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Lysed Cell Models and Isolated Chromosomes for the Study of Kinetochore/Centromere Biochemistry In Vitro

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Abstract

The centromere or kinetochore functions in both chromosome movement and in regulation of progression through mitosis. It appears likely that the signaling pathways involved are keenly dependent on solid phase cytoskeletal and karyoskeletal scaffolds that may mediate important physical signals such as tension. Understanding these pathways will be greatly aided by reconstructing the signaling in lysed cell models. Here we present approaches to the in vitro study of signaling pathways in mitotic cells, particularly those involved in protein phosphorylation changes at kinetochores that may control cell cycle progression in M phase.

Introduction

The centromere-kinetochore complex plays essential and active roles in chromosome motility and cell cycle regulation during M phase. Numerous enzymes, structural proteins, and regulatory proteins are concentrated there at mitosis. Many of these proteins are transient, concentrating at the centromeres or kinetochores only at specific times in M phase. Many also show rapid dynamic interaction, associating and dissociating with residence times of a few seconds. For simplicity the term centromere will refer to the inner region of the complex where the chromatin from the two sister chromatids is joined tightly until anaphase onset. The term kinetochore will refer to the outer “trilaminar plate” structure in classically prepared electron micrographs. Centromeres and kinetochores appear to act as scaffolds and central organizers in a number of important mitotic processes: for the motor molecules involved in chromosome movement, for signaling molecules that regulate the spindle checkpoint, for the proteins that mediate chromatid separation at anaphase, and possibly for those that initiate and maintain the cytokinetic furrow (reviewed in (1,2,3)).

The coordination of all these M phase functions involves a complex regulatory system we are only beginning to understand. One consequence of their importance in cell cycle regulation and motility is that centromeres and kinetochores alter their biochemistry during progression through M phase. Importantly, these alterations can occur at different times at the various kinetochores within a single cell. Moreover they are apparently reversible. Each centromere-kinetochore complex, even the two sister centromeres/kinetochores on a single chromosome can be biochemically distinct either in the identity and nature of transient proteins bound there or in the post-translational modifications carried by proteins resident there. This biochemical dynamism may allow the centromere/kinetochore complex to respond rapidly to changes in chromosome location and to connections to spindle microtubules within the living cell.

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However, for the researcher it also greatly complicates the study of the pathways that occur there.

One approach toward the analyzing and manipulating the complex dynamics of signaling at centromeres and kinetochores is reconstituting the biochemical dynamics in lysed cell systems in vitro. Many of the interactions within the structure of centromeres and kinetochores appear to be preserved. We have been particularly interested in changes in phosphorylation status of proteins associated with kinetochores (4,5,6). We present here approaches toward studying the kinase and phosphatase activities associated with chromosomes, particularly those at centromeres and kinetochores.

Anti-phosphopeptide antibodies

To study changes in phosphorylation at specific subcellular locales, a quantifiable, imaging marker of phosphorylation status is useful. Anti-phosphopeptide antibodies combined with fluorescence microscopy provide a high level of specificity and deliver subcellular resolution. One example is the 3F3/2 antibody. This mouse monoclonal antibody was originally produced in a screen for components of MPF (maturation-promoting factor) in *Xenopus* egg extracts. Initially this antibody was characterized as binding preferentially to proteins that had been thiophosphorylated using ATP- γ S in kinase reactions (7). We later discovered that this antibody labeled a small subset of endogenous phosphoproteins, some of which were concentrated at kinetochores (8). Intriguingly the binding of this antibody to different kinetochores within a single cell differed depending on the extent of congression of the chromosome to the metaphase plate. Labeling with this antibody showed for the first time that kinetochore biochemistry could differ among the kinetochores within a cell, even between sister kinetochores on a single chromosome.

In recent years, there has been a large increase in the availability of highly specific anti-phosphopeptide antibodies. These are monoclonal and polyclonal antibodies prepared against synthetic peptides containing phosphorylated amino acids. These antibodies can be used to track changes in phosphorylation within a single protein species.

