Identification of Novel Centromere/Kinetochore-associated Proteins Using Monoclonal Antibodies Generated against Human Mitotic Chromosome Scaffolds

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Abstract. We describe the generation of 11 monoclonal antibodies that bind to the centromere/kinetochore region of human mitotic chromosomes. These antibodies were raised against mitotic chromosome scaffolds and screened for centromere/kinetochore binding by indirect immunofluorescence against purified chromosomes. Immunoblot analyses with these antibodies revealed that all of the antigens are >200 kD and are components of nuclei, chromosomes, and/or chromosome scaffolds. Comparison of the immunolocalization of the antigens with that observed for the centromere-associated protein CENP-B revealed that each of these centromere/kinetochore proteins lies more peripherally to the DNA than does CENP-B. In cells normally progressing through the cell cycle, these antigens displayed four distinct patterns of centromere/kinetochore association, corresponding to a minimum of four novel centromere/kinetochore-associated proteins.

Mammalian chromosomes undergo complex movements as cells progress through mitosis. These movements are governed by the spindle microtubules that attach to each chromosome through the centromere-associated kinetochore. The current understanding of the structure and assembly of the kinetochore derives primarily from light and electron microscopic studies of mitotic chromosomes. These studies have defined the kinetochore as a trilaminar disk on the surface of each chromatid (Brinkley and Stubblefield, 1970; Rieder, 1982; Ris and Witt, 1981). The darkly staining inner (i.e., closest to the chromosome) disk appears to abut the centromeric heterochromatin, while the outer darkly staining disk and its associated corona seems to be the site of attachment of spindle microtubules (Ris and Witt, 1981).

Analyses of chromosome/microtubule interactions in vitro have demonstrated that the kinetochores of isolated chromosomes can capture microtubules that originate from the spindle poles (Euteneuer and McIntosh, 1981; Mitchison and Kirschner, 1985) and that chromosomes can translocate along those microtubules in a direction analogous to metaphase chromosome congression (Mitchison and Kirschner, 1985). Further studies, both in vitro (Koshland et al., 1988; Hyman and Mitchison, 1990) and in vivo (Gorbsky et al., 1987; Nicklas, 1989), have demonstrated movement of chromosomes along microtubules in the opposite direction (i.e., analogous to chromosome movement at anaphase). In fact, Rieder and Alexander (1990) have recently proven that in late prometaphase chromosomes can rapidly translocate to a pole after the attachment of only a single microtubule. From these collective data the conclusion seems inescapable that one or more microtubule-associated motors are components of the kinetochore. Indeed, Pfarr et al. (1990) and Steuer et al. (1990) have provided experimental evidence in support of this prediction through their immunolocalization of cytoplasmic dynein to the kinetochore region of mammalian metaphase chromosomes.

Despite such clear interest, the biochemical composition of the kinetochore is unknown, a situation largely due to difficulties in defining an assay for its biochemical purification. What information is available concerning specific components of the centromere/kinetochore complex derives primarily from using sera from patients with scleroderma CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia). These patients were found (Moroi et al., 1980; Fritzler et al., 1980) to produce anti-centromere antibodies (ACA) and these have been used to analyze the structure of the centromere/kinetochore region by immunoelectron and immunofluorescence microscopy (Brenner et al., 1981; Rattner et al., 1988; Cooke et al., 1990). Immunoblot analysis against chromosomal proteins revealed that sera from a majority of these patients identified proteins of 17 (CENP-A), 80 (CENP-B), and 140 kD (CENP-C) (Earnshaw et al., 1985), while other sera have identified a 50-kD protein (Kingwell and Rattner, 1987), as well as other minor species (Earnshaw et al., 1985).

CENP-A has been shown to be a histone-like protein that is predominantly associated with mono-nucleosomes (Palmer and Margolis, 1985; Palmer et al., 1987). CENP-B, 1. Abbreviation used in this paper: ACA, anti-centromere antibodies.
the most thoroughly characterized mammalian centromere/kinetochore protein (Earnshaw et al., 1987), has been shown to be enriched in chromosome scaffolds (Earnshaw et al., 1984) and immunoelectron microscopy has localized it to the heterochromatic region of the centromere but not the kinetochore itself (Cooke et al., 1990). Furthermore, biochemical evidence suggests that CENP-B, either directly or indirectly, complexes with a subset of the heterochromatic, α-satellite DNA (Matsumoto et al., 1989). Antibody reagents specific for CENP-C are not available and little is known about it except that it too is a chromosome scaffold protein (Earnshaw et al., 1984). Two additional antigens have been identified using monoclonal antibodies that decorate centromeres/kinetochores during metaphase, but whose antigens dissociate from chromosomes after anaphase and accumulate at the midplate region (Pankov et al., 1990; Yen et al., 1991).

