe an mAb 2D3, which was raised against kinetochore-enriched chromosome extract from HeLa cells (Valdivia and Brinkley, 1985). This probe recognizes two polypeptides, 180 and 210 kD, on immunoblots of whole HeLa cell extract. The detected protein, here called centrophilin, is sequentially relocated from the centromeres to the centrosomes to the midbody in a manner dependent on the mitotic phase. We demonstrate centrophilin to be a novel marker protein for spindle microtubule nucleation which potentially serves to capacitate the MTOCs.

## Materials and Methods

### Preparation of Kinetochore-enriched Extract for Immunization

HeLa cells were grown in suspension culture in McCoy's 5a medium supplemented with 7% FCS. Cells in exponential growth were synchronized by adding 2.5 mM thymidine (Sigma Chemical Co., St. Louis, MO) to the medium for 18 h, and the mitotic cells were accumulated by treatment with Colcemid at 0.06  $\mu\text{g}/\text{ml}$  in fresh medium for 16 h. A mitotic index of 98% was commonly obtained as determined by phase-contrast microscopy. The hexylene glycol method for metaphase chromosome isolation was used, starting with  $10^8$  mitotic cells (Valdivia and Brinkley, 1985). These preparations contained centrosomal material which persisted even after glycerol gradient purification.

Kinetochore enrichment involved incubating crude chromosome suspensions with 10 mM Tris-HCl, pH 7.1, 2 mM  $\text{CaCl}_2$ , 1 mM PMSF containing 200 U/ml micrococcal nuclease (MNase) (Worthington Biochemical Corp., Freehold, NJ) for 1 h at 37°C. Later, the suspension was centrifuged in a Beckman JA-20 rotor at 5,000 rpm for 10 min at 4°C. The pellet was resuspended in 10 mM Tris-HCl, pH 7.1, 1 mM EDTA, 1 mM PMSF (TEP) containing heparin (Sigma Chemical Co.) at 2 mg/ml and incubated at 4°C for 60 min. The suspension was centrifuged as above and the pellet was resuspended in TEP containing 3 M urea and incubated for 1 h at 4°C. The preparation was then centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was used as the source for immunizations.

### mAb Production

BALB/c mice immunized intraperitoneally with crude urea extracts prepared from HeLa metaphase chromosomes as described. Approximately 200–300  $\mu\text{g}$  of total protein was used per injection emulsified in Freund's adjuvant. The mice were boosted every 4 wk for several months. Blood samples obtained from the tail were screened by immunofluorescence for the presence of antibodies against mitotic spindle antigens. For immunofluorescence screening, HeLa cells were cultured on sterile glass coverslips and fixed with methanol for 10 min at 4°C. Coverslips were then incubated in undiluted mouse serum for 30 min at 37°C, washed in PBS, and incubated with a 1:20 dilution of goat antimouse IgG-FITC for 30 min at 37°C. (For more details, see "Immunofluorescence" in this section.) The spleen from the selected mouse was removed, dissociated, and the splenocytes were fused with mouse myeloma SP 2/0 cells using standard procedures as described by Kohler and Milstein (1975). Supernatants from our hybridoma cultures were again screened by immunofluorescence. Hybridomas found by the assay to secrete the desired antibody were harvested and cloned three times by limiting dilution. The hybridoma clones were then grown up in flasks and used as the source of mAbs for subsequent experiments. To maintain the stability of the mAb, the hybridoma supernatant was concentrated by tangential flow ultra-filtration (Millipore Continental Water Systems, Bedford, MA); 500 ml of supernatant would yield  $\sim 50$  ml of concentrated filtrate. Antibody filtrate was further dialyzed against a mixture of one part 2 $\times$  borate-buffered saline, pH 8.2, and one part glycerol. Dialyzed antibody was aliquoted and stored at  $-80^\circ\text{C}$ .

### Cell Culture and Microtubule Disassembly/Reassembly In Vivo

All cell lines (HeLa, WI38, human lung carcinoma, African green monkey, CHO, BHK, PtK, 3T3, Chinese Muntjac, and Indian Muntjac) were maintained in cell culture flasks containing suitable growth medium (DME, RPMI, McCoy's, or Ham's F10 [Cellgro]) supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. All cell lines were passaged with 0.1% trypsin in HBSS (Sigma Chemical Co.). HeLa cells were used exclusively in all microtubule inhibition experiments because of their fastidious growth and strong recognition by 2D3. The other cell lines were used only to determine the extent of 2D3 cross-reactivity. HeLa cells were trypsinized from culture flasks and seeded onto sterile glass coverslips in bacteriological grade 60-mm plastic petri dishes (Falcon Labware, Oxnard, CA). When coverslips reached  $\sim 75\%$  confluence, they were incubated with 10  $\mu\text{g}/\text{ml}$  nocodazole (Amersham Corp., Arlington Heights, IL) or 0.1  $\mu\text{M}$  vinblastine (Fisher Scientific Co., Pittsburgh, PA) diluted from a 10-mg/ml or 10-mM stock in DMSO stored frozen, or 0.1  $\mu\text{g}/\text{ml}$  Colcemid diluted from a 100 $\times$  stock in buffer (Gibco Laboratories, Grand Island, NY). The drugs were added directly to culture medium for 4 h. For cold treatment experiments, HeLa cells were identically cultured and incubated at 0°C for 40 min in fresh medium. Cells were recovered from mitotic inhibitor (0–5 h) or cold treatment (0–1 min) in fresh prewarmed medium (37°C) and processed for immunofluorescence. In  $\text{Ca}^{2+}$  treatment experiments, HeLa cells were rinsed in PBS or Pipes buffer, permeabilized with nonionic detergent, rinsed in buffer, incubated with  $\text{CaCl}_2$ , and subsequently fixed (see "Immunofluorescence" for details).

### Immunofluorescence

Cells on glass coverslips were rinsed in PBS and processed for single-label 2D3 immunofluorescence in one of five ways: (1) absolute methanol fixation (histological grade, Fisher Scientific Co.) for 7 min at 20°C; (2) 3% formaldehyde fixation (TEM grade, Tousimis) in PBS for 45 min at room temperature, rinsing in PBS, permeabilization with 0.5% Triton X-100 in PBS for 2 min, and rinsing in PBS; (3) permeabilization with 0.5% Triton X-100 in 0.1 M Pipes, 1 mM EGTA, 1 mM  $\text{MgCl}_2$ , pH 6.9 (PEM), rinsing in PEM, and fixation in 3% formaldehyde in PBS for 45 min; (4) permeabilization with 0.5% Triton X-100 in 0.1 M Pipes, pH 7.4, with various levels of  $\text{CaCl}_2$  (0, 1  $\mu\text{M}$ , 10  $\mu\text{M}$ , 100  $\mu\text{M}$ , 1 mM), rinsing in 0.1 M Pipes, incubation with 0.1 M Pipes containing  $\text{CaCl}_2$  for 9 min at room temperature, and fixation in 3% formaldehyde in PBS for 45 min; or (5) permeabilization with 0.5% Triton X-100 in PBS for 2 min, rinsing in 0.1 M Pipes, pH 7.4, incubation with 21 mM  $\text{CaCl}_2$  in 0.1 M Pipes for 9 min at room temperature, and fixation in 3% formaldehyde in PBS for 45 min. All coverslips were then incubated with 1:10 dilution of concentrated, dialyzed 2D3 hybridoma supernatant in PBS for 30 min at 37°C in a humidified chamber,

washed in PBS (four changes within 10 min), incubated, with a 1:20 dilution of affinity-purified goat anti-mouse IgG FITC (Boehringer Mannheim Biochemicals, Indianapolis, IN) in PBS for 30 min at 37°C, washed in PBS, optionally incubated with a 1:20 dilution of affinity-purified swine anti-goat IgG-FITC (Boehringer Mannheim Biochemicals) in PBS for 30 min at 37°C, washed in PBS, stained with 20 µg/ml Hoechst 33258 (Calbiochem-Behring Corp., La Jolla, CA) in PBS for 2 min rinsed briefly in PBS, and mounted on slides in 1:9 PBS/glycerol containing 0.1% phenylene diamine (Fisher Scientific Co.). Negative control experiments, which were performed with the omission of primary antibody, revealed only diffuse, low-level background staining.

All else unchanged, single-label antitubulin immunofluorescence was performed using a monoclonal IgG antibody specific for B-tubulin (Tu27B; L. I. Binder) diluted 1:40 on formaldehyde/Triton X-100 processed cells in place of 2D3 incubation. This probe preferentially recognizes microtubule polymer and does not require detergent extraction of soluble tubulin. An affinity-purified, monospecific sheep antitubulin antibody was originally used in early experiments.

