

Microinjection of Mitotic Cells with the 3F3/2 Anti-phosphoepitope Antibody Delays the Onset of Anaphase

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Abstract. The transition from metaphase to anaphase is regulated by a checkpoint system that prevents chromosome segregation in anaphase until all the chromosomes have aligned at the metaphase plate. We provide evidence indicating that a kinetochore phosphoepitope plays a role in this checkpoint pathway. The 3F3/2 monoclonal antibody recognizes a kinetochore phosphoepitope in mammalian cells that is expressed on chromosomes before their congression to the metaphase plate. Once chromosomes are aligned, expression is lost and cells enter anaphase shortly thereafter. When microinjected into prophase cells, the 3F3/2 antibody caused a concentration-dependent delay in the onset of anaphase. Injected antibody inhibited the normal dephosphorylation of the 3F3/2 phosphoepitope at kinetochores. Microinjection of the antibody eliminated the asymmetric expression of the phosphoepitope normally seen on sister kinetochores of chromosomes during their movement to the metaphase plate. Chromosome

movement to the metaphase plate appeared unaffected in cells injected with the antibody suggesting that asymmetric expression of the phosphoepitope on sister kinetochores is not required for chromosome congression to the metaphase plate. In antibody-injected cells, the epitope remained expressed at kinetochores throughout the prolonged metaphase, but had disappeared by the onset of anaphase. When normal cells in metaphase, lacking the epitope at kinetochores, were treated with agents that perturb microtubules, the 3F3/2 phosphoepitope quickly reappeared at kinetochores. Immunoelectron microscopy revealed that the 3F3/2 epitope is concentrated in the middle electron-lucent layer of the trilaminar kinetochore structure. We propose that the 3F3/2 kinetochore phosphoepitope is involved in detecting stable kinetochore-microtubule attachment or is a signaling component of the checkpoint pathway regulating the metaphase to anaphase transition.

IN meiotic and mitotic cells, the failure of a single chromosome to align properly at the metaphase plate activates a cell cycle checkpoint mechanism that inhibits, at least temporarily, anaphase onset and further progression of the cell cycle (reviewed in 9, 21). A quarter century ago, Zirkle, in a widely disseminated abstract (Zirkle, R. E. 1970. *J. Cell Biol.* 47:235a), proposed that kinetochores were involved in signaling the presence of misaligned chromosomes. McIntosh (19) suggested that the centromere/kinetochore regions of unattached chromosomes emit a signal that prevents premature anaphase onset. He speculated that this signal was turned off in response to tension applied to the centromere/kinetochore region by stable attachment of sister kinetochores to spindle microtubules. Recently, Li and Nicklas (16) have shown that tension applied artificially with a microneedle to misaligned chromosomes in mantid spermatocytes can turn off the checkpoint signal and allow progression to anaphase.

Early embryos appear to rely more on rigid timing of the cell cycle and appear less responsive to checkpoint controls (29). Minshull and Murray (20) found that cycling extracts from activated *Xenopus* eggs could be blocked in M phase with microtubule inhibitor only in the presence of a large number of chromosomes, again implicating a connection between kinetochores or something else on the chromosomes and the checkpoint control that inhibits anaphase onset if spindle assembly and chromosome alignment are defective.

The biochemical mechanisms underlying the ability of cells to detect and react to misaligned chromosomes have yet to be elucidated. Budding yeast possess a mitotic checkpoint that is activated in response to abnormal kinetochore-microtubule interactions (22, 30). Yeast mutants termed *mad* (mitotic arrest deficient) and *bub* (budding uninhibited by benzimidazole) have been selected that are faulty in their ability to arrest in mitosis when treated with microtubule inhibitor (11, 15). A possible complication is that cells may possess multiple checkpoints in M phase. For example, one checkpoint system may assess the assembly of the mitotic spindle microtubules while a different pathway may monitor the attachment and alignment of the chromosomes. Alternatively, since spindle disrup-

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tion by microtubule drugs necessarily results in misaligned chromosomes, it is possible that both arrests come about through the same mechanism. Biochemically, kinetochore components are best characterized in budding yeast and include a 58-kD protein for which there exists a temperature sensitive mutant allele (*ctf13-30*) that causes a temporary cell cycle arrest at the G2/M stage (see 13 for a review).

Several lines of evidence suggest that dephosphorylation events are essential for overcoming this checkpoint. Certain mutants in fungi and *Drosophila* with altered phosphatase activity result in a metaphase arrest phenotype (17, 24). The activated *Xenopus* egg extracts arrested by the addition of chromosomes and microtubule inhibitor are released from the block by the addition of a specific phosphatase that dephosphorylates and thus deactivates MAP kinase (20). Mammalian cells, when treated with the general kinase inhibitor 2-aminopurine, escape mitotic arrest induced with microtubule inhibitors (1). In cells treated with the phosphatase inhibitor okadaic acid, mitotic chromosomes undergo asynchronous entry into anaphase (14).

Recently, Rieder et al. (26) analyzed the timing of anaphase onset in Ptk1 cells, a mammalian tissue culture line. They found that anaphase onset ensues on average 23 min after bipolar attachment of the final chromosome to the mitotic spindle. Using a monoclonal anti-phosphoepitope antibody prepared by Cyert et al. (6) and designated 3F3/2, Gorbsky and Ricketts (10) identified within Ptk1 cells a phosphorylation difference among chromosomes at different stages of congression to the metaphase plate. Those chromosomes that had fully congressed to the metaphase plate showed no expression of the phosphoepitope recognized by the antibody while those some distance from alignment showed strong expression. The kinetochores of "lost" chromosomes, unattached to the mitotic spindle, exhibited particularly strong expression. The epitope was also expressed on the leading kinetochore of a chromosome congressing to the metaphase plate, but not the trailing one. They presented two hypotheses: (a) that the kinetochore phosphoepitope is involved in a checkpoint that inhibits anaphase onset in the presence of misaligned chromosomes, and (b) that the 3F3/2 kinetochore epitope regulates chromosome movement along spindle microtubules.

To test these hypotheses, we microinjected the 3F3/2 monoclonal antibody into prophase Ptk1 cells and observed subsequent mitotic events. We found that the antibody caused a concentration-dependent delay of anaphase onset. The antibody appeared to have no effect on chromosome movement. We propose that the 3F3/2 kinetochore phosphoepitope is involved in a checkpoint pathway that monitors chromosome attachment to and alignment on the mitotic spindle. This checkpoint delays anaphase until all chromosomes have aligned at the metaphase plate, thus preventing the generation of progeny cells with an abnormal complement of chromosomes.