As reviewed by others (Pluta et al., 1990), these preceding analyses have provided valuable structural detail of the centromere region. Despite this, no true kinetochore component has been identified and many kinetochore/centromere proteins must remain uncharacterized. To address this problem, we have now used human chromosome scaffolds as antigens to generate 11 monoclonal antibodies that decorate the centromere/kinetochore region of isolated human metaphase chromosomes. The combination of immunoblot analyses and immunofluorescence localization of each antigen throughout the cell cycle reveals that these 11 antibodies identify a minimum of 4 novel centromere/kinetochore components.

Materials and Methods

Isolation of Mitotic Chromosomes and Scaffolds

HeLa cells were grown in S-MEM (Gibco Laboratories, Grand Island, NY) and K562 cells were grown in RPMI 1640 both supplemented with 10% calf serum, 2 mM glutamine, 100 U/ml penicillin, and 0.1 μg/ml streptomycin. Both cell types were arrested in metaphase by treatment with 0.1 μg/ml colcemid for 16-20 h. These cells were hypotonically swollen in RSB (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, and 5 mM MgCl2) and lysed by centrifugation at 1,600 rpm (TI-6R centrifuge; Beckman Instruments, Inc., Palo Alto, CA) onto poly-L-lysine-coated coverslips. Coverslips were immediately immersed in PBS containing 3.5% paraformaldehyde for 5 min., and then transferred to 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% BSA, 0.5% Triton X-100 for 5 min, and finally to 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% BSA for 5 min. Monoclonal antibodies and CENP-B polyclonal antibodies were added to each coverslip and incubated in a humidified chamber for 30 min at room temperature. After two 5-min washes in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% BSA, the bound murine antibodies were detected using biotin-conjugated horse anti-mouse antibody (1:250; Vector Laboratories, Inc., Burlingame, CA) and Texas red-conjugated streptavidin (1 μg/ml; Sigma Co., St. Louis, MO). Bound rabbit antibodies were detected using a fluorescein-conjugated goat anti-rabbit antibody (1:30; Vector Laboratories, Inc.), and DNA was detected using 4',6-diamidino-2-phenylindole (DAPI; 0.4 μg/ml; Sigma Chemical Co., St. Louis, MO).

Intracellular localization of each antigen at different cell cycle stages was determined using HeLa cells grown on coverslips. Coverslips were immersed in stabilization buffer (4 M glycerol, 100 mM Pipes, pH 6.9, and 1 mM EGTA) for 1 min at room temperature, extracted in stabilization buffer containing 0.5% Triton X-100 for 2 min, and then returned to stabilization buffer for 2 min. Cells were then fixed in PBS containing 3.5% paraformaldehyde for 5 min and rinsed in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% BSA. Antibodies were added and incubated for 30 min in a humidified chamber. Detection of bound antibody was performed using the fluorescein-conjugated antibodies described earlier. After the final wash all coverslips were mounted with Gel-mount (Biomeda, Foster City, CA).

Microscopy was performed using an Olympus BH-2 microscope equipped for epifluorescence. Cells were photographed using Kodak FMAX 400 film.

Immunoblotting was performed on nitrocellulose strips containing protein separated by SDS-PAGE (Laemmli, 1970). Nucleosides were preincubated for 30 min in PTX (10 mM NaPO4, pH 7.5, 0.2% Triton X-100, 150 mM NaCl, 1 mM EGTA, and 1 mM Na3) containing 4% BSA. Antibodies were added to this solution and incubated with gentle agitation for 4-12 h. Unbound antibody was removed by washing the nitrocellulose in PTX five times for 3 min. The strips were then preincubated in PTX containing 4% BSA for 5 min, and bound antibody detected with [32P]-labeled goat anti-mouse antibody (1:750; Amersham Corp., Arlington Heights, IL) for 2 h. Final washes were in PTX five times for 3 min and the signals detected by autoradiography. All steps were carried out at room temperature.