For double-label 2D3/antitubulin immunofluorescence experiments, cells were rinsed in PEM, permeabilized in 0.5% Triton X-100 in PEM for 2.5 min, rinsed with several changes of PBS, fixed in 3% formaldehyde in PEM for 45 min, rinsed in PBS, blocked with TBS, pH 7.4, for 2 min, post-blocked with 1% BSA in PBS in 10 min, incubated with 2D3 diluted 1:10 in 1% BSA in PBS for 30 min at 37°C, washed in PBS, blocked with 1% BSA in PBS, incubated with goat anti-mouse IgG-biotin (Boehringer Mannheim Biochemicals) diluted 1:20 in 1% BSA in PBS for 30 min at 37°C, washed in PBS, blocked with 1% BSA in PBS, incubated with avidin-Texas Red (Boehringer Mannheim Biochemicals) diluted 1:200 in 1% BSA in PBS for 30 min at 37°C, washed in PBS, blocked with 1% BSA in PBS, incubated with monoclonal antitubulin antibody (5H1, IgM, L. I. Binder) diluted 1:40 in 1% BSA in PBS for 30 min at 37°C, washed in PBS, blocked with 1% BSA in PBS, incubated with affinity-purified u-chain-specific rabbit anti-mouse IgM (Nordic Immunology, Tilburg, The Netherlands) diluted 1:20 in 1% BSA in PBS for 30 min at 37°C, washed in PBS, blocked with 1% BSA in PBS, incubated with affinity-purified goat anti-rabbit IgG-FITC diluted 1:20 in 1% BSA in PBS for 30 min at 37°C, washed in PBS, stained with Hoechst, and mounted.

In antikinetochore/2D3 double-label immunofluorescence experiments, kinetochore localization was detected by sequential incubation with 1:100 CREST antiserum (Brenner et al., 1981), 1:20 anti-human IgG-biotin (Boehringer Mannheim Biochemicals), and 1:200 avidin-Texas Red. Centrophilin localization was highlighted by sequential incubation with 2D3, goat anti-mouse IgG-FITC, and swine anti-goat IgG-FITC.

Slide specimens were analyzed on a Leitz fluorescence microscope equipped with a Vario Orthomat II camera system. Micrographs were prepared with Kodak T-Max 400 film push processed to 1600 ASA.

### Immunoelectron Microscopy

HeLa cells were grown on Thermanox coverslips (Lux Scientific Inc., Newbury Park, CA), rinsed in PBS, permeabilized in 0.5% Triton X-100 in PBS, rinsed with several changes of PBS, fixed in 3% formaldehyde in PBS, rinsed in PBS, blocked with 1% BSA in PBS, incubated with 2D3 diluted with 1% BSA in PBS, washed in PBS, blocked with 1% BSA, incubated with anti-mouse IgG conjugated to colloidal gold, 10 nm, (Amersham Corp.) diluted 1:2 with 1% BSA in TBS for 1 h at room temperature, washed in PBS, postwashed in 0.1 M Pipes, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, pH 7.2; fixed in 3% glutaraldehyde (EMS) in Pipes for 1.5 h, washed in Pipes, postfixed in 1% osmium tetroxide in Pipes, washed in Pipes, en block stained with 2% uranyl acetate (Ted Pella, Inc., Irvine, CA) in 30% ethanol, dehydrated in a graded ethanol series, infiltrated with Spurr's low viscosity resin (EMS), embedded overnight at 70°C in flat molds and thin sectioned for electron microscopic analysis. Control experiments were performed by omitting the incubation with first antibody.

### Electrophoretic Transfer and Immunoblotting

Electrophoresis was performed on 4–16% polyacrylamide gels using the buffer system of Laemmli (1970). Exponentially growing HeLa cells were trypsinized from two T175 cultures flasks after attaining 80% confluence. The cells were washed in PBS, pelleted in a low-speed table-top centrifuge, resuspended in a small volume (200 µl) of Tris buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM CLAP) sonicated with 1 ml SDS-PAGE sample buffer, and boiled for 5 min. The boiled samples were pelleted in a microfuge, and the supernatants were collected for electrophoresis. Sample

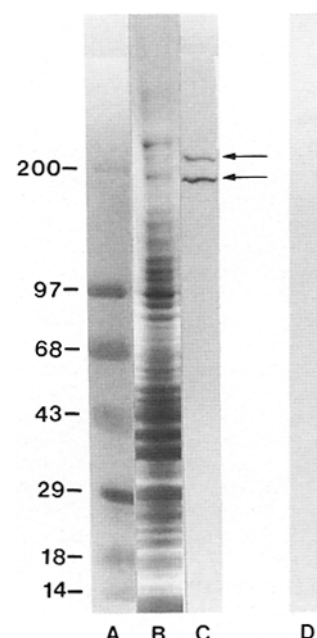
aliquots of 50–100 µl were loaded on minigels or regular gels, and electrophoresed at 100–120 V for 1.5–2 or 5–7 h, respectively. Gels were either stained with Coomassie blue R-250 or electrophoretically transferred to nitrocellulose sheets (0.2 µm pore size) at 100 V for 2 h in SDS-free transfer buffer (Towbin et al., 1979). In a procedure modified from Towbin et al., protein-bound nitrocellulose sheets were then blocked with 5% dry milk in borate-buffered saline (BBS) for 30 min with continuous agitation, incubated in 1:100 2D3 in blocking solution overnight at room temperature with agitation, washed in BBS (4×, within 30 min), incubated with 1:100 rabbit anti-mouse IgG in blocking solution for 2 h at room temperature with agitation, washed in BBS, incubated with 1:200 mouse IgG-peroxidase-antiperoxidase complex (Clono PAP; Sternberger Meyer) in blocking solution for 2 h at room temperature, washed in BBS, and developed with DAB in Tris-imidazole buffer. Occasionally, this procedure was abbreviated by using goat anti-mouse IgG directly conjugated to peroxidase (Boehringer Mannheim Biochemicals). Control trials were performed by omitting the incubation with first antibody.

## Results

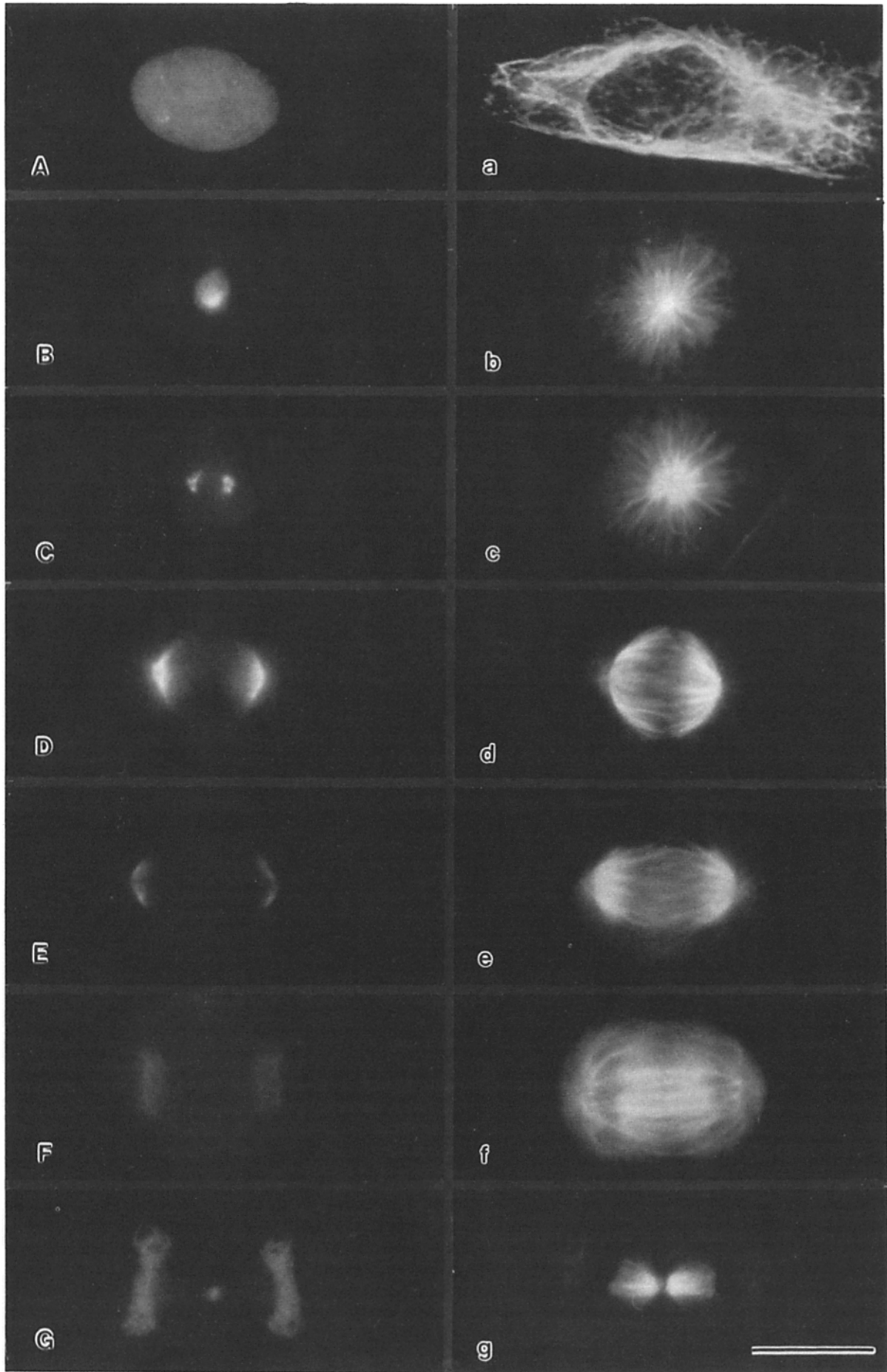
### Nature and Specificity of the 2D3 mAb