Materials and Methods

Cell Culture

Ptk1 cells were cultured in Ham's F12 medium (GIBCO BRL, Gaithers-

burg, MD) supplemented with 5% FBS (Hyclone Laboratories, Inc., Logan, UT), 5% Nu-serum (Collaborative Research, Bedford, MA), 20 mM Hepes, and 60 μ g/ml penicillin and 100 μ g/ml streptomycin. Cells for live observations and microinjections were grown on coverslips sealed with silicone grease over holes in the bottoms of 35-mm culture dishes. These chambers were sterilized by UV irradiation. A diamond scribe was used to form a simple pattern on the coverslips to aid relocation of experimental cells.

Live Cell Observations and Microinjections

Culture dishes were placed on the warmed stage of a Nikon Diaphot microscope. Temperature of the medium was maintained at $34 \pm 1^\circ\text{C}$ with a warmed air curtain incubator (Sage, Boston, MA). The medium in the dish was overlaid with mineral oil to slow evaporation.

For live cell observations, a Nikon 40 \times objective was used with a long working distance condenser and charge-coupled device camera (Dage-MTI, Michigan City, IN). Images were processed using the contrast enhancement and averaging functions of Image-1 software (Universal Imaging, West Chester, PA). Video recordings were made with an extended definition beta video cassette recorder (Sony, Cypress, CA).

For injection, the 3F3/2 and MPM-2 antibodies were purified from ascites fluid on a protein A-Sepharose column. They were then concentrated and exchanged into microinjection buffer (0.8% KCl, 0.01% NaCl, 0.115% Na_2HPO_4 , 0.02% KH_2PO_4) with a Centricon-30 concentrator (Amicon, Beverly, MA). Antibody concentrations were determined with the BCA assay (Pierce Chem. Co., Rockford, IL). All injected solutions were filtered to 0.2 μ m. Injections were performed with a micromanipulator (Narishige USA, Inc., Sea Cliff, New York), and cells were injected to 5% of cell volume.

In experiments in which nocodazole and taxol were used, cells in dishes containing one ml of medium were observed until the desired stage of mitosis. 1 ml of medium containing twice the desired final concentration of drug was then added. Nocodazole (Sigma Immunochemicals, St. Louis, MO) was used at a final concentration of 1.25 μ g/ml. Taxol (provided by Dr. Matthew Suffness at the National Cancer Institute) was used at 50 μ M.

Cell Fixation and Immunofluorescence

All manipulations were performed at room temperature. Cells on coverslips were rinsed briefly with 60 mM PIPES, 25 mM Hepes, pH 6.9, 10 mM EGTA, and 4 mM MgCl_2 (PHEM)¹ and extracted for 5 min in 0.5% CHAPS detergent in PHEM with 100 nM microcystin LR (Calbiochem Novabiochem, San Diego, CA). In some experiments, as noted, microcystin was not included in the lysis buffer. Fixation was for 15 min in 1% paraformaldehyde in PHEM followed by rinsing in 10 mM MOPS, pH 7.4, 150 mM NaCl, 0.05% Tween-20 (MBST).

Before immunolabeling, coverslips were blocked for 20 min in 20% boiled normal goat serum (NGS) in 10 mM MOPS, pH 7.4, 150 mM NaCl (MBS). 3F3/2 antibody ascites was used at a 1/4,000 dilution in 5% NGS in MBS. A CREST human autoimmune serum (a gift of J. B. Rattner, University of Calgary) was used in some experiments at a 1/500 dilution to label kinetochores. Rabbit anti-tubulin serum was used at a 1/1,000 dilution to label microtubules. Coverslips were incubated in primary antibodies for 1 h, rinsed 10 min in four changes of MBST, incubated 30 min in Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1/400 and, to visualize CREST or anti-tubulin antibodies when present, fluorescein-conjugated goat anti-human or anti-rabbit IgG (Pierce) at 1/200, and rinsed again. Cells that had been injected with 3F3/2 were treated identically except there was no 3F3/2 in the primary incubation. Coverslips were rinsed in the DNA labels Yo-Pro-1 (Molecular Probes, Inc., Eugene, OR) at 0.2 μ M or DAPI (Sigma) at 0.5 μ g/ml in water before mounting in Vectashield (Vector Labs., Inc., Burlingame, CA) with 10 mM MgCl_2 to help preserve chromatin structure.

Fluorescently labeled cells were imaged either with a Nikon Diaphot equipped with a 60 \times 1.4 NA objective, image intensifier and CCD camera (DAGE-MTI) or a Zeiss LSM 410 confocal microscope with a 63 \times 1.4 NA objective. Images for publication were taken with a film recorder (Focus Graphics, Foster City, CA).

1. Abbreviations used in this paper: MBS, 10 mM MOPS, pH 7.4, 150 mM NaCl; MBST, 10 mM MOPS, pH 7.4, 150 mM NaCl, 0.05% Tween-20; NGS, normal goat serum; PHEM, 60 mM PIPES, 26 mM Hepes, pH 6.9, 10 mM EGTA, and 4 mM MgCl_2 .

The Stages of Mitosis

To establish a baseline for testing the effects of microinjected 3F3/2 antibody, normal, uninjected cells were observed to determine the timing of mitotic events. Prometaphase was defined as starting with the first evidence of nuclear envelope breakdown. Because no similar morphological event clearly signifies the start of metaphase, we used our own working definition. Using Image-1 software, we superimposed a rectangular box extending 3 μm on either side of the midplane of the spindle. We defined the beginning of metaphase as the time at which all the kinetochore regions of the chromosomes were within the box, in other words within 3 μm of the spindle equator (Fig. 1 A). This moment correlated well with the point at which 3F3/2 labeling is no longer seen at the kinetochores (Fig. 1, B and C). For comparison, the length of the spindle ranges from \sim 13–16 μm measured in cells labeled with 3F3/2, which also binds to centrosomes. Anaphase onset was defined as the first evidence of sister chromatid separation as seen with phase contrast microscopy.

Mean metaphase transit times for different experimental treatments were compared by one way analysis of variance and Tukey's range test. Significant differences were determined at 95% or greater probability.

Immunoelectron Microscopy and Analysis

To accumulate mitotic cells, cultures were blocked 4 h with 4.5 $\mu\text{g}/\text{ml}$ vinblastine (Sigma). Cells grown on coverslips were rinsed with PHEM and treated 1 min with 0.5% CHAPS in PHEM with 100 nM microcystin LR. An equal volume of 1% glutaraldehyde in PHEM was then added and cells were incubated for 10 more min. Free aldehyde groups were quenched by treatment for 15 min in 10 mg/ml NaBH_2 in water. Immunolabeling was carried out as described above through the incubation in 3F3/2 primary antibody. Secondary incubation was for 1 h in Nanogold 1.4 nm gold-conjugated goat anti-mouse F(ab') (Nanoprobes, Stony Brook, NY) at a dilution of 1/25. Samples were then postfixed 10 min in 2% glutaraldehyde in MBS, rinsed in MBST and then water for 25 min, followed by silver enhancement for 5 min as described in detail by Burry et al. (4). After enhancement, coverslips were rinsed several times in water, incubated 15 min in 0.2% tannic acid, rinsed in water, and stained in 0.14% osmium tetroxide, 0.6% uranyl acetate for 15 min. Samples were dehydrated through a graded series of ethanols, infiltrated, and flat embedded in EPON. Hardened blocks were examined by light microscopy and mitotic cells were selected and marked for sectioning. Samples were examined and photographed in a JEOL 100CXII electron microscope.