Phosphatase experiments were performed essentially as described by Davis et al. (1983). Briefly, nitrocellulose membranes were rinsed in 0.1 M citrate, pH 5.6 for 5 min, and then incubated with 5 U/ml potato acid phosphatase (Boehringer Mannheim Diagnostics, Inc., Houston, TX) in 0.1 M citrate, pH 5.6 at 37°C for 60 min. Membranes were then rinsed in 0.1 M citrate, pH 5.6 for 10 min, and subjected to immunoblotting as described earlier.

Results

Identification of Monoclonal Antibodies that Bind to the Centromere/Kinetochore Region of Chromosomes from Metaphase-arrested Cells

To generate monoclonal antibodies to novel centromere/kinetochore proteins, we chose to use chromosome scaffolds as an initial antigen. This complex subcellular fraction was prepared from metaphase chromosomes after removal of most of the DNA with micrococcal nuclease and most of the histones with 2 M NaCl. Since the final scaffold preparation,
which contains 0.1–0.2% of whole cell protein, quantitatively retains two previously identified centromeric components (CENP-B and CENP-C; Earnshaw et al., 1984), our rationale was that at least a portion of the centromere/kinetochore complex remained in the scaffold fraction.

After immunization of mice and production of hybridomas, supernatants from the initial pools of antibody producing cells were assayed for centromere/kinetochore staining by indirect immunofluorescence microscopy. An example of the staining pattern observed with one initial uncloned culture supernatant (1H1) against chromosomes prepared from colcemid-treated K562 cells is shown in Fig. 1 A. This antibody localizes to the centromere region of each chromosome revealing the classic "double-dot" appearance. From a total of 760 independent culture supernatants assayed in this manner, twenty-nine yielded centromere/kinetochore staining similar to that in Fig. 1 A. 11 of these 29 continued stably to produce antibody through cloning by limiting dilution.

Immunofluorescence localization of the antigens recognized by all 11 cloned antibodies revealed a double-dot appearance on isolated mitotic chromosomes (Fig. 2). (The examples shown are of chromosomes from mitotic cells obtained by treatment with colcemid, but indistinguishable staining was also observed on chromosomes from cells blocked with vinblastine [not shown].) In each case, while the staining was reminiscent of that seen with ACA, simultaneous localization of the centromeric DNA-associated CENP-B demonstrated two important differences between CENP-B and the antigen recognized by each monoclonal. First, the intensity of staining of the centromeres of different chromosomes was more uniform with each mAb than was the staining with anti-CENP-B serum (as shown for mAb 1F1 in Fig. 1 B, arrows). Second, although all of the monoclonal antibodies bound to the centromere domain, all bound to sites peripheral to the localization of CENP-B (e.g., Fig. 2 A, arrows). For example, measurement of the center-to-center spacing of the "dots"
Figure 2. Double immunofluorescence staining of isolated mitotic chromosomes with rabbit anti-CENP-B and a series of monoclonal antibodies prepared against chromosome scaffolds. (A-K) Staining pattern with each of 11 monoclonal antibodies, as indicated on each portion of the figure. (Left) Chromosomes are visualized with DAPI staining. (Center) The same fields shown with DAPI staining are shown by immunofluorescence staining with CENP-B and (right) each monoclonal antibody. Bars, 2 μm.

Identified by mAb 154 revealed a spacing of 0.8 μm (SD = 0.1), whereas the more poorly resolved CENP-B dots were spaced 0.5 μm (SD = 0.1). Although the actual separation of the CENP-B dots varied among different preparations of chromosomes (possibly as the result of differences in preparation or fixation), in all cases the monoclonal binding was farther from the chromosomal axis. Both the more uniform intensity and the more peripheral localization are similar to staining with whole ACA that react with multiple centromere-related antigens (CENP-A, CENP-B, and CENP-C), while all antibodies specific for CENP-B reveal less distinct dots on metaphase chromosomes (Earnshaw et al., 1987; Fig. 2 A). These data are consistent with the growing body of evidence that localizes CENP-B within or immediately adjacent to the centromeric heterochromatin (Cooke et al., 1990; Matsumoto et al., 1989) and suggest that the anti-
Figure 3. Immunoblot analyses with six centromere/kinetochore-reactive monoclonal antibodies. 40 μg of protein from (lanes 1) a total cell extract, (lanes 2) isolated nuclei, (lanes 3) chromosomes, and (lanes 4) chromosome scaffolds were immunoblotted after fractionation on a 4% polyacrylamide gel. The monoclonal antibody used in each case is listed at the bottom of each immunoblot. Migration position of myosin (205 kD) is shown at the left.