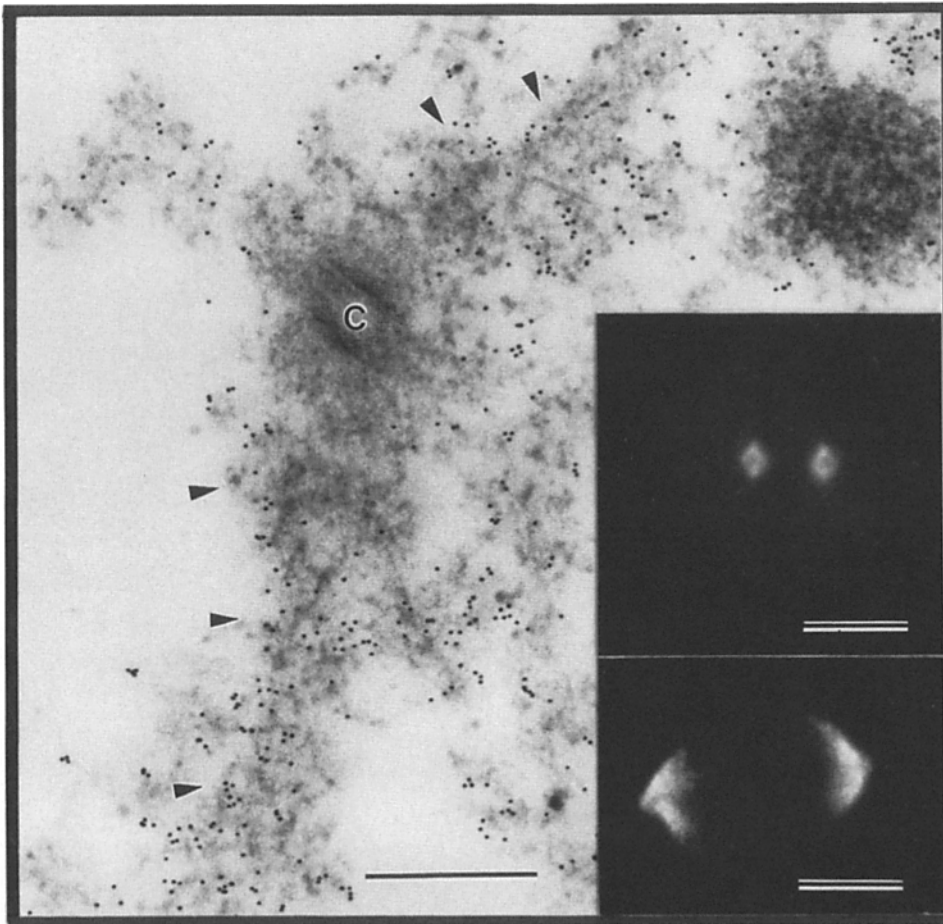
The production of 2D3 involved the immunization of mice with kinetochore-enriched chromosome extracts from HeLa mitotic cells (prepared according to Valdivia and Brinkley, 1985). The fact that 2D3 recognizes centrosomes as well as centromeres is not surprising considering that the original immunogen in part contains centrosomal material that transiently associates with kinetochores during mitosis.

The 2D3 antibody was classified as an IgG by Ouchterlony double immunodiffusion. In this assay, 2D3 hybridoma supernatant was run against specific subclasses of mouse immunoglobulins (data not shown). Our antibody cross-reacts with all primate cell lines tested, including HeLa, WI 38, human lung carcinoma cells, and African green monkey. Non-primate lines such as CHO, PtK1, Indian muntjac, and Chinese muntjac yield negative results. Although the particular epitope recognized by 2D3 is restricted to primates, the protein is likely to be common to the mitotic spindles of other species. Two polypeptides which respectively migrate at 180 and 210 kD are resolved on electrophoretic transfers of whole HeLa cell extract immunoblotted with 2D3 (Fig. 1).



**Figure 1.** Immunoblot of whole HeLa cell extract with 2D3. (A) PAGE migration pattern of molecular weight standards. (B) Coomassie blue stain of PAGE-separated proteins present in HeLa extract. (C) Two high-molecular mass polypeptides, 180 and 210 kD are revealed upon probing with 2D3 (arrows). (D) Corresponding negative control assay in which 2D3 was omitted.





**Figure 3.** Immunogold electron microscopic localization of centrophilin at the mitotic poles in HeLa cells. Centrophilin is localized in the pericentriolar material as a diffuse electron-dense crescent (arrowheads) but does not bind to the centriole (c) or the immediately surrounding region. Bar, 0.5  $\mu\text{m}$ . This pattern correlates with the immunofluorescence localization of centrophilin (insets). Bars, 5  $\mu\text{m}$ .

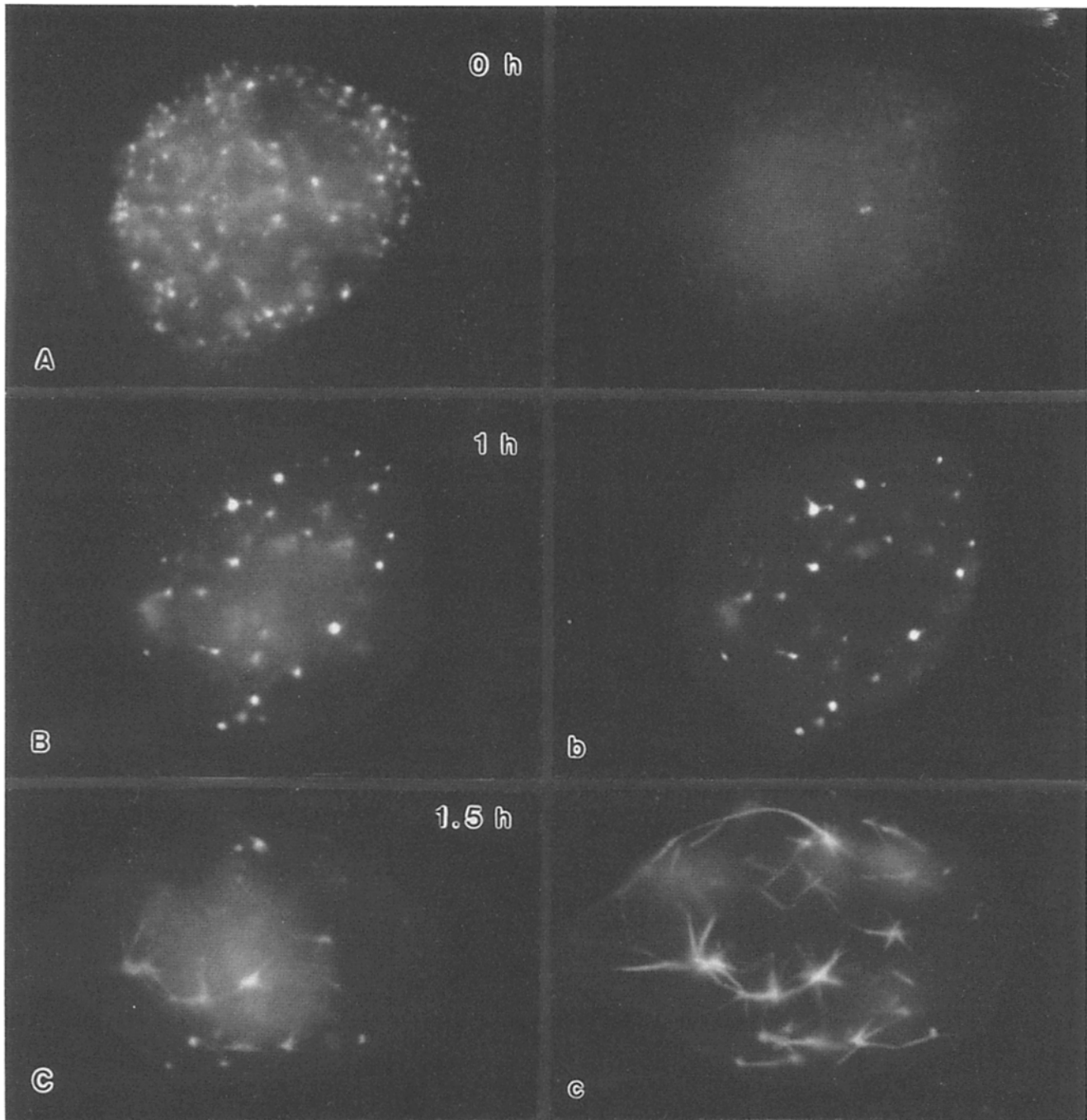
Corresponding Coomassie-stained gels of HeLa extract showing the full complement of extract proteins and negative control assays in which 2D3 was excluded together confirm the blotting immunospecificity. The two polypeptides might either be subunits of centrophilin, or one of the polypeptides may be a distinct protein that shares a common epitope with centrophilin. The first possibility is more likely, for no anomalous staining pattern indicative of another localized protein is seen in immunofluorescence assays with 2D3 (see Fig. 2). It is also plausible that the 180-kD polypeptide is a proteolytic fragment of the 210-kD polypeptide although a "ladder pattern" between the two bands indicative of proteolysis is not observed.