While qualitative information about comparative intensity of labeling and subcellular localization often provide meaningful information, it is essential that analyses of signaling pathways be quantifiable. The advent of highly sensitive digital cameras, imaging software, and the ability to collect images at multiple focal planes by confocal and widefield microscopy have made the collection of quantitative information from fluorescence microscopy routine.

Whole lysed cell models

The study of centromere/kinetochore biochemistry in whole mount, lysed mitotic cell models is greatly facilitated by using cells that remain relatively flat during division. In addition species with a small number of chromosomes tend to have larger chromosomes, generally with correspondingly larger kinetochores that are imaged and measured easily. The Ptk1 and Ptk2 cell lines derived from the rat kangaroo, *Potoroo tridactylis*, are particularly favored by workers studying mitosis for these qualities, but any cell type may be used as long as the mitotic cells are retained on the substrate during detergent lysis and buffer changes. Care should be exercised while adding and aspirating solutions and during manipulation of coverslips in order to avoid dislodging mitotic cells, which are often weakly attached to the substrate.

Isolated chromosomes

Manipulating kinetochore biochemistry with isolated chromosomes provides complimentary information to that gained through use of lysed whole cell models. One advantage is that

chromosomes can both be studied microscopically (by centrifuging them onto coverslips) and they can be studied by biochemical means (by SDS gel electrophoresis) using either anti-phosphoepitope antibodies or by changes in electrophoretic mobility induced by phosphorylation. For these purposes cells that are easy to grow in large quantities and which can be easily synchronized in culture are preferred. HeLa cells fulfill this requirement. Various sublines are adapted to grow in suspension or as adherent cells. They can be arrested in M phase with high efficiency in a single step with an overnight treatment with a microtubule depolymerizing drug such as nocodazole or colcemid. Higher yields of cells can be obtained with suspension cultures. Lower yields are obtained with adherent cultures but the proportion of mitotic cells is much higher often approaching 100%.

Other cell lines can also be used. Suspension cultures of rodent lines are often useful. Many rodent cell lines arrest temporarily in M phase when treated with microtubule drugs. They escape (a process sometimes termed adaptation) the spindle checkpoint and reenter interphase after several hours without undergoing normal mitosis. Obtaining a high mitotic index with such cells requires a two step synchronization protocol. The cells are first synchronized S phase using excess thymidine or with aphidicolin, an inhibitor of DNA polymerase α . After release from the S phase block, the cells are then incubated with a microtubule drug to induce arrest in M phase (9).

“Endogenous” and “exogenous” kinases in kinetochore rephosphorylation assays

The protocols detailed below provide a framework for the analysis of kinase activities in lysed cell systems, exemplified here in the manipulation of the 3F3/2 phosphoepitope at kinetochores of mitotic cells. The kinetochore phosphoepitopes recognized by the 3F3/2 anti-phosphoepitope antibody are extremely labile and very sensitive to rapid dephosphorylation by endogenous phosphatase upon lysis of cells with detergent. Thus the 3F3/2 phosphoepitopes provide a highly sensitive readout for regulating kinetochore phosphorylation. Because of its high lability, endogenous phosphatases are generally sufficient to completely eliminate the 3F3/2 phosphoepitopes at kinetochores of mitotic cells. Other phosphoepitopes may require addition of exogenous phosphatases. A useful reagent is lambda phosphatase, a broad specificity phosphatase with activity toward phosphorylated serine, threonine and tyrosine. It is available commercially from New England Biolabs.

Regeneration of phosphoepitopes can be catalyzed in two ways. First inhibiting phosphatase activity in the lysed cell model and supplying ATP will reveal the activities of endogenous kinases that are bound to insoluble elements within the cytoskeleton. Second by irreversibly inactivating the bound endogenous kinases (generally using N-ethyl maleimide), we can analyze activities the ability of cell extracts or purified kinases to regenerate phosphoepitopes on the cytoskeleton (Fig 1).

Stock Solutions

Protease inhibitor stock (200 x)—25 mg pepstatin (Sigma) dissolved in 18.75 ml of DMSO, 25 mg leupeptin (Sigma) and 25 mg Pefabloc SC (Boehringer Mannheim) dissolved in 6.24 ml of ddH₂O. Combine, aliquot 1 ml per tube, and store at -20°C .