Detection of Centromere/Kinetochore Antigens by Immunoblot Analysis

To begin a characterization of the protein(s) recognized by each centromere antibody, we used immunoblot analysis of equal amounts of protein from a total cell extract, nuclei, chromosomes, and chromosome scaffolds. Representative results for six of the antibodies are shown in Fig. 3. Monoclonal antibodies 154 and 9H8 bind to proteins with approximate molecular mass of ~250 and ~275 kD, respectively (Fig. 3 A and B). The distribution of these antigens among the various fractions was precisely as predicted for mitotic chromosome scaffold proteins. Each is most abundant in the scaffold fraction (Fig. 3, lanes 4), still detectable in the chromosome fraction (observable on overexposure of the autoradiograms; data not shown), but undetectable in nuclear and total cell fractions (lanes 1 and 2). Enrichment of these antigens from the chromosome fraction to the chromosome scaffold fraction is ~10-fold. This corresponds closely to the fraction of mitotic chromosomal proteins that are retained in the scaffold (based on the bicinechonic acid assay [Smith et al., 1985]). Presumably, both antigens recognized are mitosis-specific scaffold components and are either not present in interphase or present at levels that are undetectable with these antibodies.

The remaining immunoblots (Fig. 3, C and F) show the cellular distribution and approximate molecular weights of antigens detected by four other monoclonal antibodies. The apparent molecular masses of these antigens are all >200 kD (as are the antigens detected by all 11 antibodies). The antigens recognized by these latter antibodies are enriched in the chromosome scaffold fraction, and are also apparent in the nuclear fraction as well. The proportional increase in these antigens in going from chromosomes to scaffolds is not as dramatic as that observed with 154 and 9H8, demonstrating that these antigens are only partially retained in the scaffold fraction.

Cellular Distribution of Kinetochore-associated Antigens during the Cell Cycle

To determine the distribution of each antigen throughout the cell cycle, we used immunofluorescence microscopy on normally cycling HeLa cells. Chromosome condensation, determined by DAPI staining, and nuclear envelope breakdown were used to assess cell cycle position. In all cases, antibody concentrations were the same as used for immunoblotting. Cells extracted under conditions that stabilize microtubules and leave the mitotic spindle intact and cells fixed without prior extraction of soluble components gave similar immunofluorescence patterns, although the data presented are from extracted cells because of slightly lower background fluorescence. The 11 antibodies revealed 6 distinct patterns of cell cycle–dependent localization, although only 4 of these patterns included unambiguous localization to centromeres/kinetochores.

The first of these patterns is represented by mAb 154 (Fig. 4). Immunofluorescence was simultaneously performed with a rabbit anti-CENP-B serum to provide a known reference point for the centromere region on each chromosome. Consistent with the immunoblot (Fig. 3 A), no staining was detectable with mAb 154 in either interphase (Fig. 4 A) or prophase (Fig. 4 B) cells despite the expected punctate pattern detected with the CENP-B antiserum. Hence, either the
Figure 4. Localization of CENP-B and the mAb154 antigen(s) in HeLa cells at various stages of the cell cycle. (Left) DAPI staining showing chromosome location; (center) CENP-B staining; and (right) mAb 154 staining. Cells shown are in (A) interphase, (B) prophase, (C) prometaphase, (D) metaphase, and (E) anaphase. Bar, 10 μm.
Figure 5. Localization of CENP-B and the mAb 9H8 antigen(s) in HeLa cells at various stages of the cell cycle. (Left) DAPI staining showing chromosome location; (center) CENP-B staining; and (right) mAb 9H8 staining. Cells shown are in (A) interphase, (B) prophase, (C) metaphase, and (D) anaphase. Bar, 10 μm.

The epitope for mAb 154 is masked before prophase or the protein is too low in abundance or too diffusely localized to be detected by immunofluorescence. The mAb 154 antigen first appears at prometaphase when it localizes to a pair of centromeric dots on each newly condensed chromosome (Fig. 4 C). At metaphase, the antigen remains localized to the aligned chromosomes (Fig. 4 D) in a pattern nearly superimposable with that observed with the CENP-B antiserum. At anaphase, when the chromosomes begin to move toward the poles, CENP-B remains attached to the centromeres, but the mAb 154 antigen quantitatively dissociates from the chromosomes and localizes to the midzone (Fig. 4 E). (The faint midzone staining visible at anaphase in the CENP-B image [Fig. 4 E] is an artifact arising from weak detection of the strong mAb 154 image in the Texas red fluorescence channel.) Ultimately, at telophase, the mAb 154 antigen concentrates in the midbody where it is presumably discarded after completion of cytokinesis (data not shown).