#### **Localization of Centrophilin during the Cell Cycle**

Centrophilin forms crescents at the poles of the mitotic spindle and bands the center of the midbody during cell division, as revealed by immunofluorescence with 2D3 (Fig. 2). The formation and breakdown of the spindle and midbody is synchronous with the appearance and disappearance of centrophilin. Formaldehyde/Triton X-100 fixation and permeabilization was employed in our initial immunofluorescence assays. This protocol was ideal for demonstrating the prominent association of centrophilin with the centrosomes and

midbody. However, diffuse, punctate staining of chromosomes and kinetochore tubules in prometaphase, and subtle staining of interzonal fibers in anaphase was also observed. This low-level staining was particularly enhanced using other fixation protocols, as will be shown. Fig. 2, A-G provides an abbreviated profile of centrophilin localization throughout the cell cycle; a-g show the corresponding microtubule pattern in each respective phase. During interphase, centrophilin is diffusely localized in the nucleus (A, a) and excluded from the cytoplasm. In prometaphase, the protein concentrates into a central focus correlating in time and space with the developing monaster (B, b). Due to the close proximity of the double centrosomes, the diplosome is not yet clearly visible. Later in prometaphase, the centrophilin focus becomes resolvable as two foci, corresponding with the clear separation of the diplosome (C, c). In metaphase, prominent crescents of centrophilin appear at the spindle poles (D, d). This pronounced staining correlates with the increasing accumulation of dense pericentriolar material seen by electron microscopy (Rieder and Borisy, 1982). As the kinetochore microtubules shorten in anaphase, the centrophilin crescents gradually disappear (E, e; F, f). In telophase, centrophilin reappears in the center of the forming midbody (G, g). Again, this localization corresponds with the aggregation of electron-dense material.

**Figure 2.** Profile of centrophilin localization during the cell cycle. A-G show immunofluorescence with 2D3. a-g show corresponding immunofluorescence with antitubulin antibody. After its nuclear deposition (A, a), centrophilin associates with the prometaphase centrosomes (B, b; C, c), forms crescents at the poles of the metaphase spindle (D, d), gradually vanishes during anaphase (E, e; F, f), and reappears as a band at the center of the midbody (G, g). Upon reversion to its nuclear localization, the centrophilin cycle is complete. Bar, 10  $\mu\text{m}$ .



**Figure 4.** Centrophilin foci nucleate spindle tubule growth. Treatment with 10  $\mu\text{g/ml}$  nocodazole results in the reversible disaggregation of centrophilin into detergent-insoluble foci throughout the cytoplasm (A) and induces the complete breakdown of spindle microtubules into soluble tubulin (a). This tubulin is completely detergent extractable except for the tiny double focus of tubulin characteristic of the centrioles. Within 1 h after removal of nocodazole, tubulin (b) focuses on insoluble centrophilin nuclei (B). Roughly 30 min later, microtubules (c) are seen to extend from these nuclei (C). Centrophilin often appears to coat growing spindle tubules, taking on a fibrous appearance. As centrophilin nuclei gradually aggregate into polar crescents over a 5-h recovery period (D–F), corresponding microtubule centers interconnect and converge to form the bipolar spindle (d–f). Visualization by double label immunofluorescence. Bar, 5  $\mu\text{m}$ .

### ***Immunoelectron Microscopic Localization of Centrophilin***

Immunogold EM with 2D3 in permeabilized metaphase HeLa cells confirms that centrophilin is part of the electron-dense pericentriolar material, aggregating into crescents (Fig. 3). The protein does not however reside on centrioles

or a narrow zone surrounding the centriole. These observations correlate with both the polar “doughnut” pattern (*top inset*) and the diffuse crescent localization seen by immunofluorescence (*bottom inset*). The fact that centrophilin plaques variably appear as globular densities or as defined or diffused crescents (Figs. 2 and 3), most likely reflects changes in spindle dynamics during metaphase alignment.