The distribution of silver particles on electron micrographs was determined with Image-1 software (Universal Imaging Corp., West Chester, PA). Tracings were made on 10 images of kinetochores to divide them into three regions corresponding to the electron-lucent middle zone between the inner and outer plates, generally 20–25 nm wide, the outer plate and fibrous corona, extending 100–140 nm above the middle zone, and the inner plate and underlying chromatin extending \sim 150 nm beneath the middle zone. The middle zone was demarcated where a 40% or greater change in pixel intensity occurred upon scanning outward from the inner plate to the middle zone and inward from the outer plate to the middle

zone. Since many particles overlapped, a mean particle size of 100 nm^2 was assigned based on clear single particles, and particles smaller than 50 nm^2 were not included in measurements. With the Image-1 software we then calculated the number of particles within each region. A particle was included in a region if its centroid (center of mass) lay within that region.

Results

Microinjected 3F3/2 Binds Rapidly to Kinetochores

To determine whether the injected antibody would bind readily to kinetochores in living cells, the antibody was injected into the nuclei of prophase cells or into the cytoplasm of prometaphase cells. The cells were immediately removed from the microscope stage, detergent extracted, fixed, and treated with fluorescent secondary antibodies. The time between injection and extraction was \sim 12 s. As shown in Fig. 2 (A and B), injected antibody specifically bound to kinetochores within this short time. To eliminate the possibility of antibody binding during the extraction, other cells were fixed before detergent extraction. Kinetochores in cells so treated were clearly labeled, although higher background staining was seen, presumably due to unbound antibody in the cytoplasm (Fig. 2, C and D).

Microinjection of 3F3/2 Antibody Delays Anaphase Onset

To learn whether binding of the 3F3/2 antibody in living cells would perturb mitotic events, antibody was microinjected into the nuclei of early to mid-prophase Ptk1 cells and subsequent events were observed. Nuclear envelope breakdown, prometaphase chromosome movement, and metaphase alignment appeared normal. However, microinjection with 3F3/2 antibody significantly prolonged metaphase in comparison to control cells (Table I). The length of the delay of anaphase onset increased in proportion to the concentration of antibody injected. The longest times spent in metaphase, after injection with 3F3/2 antibody at 11 mg/ml in the micropipette, ranged from 44 to 91 min, compared to \sim 11 min in a comparable untreated cell. A second batch of more recently purified 3F3/2 antibody consistently caused even longer delays in metaphase, up to 135 min, when injected at 11 mg/ml. Cells injected with

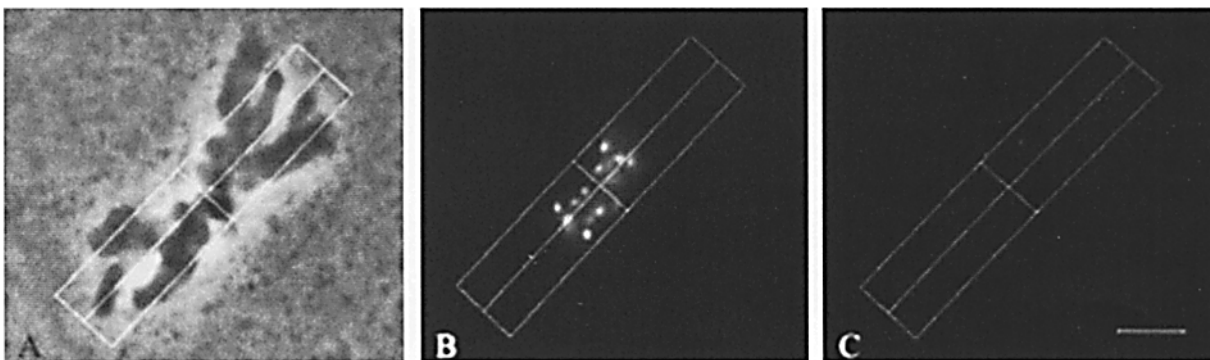


Figure 1. Definition of the start of metaphase. A Ptk1 cell was extracted and fixed when kinetochore regions of chromosomes were within a box extending 3 μm on either side of the spindle midplane when observed by phase contrast microscopy (A). The same cell was then immunolabeled with CREST serum (B), which labels kinetochores at all stages of the cell cycle, and 3F3/2 antibody (C), which labels kinetochores only before metaphase. The 3F3/2 antibody also labels centrosomes through late anaphase, but they are out of the plane of focus in C. Bar, 5 μm .

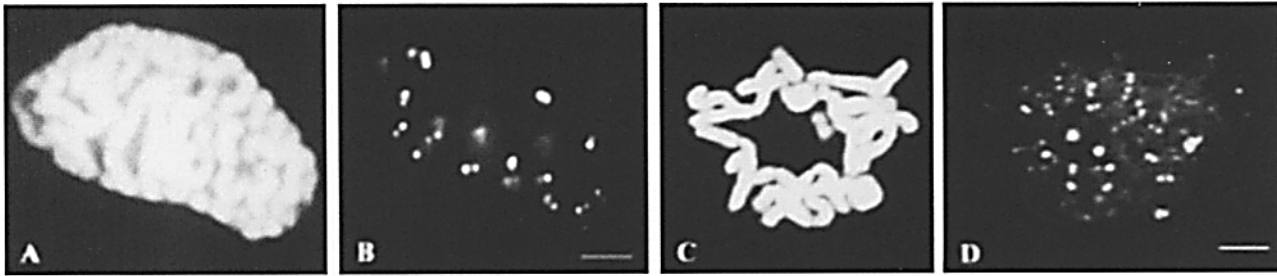


Figure 2. Microinjected 3F3/2 antibody binds rapidly to kinetochores. Immediately after injection with 3F3/2 antibody, cells were quickly rinsed in PHEM and either extracted with detergent then fixed (*A* and *B*), or fixed and then extracted with detergent (*C* and *D*). Cells were then treated with fluorescent anti-mouse IgG secondary antibodies to visualize injected 3F3/2 antibody (*B* and *D*) and Yo-Pro1 to label chromosomes (*A* and *C*). *C* and *D* are confocal micrographs. Bars, 5 μ m.