The second class of cell cycle-dependent centromere/kinetochore association is provided by mAb 9H8 (Fig. 5). Similar to mAb 154, no staining is detectable in cells at interphase (Fig. 5 A). Unlike mAb 154, however, staining with mAb 9H8 first appears during prophase, both at the periphery of the nucleus and in two brightly staining, nuclear-asso-
Figure 6. Localization of CENP-B and the mAb 3G3 antigen(s) in HeLa cells at various stages of the cell cycle. (Left) DAPI staining showing chromosome location; (center) CENP-B staining; and (right) mAb 3G3 staining. Cells shown are in (A) interphase, (B) prophase, (C) metaphase, and (D) anaphase. Bar, 10 µm.

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mAb 3G3 and anti-CENP-B and intense staining of the pericentrosomal regions (Fig. 6 C). The pericentrosomal staining remains unchanged at anaphase, while most of the chromosome-bound antigen recognized by mAb 3G3 dissociates from the chromosomes and remains at the midplate (Fig. 6 D). In addition, there is also a small proportion of staining detected between the midplate and the now segregated chromosomes. Ultimately, the antigen that accumulates at the midplate becomes restricted to the midbody. The overall pattern is similar to that seen with the first class (mAb 154) except that mAb 3G3 also identifies an antigen(s) localized to the pericentrosomal region throughout metaphase and anaphase.

Yet another pattern of cell cycle-dependent localization was seen with the antigen recognized by mAb 3H1 (Fig. 7). In interphase cells, this antibody stains not the nucleus but rather an unusual region adjacent to it (Fig. 7 A). What restricts this staining to a defined, perinuclear domain is not proven but the staining is centered directly over the centrioles (as judged by simultaneous staining with the centrosome-specific autoimmune sera, not shown). At prophase, this antibody intensely stains the nuclear envelope in a dif-

Figure 7. Localization of CENP-B and the mAb 3H1 antigen(s) in HeLa cells at various stages of the cell cycle. (Left) DAPI staining showing chromosome location; (center) CENP-B staining; and (right) mAb 3H1 staining. Cells shown are in (A) interphase, (B) prophase, (C) metaphase, and (D) anaphase. Bar, 10 μm.
fuse pattern clearly distinct from CENP-B (Fig. 7 B). At metaphase, both centromeres/kinetochores and pericentrosomal material are stained (Fig. 7 C), a pattern resembling that seen previously with mAbs 9H8 and 3G3. At anaphase, the mAb 3H1 antigen remains attached both to the pericentrosomal regions and to the centromere domains of the translocating chromosomes (Fig. 7 D).

Monoclonal antibody 5E3 offers a fifth variation on antigen distribution during the cell cycle. This antibody yields punctate nuclear staining in interphase and prophase cells, but the pattern is distinct from CENP-B (Fig. 8, A and B). By metaphase, only centrosomes and the pericentrosomal regions are identified (Fig. 8 C). Oddly, despite its initial identification by binding to chromosomes from cells arrested in mitosis, little or no detectable staining is observable at the centromeres/kinetochores of normally cycling cells. However, it is plausible that some of the antigen may actually be present at the centromeres/kinetochores, but obscured or occluded during metaphase, since faint midplate staining becomes detectable after the chromosomes have segregated at anaphase (Fig. 8 D).

Finally, mAb 1H1 identifies a 205-kD antigen that shows intense nuclear staining during interphase and prophase (Fig. 9, A and B), followed by redistribution during metaphase.
Figure 9. Localization of CENP-B and the mAb 1H1 antigen(s) in HeLa cells at various stages of the cell cycle. (Left) DAPI staining showing chromosome location; (center) CENP-B staining; and (right) mAb 1H1 staining. Cells shown are in (A) interphase, (B) prophase, (C) metaphase, and (D) anaphase. Bar, 10 μm.

and anaphase to the pericentrosomal region. Like 5E3, no centromere/kinetochore staining is seen at any cell cycle point (Fig. 9, C and D), even though such staining is easily seen after treatment with microtubule-destabilizing drugs (Fig. 2 K).