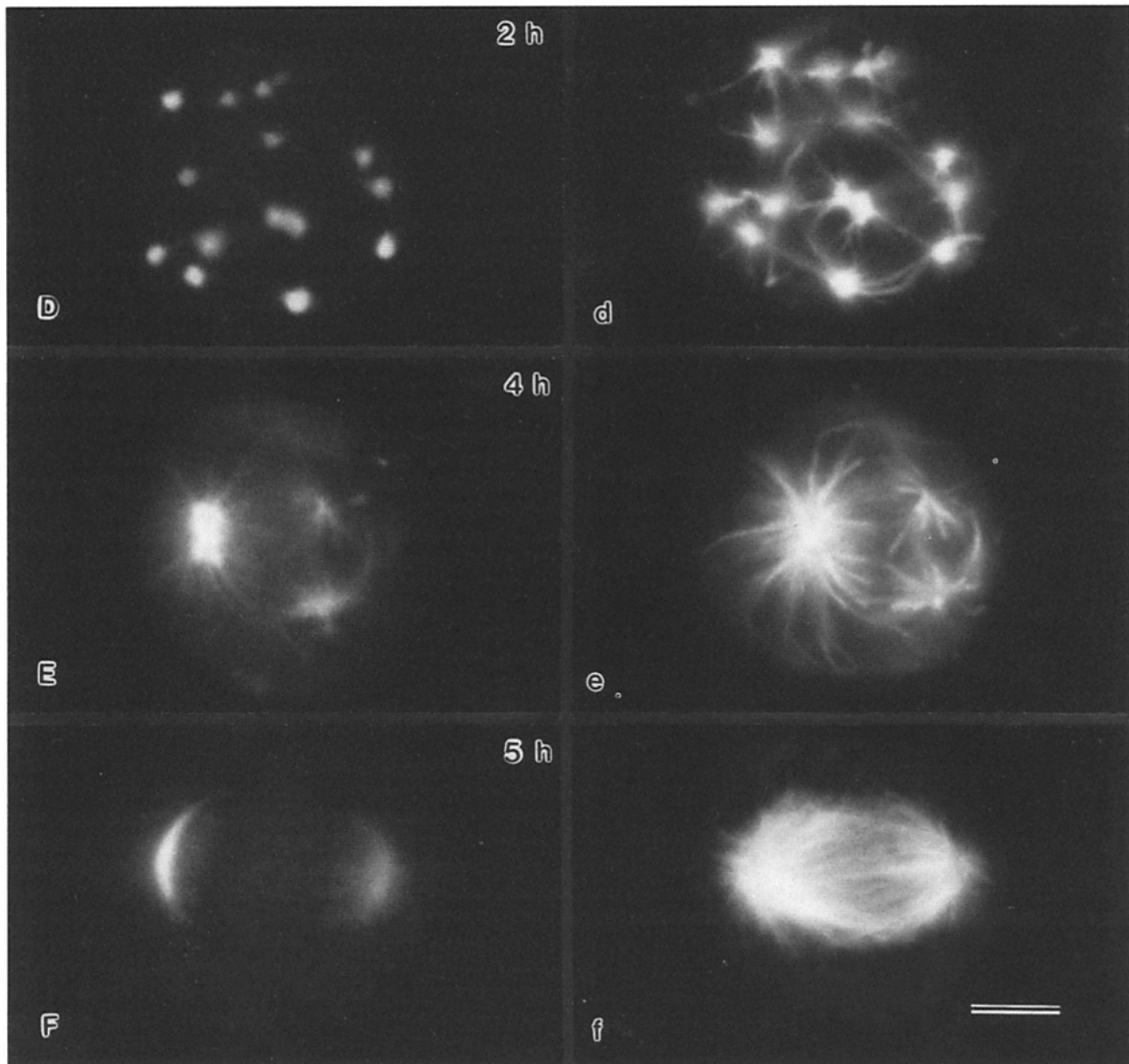


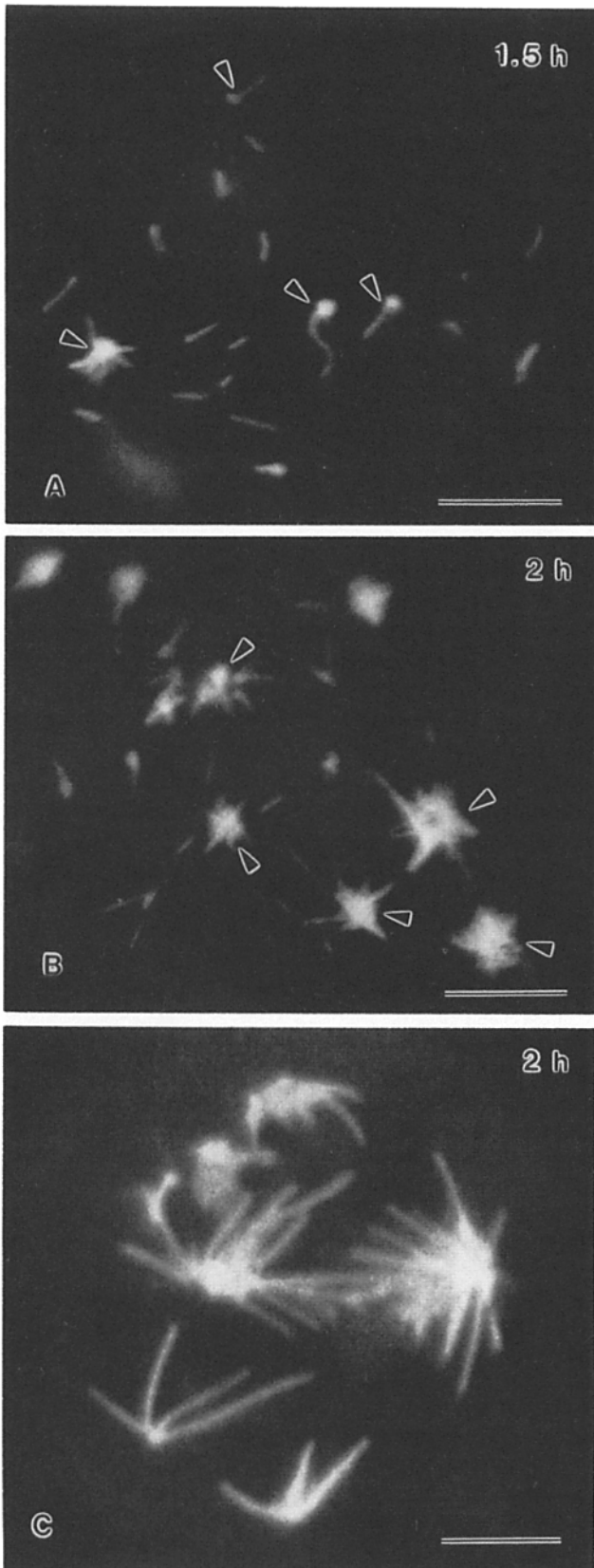
Figure 4 continued.

#### ***Effect of Microtubule Inhibitory Agents on Centrophilin Localization in Arrested Mitotic Cells***

From its cell cycle colocalization with spindle tubules, it is clear that centrophilin is intimately associated with microtubules. Treatment with nocodazole, colcemid, vinblastine, or cold results in the disaggregation of centrophilin and the simultaneous dissolution of spindle tubules (Fig. 4, A, a). The cells in Fig. 4 were permeabilized in Triton X-100 before fixation and processing for immunofluorescence. No remaining tubulin foci are observed, except for a tiny double spot characteristic of the centrioles (Fig. 4 a). Centrophilin foci, however, resist both detergent extraction and dissolution by microtubule inhibitors, as shown in Fig. 4 A.

#### ***Centrophilin Foci Are Nucleation Centers for Spindle Reassembly after Removal of Microtubule Inhibition***

HeLa cells recover very slowly from mitotic arrest. This sluggish recovery is not a disadvantage because it allows for easy visualization of otherwise fleeting phases of spindle regrowth (Fig. 4). Within 1 h after removal of microtubule inhibitor, tubulin (Fig. 4 b) aggregates onto persistent centrophilin centers (Fig. 4 B). Shortly afterward, nascent microtubules (Fig. 4 c) emanate from these centers (Fig. 4 C). Note that centrophilin centers serve as authentic nucleation sites for microtubule assembly. This is not merely a passive colocalization since tubulin, originally in a soluble diffuse state, is seen to aggregate at insoluble centrophilin



**Figure 5.** Single-label immunofluorescence pattern of centrophilin morphogenesis with 2D3 and spindle microtubule reformation with antitubulin antibody after recovery from mitotic block. Within 1.5–2 h after recovery, centrophilin foci (*arrowheads*) become rod-like and progressively more fibrous, resulting in astral arrays (*A* and *B*).

sites. With the gradual consolidation of centrophilin from multiple nuclei to polar crescents (Fig. 4, *B–F*), corresponding microtubule foci interconnect and associate to form the bipolar spindle in absolute spatial and temporal harmony (Fig. 4, *b–f*). The growth of microtubules off of centrophilin nuclei after recovery from cold treatment occurs similarly (data not shown).

#### ***Centrophilin Coats Nascent Microtubules***

In double-label immunofluorescence of spindles reforming after removal of microtubule inhibitor, centrophilin foci often appear indistinguishable from growing microtubule centers (Fig. 4, *C–c*, *E–e*). The possibility of image “bleed through” exists, however, resulting from the potential overlap in the wavelengths for fluorochrome excitation selected by the fluorescein and Texas red filters. We consequentially ran corresponding single-label experiments to more accurately document centrophilin morphogenesis during spindle tubule reassembly. After 1–2 h recovery from microtubule inhibitor, centrophilin nuclei become rod-like and progressively more fibrous, resulting in astral formations (Fig. 5, *A* and *B*, *arrowheads*). These astral formations develop synchronously with nascent astral microtubule centers (Fig. 5 *C*). Comparable patterns are also seen during recovery from cold treatment. In light of our double-label 2D3/antitubulin experiments, it is clear that centrophilin coats forming microtubules.

#### ***Centrophilin Associates with Kinetochores during Prometaphase***

The multiple foci of centrophilin seen after cell exposure to microtubule disassembly agents are for the most part non-randomly arranged. Treatment with nocodazole, vinblastine, colcemid, or exposure to cold results in a deployment in which most centrophilin foci are intimately associated with kinetochores of chromosomes, as determined by double-label immunofluorescence with 2D3 and antikinetochore antiserum from patients with scleroderma CREST autoimmune disease. After removal of microtubule inhibitor, chromosomes begin to form small clusters with centrophilin foci at the centers of resulting kinetochore rosettes. Considering that spindle microtubules nucleate from centrophilin foci (Fig. 4) and that centrophilin foci associate with kinetochore rosettes (Fig. 6), it follows that microtubule foci are also at the centers of kinetochore rosettes. During later steps of recovery from microtubule inhibition, interkinetochore microtubule foci associate into short-lived multipolar spindles, as centrophilin is transported from the kinetochores and gradually takes on its polar status (Fig. 4, *D–d*, *E–e*, *F–f*).

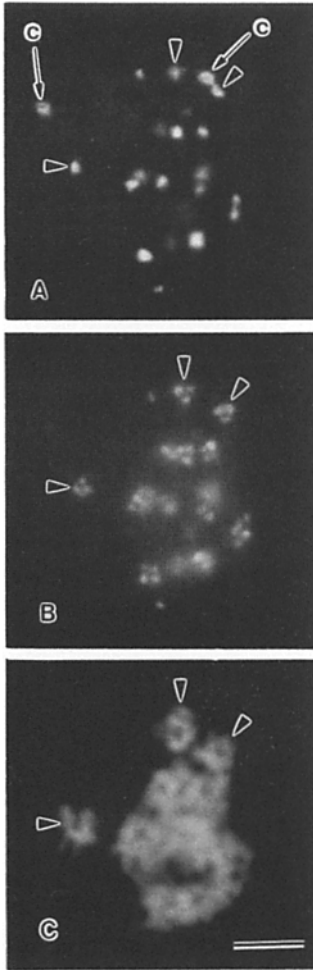
#### ***Centrophilin Is Detected at the Interphase Centrosome after Microtubule Inhibition***

Centrophilin is not normally detected at the interphase centrosome by routine immunofluorescence. However, in the presence of nocodazole or immediately after recovery, inter-

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These astral arrays of centrophilin develop synchronously with forming astral microtubule centers (*C*), taking on their characteristic appearance. Bars, 5  $\mu$ m.