Table 1. Minutes Spent in Metaphase by Cells Microinjected with 3F3/2 Antibody

Injection	Average (SD)	Range	Number of cells
None	10.4 (4.4)*	6.5–17.5	10
Buffer	14.7 (5.4)*	9.5–22	5
Boiled 3F3/2 11 mg/ml	14.9 (4.5)*	10–21	5
MPM-2 11 mg/ml	10.9 (3.8)*	4.5–15.5	9
3F3/2 5.5 mg/ml	31.9 (7.7)‡	19–43	5
3F3/2 11 mg/ml	65.4 (13.8)§	44–91	13

* Not significantly different from each other.

‡ Significantly different from other values, $P < 0.05$.

§ Significantly different from other values, $P < 0.01$.

3F3/2 antibody eventually entered anaphase after the delay and completed cytokinesis normally. Fig. 3 shows a cell injected with 3F3/2 at 11 mg/ml from postinjection through anaphase. Control cells included those receiving no micro-

injection, injection with buffer alone, with 3F3/2 antibody that had been boiled for 10 min, and with the MPM-2 antibody, which has also been shown to bind to kinetochores by immunofluorescence and immunoelectron microscopy (31, 33, 34). Cells injected with MPM-2 antibody fixed, and labeled with fluorescent secondary antibody showed strong labeling of kinetochores (not shown). No effects on chromosome movement or the progression of mitosis were noted in control cells.

Microinjected Antibody Inhibits Dephosphorylation of the 3F3/2 Phosphoepitope

To understand the mechanism by which microinjected 3F3/2 antibody delayed anaphase onset, we determined the status of the phosphoepitope in injected cells. Normally, at metaphase, when all chromosomes are properly aligned, the epitope is no longer expressed at kineto-

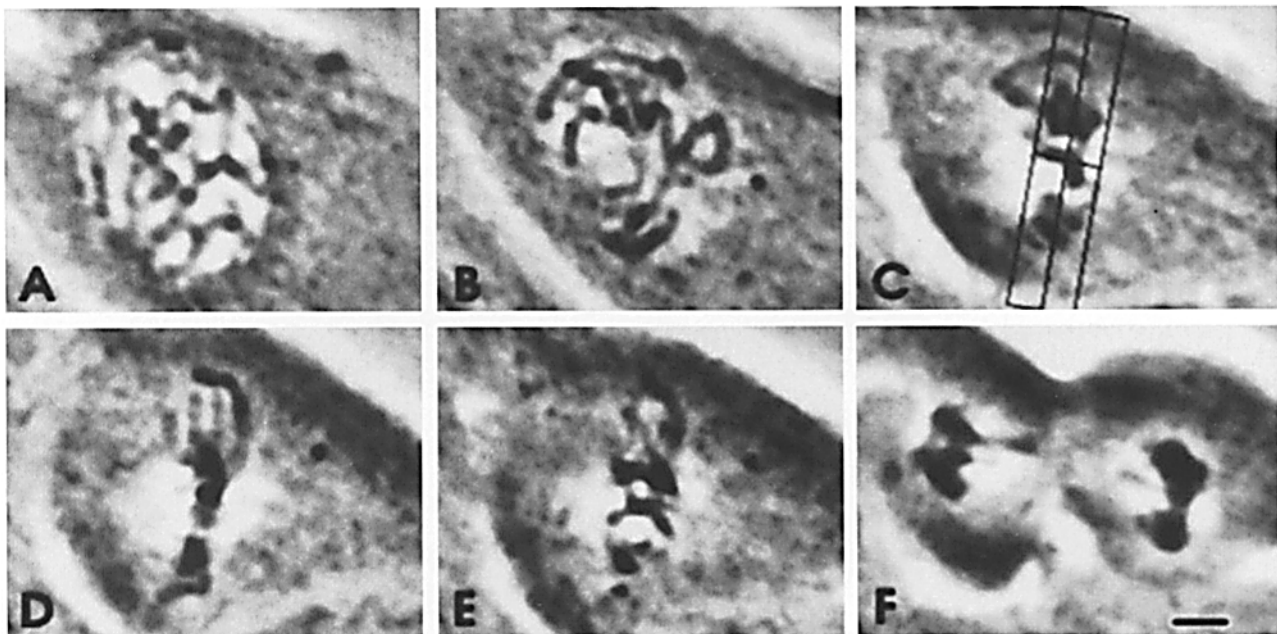


Figure 3. Microinjected 3F3/2 antibody delays anaphase onset. Phase contrast images of a cell injected with 3F3/2 antibody at 11 mg/ml at 0 min. 2 min later (*A*), the cell was still in prophase. NEB occurred at 7 min and (*B*) shows the cell at 9 min. Metaphase was reached at 20.5 min (*C*) and the cell was still in metaphase at 62 min (*D*). Anaphase onset occurred at 93.5 min and (*E*) shows the cell at 94.5 min. By 103 min (*F*) the cell reached late telophase and went on to complete a normal cytokinesis. Prometaphase lasted 13.5 min, comparable to untreated cells in which it is very variable but generally lasts 10–30 min. Metaphase lasted 73 min, compared to 10–13 min in untreated cells. Bar, 5 μ m.

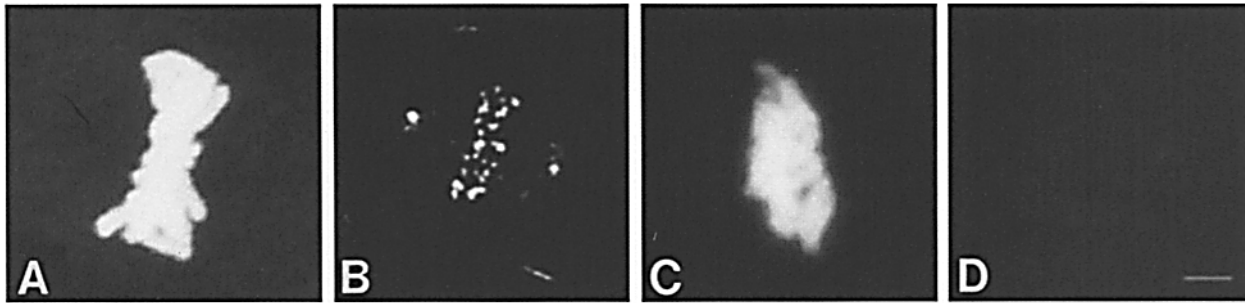


Figure 4. Retention of 3F3/2 phosphoepitope in metaphase but not anaphase in cells previously microinjected with 3F3/2 antibody. After injection with 3F3/2 antibody, cells were allowed to progress to metaphase (*A* and *B*) or early anaphase (*C* and *D*) and were then extracted, fixed, and labeled with fluorescent anti-mouse secondary antibodies (*B* and *D*). Fluorescent images of chromosomes are shown in *A* and *C*. One centrosome is faintly visible in *D*; the other is in a different plane of focus. *A* and *B* are confocal micrographs. Bar, 5 μm .

chores. Cells injected with 3F3/2 antibody were allowed to reach metaphase and then were extracted, fixed, and labeled with fluorescent secondary antibody. The 3F3/2 phosphoepitope was expressed at the kinetochores of metaphase chromosomes in injected cells (Fig. 4, *A* and *B*). However, once the 3F3/2 antibody-injected cells started anaphase, the epitope was no longer expressed at the kinetochores of the anaphase chromosomes (Fig. 4, *C* and *D*).