Since all antibodies were initially screened for binding to mitotic chromosomes, the failure of some (1F1 and 1H1) to reveal centromeric staining in normally cycling cells was surely surprising and raised the possibility that the observed pattern arose from a redistribution of the antigen(s) during cell extraction or fixation. However, this does not seem likely since, as expected, double immunofluorescence with each monoclonal and a polyclonal antibody to tubulin revealed that the mitotic spindle microtubules were preserved under our conditions, even though mAb 1H1 (not shown) and 1F1 (Fig. 10 B) localized only to the pericentrosomal domains of these spindles. Under these conditions, the anticipated centromere staining was easily observed with other antibodies (such as mAb 154; Fig. 10 A). In addition, the patterns of antibody binding were essentially indistinguishable in cells fixed in other ways (paraformaldehyde without extraction, or extraction under microtubule stabilizing conditions, followed by fixation with methanol; not shown).
Figure 10. Simultaneous localization of tubulin and the antigens recognized by mAbs 154 and IF1. (A) Localization of tubulin and mAb 154; (B) localization of tubulin and mAb IF1. (Left) DAPI staining showing chromosome location; (center) tubulin staining; and (right) mAb staining. Bar, 5 μM.

Figure 11. Immunoblot assay for phosphatase-sensitive epitopes recognized by monoclonal antibodies. Nitrocellulose strips containing chromosome scaffold proteins fractionated by 4% SDS-PAGE were incubated at 37°C for 60 min in 0.1 M citrate, pH 5.6 in the (+) presence or (−) absence of 5 U/ml potato acid phosphatase, and subjected to immunoblot analysis with the indicated antibodies. (A) MPM-2, (B) 9H8, (C) 3G3, and (D) 1H1 monoclonal antibodies.
**Table I. Summary of Monoclonal Characteristics**

| mAb | Class/Ig | Phosphorylated epitope | CHO cell staining | Molecular mass (kD) | Immunofluorescence staining |
|-----|----------|------------------------|-------------------|---------------------|-----------------------------|
| I   | 154 IgM  | −                      | −                 | 250                 | Interphase, Kinetochore     |
|     | 9H8      | −                      | −                 | 275                 | Metaphase, Kinetochore/centrosomes |
| II  | 9G9 IgG1 | +                      | +                 | 205                 | Nucleus, Kinetochore/centrosomes |
|     | 9G6      |                        |                   | 205                 | Juxtanuclear, Kinetochore/centrosomes |
| III | 3G3 IgG1 | −                      | −                 | 205                 | Nucleus, Centrosomes        |
| IV  | 3H1 IgM  | −                      | −                 | 205                 | Nucleus, Centrosomes        |
| V   | 2D3 IgG1 | −                      | −                 | 205                 | Nucleus, Centrosomes        |
|     | 5E3 IgM  |                        |                   |                     |                             |
| VI  | 1F1 IgG1 | −                      | −                 | 205                 | Nucleus, Centrosomes        |

**Several of the Centromere/Kinetochore Antibodies Recognize a Phosphorylated Epitope**

Recently an enormous body of evidence has emerged implicating phosphorylation cascades in the control of mitosis (for example see Murray and Kirschner, 1989). Since some of the staining patterns observed with the monoclonal antibodies described here appear mitosis-specific and/or show mitosis-specific redistributions, we analyzed each epitope for sensitivity to phosphatase. Immunoblotted strips containing mitotic chromosome scaffold proteins were incubated with or without potato acid phosphatase and subsequently analyzed with each monoclonal antibody. Fig. 11 shows a representative experiment. The mAb MPM-2, which has been reported to recognize mitosis-specific phosphorylated epitopes, was used as a phosphorylation specific control (Davis et al., 1983). Immunoblot analysis with MPM-2 against chromosome scaffolds yielded predominantly two bands (>200 kD) that were nearly eliminated by acid phosphatase treatment (Fig. 11 A). Similar analysis using mAb 9H8 indicated that the epitope recognized was also acid phosphatase sensitive (Fig. 11 B). Three other mAbs (9G6, 9G9, and 9H8) that identified a similar-sized polypeptide and yielded indistinguishable staining throughout the cell cycle also showed phosphatase sensitivity (not shown). (It is possible that these monoclonal antibodies are directed against an epitope(s) on one of the many proteins detected by MPM-2. Unfortunately, all four monoclonal antibodies are of the same class and isotype as MPM-2, precluding a direct test of this possibility.) All of the remaining epitopes were insensitive to phosphatase (e.g., Fig. 11, C and D for mAb 3G3 and 1H1). Since these antibodies detect their respective scaffold proteins in the acid phosphatase-treated sample, this also supports the view that the diminution in signal observed with MPM-2 and 9H8 is not due to proteolysis during phosphatase treatment.