Microcystin-LR stock (2000 x)—Dissolve to 400 μM in 100% ETOH that has been stored over molecular sieve to protect from moisture. Aliquot in 100 μl amounts and store at -80°C . Microcystin is labile and should be added to working solutions just before use. (Caution: microcystin-LR is highly toxic and should be handled with care).

ATP stock (100 mM)—Dissolve ATP to 100 mM in 200 mM Tris base (Do not adjust pH of Tris base after dissolving). Aliquot 100 μ l/tube and store at -20°C .

DTT stock (1 M)—in ddH₂O, aliquot 100 μ l/tube and store at -20°C .

PBS (10 x)—90 g NaCl, 7.95 g (29.6 mM) Na₂HPO₄•7H₂O, 1.44 g (10.6 mM) KH₂PO₄

PHEM (2 x)—120 mM Pipes, 50 mM Hepes, pH 7.0, 20 mM EGTA, 8 mM MgSO₄

MBS (10 x)—100mM MOPS, pH 7.3, 1.5M NaCl

Swelling Buffer (10x)—100 mM Hepes, pH 7.4, 400 mM KCl, 50 mM EGTA, 40 mM MgSO₄

Working Solutions

TM—50 mM Tris-HCl, pH 7.5, 4 mM MgSO₄

Extraction Buffer—TM, 0.5% CHAPS detergent, 1 mM DTT, 1x protease inhibitors. (0.5% Triton X-100 may be used in place of CHAPS)

Propidium Iodide Solution—25 μ g/ml propidium iodide, 0.5 mg/ml sodium citrate, 0.1% (v/v) NP-40 in 0.5 x PBS (mix 1:1 with cell suspension to label chromosomes).

Dephosphorylation Buffer—TM, 1 mM DTT, 1x protease inhibitors

Rinse Buffer—TM, 1x protease inhibitors.

Kinase Inactivation Buffer—TM, 5 mM N-ethylmaleimide (NEM), 1x protease inhibitors (N-ethylmaleimide is added from powder just before use).

Phosphorylation Buffer—TM, 1mM DTT, 200 nM Microcystin-LR, 1 mM ATP, 1x protease inhibitors.

Fixation Solution (2% formaldehyde in PHEM)—Add 0.8 g and 25 μ l of 1 N NaOH to 20 ml ddH₂O, boil in water bath in chemical fume hood to dissolve paraformaldehyde, cool, add 20 ml 2X PHEM.

MBST—10mM MOPS, pH7.3, 150mM NaCl, 0.05% Tween 20

Blocking Solution - 20% Boiled Normal Goat Serum^a—add 20 ml normal goat serum to 90 ml ddH₂O, boil in water bath for 10 min, cool, add 10 ml 10x MBS and sodium azide to 0.05%, spin 25,000 x g in refrigerated centrifuge for 1 hour, filter supernatant through 0.22 μ m pore filter, store at 4°C .

Antibody Solution - 5% Boiled Goat Serum—5 ml Blocking Solution, 1.5 ml 10 x MBS, 13.5 ml ddH₂O, store at 4°C .

Swelling Buffer—10 mM Hepes, pH 7.4, 40 mM KCl, 5 mM EGTA, 4 mM MgSO₄, 0.2 x protease inhibitors.

^aThe Blocking Solution and Antibody Solution contain serum from the species that is the source of the secondary antibodies used. Boiling helps to inactivate endogenous enzymes (e.g. phosphatases) that might be present in serum.

Chromosome Buffer—1 x PHEM, 1% Chaps (or 0.5 % Triton X100) 1 mM DTT, 1 x protease inhibitors.

Protocol for Endogenous Kinase Assay (Fig. 2)

1. Grow Ptk1 cells on coverslips in plastic Petri dishes until about 70 - 85% confluent in 6 well culture dishes.
2. Aspirate culture medium and rinse coverslips very quickly with TM buffer.
3. Immediately add 2 ml extraction buffer and incubate for 5 min at room temperature.
4. Withdraw extraction buffer and add 2 ml dephosphorylation buffer for 5 to 7 min.
5. Optional: treat with phosphatase^a.
6. Remove coverslip from dish, place cell side down onto 100 μ l droplet of phosphorylation buffer on parafilm in a humidified chamber^b. Incubate for desired length of time (usually 3 - 20 min) at room temperature or 37°C.
7. Proceed to fixation protocol below^c.

