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CENP-E, a novel human centromere-associated protein required for progression from metaphase to anaphase

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We have identified a novel human centromere-associated protein by preparing monoclonal antibodies against a fraction of HeLa chromosome scaffold proteins enriched for centromere/kinetochore components. One monoclonal antibody (mAb177) specifically stains the centromere region of mitotic human chromosomes and binds to a novel, ~250-300 kd chromosome scaffold associated protein named CENP-E. In cells progressing through different parts of the cell cycle, the localization of CENP-E differed markedly from that observed for the previously identified centromere proteins CENP-A, CENP-B, CENP-C and CENP-D. In contrast to these antigens, no mAb177 staining is detected during interphase, and staining first appears at the centromere region of chromosomes during prometaphase. This association with chromosomes remains throughout metaphase but is redistributed to the midplate at or just after the onset of anaphase. By telophase, the staining is localized exclusively to the midbody. Microinjection of the mAb177 into metaphase cells blocks or significantly delays progression into anaphase, although the morphology of the spindle and the configuration of the metaphase chromosomes appear normal in these metaphase arrested cells. This demonstrates that CENP-E function is required for the transition from metaphase to anaphase.

Key words: cell cycle/centromere-associated protein/kinetochore/mitosis

Introduction

The kinetochore complex is a microscopically defined structure on the mitotic chromosome which is situated on either side of the primary constriction (the centromere). The kinetochore serves as the site of attachment for spindle microtubules, which facilitate the alignment and subsequent separation of chromosomes during mitosis. Electron microscopic images of the kinetochore reveal a trilaminar disk-shaped structure stacked above the centromeric heterochromatin with microtubules terminating at or within the outer disk (see Brinkley, 1990; Pluta *et al.*, 1990; Rieder, 1982 for reviews). Functionally, kinetochores can capture microtubules that originate from the spindle poles (Euteneuer and McIntosh, 1981; Mitchison and Kirschner, 1985). Moreover, from *in vitro* (Koshland *et al.*, 1988) and *in vivo* (Gorbsky *et al.*, 1987; Nicklas, 1989) experiments, it seems likely that the poleward directed movement of chromosomes during anaphase A is driven either by depolymerization of microtubules at the kinetochore or by a kinetochore-associated motor. Similarly, the observations by Rieder and Alexander (1990) have shown that upon attachment to a single polar microtubule during prometaphase, chromosomes rapidly translocate towards the pole followed by slower congression to the metaphase plate. The predicted existence of a motor(s) at the kinetochore has gained recent experimental support since antibodies raised against cytoplasmic dynein stain the centromere region of human, chicken, hamster and rat kangaroo chromosomes (Pfarr *et al.*, 1990; Steuer *et al.*, 1990).

The ability to identify and characterize the molecular components of the kinetochore may provide the basis for a biochemical understanding of the processes that occur at the complex during mitosis. The discovery that many human autoimmune sera from patients with the CREST (calcinosis, Raynaud's phenomenon, esophageal dismotility, sclerodactyly, telangiectasiae) syndrome of progressive systemic sclerosis recognize antigens localized to the centromere region has stimulated efforts towards biochemical analysis of the structure (Moroi *et al.*, 1980; Brenner *et al.*, 1981; Earnshaw and Rothfield, 1985). The use of these autoimmune sera has led to the identification of three human centromere-associated proteins (Earnshaw and Rothfield, 1985; Earnshaw *et al.*, 1985) of 140 kd (CENP-C), 80 kd (CENP-B) and 17 kd (CENP-A) along with the cloning of a near full length cDNA which encodes CENP-B (Earnshaw *et al.*, 1987). CENP-A (17 kd) has been shown to be a histone H3-like protein, a portion of which remains associated with mono-nucleosomes following nuclease digestion of whole chromosomes (Palmer and Margolis, 1985; Palmer *et al.*, 1987). CENP-B is an 80 kd highly acidic chromosome scaffold protein (Earnshaw *et al.*, 1987) which is associated with a subset of the repetitive alphoid DNA sequences found at most human centromeres (Masumoto *et al.*, 1989). Immunoelectron microscopy using antibodies specific for CENP-B showed that this protein is localized to the alphoid rich heterochromatic region underlying the kinetochore plates in HeLa chromosomes (Cooke *et al.*, 1990). A fourth (50 kd) centromere-associated protein (which we will refer to as CENP-D) has also been identified using CREST sera (Kingwell and Rattner, 1987). In addition, using a monoclonal antibody (named 37A5) raised to a histone depleted nuclear extract, Pankov *et al.*

(1990) have identified a 140–155 kd doublet of proteins that attach to the centromere/kinetochore domain during metaphase, but redistribute to the mid-zone at anaphase.

The biochemical and molecular analysis of centromeric auto-antigens has provided new insights toward our understanding of the organization of the centromere region of chromosomes. However, no component has been proven to be a true constituent of the kinetochore. One approach to this problem would be to attempt a biochemical purification of kinetochores. To this end, we have fractionated chromosomal proteins using the assumption that any centromeric/kinetochore complex would retain the known centromeric antigens CENP-B and CENP-C. We have used a chromosome scaffold fraction to enrich for putative centromere/kinetochore components by ~4000- to 5000-fold and have used this fraction as an immunogen to generate monoclonal antibodies. We now report the identification of one monoclonal antibody (mAb177), which recognizes a 250–300 kd human centromere associated protein that localizes to centromeres only during the early stages of mitosis and undergoes a dramatic redistribution following the onset of anaphase. Microinjection experiments using antibodies specific for CENP-E (mAb177) suggest that CENP-E may be important for chromatid separation at the onset of anaphase.

Results

Strategy for generating antibodies to human kinetochore components

In order to obtain an enrichment for centromere/kinetochore complexes, we digested chromosomes obtained from colcemid blocked HeLa cells with micrococcal nuclease and extracted most of the histones with 2 M NaCl. The resultant insoluble fraction, called the chromosome scaffold, contains ~0.05% of cell protein but retains both the architecture of the chromosome (Earnshaw and Laemmli, 1983) and at least a part of the centromere/kinetochore including CENP-B and CENP-C (Earnshaw *et al.*, 1984; see also Figure 1).

To separate the solubilized DNA and histones from the scaffold fraction, a sucrose step gradient was employed to pellet the scaffolds gently. The gradient fractions were assayed for the presence of CENP-B and CENP-C using anticentromere autoantibodies (ACA). SDS–PAGE and immunoblotting revealed that CENP-B and CENP-C were both quantitatively recovered in the glassy, gelatinous pellet at the bottom of the tube, while CENP-A was found exclusively in the top fraction along with the solubilized chromatin and histones (data not shown). The intermediate portion of the gradient was devoid of proteins as determined by silver-stained SDS–PAGE.

Scaffolds were next subjected to further extraction to achieve additional enrichment for a putative centromere/kinetochore complex. A variety of detergents and chaotropes were tested for their ability to solubilize scaffold components while leaving the residual centromere intact. The most satisfactory results were obtained with urea. Following extraction of purified scaffolds with either 2 M or 4 M urea (Figure 1, lanes 5–8) increasing amounts of scaffold proteins were solubilized (Figure 1A, lanes 5,7). Both CENP-B and CENP-C remained exclusively in the insoluble fraction (Figure 1B, lanes 6 and 8), even though 4 M urea released roughly one-half of the scaffold proteins into the supernatant.