**Discussion**

**Summary of Monoclonal Antibody Characteristics**

The 11 monoclonal antibodies we have identified here (whose characteristics are summarized in Table I) recognize a minimum of 6 proteins that associate with centromeres of isolated metaphase chromosomes. Four of these antigens represent novel centromere/kinetochore proteins as judged by molecular weight, subcellular distribution, and cell cycle-dependent localization. The remaining two proteins are associated with centromeres on isolated chromosomes, although we have not unambiguously localized them to centromeres in cycling cells. At the least, the successful isolation of monoclonal antibodies to centromere/kinetochore associated proteins confirms that additional centromere/kinetochore components are contained in the chromosome scaffold fraction. But more importantly, these antibodies provide reagents that will now permit detailed analysis of at least four (and possibly as many as six) kinetochore/centromere associated components.

**None of the 11 Monoclonal Antibodies Recognize Previously Identified Centromere/Kinetochore Proteins**

Despite our use of a chromosome scaffold fraction enriched in the centromere proteins CENP-B and CENP-C (Earnshaw et al., 1987), we did not isolate any new monoclonal antibodies recognizing these proteins. Such antibodies might well have been anticipated for CENP-B, since there are an estimated 30,000-50,000 CENP-B molecules/cell (Bernat et al., 1990), which comprise between 0.1 and 1% of scaffold proteins. Since we did not obtain new CENP-B antibodies and since for four of the six proteins now identified we isolated only one antibody recognizing each protein, we infer that by no means have we saturated all of the centromere/kinetochore antigens in scaffold proteins and others should also be forthcoming using this approach. Still, since CENP-B is relatively abundant in the initial scaffold antigen and is highly antigenic both in humans (Earnshaw and Rothfield, 1985) and in rabbits (Earnshaw et al., 1987), the absence of new CENP-B antibodies is a little surprising. Of course, this could reflect our small sample size of 11 monoclonals, the large number or abundance of other centromere-associated components or differential immunogenicity between CENP-B (and also CENP-C) and the proteins to which our antibodies were generated.
Which, If Any, of the Antigens Are Kinetochore Components?

All 6 different antigens detected by the 11 identified monoclonals localize to the centromeric domain of chromosomes in cells blocked at metaphase, but display a pattern that is clearly distinguishable from that previously documented for the centromere-associated protein CENP-B. When attached to centromeres, each new antigen is clearly localized peripherally to CENP-B and is more constant in amount at the centromeres of different chromosomes (although some variation in amount of antigen present is apparent for some antibodies; see Figs. 1 B and 2). The peripheral localization of these antigens is easily distinguished from that of CENP-B, yielding a measured spacing of ~0.8 \( \mu \)m between the antigens localized on each of the two chromatids, while CENP-B spacing is only ~0.5 \( \mu \)m. The increased spacing on isolated chromosomes is not readily reconcilable with association with centromeric DNA, but rather is appropriate for an intrinsic component of the kinetochore or its surrounding corona. For example, previous electron microscopic views of the distances between the opposite kinetochores on paired mitotic human chromosomes have revealed a kinetochore-to-kinetochore spacing of ~0.7 \( \mu \)m (for example see the micrographs of Cooke et al. [1990]). Since immunogold methods with antibodies directed specifically against CENP-B have revealed that CENP-B is tightly associated with the heterochromatin that underlies the kinetochore (Cooke et al., 1990), it seems likely that some (or all) of the antigens identified by these new monoclonals, whose antigens lie more peripherally to the chromosome axis than does CENP-B, are transiently involved with the kinetochore per se or with its associated corona. Obviously, immuno-electron microscopy with each monoclonal antibody will be required to document more thoroughly this putative kinetochore localization.

The Antigen Most Likely To Be a True Kinetochore Component Is Identified by mAb 154

Of the antigens identified by the 11 monoclonals reported here, the one detected by mAb 154 seems most likely to identify a genuine kinetochore component. This antigen, which is absent or diffusely localized prior to the onset of prometaphase, is exclusively associated with centromeres/kinetochores during prometaphase, remains aligned at the centromeres of metaphase chromosomes, but unexpectedly dissociates from chromosomes at the onset of anaphase movement and remains behind at the mid-zone. This cell cycle-dependent behavior is similar to that of a 140–155-kD doublet previously identified by monoclonal 37A5 (Pankov et al., 1990) and to CENP-E, the antigenic target of another monoclonal antibody prepared to a similar subcellular fraction (Yen, T. J. et al., 1991). The common dissociation of CENP-E, the 37A5 antigen and the mAb 154 antigen from chromosomes at, or just after, the onset of anaphase demonstrates that several, and possibly many, such proteins follow a similar pathway. Given the known cascade of protein phosphorylation events that govern cell cycle progression (for example see Murray and Kirschner, 1989), it is tempting to speculate that such changes in phosphorylation may trigger dissociation or disassembly of multiple chromosome associated complexes at, or just after, the anaphase transition.