Differential Expression of the Phosphoepitope on Sister Kinetochores Is Not Essential for Directed Chromosome Movement Toward the Metaphase Plate

Based on the asymmetric immunolabeling of sister kinetochores in chromosomes congressing toward the metaphase plate, Gorbisky and Ricketts (10) postulated a role for the 3F3/2 phosphoepitope in regulating chromosome movement in prometaphase. However, as noted, prometaphase chromosome movements appeared unimpaired in cells microinjected with 3F3/2 antibody. Moreover, in microinjected cells fixed during chromosome congression in prometaphase, expression of the phosphoepitope was invariably equal on both kinetochores of each chromosome. Chromosomes actively moving toward the metaphase plate were brightly labeled on both kinetochores (Fig. 5). Thus, asymmetric expression of the phosphoepitope on sister kinetochores of a single chromosome is not essential for movement of chromosomes to the metaphase plate.

3F3/2 Antibody Preserves the Phosphoepitope During Cell Extraction in the Absence of Phosphatase Inhibitors

The kinetochore phosphoepitope recognized by the 3F3/2 antibody is very labile and normally is preserved for immunofluorescence labeling only when the potent phosphatase inhibitor microcystin LR is included in the extraction buffer (10). To determine if microinjected antibody alone could preserve the epitope by inhibiting phosphatase activity, injected cells that had reached prometaphase were extracted in the absence of microcystin, fixed, and labeled with fluorescent secondary antibody. As seen in Fig. 6, in the microinjected cells the 3F3/2 antibody remained bound at the kinetochores in the absence of phosphatase inhibitor, suggesting that the presence of the antibody pro-

tected the phosphoepitope from dephosphorylation by endogenous phosphatases.

Microtubule Perturbations Affect Expression of the 3F3/2 Kinetochore Phosphoepitope

Agents that promote disassembly of microtubules and disturb spindle organization delay cells in mitosis (reviewed in 25). Since the continued expression of the 3F3/2 epitope on kinetochores was correlated with the delay of anaphase onset in injected cells, it was of interest to investigate the effects of microtubule-disrupting agents on the expression of the 3F3/2 phosphoepitope.

Ptk1 cells were observed until early metaphase, by which point the kinetochores have lost expression of the 3F3/2 phosphoepitope. Nocodazole was added to the medium for various lengths of time, and the cells were prepared for immunofluorescence labeling with 3F3/2 antibody. We found that the 3F3/2 phosphoepitope reappeared at the kinetochores of metaphase chromosomes in cells treated with nocodazole. Expression was first detectable by immunofluorescence labeling after only 3 min of incubation in nocodazole and was maximal at 5 min (Fig. 7, *A* and *B*). At these times, anti-tubulin immunofluorescence showed that kinetochore fiber bundles extending from the cen-

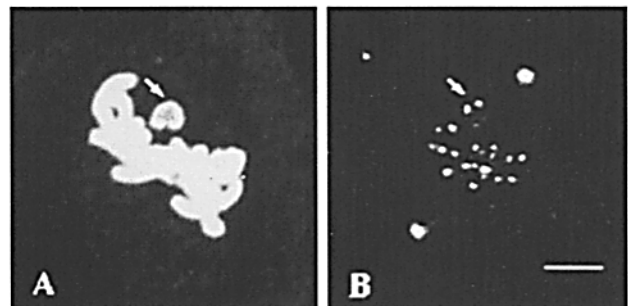


Figure 5. Microinjection of 3F3/2 antibody eliminates differential expression of the 3F3/2 phosphoepitope between sister kinetochores. This cell was injected in prophase and prepared for immunofluorescence in late prometaphase as the indicated chromosome (*arrow*) was approaching the metaphase plate. 3F3/2 (*B*) was visualized with fluorescent secondary antibodies and chromosomes (*A*) with Yo-Pro1 on a confocal microscope. Bar, 5 μm .

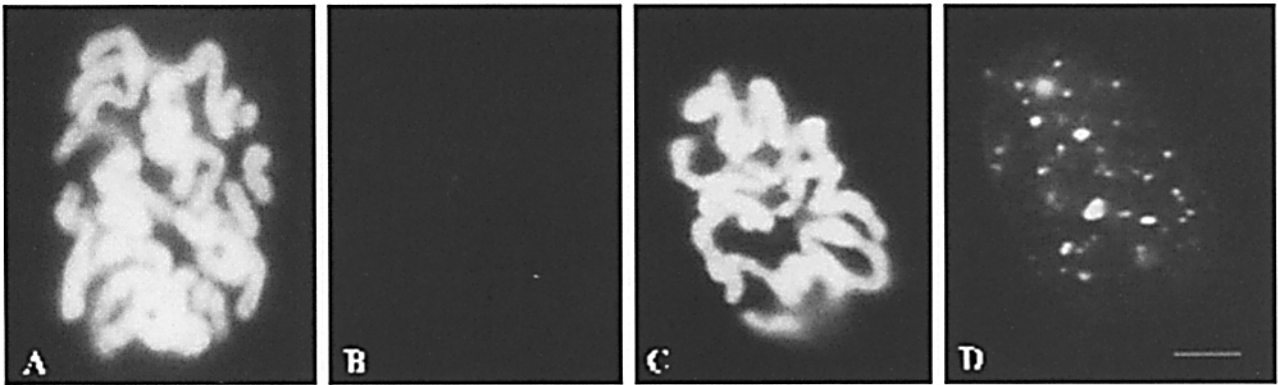


Figure 6. The 3F3/2 antibody protects the phosphoepitope from dephosphorylation. Control uninjected (*A* and *B*) and 3F3/2 injected (*C* and *D*) cells were extracted in buffer without microcystin and then fixed. The uninjected cell was then labeled with 3F3/2 antibody and fluorescent anti-mouse secondary antibodies (*B*), and the injected cell with secondary antibodies only (*D*). The larger labeled spots in *D* are not kinetochores, but unidentified nuclear structures typically labeled in interphase and early prophase cells. Fluorescently labeled chromosomes are shown in *A* and *C*. Bar, 5 μm .

microtubules to the kinetochores had not yet been disrupted (Fig. 7 *C*). Cells at various later times in metaphase and cells that had progressed into anaphase were also tested. After a 12-min incubation in nocodazole, the cells were extracted, fixed, and immunolabeled with 3F3/2 antibody. As seen in Fig. 7, *D–G*, nocodazole treatment leads to the reappearance of the 3F3/2 phosphoepitope on kinetochores when applied throughout metaphase, but not after anaphase has begun.