Preparation of Mitotic Cell Extracts

1. HeLa S3 cells^a are grown in 500 ml of medium in 1 liter spinner flasks to a density of 5×10^5 cells/ml.
2. Add Colcemid^b to 0.15 μ g/ml for 14 - 18 hours to arrest cells in M phase.
3. Collect cells by centrifugation with a clinical centrifuge at 300 xg.
4. Wash cells twice by centrifugation in PBS, pH 7.4.
5. Cell pellets may be flash frozen in liquid nitrogen and stored at -70°C.
6. Suspend cell pellets containing approximately 5×10^6 cells in 1 ml of Extraction Buffer on ice.
7. The tube is intermittently vortexed and kept on ice for 5 min.
8. Clarify by centrifugation at 15,000 xg for 15 min at 4°C.
9. Aliquot, flash freeze in liquid nitrogen and store at -70°C.

^aEndogenous cellular phosphatases are present at high concentration on chromosomes and at kinetochores (10,11). For highly labile phosphoepitopes such as those at kinetochores recognized by the 3F3/2 anti-phosphoepitope antibody, endogenous phosphatases are sufficient to completely abolish immunoreactivity. For other phosphoepitopes and for those present at locations in the cell where endogenous phosphatases are not concentrated, dephosphorylation may be enhanced by treatment at step 4 with exogenously applied phosphatase. If so, at step 4, transfer coverslips for 10 - 20 min at 37°C. to 100 μ l drops containing phosphatase PP1 (0.1 u/ml) or lambda phosphatase (100 u/ml), both from New England Biolabs, prepared in buffer supplied by the manufacturer.

^bIncubations are carried out on droplets on parafilm to conserve expensive or limited enzymes, reagents and antibodies. Humidified chambers are may be constructed from 150 mm diameter, plastic or glass Petri dishes, with a piece of wet filter paper at the bottom to provide humidity.

^cControls should include

1. Cells lysed in the presence of microcystin-LR during step 2 and then fixed demonstrating initial starting phosphorylations.
2. Cells fixed after steps 4 or 5 to demonstrate effectiveness of dephosphorylation.
3. Cells treated with rephosphorylation buffer lacking ATP and then fixed to demonstrate that recovery of immunolabeling is due to phosphorylation.

^aMitotic extracts can be prepared from other suspension grown cells or from monolayer arrested in M phase with anti-microtubule drugs and collected by washing off the rounded mitotic cells.

^bOther anti-microtubule drugs such as nocodazole, vinblastine, or paclitaxel may also be used.

Protocol for Exogenous Kinase Assay

1. Ptk1 cells are grown on coverslips until about 70 - 85 % confluent in 6 well culture dishes.
2. Culture medium is aspirated and coverslips are rinsed briefly with TM buffer.
3. Immediately add 2 ml Extraction Buffer and incubate for 5 min at room temperature.
4. Withdraw Extraction Buffer and add 2 ml Dephosphorylation Buffer for 5 to 7 min.
5. Optional: treat with phosphatase.
6. Aspirate Dephosphorylation Buffer. Add 2 ml of Rinse Buffer (to remove DTT).
7. Aspirate Rinse Buffer. Add 2 ml of Kinase Inactivation Buffer and incubate for 10 min at room temperature.
8. Aspirate, add 2 ml of Rinse Buffer, repeat.
9. Remove coverslip from dish, place cell side down onto a 100 μ l droplet of phosphorylation buffer containing the kinase or extract^a to be tested for kinase activity. The droplet is placed on parafilm in a humidified chamber. Incubate for desired length of time (usually 3 - 20 min) at room temperature or 37°C.
10. Proceed to fixation protocol below.