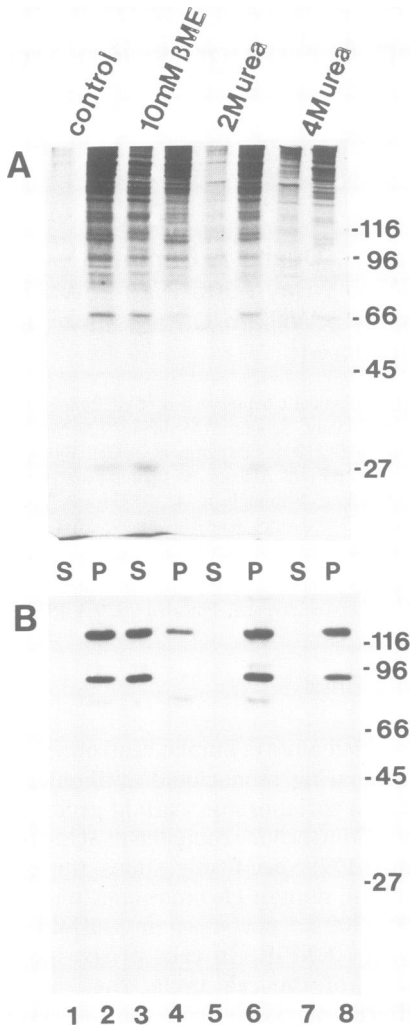

Fig. 1. Fractionation of chromosome scaffold following enrichment by sucrose gradient centrifugation. Scaffolds were resuspended in 2 M NaCl lysis mix and extracted at 4°C for 20 min as described below. Samples were centrifuged at 15 000 *g* in a Brinkman microcentrifuge for 10 min at 4°C and supernatant and pellet fractions were separated. Proteins in supernatant fractions were concentrated by precipitation with ice-cold trichloroacetic acid (added to 15% final concentration). All samples were resuspended in SDS sample buffer, boiled and loaded onto an 8.5% SDS polyacrylamide gel. (**A**) Silver-stained SDS–PAGE of various scaffold fractions; (**B**) immunoblot of identical samples probed with CREST serum NR (used at 1:2500 dilution—see Earnshaw and Rothfield, 1985). Lanes 1 and 2: unextracted scaffolds; lanes 3 and 4: scaffolds extracted with 10 mM β-mercaptoethanol; lanes 5 and 6: scaffolds extracted with 2 M urea; lanes 7 and 8: scaffolds extracted with 4 M urea. S, supernatant fraction; P, pellet fraction.

The final urea extracted scaffold represents an ~4000- to 5000-fold enrichment for CENP-B compared with whole cell proteins.

Immunoblotting of the various cell fractions with ACA or with a rabbit antibody generated against CENP-B expressed in bacteria (Earnshaw *et al.*, 1987) revealed that CENP-B was recovered in the final urea-extracted scaffold at nearly 100% yield. Furthermore, immunoblotting using known amounts of bacterially derived CENP-B as quantification standards, together with the estimate of 20 000–50 000 CENP-B molecules/cell (R.Bernat and W. Earnshaw, unpublished), revealed that CENP-B comprises ~1% of the final urea extracted scaffolds.

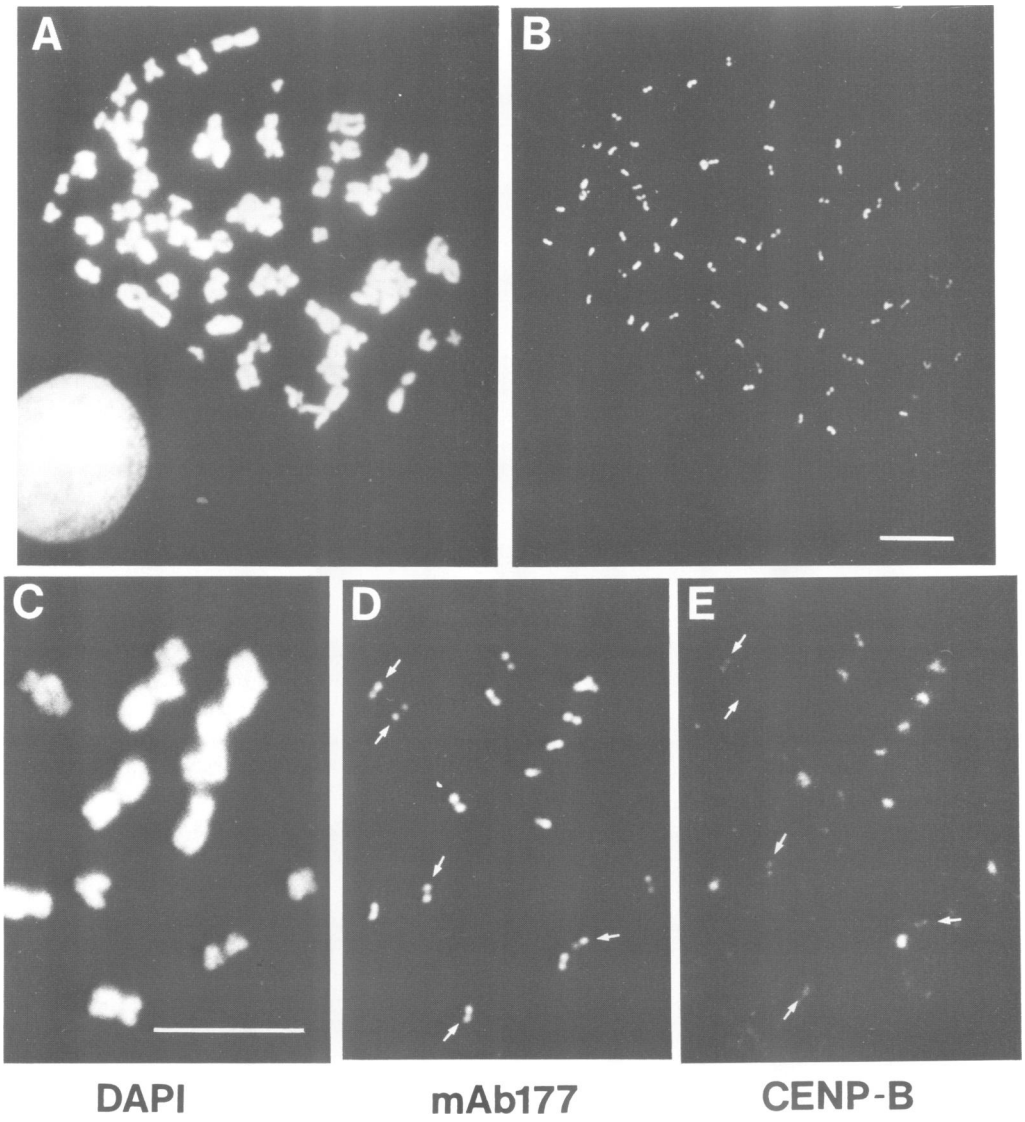

Fig. 2. Localization of mAb177 staining to the centromere of HeLa chromosomes. Indirect immunofluorescence of HeLa chromosomes from cells blocked with colcemid was used to localize antigens recognized by mAb177. (**A**) DAPI staining. (**B**) Immunofluorescence of the same field using mAb177 (1:200 dilution of a culture supernatant) and an appropriate fluorescent secondary antibody. (**C**–**E**) Double immunofluorescent localization of the mAb177 antigen and CENP-B on purified HeLa chromosomes. (**C**) DAPI image; (**D**) localization of mAb177; and (**E**) localization of CENP-B. Arrows denote chromosomes that have little CENP-B, but substantial amounts of the antigen recognized by mAb177. Bar, 10 μm.