Defining Common Components of Kinetochores/ Centromeres and Centrosomes

All but 1 of the 11 monoclonal antibodies selected for kinetochores/centromere binding displays the surprising feature that they bind not only to kinetochores but also to centrosomes and/or the pericentromeral region, a property that antibodies raised against Drosophila microtubule associated proteins had shown earlier (Kellogg et al., 1989). Possibly each of the corresponding antigens is itself bifunctional, participating both in centromere activity and in some pericentromeral function as well. Regardless, an even bigger surprise was the frequency with which the centromere/kinetochore-associated antigens dissociated from chromosomes after the anaphase transition and remained at the mid-plate, ultimately to be concentrated in the midbody. Three of the newly identified proteins behaved in this manner. This suggests that the resulting midbody is a rich source of centromere/kinetochore proteins (or, more accurately, postkinetochore proteins). Why a kinetochore/centromere component would be sequestered to the midbody is far from clear, but two scenarios are satisfying: either such proteins are multi-functional and participate not only during chromosome movement but also during some step in cytokinesis, or restriction to the midbody serves as a means to discard kinetochore/centromere components once their function is completed.

A troublesome aspect of our results emerges from the four antibodies (IHI, IF1, 2D3, and 5E3) representing classes V and VI (Table I). The antibodies intensely decorate the centromere/kinetochore region of mitotic chromosomes isolated from colcemid-treated cells (Fig. 2, H–K), but fail to stain the centromere/kinetochores of normally cycling mitotic cells (Figs. 8 and 9). For the antigens recognized by 2D3 and 5E3 (which appear at the midplate just after anaphase A begins; Fig. 8 D), it is plausible to suggest that a portion the protein(s) recognized is actually present at the mitotic centromeres/kinetochores of cycling cells, but that antibody binding is occluded when the antigen is chromosome associated. Only when the antigen is released at the anaphase transition is its position at the mid-plane observable. Antibodies IF1 and IHI are, however, more problematic because no centromere/kinetochore-associated or midplate staining is seen in cells not treated with anti-microtubule drugs. Hence, if these antigens are present at the centromeres/kinetochores of normal cells, their epitopes must be blocked from antibody binding throughout their attachment to chromosomes. For these antigens, the (clearly less interesting) alternative possibility that centromere/kinetochore association arises artefactually after colcemid-induced disruption of normal cell structure cannot yet be discounted.

What Roles Do these Kinetochore/Centromere Components Play?

As is generally true, it is difficult to extrapolate from immunofluorescence localization to function. For the mAb 154 antigen, given its attachment to chromosomes during prometaphase and its peripheral localization, the most plausible function would be in assembly of an active kinetochore that can capture microtubules nucleated from the spindle poles. Alternatively, its dissociation from chromosomes during anaphase and its corresponding deposition at the mid-zone could
indicate that functions during anaphase B movement or even later during cytokinesis, although it is difficult to rationalize why transient chromosomal localization would be necessary unless the protein was used for some chromosomal function as well. Perhaps this antigen (and some of the others as well) function in initiating and/or stabilizing spindle microtubule attachment to kinetochores. In any event, the availability of these antibodies potentially permits several experimental approaches to examine the corresponding in vivo functions. First, microinjection of each monoclonal antibody into living cultured cells, as has been done recently by other groups (Nislow et al., 1990), may reveal the consequence of intracellular depletion of the corresponding antigen at different cell cycle points. For example, ACA injection has yielded either prometaphase or metaphase arrest, depending on when in the cycle the antibody was injected (Bernat et al., 1990), a finding demonstrating that an antigen recognized by most ACA (presumably CENP-B) is required at two cell cycle points. A complimentary approach of equal potential interest will be to examine any of these antibodies affect in vitro microtubule capture (e.g., as in the experiments of Mitchison and Kirschner, 1985), and for those antibodies that also identify pericentrometal antigens, whether microtubule nucleation by isolated centrosomes is affected.

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