The drug taxol promotes the stability and assembly of

microtubules, and has also been shown to delay cells in metaphase (7, 26). Cells in early metaphase were treated with 50 μM taxol for 12 min. As seen in Fig. 8, *A* and *B*, taxol also caused the reappearance of the 3F3/2 phosphoepitope on the kinetochores of metaphase chromosomes. Again, kinetochore microtubules were still present (Fig. 8 *C*).

Ultrastructural Localization of the 3F3/2 Kinetochore Phosphoepitope

To determine the precise ultrastructural location of the

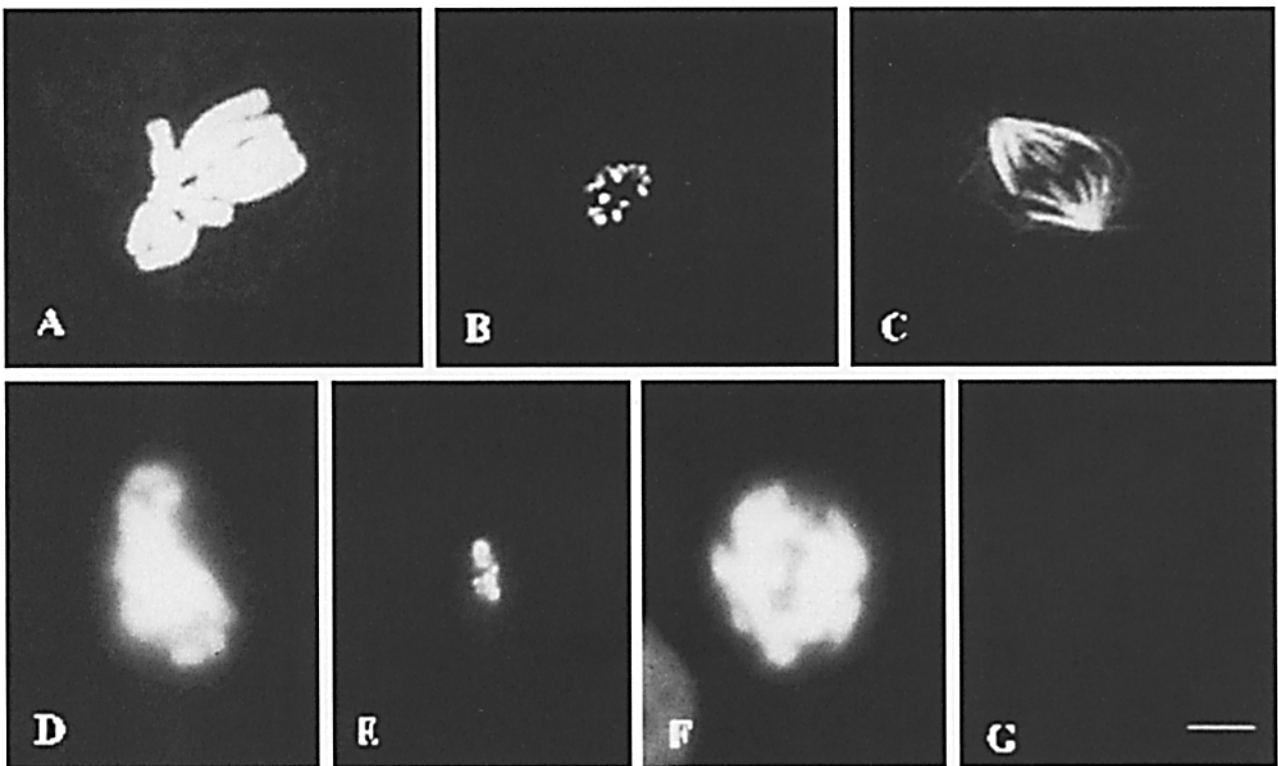


Figure 7. Nocodazole causes regeneration of the 3F3/2 kinetochore phosphoepitope during metaphase but not anaphase. (*A–C*) 2 min after chromosomes reached the metaphase plate (early metaphase) the cell was treated with nocodazole for 5 min and labeled for DNA (*A*), 3F3/2 (*B*), and microtubules (*C*). (*D–G*) A cell was treated with nocodazole for 12 min beginning 12 min after the start of metaphase (late metaphase) (*D* and *E*) or shortly after the start of anaphase (*F* and *G*). The 3F3/2 phosphoepitope can be regenerated well into metaphase (*E*), but not after anaphase has begun (*G*). *A–C* are confocal micrographs. Bar, 5 μm .

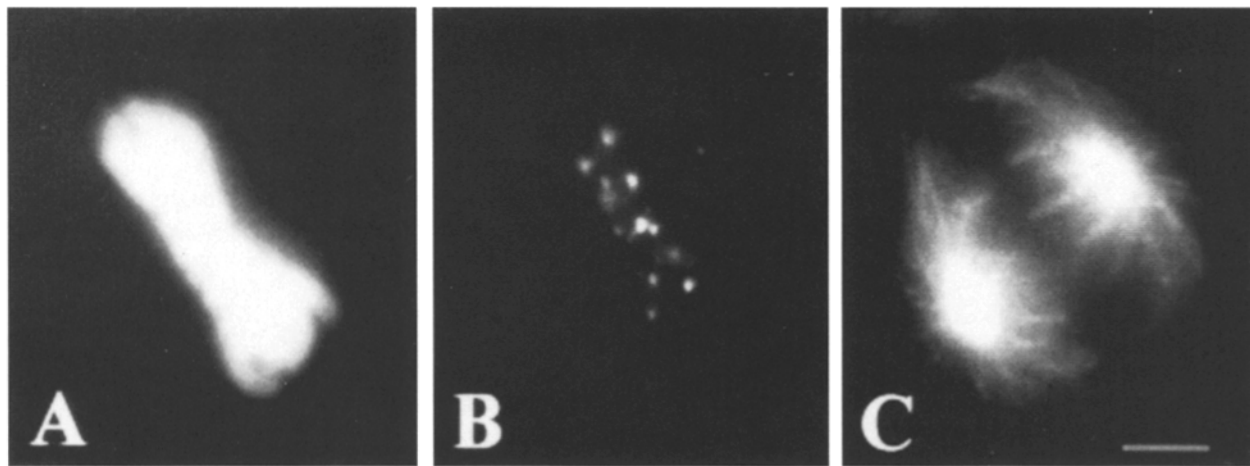


Figure 8. Taxol causes regeneration of the 3F3/2 kinetochore phosphoepitope. Early metaphase cells were treated for 12 min with taxol and labeled for chromosomes (A), 3F3/2 phosphoepitope (B), and microtubules (C). Bar, 5 μ m.