Isolation of a monoclonal antibody that binds to centromeres of mitotic HeLa chromosomes

The insoluble fraction obtained from treatment of scaffolds with 4 M urea was used to immunize mice and the resultant hybridomas were screened initially by ELISA using whole chromosomal proteins. Supernatants from ELISA-positive wells were then analyzed by indirect immunofluorescence microscopy for binding to the centromere region of mitotic HeLa chromosomes. Using this screening procedure, six hybridomas secreting anti-centromere antibodies were initially identified, one of which continued to produce antibodies through subsequent cloning. This hybridoma (mAb177) was cloned twice by limiting dilution (using centromere staining to monitor positive clones). This monoclonal antibody was identified to be an IgG_1 subtype.

Figure 2A shows the DAPI staining of a HeLa mitotic cell and Figure 2B displays the indirect immunofluorescence pattern obtained using mAb177. As can be seen, the monoclonal antibody specifically localizes to the centromere region of these chromosomes, revealing a characteristic pair of dots on each set of paired chromosomes. To compare this staining directly with that found for CENP-B, double immunofluorescence was performed to localize the mAb177 antigen and CENP-B in the same chromosomes. Three differences between the mAb177 and CENP-B were apparent (Figure 2D and E).

First, as noted earlier (Earnshaw *et al.*, 1987), while the abundance of CENP-B varies by at least an order of magnitude amongst different chromosomes [a variability attributed (Cooke *et al.*, 1990) to variable numbers of alphoid DNA repeats containing the CENP-B binding sequence at different centromeres (Willard and Waye, 1987)], the overall centromere staining of each chromosome by mAb177 is of similar intensity.

In addition to this quantitative difference with CENP-B, a second difference is the nearly complete absence of

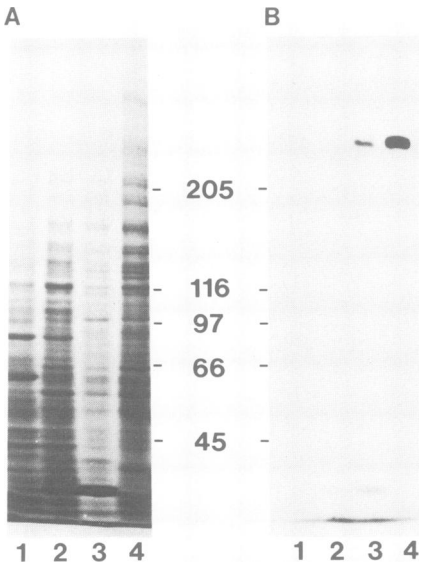

Fig. 3. Immunoblot analysis of subcellular fractions of HeLa cells probed with mAb177. Equal amounts (40 μg) of protein from a whole cell extract (lane 1), nuclei (lane 2), chromosomes (lane 3) and scaffolds (lane 4) were boiled in SDS sample buffer and separated on a 4–10% gradient polyacrylamide gel. (**A**) Coomassie blue stained gel. (**B**) A parallel gel immunoblotted with mAb177 (1:200 dilution).

detectable mAb177 antigen in interphase cells (note cell at lower left in Figure 2A, B). The failure to observe mAb177 staining in interphase cells lysed with a Triton-containing, microtubule stabilizing buffer prior to fixation could be due to the absence of the mAb177 antigen, to diffuse localization, to occlusion of the antibody binding site during interphase, or to extraction of soluble antigen prior to fixation. Although we attempted to address a part of this question by fixation with formaldehyde or by fixation with −20°C methanol without prior extraction, all such efforts yielded weak fluorescence distributed throughout the interphase cells (not shown). We cannot be certain whether this represents a true diffuse interphase localization of the mAb177 antigen or residual, non-specific background staining.

A final difference between mAb177 and CENP-B staining is that on each chromosome the pair of dots stained by mAb177 invariably showed a greater center to center spacing than the pair stained by CENP-B (compare Figure 2D and E). Measurement of ~100 chromosomes revealed this spacing to be 1.3 μm (SD = 0.2) for mAb177, while CENP-B yielded a spacing of only 0.8 μm (SD = 0.3). We interpret this to mean that the mAb177 antigen lies more peripheral to the centromeric DNA than does CENP-B, a location expected for a component of the kinetochore or its associated corona.

Identical staining patterns were observed when primary human foreskin fibroblasts or cells from a human erythroleukemic line (K562) were examined. However, the epitope recognized by mAb177 is unique to human centromere/kinetochore complexes inasmuch as no chromosomal staining could be detected in Indian muntjac, hamster (CHO) or mouse (3T3 and L) cell lines (not shown).

mAb177 recognizes a centromere-associated protein of ~250–300 kd

In order to reveal the identity of the protein(s) recognized by mAb177, various subcellular fractions obtained from HeLa cells were examined by immunoblotting. Equal amounts of proteins derived from a whole cell extract, nuclei, purified mitotic chromosomes and scaffolds (Figure 3A, lanes 1–4, respectively) were examined. The results show that the antibody recognizes an ~250–300 kd protein in purified chromosomes and in scaffold fractions (Figure 3B, lanes 3 and 4), a finding consistent with the use of scaffold proteins as antigens to generate the antibody. The failure to detect a signal in whole cell extracts (Figure 3B, lane 1) or nuclear extracts (Figure 3B, lane 2) supports the failure of immunofluorescence to reveal distinct staining in interphase nuclei and suggests that in interphase this protein is absent or accumulated to a level below the detection limit of mAb177. Alternatively, the epitope recognized by mAb177 may itself be absent or occluded in interphase. In any event, the immunoblot results with chromosomal proteins suggest that mAb177 identifies a novel human centromere associated protein. Following the present nomenclature (Earnshaw and Rothfield, 1985), we will refer to this ~250–300 kd protein as CENP-E.