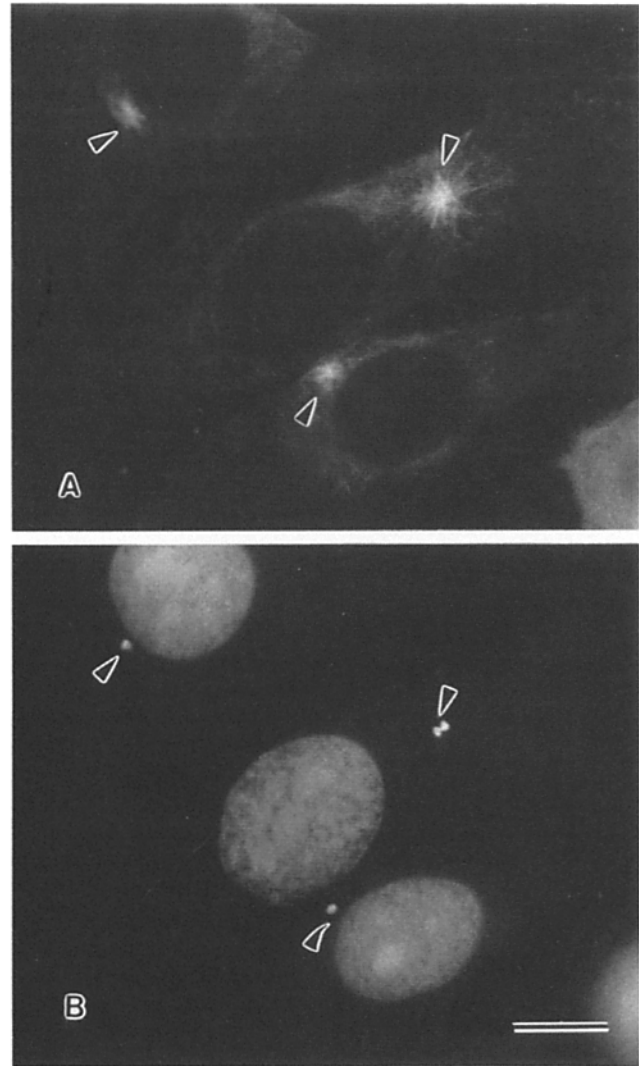
**Figure 6.** Double-label immunofluorescence with 2D3 and CREST antiserum in prometaphase after a 2-h recovery from mitotic block. Centrophilin foci (A) are seen at the centers of kinetochore rosettes (B) as indicated by arrowheads (B). The two "doughnut-shaped" foci in A which do not colocalize with kinetochores are presumably the centrosomes (c). This stage of recovery corresponds with that in Fig. 4 D, d. Chromosomes in the same cell were stained with Hoechst (C). Their clustered appearance corresponds with kinetochore rosettes. Bar, 5  $\mu$ m.

phase cells exhibit centrosomal staining with 2D3 (Fig. 7). This phenomenon is also observed in permeabilized cells treated with millimolar levels of  $\text{CaCl}_2$ . Either the diminished CMTc or the induced disaggregation of other centrosomal proteins appears to allow for greater accessibility of the relevant centrophilin epitope to 2D3. As such, a basal level of centrophilin may persist at the interphase centrosome.

#### ***Centrophilin Transiently Associates with Centromeres and Is Transported on Kinetochore Microtubules to the Mitotic Poles***

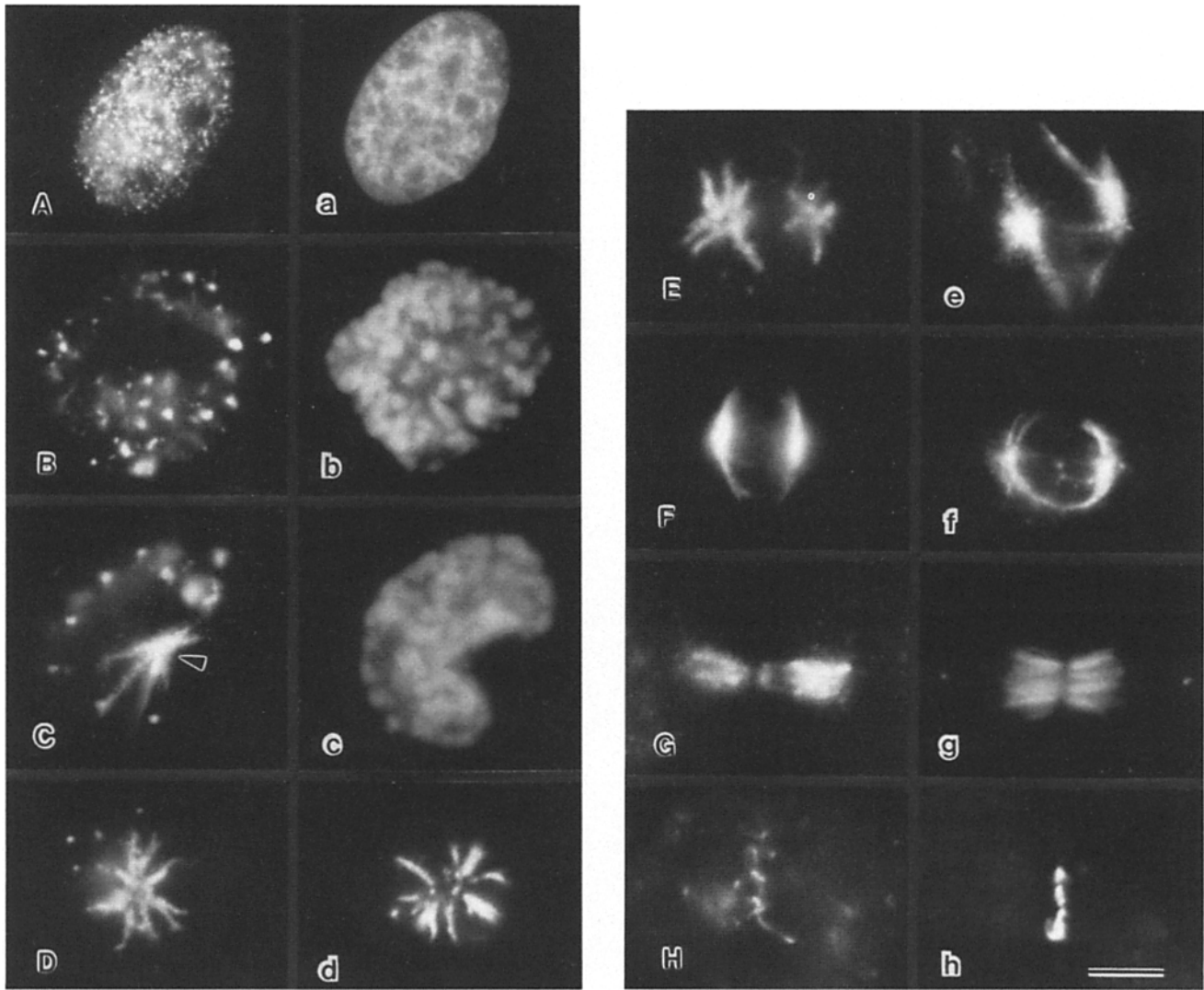
Immunofluorescence assays in which normal, undrugged HeLa cells were permeabilized with Triton X-100 before formaldehyde fixation results in enhancement of the transitory, diffuse low-level fluorescence patterns seen with the formaldehyde/Triton X-100 protocol. These elusive patterns become progressively most prominent when the cells are treated with increasing levels of  $\text{CaCl}_2$  (Fig. 8). It is likely that these permeabilization conditions help to expose antigenic sites for 2D3 recognition. With the exception of millimolar  $\text{CaCl}_2$  addition, these treatments have no visible effect on the structure of the CMTc or mitotic spindle (data not shown, but see Olmsted and Borisy, 1975).

During interphase, centrophilin (Fig. 8 A) exhibits a diffuse punctate pattern in the nucleus (Fig. 8 a). In



**Figure 7.** A basal level of centrophilin is present at the interphase centrosome. (A) Forming CMTcs after removal of nocodazole, shown here to mark location of MTOCs. (B) Centrophilin localization in same cells. Bar, 5  $\mu$ m.

prophase, centrophilin condenses down with the compacting chromosomes into discrete foci. These foci assume a characteristic kinetochore pattern (Fig. 8 B-b) and are clearly seen to colocalize with kinetochores in normal prometaphase as determined by double-label immunofluorescence with 2D3 and CREST antiserum (Fig. 9). Later during prometaphase, centrophilin eventually disappears from kinetochores. It subsequently appears along kinetochore fibers and gradually concentrates at the poles (Fig. 8 C-c, D-d, E-e, F-f; Fig. 9). This general pattern is also seen in cells recovering from microtubule inhibition (Figs. 4 and 6) and can be rapidly reversed by cold treatment (data not shown). By late anaphase, centrophilin associates with interpolar and midbody microtubules (Fig. 8 G-g), progressively becoming restricted to their equatorial ends (Fig. 8 H-h). The simplest interpretation of these events is that centrophilin is transported from kinetochores to the poles along kinetochore