3F3/2 epitope within the kinetochore, immunoelectron microscopy was performed on Ptk1 cells with the 3F3/2 antibody. The kinetochore is a very dense complex, and penetration of such structures with large gold probes has been problematic (32). Thus, we elected to use secondary anti-

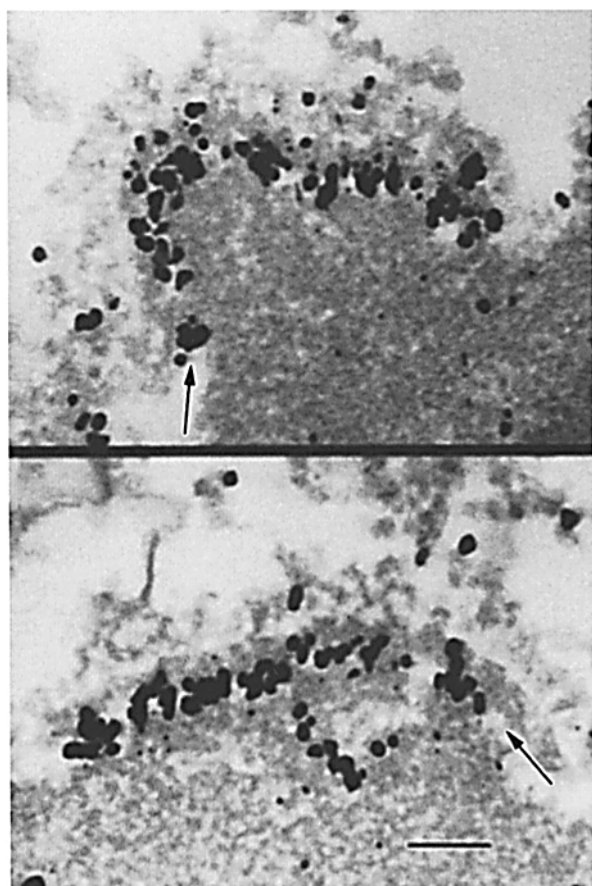


Figure 9. Ultrastructural localization of the 3F3/2 kinetochore phosphoepitope. The 3F3/2 epitope was labeled with 1.4-nm gold particles and then silver-enhanced, resulting in a range of particle sizes. The silver particles are located primarily in the middle zone (arrows), between the inner and outer kinetochore plates. Bar, 0.1 μ m.

bodies covalently linked to small, 1.4-nm gold particles followed by silver enhancement. This combination resulted in good penetration of the kinetochore and dense, unmistakable labeling. As seen in Fig. 9, the 3F3/2 phosphoepitope appears localized primarily to the middle, electron-lucent layer of the trilaminar kinetochore structure. The distribution of silver particles was counted in ten electron micrographs of labeled kinetochores. Some 62% of the particles were found in the electron lucent middle zone of the kinetochore (Table II).

Discussion

Expression of the 3F3/2 Kinetochore Phosphoepitope Is Associated With the Activation of a Checkpoint That Blocks Anaphase Onset in the Presence of Misaligned Chromosomes

In cells microinjected with the 3F3/2 antibody, chromosomes continued to express the phosphoepitope for an extended time after they reached the metaphase plate. At the same time, the cells were inhibited from entering anaphase. Only after a significant delay, the length of which depended on the amount of antibody injected, did the phosphoepitope finally disappear from the kinetochores of the metaphase chromosomes. Coincident with the time of disappearance of the phosphoepitope, the cells overcame the metaphase block and entered anaphase.

The phosphoepitope reappears on metaphase kinetochores within minutes of treatment with nocodazole or taxol, before disappearance of kinetochore microtubule fibers is detectable by light microscopy. Using electron microscopy, Cassimeris et al. (5) have shown that kinetochore microtubules completely disappear from Ptk1 cells

Table II. Distribution of Silver Particles in Electron Micrographs of 3F3/2 Antibody-labeled Kinetochores

	Inner plate	Middle zone	Outer plate and corona	Totals
No. particles	107	686	314	1107
% total	9.7	62	28.3	100

only after a 20-min treatment with nocodazole at a concentration eight times greater than that used here. The results of our experiments with microtubule drugs suggest that the disruption of normal microtubule dynamics is sufficient to cause reexpression of the 3F3/2 phosphoepitope at metaphase kinetochores, even though they still contain many microtubules. This evidence suggests that simple occupancy of the kinetochore by microtubules is not sufficient to signal a stable attachment and turn off the phosphoepitope.

In mammalian tissue culture cells microinjection of CREST autoimmune antibodies during interphase caused a metaphase arrest during the subsequent mitosis (3). In separate studies using a different source of CREST autoimmune IgG, microinjection of antibody into mouse eggs disrupted chromosome congression when injected during interphase or during early prometaphase in meiosis and mitosis (27). The microinjection of antibodies to CENP-E, a kinesin-like protein found at kinetochores, was originally thought to arrest mammalian cells at metaphase (35). However, recent studies have shown that antibodies to CENP-E have profound effects on the normal movement of chromosomes to the metaphase plate (Mol. Biol. Cell, 5 (suppl.):255a, abstract 1483). Thus, the 3F3/2 antibody is unique in that, when injected during mitosis, it inhibits mitotic progression while allowing normal chromosome congression to the metaphase plate.

Other treatments, such as the application of low concentrations of microtubule inhibitors (12) or stabilizers (26), similarly lead to mitotic arrest with all the kinetochores and chromosomes aligned at a metaphase plate. These results suggest that the mere positioning of kinetochores at the metaphase plate is not, by itself, sufficient to relieve the checkpoint preventing entry into anaphase.

Finally, although expression of the 3F3/2 phosphoepitope at the mitotic poles diminishes only slightly from prometaphase to late anaphase (10), we can not exclude the possibility that antibody binding at the poles plays a role in the induction of the metaphase arrest. Furthermore, it is also possible that the mechanism of the metaphase arrest caused by the antibody is more indirect, perhaps involving interference with kinetochore/microtubule interactions.

Differential Expression of the 3F3/2 Phosphoepitope at Sister Kinetochores Is Not Essential for Chromosome Movement to the Metaphase Plate

Injection of the 3F3/2 antibody eliminated the asymmetric expression of the phosphoepitope on the sister kinetochores of moving chromosomes in prometaphase. Although we can not rule out very subtle effects, when comparing control cells to those microinjected with the 3F3/2 antibody, we found no differences in chromosome movement or in alignment at the metaphase plate. Therefore, we conclude that the 3F3/2 phosphoepitope is not likely to signal the activation or deactivation of kinetochore microtubule motors and does not play a direct role in regulating the directionality of chromosome movement.