CENP-E staining first appears at late prometaphase

The apparent absence of CENP-E during interphase and its presence at mitosis suggested that expression of the CENP-E epitope is cell cycle regulated. To determine the time during the cell cycle at which mAb177 staining first appears, we used double immunofluorescence microscopy to follow both CENP-E and CENP-B in cells at various points in the cell cycle. Chromosome condensation and positioning, as viewed by DAPI staining, as well as nuclear envelope breakdown, were used to identify cell cycle position. Cells in interphase (cell at upper right in Figure 4A) and in prophase (cell at lower left in Figure 4A) failed to stain with mAb177 (Figure 4B), although CENP-B staining was easily observable as punctate dots in such cells (Figure 4C). However, cells that had progressed into prometaphase showed CENP-E localization at either side of the centromere region, producing a characteristic double dot pattern that was more easily seen for CENP-E than for CENP-B (see arrows in Figure 4E and F, respectively). As noted earlier for chromosomes isolated from colcemid-blocked cells, although both CENP-B and CENP-E localize to the centromere region, CENP-E staining in chromosomes from normally cycling cells always appeared more peripheral to CENP-B [note the difference in spacing between pairs of CENP-B stained centromeres with those stained for CENP-E (Figure 4E, F)].

CENP-E is redistributed during anaphase, then restricted to the midbody at telophase

Following the appearance of CENP-E at prometaphase, we used double immunofluorescence staining to follow the distribution of CENP-E throughout subsequent mitotic phases. At metaphase, CENP-E and CENP-B (Figure 5B and C, respectively) are localized to the aligned chromosomes, presumably at the centromeric region. For example, in Figure 5A–C, one chromosome that had not yet congressed to (or during preparation had been dislodged from) the metaphase plate (Figure 5A, arrow) revealed specific staining at the centromere region by both mAb177 (Figure 5B) and CENP-B (Figure 5C) antibodies. Figure 5D–F shows another metaphase example, this time viewed along the spindle pole axis where chromosomes are organized in a ring. Again, both CENP-E and CENP-B

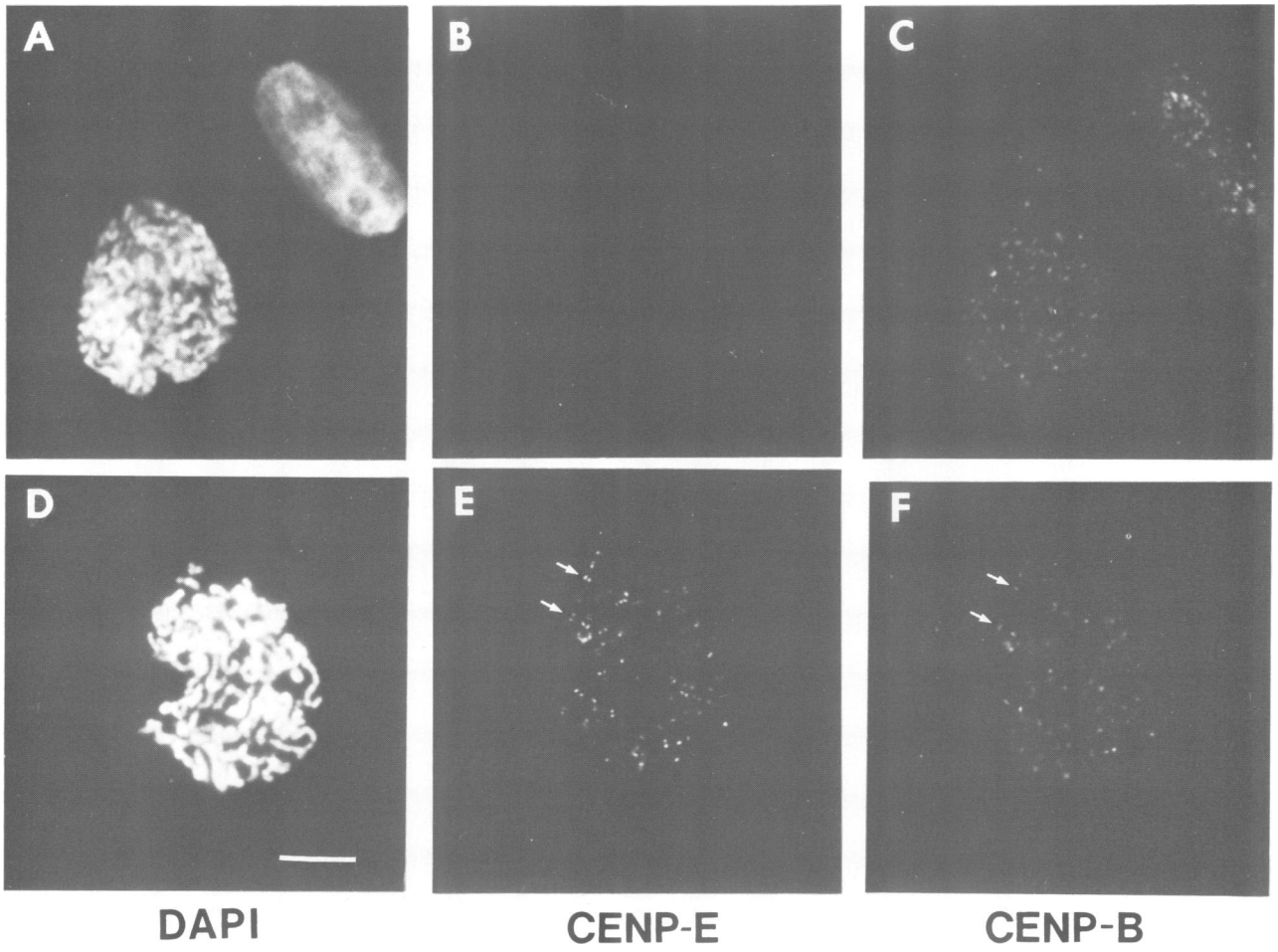

Fig. 4. Double immunofluorescence localization of CENP-E and CENP-B during the earliest mitotic stages. (**A**–**C**) HeLa cells in interphase and early prometaphase. (**D**–**F**) Cell in prometaphase. (**A** and **D**) DAPI image. (**B** and **E**) CENP-E localized with mAb177 (used at 1:200) and visualized with biotinylated goat anti-mouse Ig (used at 1:250) coupled with streptavidin–Texas Red (1:1000); (**C** and **F**) rabbit polyclonal antibody rat-ACA1 (1:500 dilution) raised against bacterially produced CENP-B and visualized with fluorescein-conjugated goat anti-rabbit Ig (used at 1:30). Bar, 10 μm.

staining are found to be localized to chromosomes. At the resolution afforded by light microscopy, the overall metaphase distribution of CENP-E is similar to that of CENP-B, although as mentioned earlier there are obvious differences between chromosomes in the intensity of staining.

When chromosomes begin to separate during early anaphase (anaphase A), CENP-B (Figure 5I) remains fully associated with the chromosomes as does the majority of CENP-E (Figure 5H). However, a faint residual staining is visible (Figure 5H) in the region between the separated chromosomes, indicating that some CENP-E has been left behind at (or migrated to) the midzone. In contrast, no CENP-B staining is apparent in this zone (Figure 5I). The redistribution of CENP-E is seen most dramatically later in anaphase when it is completely absent from the separated chromosomes, and is found localized exclusively to a prominent fibrous zone at the original midzone region (Figure 5K). In contrast, CENP-B continues to migrate with the chromosomes, as would be expected for a protein which is associated with centromeric DNA (Figure 5L). Finally, at telophase, the fibrous midplate staining seen for CENP-E is restricted to the midbody (Figure 5N). Presumably, CENP-E is discarded after cytokinesis, while the chromosome-bound CENP-B is retained in the newly forming nuclei (Figure 5O).