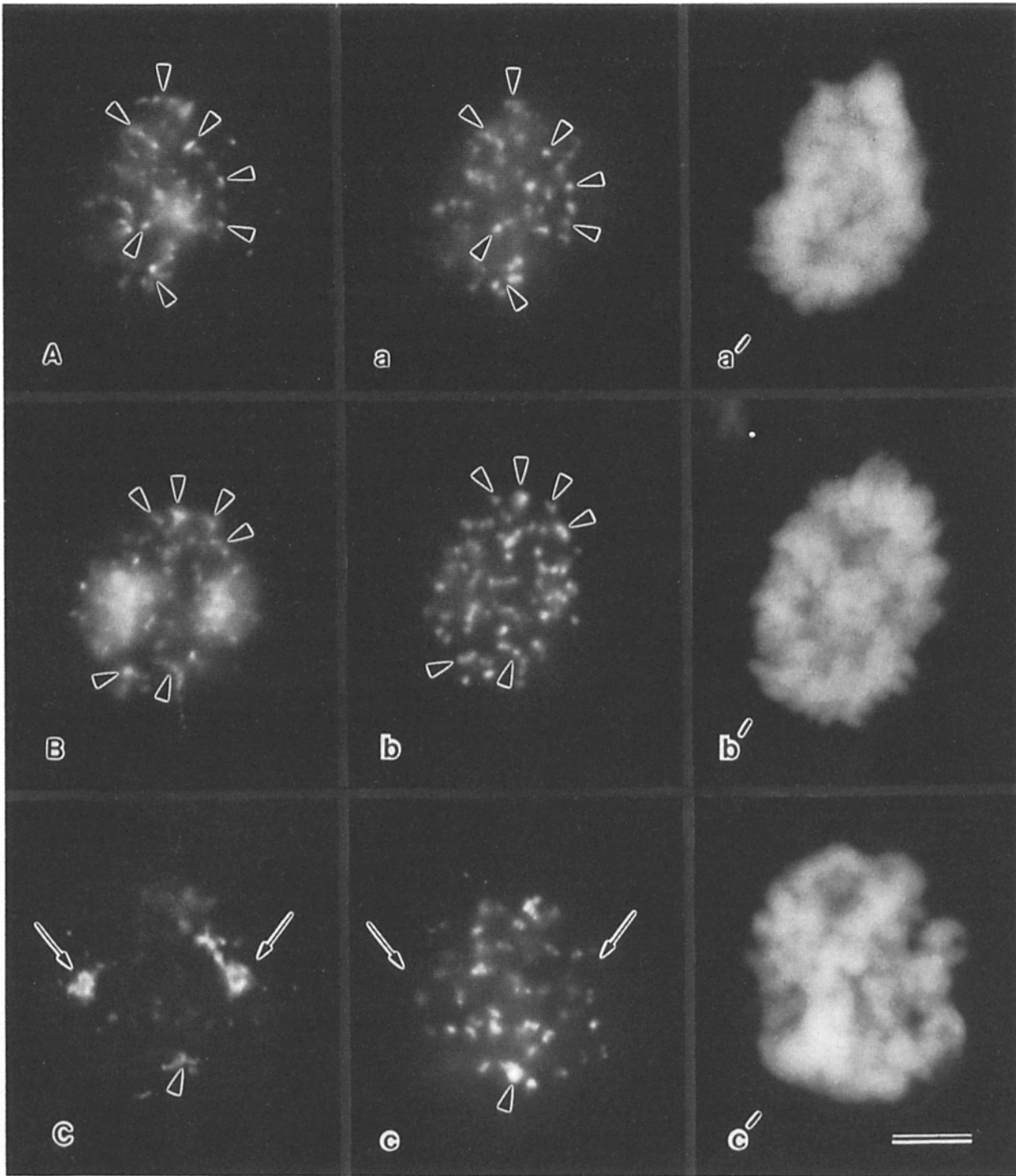
**Figure 8.** Profile of centrophilin relocation during the cell cycle in cells which were Triton X-100 permeabilized in 0.1 M Pipes, pH 6.9, with 21 mM  $\text{CaCl}_2$  before fixation. Comparable images, but with reduced sharpness, are obtained when  $\text{CaCl}_2$  is decreased to micromolar levels or when it is omitted from the permeabilization buffer. (A-H) Centrophilin pattern. (a-c) Visualization of chromatin and chromosomes by Hoechst staining in the same cells shown in A-C. (d-h) Corresponding single-label spindle microtubule pattern. Cells shown are deliberately nonidentical to those shown in D-H to discount the possibility of artifactual image "bleed through." Diffuse granules of centrophilin in the interphase nucleus (A, a) condense down progressively to form discrete foci on the prometaphase chromosomes (B, b). From prometaphase to metaphase, centrophilin dissociates from the chromosomes and appears to be transported to the mitotic poles (arrowheads) along kinetochore microtubules (C-c, D-d, E-e, F-f). In late anaphase to telophase, centrophilin (G,H) is apparently transported to the center of the forming midbody along interzonal microtubules (g,h). Bar, 5  $\mu\text{m}$ .

microtubules in prometaphase, and from the poles to the center of the developing midbody along interpolar microtubules in late anaphase to telophase.

### Discussion

We have described a mitotic protein, centrophilin, which sequentially relocates from the centromeres to the centrosomes, to the midbody, presumably along spindle microtubules. Although the foci stained with 2D3 nucleate spindle microtubule growth, our data do not demonstrate that centrophilin is actually the principal nucleating factor at these sites. It is very possible that other proteins present at these foci in concert with centrophilin nucleate microtubule as-

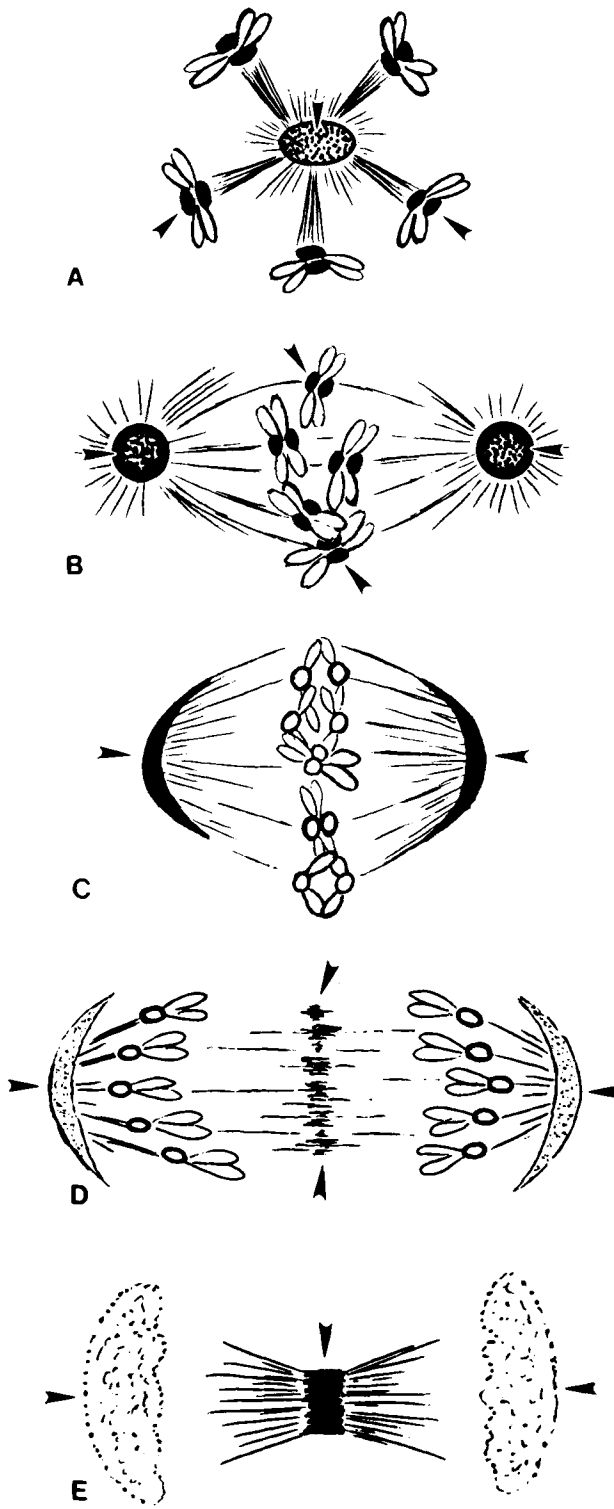
sembly. It is improbable that centrophilin is passively associated with these spindle nucleating foci since it undergoes morphogenesis and redistribution in a manner dependent on the mitotic phase. Furthermore, there is a tenet in biology that structure and location implies function. Proteins that are integral components of mitochondria, for example, invariably affect the function of mitochondria, as is true for proteins of the lysosomes, ribosomes, chloroplasts, and the plasma membrane. This is also valid for proteins associated with microtubules. There is no known MAP that does not influence microtubule assembly or function. At the evolutionary level, selective pressures impede the persistence of nonfunctional or passive proteins since the organisms' limited resources otherwise become allocated for needless synthesis.



**Figure 9.** Centrophilin transiently associates with kinetochores in normal prometaphase. Cells were Triton X-100 permeabilized in 0.1 M Pipes, pH 6.9, with 1 mM EGTA and 1 mM MgCl<sub>2</sub> before fixation. (A-C) Centrophilin localization. (a-c) Respective kinetochore localization in same cells. (a'-c') Chromosome pattern. Arrowheads indicate regions of centrophilin colocalization with kinetochores. Centrophilin-kinetochore association occurs even in the absence of the microtubule inhibitory effects of CaCl<sub>2</sub>. Comparable patterns are also seen with omission of MgCl<sub>2</sub>. Later in prometaphase (C-c'), centrophilin relocates to the poles (arrows). Image bleed-through is minimal, thereby authenticating the colocalization of centrophilin with kinetochores in A-a and B-b.