Fixation and Immunofluorescence

1. In 6 well dish rinse coverslips briefly with PHEM containing 200 nM microcystin-LR. Aspirate and add 2 ml Fixation Solution, incubate 10 min.
2. Aspirate and add 2 ml MBST, repeat.
3. Place coverslips, cell side down, onto 100 μ l droplets of Blocking Solution for 20 min at room temperature.
4. Rinse coverslips with MBST
5. Place coverslips on droplets of primary antibody diluted in Antibody Solution, incubate for 30 - 45 min at room temperature.
6. Place coverslips in 6 well dish with MBST, incubate 5 min with gentle agitation on shaker, repeat wash 2x.
7. Place coverslips on droplets of fluorescent secondary antibody diluted in Antibody Solution for 30 - 45 min at room temperature.
8. wash with MBST 3x 10 min
9. Incubate few seconds with 0.5 μ g/ml Dapi in MBST
10. Rinse coverslips with three changes of dH₂O
11. Mount in Vectashield (Vector laboratories) with added 10mM Mg (for better preservation of chromosome structure).
12. Seal edges of coverslip with nailpolish.

^aFor phosphorylation of kinetochores to generate the 3F3/2 phosphoepitope, mitotic extracts are commonly diluted 10 to 50 fold in rephosphorylation buffer.

Isolation of Mitotic Chromosomes

1. Grow 250 ml of Hela S3 cells in suspension to a density of 1.0×10^6 cells per ml^a.
2. Dilute cells 1:1 with fresh medium and return to incubator for 6 to 8 hours.
3. Treat cells overnight (17 - 20 hours) with colcemid at 0.15 µg/ml.
4. Collect cells by centrifugation in two 250 ml polypropylene conical centrifuge tubes by centrifugation at 250 x g for 5 min at room temperature.
5. Aspirate medium and resuspend cells in 50 ml of Swelling Buffer that has been prewarmed to 37°C.
6. Centrifuge cells at 200 x g for 7 min in two 50 ml polypropylene conical centrifuge tubes at room temperature.
7. Aspirate supernatant and resuspend cells in a total of 50 ml warm Swelling Buffer^b.
8. Centrifuge at 200 x g for 7 min at room temperature.
9. Aspirate supernatant and place tubes containing cell pellets on ice for 5 min. The remaining steps are performed at 4°C.
10. Lyse cells with 10 ml of Chromosome Buffer by repetitive, aggressive pipetting. Fluid that is expelled from the pipet is forced between the tip of the pipet tip and the centrifuge tube. Repeat at least 10 times using 5 ml **plastic** pipettes.
11. Centrifuge at 200 x g for 5 min in 15 ml polypropylene conical centrifuge tubes.
12. Carefully remove supernatant (containing chromosomes) and save on ice.
13. Repeat lysis and centrifugation steps with 5 ml of Chromosome Buffer 2 to 3 more times to release more chromosomes from pelleted material.
14. Combine chromosome fractions into 50 ml conical centrifuge tube.
15. Centrifuge at 400 x g for 7 min to pellet nuclei and larger pieces of cellular debris.
16. Recover supernatant and centrifuge at approximately 1200 x g for 10 min to pellet chromosomes.
17. Carefully remove supernatant containing smaller organelles and soluble cytoplasmic proteins and discard.
18. Resuspend pellet in 5 ml of Chromosome Buffer in 15 ml tube.
19. Centrifuge at 1200 x g, discard supernatant.
20. Resuspend in 5 ml of Chromosome Buffer.
21. Chromosomes isolated by the differential centrifugation protocol described to this point are sufficient for both biochemical and morphological studies. Further purification by centrifugation on density gradients may be performed, but may result

^aFor suspension cultures, it is important that cells be in log phase growth. Generally the protocol described results in a mitotic index of approximately 85%. Other suspension cell lines mitotic cells obtained by shake-off of adherent cultures may be used with appropriate scaling of buffer volumes.