Microinjection of mAb177 into metaphase cells inhibits the transition from metaphase to anaphase

In order to test the functional role of CENP-E during the transition from metaphase onward, human pancreatic epithelial cells in late prometaphase were injected with mAb177 and observed by time-lapse video microscopy. As controls, other cells were followed after no injection or mock injection with a comparable amount of an unrelated monoclonal antibody (to β-galactosidase). In eleven out of twelve prometaphase cells injected with the antibody directed against CENP-E, the transition from metaphase to anaphase was slowed or stopped, while in all mock injected cells this transition occurred within 16 min of injection. Figure 6 displays a series of video images of two cells injected with mAb177 and one mock injected cell. The cell in Figure 6A was followed for 38 min post-injection, but no movement of the chromosomes away from the midplate was observed. A second cell injected with mAb177 was followed for 63 min post-injection, but again no transition into anaphase was observed (Figure 6B). During this period, there was no

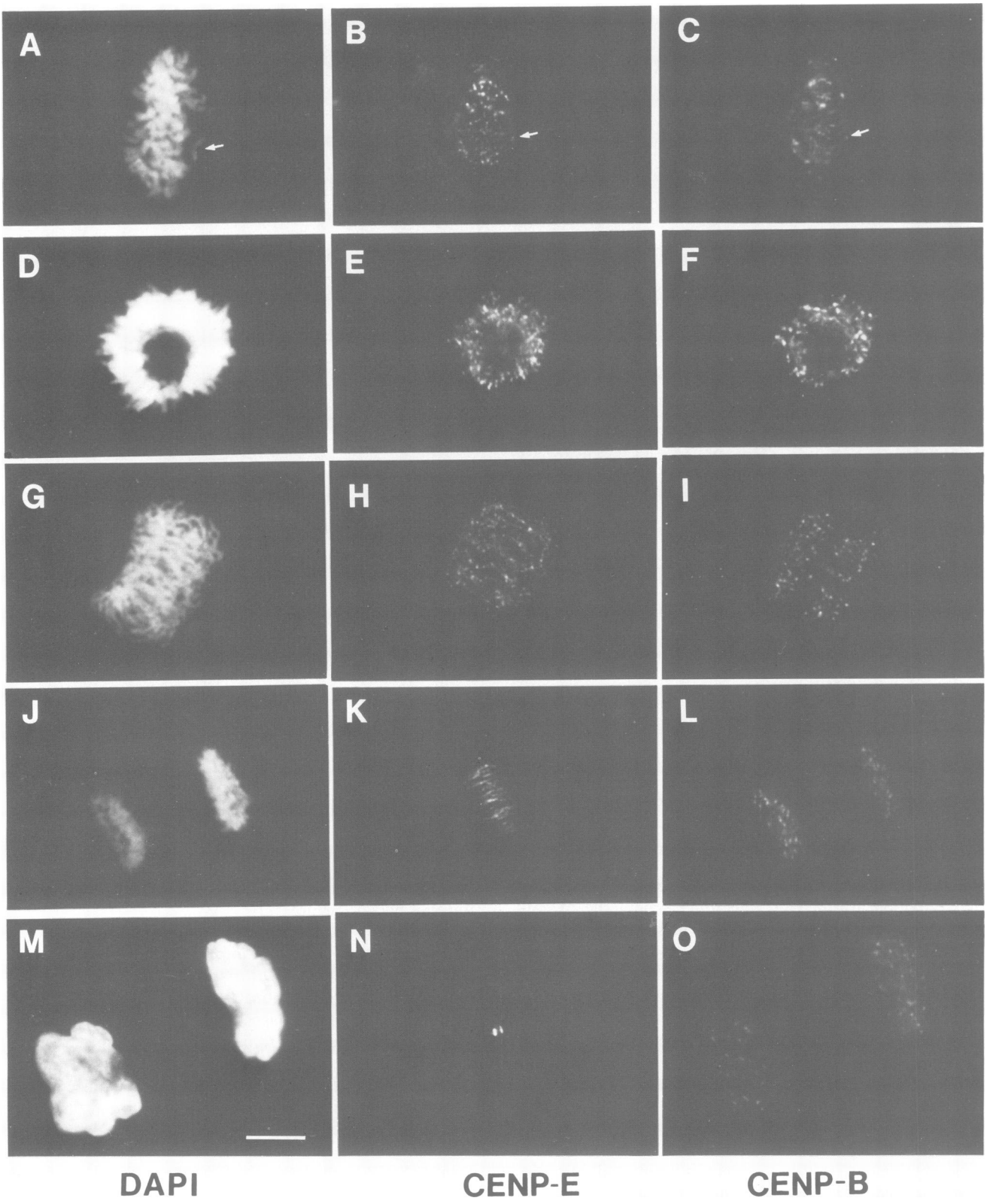

Fig. 5. Double immunofluorescence staining pattern of CENP-E and CENP-B in HeLa cells progressing from metaphase through telophase. (A,D,G,J,M) DAPI image; (B,E,H,K,N) CENP-E localized with mAb177 (used at 1:200); (C,F,I,L,O) CENP-B detected with a rabbit polyclonal antibody (used at 1:500). (**A–C**) Metaphase cell: note arrow pointing to a single chromosome that has not congressed to the metaphase plate; (**D–F**) view of metaphase along the spindle pole axis; (**G–I**) early anaphase; (**J–L**) anaphase; (**M–O**) telophase. Bar, 10 μm.

significant alteration in the configuration of the aligned chromosomes when visualized at the light microscopic level. Since the normal length of metaphase in these cells is ~10–15 min, this indicates a substantial delay at metaphase induced by the anti-CENP-E antibody. Cell cycle progression was unaffected by injection of a non-specific monoclonal