Whatever the exact function of centrophilin, its presence as detected with 2D3 appears to be a reliable marker for spindle microtubule nucleation based on the following data (a) The formation and breakdown of the spindle in both normal and drug-recovered mitosis corresponds in time and

space with the aggregation and disaggregation of centrophilin foci. (b) The regions of the spindle reported to exhibit net incorporation of subunits (Mitchison, 1989; Nicklas, 1989; Wadsworth et al., 1989; Gorbsky and Borisy, 1989; Gorbsky et al., 1987; Masuda and Cande, 1987; Mitchison et al.,



**Figure 10.** Model showing how centrophilin, a relocating marker protein for microtubule nucleation, may systematically help to modulate spindle tubule turnover throughout mitosis. This model is completely consistent with reported observations of spindle tubule dynamics (see Discussion). Shaded or blackened regions (indicated by *arrowheads*) represent the variable distribution of centrophilin in the mitotic apparatus. Lines represent spindle fibers. Centrophilin distribution indicates regions of net localized subunit incorporation in the spindle. Lightly shaded or white areas respectively signify reduction or absence of centrophilin and consequently mark zones of lessening assembly or disassembly of spindle

1986) correlate ideally with the localization of centrophilin throughout mitosis. (c) Centrophilin is a major component of electron-dense pericentriolar material (PCM) in mitosis. (d) PCM has been shown to nucleate microtubule assembly in vitro (Gould and Borisy, 1977). (e) During recovery from microtubule inhibition in vivo, soluble tubulin protein aggregates at centrophilin sites and assembles into microtubules that radiate from these sites. (f) Centrophilin foci characteristically decondense and coat nascent microtubules during recovery from microtubule inhibition.

### **Centrophilin as a Marker for End-associated Microtubule Dynamics**

The distribution of centrophilin, a protein tightly coupled to sites of nucleation, corresponds precisely with observed high-turnover regions of spindle microtubules during the course of mitosis (Fig. 10). Prometaphase congression and alignment of chromosomes at metaphase is accompanied by a net incorporation of tubulin subunits at the kinetochore-associated end of microtubules (Mitchison et al., 1986; Gorbisky and Borisy, 1989; Mitchison, 1989; Wise et al., 1990). This correlates with the transient localization of centrophilin at the centromere during prometaphase.

The gradual diminution of centrophilin immunofluorescence at the kinetochore and the concomitant accumulation of staining at the poles throughout metaphase corresponds to a shift in end-associated microtubule dynamics by early anaphase. The poleward movement of chromosomes at anaphase is characterized by a net disassembly of microtubules from their kinetochore-associated ends (Mitchison et al., 1986; Gorbisky et al., 1987; Nicklas, 1989) and de novo assembly from the poles (Wadsworth et al., 1989).

As the polar accumulation of centrophilin gradually diminishes during anaphase chromosome migration, kinetochore microtubules correspondingly cease to turnover (Wadsworth et al., 1989). At this stage, spindle microtubule dynamics shift again to the interzone where overlapping inter-polar microtubules incorporate subunits and the midbody begins to form (Masuda and Cande, 1987). This emerging MTOC activity corresponds with the relocation of centrophilin to the plus ends of inter-polar microtubules.

### **Transport of Centrophilin along Microtubules**

The concept of a nucleation factor which is relocated in a manner dependent on the mitotic phase can help explain the rather complicated shifts in spindle tubule turnover. It is not clear, however, how such a protein is deployed or how its potential nucleation activity is regulated. From the cell cycle profile of centrophilin localization presented in Fig. 8 in which centrophilin transiently spans across spindle

tubules. The transient association of centrophilin with the centromeres in prometaphase (A and B) correlates with the net incorporation of tubulin subunits at the kinetochore-associated end of microtubules. The relocation of centrophilin from the centromeres to the poles in late metaphase corresponds in turn with the net disassembly of microtubules from their kinetochore-associated ends and with de novo assembly from the poles in early anaphase (C). The developing MTOC activity of the forming midbody corresponds with the relocation of centrophilin from the poles to the equatorial ends of inter-polar microtubules in late anaphase (D) and telophase (E).

bules before concentrating at the poles or the center of the midbody, it appears that centrophilin is transported along microtubules. This notion is supported by the observation that prevention of HeLa spindle formation with microtubule inhibitors or the complete dissolution of the spindle by cold treatment results in the interruption of centrophilin aggregation or in the deseminantion of centrophilin onto kinetochores, respectively. Reaggregation only occurs after recovery from microtubule inhibition (Fig. 4). In late interphase, during the G<sub>2</sub>/M transition, the condensation of centrophilin onto kinetochores is probably coupled to chromosome condensation.

The observation that certain mitotic factors shuttle along spindle microtubules is not new. In early reports on mitotic movements, electron dense material and vesicles were seen to translocate to the poles along microtubules in prometaphase, reaching a maximum polar concentration at metaphase, and to translocate to the center of the forming midbody along interpolar microtubules in anaphase (Bajer, 1967; Rebhun, 1972). Centrophilin may be a key component of this material.

The association of centrophilin with spindle microtubules (Fig. 8) is not likely to result from the artifactual deposition of the protein on microtubules caused by the permeabilization conditions of our immunofluorescence assays since centrophilin-microtubule association is indicated in mitotic cells that have not been detergent-permeabilized before fixation (i.e., Fig. 5). Furthermore, centrophilin association with microtubules becomes progressively restricted to the poles during mitosis (Fig. 4, *E-e* and *F-f*). If this localization was an artifact due to permeabilization, centrophilin would be detected along the entire spindle or CMTC. However, this is never seen. To our knowledge there is no other intracellular mitotic transport system aside from a spindle microtubule complex that can accommodate centrophilin relocation. Centrophilin does not colocalize with either microfilaments or intermediate filaments.

It is unlikely that the apparent transport of centrophilin during mitosis is actually the result of timed posttranslational modifications and "counter demodifications" of persistent centrophilin at each site that affect recognition of the antigen by 2D3. If this were true, microtubule inhibition would not be seen to stabilize the association of centrophilin with centromeres, cold treatment would not cause centrophilin at the poles of the metaphase spindle to reassociate with the centromeres, and centrophilin would not transiently span across microtubules before concentrating at each site. Furthermore, we would still have to evoke a transport model to explain how the hypothetical posttranslational modifier, itself, can be shuttled, as well as elaborating an explanation for the "counter demodifications" of centrophilin. The sheer clumsiness of this three-part model renders it improbable. The possibility that centrophilin is relocated during mitosis simply by diffusion is also not tenable because centrophilin progressively aggregates at each new location, ever against the concentration gradient. This implies an active, energy-consuming transport.

Considering that the interaction of centrophilin with microtubules appears dynamic, it is probable that some microtubule-associated mechanochemical ATPase such as kinesin (Neighbors et al., 1988) or dynein (Pfarr et al., 1990) impels centrophilin translocation. It is also likely that

centrophilin binds to other MAPs such as centrosomal MAP 1A (Bonifacino et al., 1985; Sato et al., 1983) or directly binds to tubulin. The presence of both phosphoproteins (Vandre et al., 1986) and cell cycle-dependent protein kinase activity at the centrosome (Bailey et al., 1989) suggests that phosphorylation may play a part in centrophilin regulation. Of all the reported centrosome-associated proteins that might interact with centrophilin, including POPA (Sager et al., 1986), CHO3 antigen (Kuriyama and Sellitto, 1989), NuMA (Leyderson and Pettijohn, 1980), centrin (Baron and Salisbury, 1988), and MPM 13 antigen (Rao et al., 1989), calmodulin (Welsh et al., 1978) seems to be the most likely candidate. Calmodulin appears as polar crescents in the metaphase spindle and is found in midbodies in a pattern which closely resembles centrophilin localization. Calmodulin has also been shown to colocalize with kinetochore microtubules (Sweet and Welsh, 1988; Sweet et al., 1988) and is found associated with growing microtubule foci during recovery from nocodazole treatment (Sweet et al., 1989).

Although the operation and regulation of the mitotic apparatus remains unclear, the 2D3 probe may help add a third dimension to our somewhat two-dimensional understanding of spindle dynamics.

We extend our thanks to Bill Mollon for his assistance in EM and Ray Zinkowski and Tamas Yilling who graciously helped us with image analysis. We are grateful to Gary Martin, Jennifer Kidd, and Ric Villani for maintaining our cell cultures; and Harry Johnson and L. I. Binder for providing guidance in hybridoma cell culture and antibody production. We thank Arnold Rivera and Patty Loomis for helpful tips in electrophoresis and immunoblotting. We are also grateful to Sherry Crittenden for typing and Ann Harrell for proofreading the manuscript.

This work was sponsored by NIH grant CA 41424 to B. R. Brinkley.

Received for publication 7 May 1990 and in revised form 11 October 1990.

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