If not involved in regulating chromosome directionality, why is the phosphoepitope often expressed at different levels on sister kinetochores? One explanation is that the

difference in timing of sister kinetochore attachment to spindle microtubules is responsible for the asymmetric expression of the 3F3/2 phosphoepitope (see Fig. 10). A repeat subunit model of kinetochore structure has been proposed wherein the kinetochore is composed of multiple functionally self-contained domains, each containing a microtubule binding site (36). Stable attachment of a microtubule to the kinetochore might down-regulate 3F3/2 epitope expression in one subunit by locally turning off a kinase or activating a phosphatase. Differential expression of the phosphoepitope would be expected on chromosomes in which each kinetochore was attached to its pole by different numbers of microtubules. Chromosomes mono-oriented to one pole begin to move toward the metaphase plate after capturing only one or a few microtubules from the opposite pole. Careful electron microscopic analysis of moving chromosomes should show that kinetochores expressing intermediate levels of the phosphoepitope contain fewer microtubules than those in which 3F3/2 phosphoepitope expression is absent.

The 3F3/2 Phosphoepitope Resides on the Structural Connection Between the Inner and Outer Kinetochore Plates

Using computerized reconstructions of thick sections, McEwen et al. (18) proposed a model of kinetochore structure in which the outer, electron dense kinetochore plate, composed of a mat of intertwined 10–20-nm fibers, was connected to the inner plate by an array of connecting pillars. Our quantitative analysis revealed that the 3F3/2 phosphoepitope is located primarily in the middle, electron-lucent, zone of the kinetochore, where these connecting structures are found. The protein in which the 3F3/2 phosphoepitope resides may itself constitute, or be associated with, a structural protein linking the inner and outer electron dense kinetochore plates.

Linking Mechanical Signals at the Kinetochore and the Regulation of the 3F3/2 Phosphoepitope

Dietz (8) first hypothesized that stable chromosome attachment to the spindle is dependent upon proper tension, a hypothesis later verified by chromosome micromanipulation studies in insect spermatocytes (2, 23). McIntosh (19) proposed that a diffusible inhibitory signal from the kinetochores or centromeres of unattached chromosomes is down regulated by microtubule-generated tension. In meiotic mantid spermatocytes, Li and Nicklas (16) have directly shown that tension, artificially placed on a mal-oriented chromosome, induces cells with a misaligned chromosome to enter anaphase.

We hypothesize that the mechanism detecting tension at the kinetochore or the system that broadcasts the signal controlling the progression of mitosis involves expression of the 3F3/2 phosphoepitope. Skibbens et al. (28) have demonstrated that during prometaphase the kinetochore region undergoes stretching. The ultrastructural location of the 3F3/2 phosphoepitope within the kinetochore is consistent with a role in sensing or responding to tension from the attached microtubules.

The careful video analysis of Rieder et al. (26) suggests that stable attachment of microtubules to the last kineto-

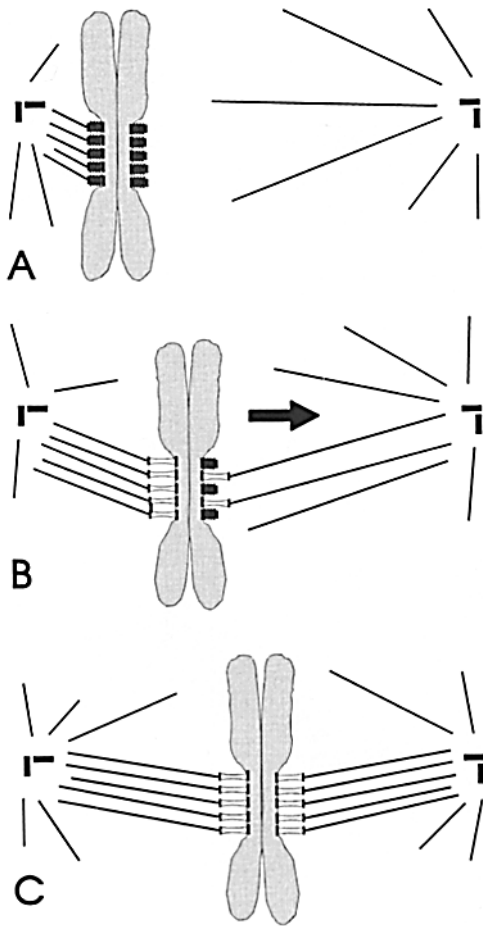


Figure 10. A model, based on the repeat subunit model of kinetochore structure, linking mechanical signals at the kinetochore to differential expression of the 3F3/2 phosphoepitope. Often, one sister kinetochore of a chromosome attaches to spindle microtubules before the other (A). Once bipolar attachment is established, the chromosome begins moving toward the metaphase plate (B). At the same time, kinetochore subunits attached to microtubules are subjected to tension, which stretches those subunits and initiates downregulation of the 3F3/2 kinetochore phosphoepitope (3F3/2 epitope expression is represented by dark shading in the kinetochore subunits). As the chromosome reaches the metaphase plate (C), all kinetochore microtubule binding sites are occupied, the kinetochores are fully under tension, and 3F3/2 epitope is absent.

chore starts a timer that counts down to anaphase onset. The time delay provides a window for the final chromosome, after attachment to spindle microtubules, to move to the metaphase plate. We speculate that the slow decay of the 3F3/2 phosphoepitope from the kinetochores of chromosomes as they move to the metaphase plate may be an early component of this timing mechanism. However, given that the 3F3/2 phosphoepitope is normally undetectable for some minutes before anaphase onset, it may be that the decay of other, downstream elements of the pathway is more proximally responsible for finally releasing the inhibition of anaphase onset.

The time delay between stable attachment of the final kinetochore and anaphase onset also provides a last quality control check for accurate chromosome segregation. If,

during this period, any kinetochores lose their normal attachment from the spindle microtubules, the timer must be reset. We suggest that the treatment of cells with microtubule drugs mimics such an event. Normal kinetochore-microtubule interactions are disrupted. Immediately the 3F3/2 phosphoepitope reappears at the kinetochores and the checkpoint block is reestablished.

When all the chromosomes are aligned at the metaphase plate and the timer has run out, anaphase is initiated. Anaphase cells treated with nocodazole fail to regenerate the 3F3/2 phosphoepitope. Thus it is likely that the machinery responsible for generating the 3F3/2 phosphoepitope at kinetochores is irreversibly turned off at this transition.

Due to the extreme lability of the phosphorylation involved, the identity of the kinetochore protein that contains the 3F3/2 epitope is not yet known. However, the experiments presented here provide important evidence that the phosphoepitope plays a role in regulating cell cycle progression through mitosis.

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