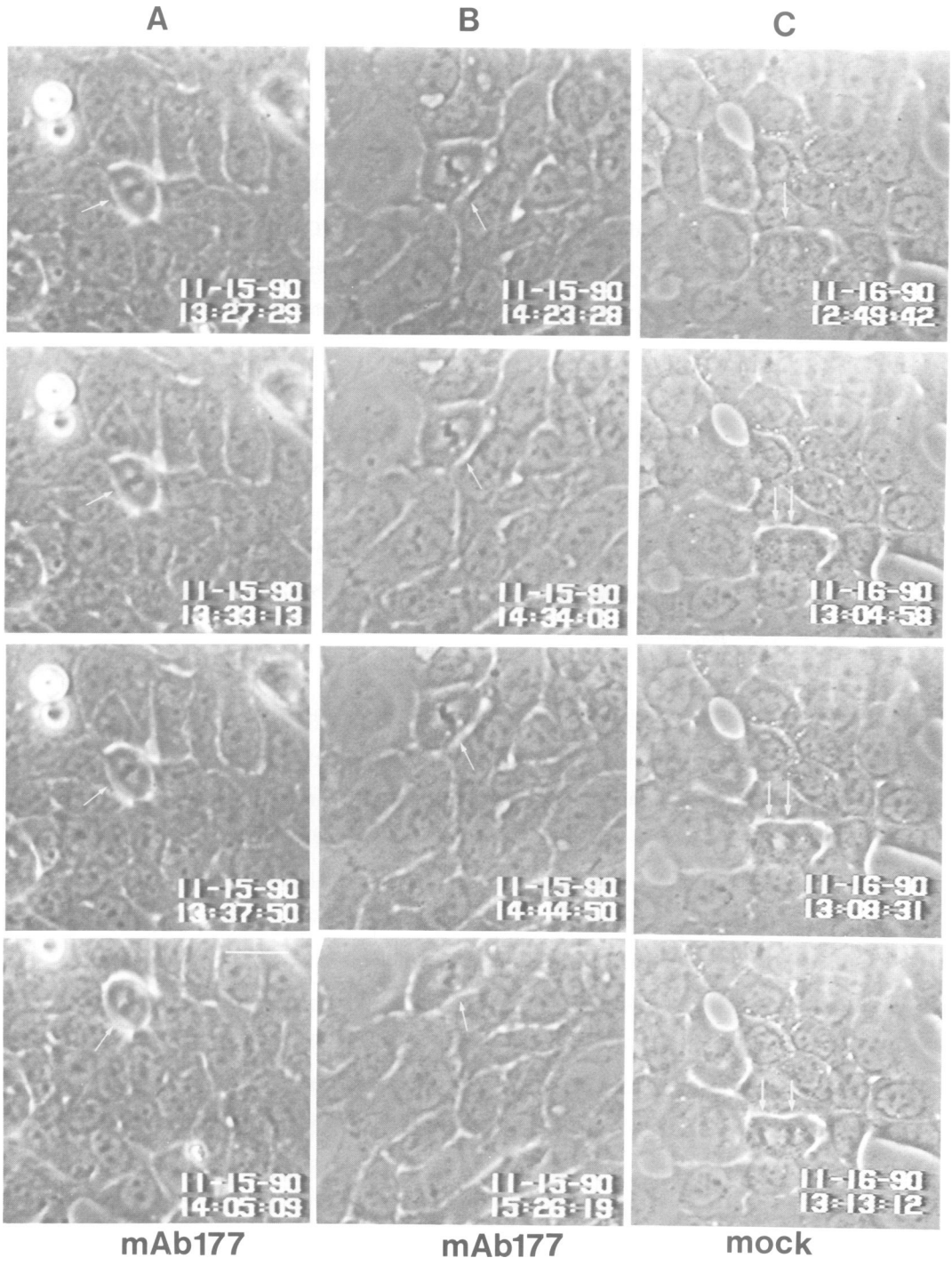

Fig. 6. Time-lapse video microscopy of metaphase cells microinjected with antibodies. Columns A and B: cells injected with monoclonal antibody (mAb177) against CENP-E; (column C): cells mock injected with monoclonal antibody against β-galactosidase. Arrows indicate injected cells as well as location of chromosomes in these cells. Bar, 30 μm.

antibody, as is evident in the cell in Figure 6C which entered early anaphase within 16 min post-injection (second panel) and entered anaphase B within 24 min (bottom panel).

The localization of injected mAb177 in metaphase cells was determined by fixing cells and staining with fluorescein-conjugated goat anti-mouse antibody. Indirect immunofluorescence microscopy revealed intense, punctate staining over the chromosomes (presumably at the centromeres/kinetochores) of each injected metaphase cell, as well as diffuse staining from the excess injected antibody (Figure 7A, left). This staining is absent in an uninjected metaphase cell (Figure 7A, right). Simultaneous staining for tubulin revealed that the binding of mAb177 to the centromere/kinetochore associated CENP-E had no observable effect on spindle morphology when compared with an adjacent, uninjected metaphase cell (Figure 7B, compare left and right). Additionally, there was no apparent difference in the configuration of the chromosomes aligned at the metaphase plate between injected and uninjected cells (Figure 7C, compare left and right).

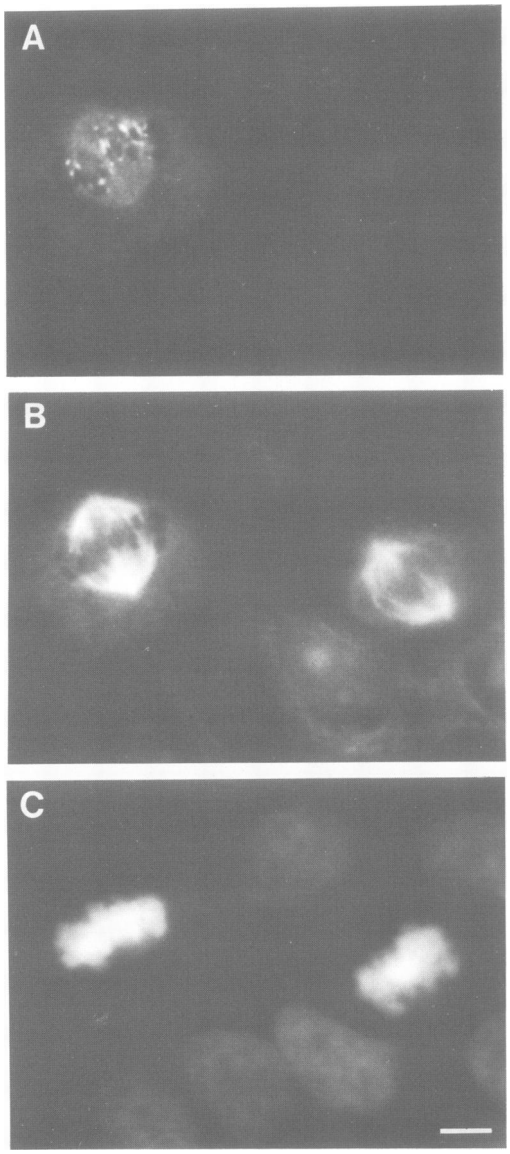

Fig. 7. Double immunofluorescence staining of a cell arrested at metaphase following microinjection of anti-CENP-E antibodies. (**A**) Microinjected mAb177 visualized using fluorescein conjugated anti-mouse secondary antibody (1:25); (**B**) array of microtubules visualized with a sheep polyclonal antibody to tubulin (1:2), followed by Texas Red conjugated anti-sheep antibody. (**C**) Hoechst image of chromosomes aligned at the metaphase plate. Cells were fixed ~25 min after injection. Bar, 10 μm.

Discussion

The discovery of monoclonal antibody mAb177 has allowed us to identify CENP-E, an ~250–300 kd chromosome scaffold protein that transiently associates with the centromere/kinetochore. CENP-E tightly associates with chromosomes in prometaphase and remains bound to the centromere throughout metaphase chromosome movements, but then dissociates from chromosomes at the onset of anaphase, followed by localization to a series of fibers in the midzone and restriction ultimately to the midbody. From its large apparent size and its localization through mitosis, we conclude that CENP-E is a novel human centromere/kinetochore protein.

Two other antigens have previously been shown to have similar cell cycle dependent chromosome association. The first of these are in the INCENPs, two chromosomal proteins identified in chicken scaffolds and localized between the paired sister chromatids at metaphase (Cooke *et al.*, 1987). Furthermore, the localization of the INCENPs in the central and pairing domains between chromatids suggests a role in chromosomal pairing, although other possibilities (see discussion in Cooke *et al.*, 1987) cannot yet be excluded. The second protein (Pankov *et al.*, 1990) known to be transiently associated with centromeres is defined by a monoclonal antibody (37A5) which shows the antigen to be a nuclear protein until late prometaphase when it (like CENP-E) associates with centromeres. Like CENP-E, both the INCENPs and the 37A5 antigen dissociate from chromosomes at anaphase and concentrate in the midzone. However, while INCENPs appear to dissociate completely from chromosomes during late metaphase (W.C.Earnshaw, unpublished), CENP-E dissociation is apparently slower, with a portion remaining chromosome-associated during the earliest anaphase movement (Figure 5H). (The timing of dissociation of the 37A5 antigen has not been reported.) The release of the majority of CENP-E could coincide with the beginning of anaphase B, when the poles move apart, but this needs to be examined more carefully. In any event, the migration of CENP-E to the midplate after release from the centromere during anaphase suggests that CENP-E (or a complex containing it) may translocate to the midzone along interpolar microtubules.

The common dissociation of CENP-E, the INCENPs and the 37A5 antigen from chromosomes at, or just after, the onset of anaphase demonstrates that several, possibly many, such proteins follow a similar pathway. Given the known cascade of protein phosphorylation events that govern cell cycle progression (e.g. Murray and Kirschner, 1989), it seems reasonable to speculate that such changes in phosphorylation trigger dissociation or disassembly of multiple chromosome associated complexes at, or just after, the anaphase transition.

The ability of an antibody directed against CENP-E injected during metaphase to delay (or block) cells from entering into anaphase suggests that CENP-E may play a key role in this transition. Precisely how antibody binding blocks normal CENP-E function is not certain but two possibilities are apparent. In the first, the antibody may occlude an essential interaction of CENP-E with other components or with components physically adjacent to CENP-E in the centromere/kinetochore. Such interactions could include binding to microtubules [although spindles look normal (Figure 7)], microtubule motors (e.g. cytoplasmic dynein), or other centromere/kinetochore components or could involve cell cycle dependent modification of CENP-E (e.g. phosphorylation) that is essential for activating (or allowing) the metaphase–anaphase transition. The alternative possibility is that antibody binding directly blocks an intrinsic activity of CENP-E (an enzymatic activity, for example) and that such activity is necessary for exit from metaphase.

In either case, it is unclear how the apparent release of CENP-E *after* anaphase A has begun could be related to a metaphase block, unless in normally cycling cells the association of CENP-E with the centromere is altered at the metaphase–anaphase transition. As mentioned above, such

an alteration could include binding to other components (or release of them) or post-translational modification of CENP-E itself. Why CENP-E is then released slowly from centromeres and why it then localizes to the midzone is not obvious, but may indicate additional functions for CENP-E during anaphase and/or cytokinesis. If this is true, association with chromosomes may serve as a way of depositing CENP-E at the midzone where it could somehow participate in anaphase B movement (i.e. when the spindle poles separate, presumably as a consequence of microtubule-associated motor proteins that interact with the interdigitated arrays of microtubules arising from the two spindle poles). A final possibility is that the deposition of CENP-E (and/or the INCENPs and the antigen recognized by the 37A5 antibody) at the site where the cleavage furrow will form could reflect a function during the subsequent cytokinesis, although the necessity for a chromosome derived component in cleavage formation is made less likely by Rappaport's collective experiments in marine embryos demonstrating that cleavage furrows can form between any two spindle poles even when chromosomes are absent (Rappaport, 1971, 1975). In any event, these potential post-metaphase functions have not yet been examined, since using a microinjection approach (as we have here), antibody injection leads to metaphase arrest.

We are not certain as to whether the sudden appearance of CENP-E at centromeres during prometaphase represents a cell cycle dependent localization from a previously dispersed distribution or modification of CENP-E that unmasks (or creates) the epitope recognized by mAb177. Although the failure to detect CENP-E on immunoblots of cytoplasmic or nuclear proteins certainly suggests that the epitope is not present prior to metaphase (and hence its abrupt appearance may involve a post-translational modification), an equally plausible alternative explanation is that the amount of CENP-E in such extracts is below the detection limit of mAb177. In either case, immunoblots of chromosomal proteins treated with acid phosphatase retain the mAb177 epitope (D.A.Compton and D.W.Cleveland, unpublished), an outcome suggesting that mAb177 does not recognize a phosphoepitope as does, for example, the widely used mpm-2 monoclonal antibody (Davis *et al.*, 1983). The finding that mAb177 recognizes proteins in bacterial expression libraries also argues against the epitope containing a eukaryotic-specific post-translational modification (T.J.Yen and D.W.Cleveland, unpublished).

While both CENP-B and CENP-E are localized to the centromeric domain at metaphase, their patterns are clearly distinguishable: CENP-E is both localized peripherally to CENP-B and is more constant in amount at the centromeres of different chromosomes. Although an initial electron microscopic experiment had shown that autoimmune anti-centromere sera yielded some kinetochore staining using an immunoperoxidase method (Brenner *et al.*, 1981), a more recent analysis using immunogold methods and antibodies directed specifically against CENP-B revealed that CENP-B is tightly associated with the heterochromatin that underlies the kinetochore (Cooke *et al.*, 1990). That CENP-E lies peripheral to CENP-B is thus consistent with the transient involvement of CENP-E either with the kinetochore *per se* or with its associated corona, although electron microscopy is obviously required to document this putative kinetochore localization.

Materials and methods

Isolation of mitotic chromosomes and scaffolds

Mitotic chromosomes were isolated from HeLa cells grown in S-MEM (Gibco, Grand Island, NY) supplemented with 10% calf serum and blocked overnight with 0.1 μg/ml colcemid as described by Lewis and Laemmli (1982). Scaffolds were obtained from chromosomes by digestion with micrococcal nuclease (Worthington, Freehold, NJ) and extraction with lysis mix [10 mM Tris−HCl, pH 9, 10 mM EDTA, 0.1% amonyx, 2 M NaCl (Lewis and Laemmli, 1982)]. Isolation of scaffolds for extractions was performed by layering the nuclease and salt extracted chromosomes onto a sucrose step gradient (30, 40, 60 and 80% steps) which was formed in a 15 ml Corex tube. The gradient was spun in a swinging bucket rotor at 10 000 *g* for 1 h at 4°C. The gradient fractions were collected initially for SDS−PAGE and immunoblot analysis to identify fractions containing CENP-B or CENP-C. The glassy pellet at the bottom of the tube (the scaffolds) was resuspended by vigorous pipetting in lysis mix. The resultant fine suspension was then diluted with an equal volume of lysis mix (without salt) but containing various agents to be tested for their ability to solubilize scaffold proteins.

Generation and screening of monoclonal antibodies

Scaffolds from ~5 × 10^8 HeLa cells were extracted with 4 M urea and the remaining insoluble fraction was emulsified in complete Freund's adjuvant and then injected i.p. into two 6-week-old BALB/c mice. One month after the first injection, each mouse was boosted with an equivalent amount of immunogen emulsified in incomplete Freund's adjuvant. Each mouse received a third immunization 2 weeks later and sera were collected by tail bleed 7 days after that. Serum titers of anti-centromere antibodies of 1:2000 were obtained (using immunofluorescence microscopy). The mice were boosted a final time and their spleen cells were fused to P3-X63 myeloma cells 4 days later according to the published procedure of Harlow and Lane (1988). Fused cells were plated into 96-well microtiter dishes at a density of 2 × 10^5 cells per well containing Iscove's MEM (Sigma, St Louis, MO) supplemented with 10% fetal calf serum, penicillin, streptomycin, fungizone and HAT (100 mM hypoxanthine, 0.4 mM aminopterin and 20 mM thymidine). Hybridomas were grown in a humidified 37°C incubator in the presence of 7% CO_2.

Culture supernatants were tested by an ELISA in which ~0.5 mg of solubilized HeLa chromosomal proteins were adsorbed to each well of a 96-well microtiter dish (Nunc-Immuno Plate, Roskilde, Denmark), rinsed with PBS and blocked overnight at 4°C in Tween buffer with bovine albumin (3% albumin, 10 mM Tris−HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 0.005% Thimerosal). The blocking solution was removed and hybridoma supernatants were incubated in the wells for 2 h at room temperature. The wells were washed three times with Tween buffer (−BSA) and incubated for 90 min with a 1:2500 dilution of sheep anti-mouse Ig conjugated with horseradish peroxidase (Amersham, Arlington Heights, IL). The wells were washed three times with Tween buffer (−BSA) and the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) was added to identify positive wells. ELISA positive wells were retested by immunofluorescence microscopy for binding at the centromere of HeLa chromosomes. Wells secreting anti-centromere antibodies were expanded and cloned by limiting dilution. Monoclonal antibodies were classified with an isotyping kit (Amersham, Arlington Heights, IL).

Large-scale preparations of monoclonal antibodies were obtained by ammonium sulfate precipitation of tissue culture supernatants. Antibodies were purified away from serum proteins by affinity chromatography using DEAE Affi-Gel Blue (BioRad, Richmond, CA). Antibodies were eluted with a linear salt gradient (0−2 M NaCl in 50 mM Na-phosphate, pH 7.2), concentrated by precipitation with 50% ammonium sulfate, resuspended in PBS and dialyzed against PBS. The final concentration of the antibodies after dialysis was 5−10 mg/ml although only a fraction contains monoclonal antibody while the remainder consists of IgG derived from serum used in tissue culture medium. Purified antibodies were stored in aliquots at −20°C.

Immunological techniques

Immunofluorescence microscopy was performed as described earlier (Earnshaw and Rothfield, 1985). Staining with mouse monoclonal antibodies was visualized using affinity purified, biotinylated goat anti-mouse Ig (used at a 1:250 dilution; Vector Labs, Burlingame, CA) followed by incubation with streptavidin−Texas Red (used at 1 μg/ml; Sigma, St Louis, MO). Rabbit antibodies directed against CENP-B were visualized using a goat anti-rabbit Ig antibody conjugated to fluorescein (used at 1:30, Vector Labs). Samples were examined with an Olympus BH-2 microscope equipped with epifluorescence. Cells and chromosomes were viewed under oil (using a

100× objective, N.A. 1.4) and photographed using Kodak TMAX 400 film.

Immunoblotting experiments were performed essentially as described (Earnshaw *et al.*, 1987) except that bound mouse monoclonal antibodies were detected with ^{125}I-labeled sheep anti-mouse Ig used at 1:1000 (Amersham, Arlington Heights, IL).

Microinjection

Human CF-PAC (cystic fibrosis pancreatic cancer) cells were grown in McCoy's modified Dulbecco's medium containing 25 mM HEPES in 5% CO_2 at 37°C. The cells were grown on coverslips pretreated with placental collagen. Prior to injection, the coverslips were transferred to a 30 mm Petri dish in the same medium and kept at 37°C in a Nikon forced air microscope chamber. Living cells in division were viewed with a 40× phase-contrast objective and images were recorded on video tape using a Panasonic AG-6010s time-lapse video cassette recorder. Micropipettes, pulled on a vertical pipette puller (Narishige Instruments, Greenvale, NY) to a tip diameter of ~1 μm were backloaded with mAb177 or monoclonal anti-β-galactosidase (10 mg/ml) in PBS using a Hamilton syringe. The micropipette was positioned over the target cell using a Narishige micromanipulator and ~10% (i.e. 0.1 pl) was injected into the cytoplasm of cells in late prometaphase, using a nitrogen-driven pressure injection system (Medical Systems Corp., Greenvale, NY). Injected cells were recorded for at least 1 h post-injection on video tape. For analysis, time-lapse video tapes were played at 48× the recorded speed.

The localization of injected mAb177 as well as the spindle morphology was determined by indirect immunofluorescence microscopy. Coverslips containing injected and uninjected cells were rinsed in PEM (80 mM PIPES, pH 7.2, 1 mM EGTA, 1 mM $MgCl_2$), fixed in 3% paraformaldehyde in PEM for 45 min, permeabilized in 0.5% Triton X-100 in PEM for 2 min, rinsed in PEM, blocked with 0.5 mg/ml of $NaBH_4$ in PEM for 10 min, rinsed in TBS (25 mM Tris−Cl, pH 7.4, 135 mM NaCl, 40 mM KCl) for 2 min, and post-blocked with 1% BSA in PBS for 10 min. Injected cells were rinsed, incubated with fluorescein labeled goat anti-mouse IgG (1:20; Boehringer-Mannheim Biochemicals, Indianapolis, IN) for 30 min at 37°C, rinsed, and incubated for 30 min at 37°C with fluorescein labeled swine anti-goat IgG (1:20; Tago Inc., Burlingame, CA). A sheep polyclonal anti-tubulin antibody (1:2) was then added and incubated for 30 min at 37°C, rinsed, incubated with rabbit anti-sheep IgG−Texas Red (1:20; Accurate Chemical and Scientific Co., Westbury, NY), rinsed, stained with 1 μg/ml Hoechst, and mounted in 1:10 (v/v) PBS:glycerol containing 1 mg/ml of *p*-phenylenediamine.

Computer enhanced video microscopy and pseudocoloring of double-stained cells was performed using a Nikon Optiphot microscope equipped with epifluorescence and a DAGE MTI series 65 SIT video camera (DAGE-MTI, Michigan City, IN) coupled to an Image 1-AT (Universal Imaging, Media, PA) image analysis system. Computer enhanced video microscope images were printed using a Sony UP-5000 color video printer.

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