^bSwelling of cells in hypotonic buffer is essential to ensure dispersed chromosomes. During this time, the mitotic index of the culture is calculated by mixing a drop of the cell suspension with a drop of propidium iodide solution on a glass slide. This is observed using an epifluorescence microscope with a dry 20x objective without using a coverslip (to prevent bursting the cells). While standardizing the protocol for the first time it is good practice to take samples at many stages and label with propidium iodide to track the dispersion and preservation of the isolated chromosomes and contamination by interphase nuclei.

in reductions in yield as well as disruption of chromosome morphology. If further purification is not required skip to step 30 below.

22. For further purification, prepare glycerol step gradients. In 15 ml clear plastic tube add 2 ml 80% glycerol/20% Chromosome Buffer. Over layer with 2 ml 40% glycerol/60 % Chromosome Buffer.
23. Over layer 40% glycerol with chromosome suspension from step 20.
24. Centrifuge in swinging bucket rotor at 2600 x g for 30 min at 4°C.(E.g. Sorvall HB-4 rotor, 4000 rpm with adaptors for 15 ml tubes).
25. Carefully aspirate and discard the upper buffer phase and most of the 40% glycerol phase.
26. Carefully collect chromosomes from interface of 40% and 80% glycerol phases. (Often the chromosomes will have entered the top of the 80% glycerol phase so it is useful to collect the upper portion of the 80% phase.)
27. Add 5 ml of Chromosome Buffer to dilute the glycerol.
28. Centrifuge 1200 x g to pellet chromosomes
29. Resuspend in 5 ml of Chromosome Buffer.
30. Centrifuge 1200 x g to pellet chromosomes.
31. Resuspend in 1 ml of Chromosome Buffer.
32. For morphological studies it is best to use the chromosomes immediately. For biochemical studies they can be aliquoted, flash frozen and stored at -80°C.

Phosphorylation of Isolated Chromosomes and Preparation for SDS-PAGE (Fig 3)

1. For phosphorylation, add ATP to a final concentration of 1 mM and Microcystin-LR to a final concentration of 200 nM in 50 - 100 µl aliquots of isolated chromosomes in Chromosome Buffer.
2. Incubate in microfuge tubes 5 - 30 min at 37°C.
3. Centrifuge for 10 sec at 15000 x g to pellet the chromosomes.
4. Aspirate supernatant.
5. Resuspend in 50 µl of Chromosome Buffer containing 200 nM Microcystin-LR and 1 µg/ml DNase.
6. Incubate 5 min at 37°C.
7. Add 50 µl of 2x SDS-PAGE Sample Buffer.

Phosphorylation of Isolated Chromosomes and Preparation for Immunolabeling

1. Incubate 18 mm round coverslips on droplets of 0.1% (w/v) poly-L-lysine (Sigma P8920) on parafilm for 10 min at room temperature.
2. Rinse briefly with water and place coverslips in an appropriate chamber that can be centrifuged in a swinging bucket centrifuge. We routinely use 4 well chambers that are cut from 12 well tissue culture plates. These chambers fit into buckets of large swinging bucket rotors^a.

^aVaried approaches to centrifuging chromosomes onto coverslips may be used including cytospin centrifuges or plastic adapters that create a flat bottom in centrifuge tubes.

3. For phosphorylation, add ATP to a final concentration of 1 mM and Microcystin-LR to a final concentration of 200 nM in 10 - 50 μ l aliquots of isolated chromosomes in Chromosome Buffer.
4. Incubate in microfuge tubes 5 - 30 min at 37°C.
5. Add 1 ml of Chromosome Buffer to chromosomes and place into chambers on top of poly-L-lysine-treated coverslips.
6. Centrifuge chromosomes onto coverslips at 1500 x g for 10 min at 4°C or at room temperature.
7. Fix and process coverslips for immunofluorescence as described above.

Future Directions

The ultimate goal of the described approaches are to develop in vitro systems for mammalian cells that both retain much of the complex signaling attributes of the in vivo condition and that can be manipulated biochemically. One of the most important aspects of working with lysed cell models is the retention of the solid phase cytoskeletal and karyoskeletal elements that act as insoluble scaffolds for signaling protein complexes. In the future, dynamic imaging strategies will aid in providing real time assessment of biochemical changes. One ultimate goal in our laboratory is recapitulating the signaling of the spindle checkpoint. An example of a developing approach to real time analysis of phosphorylation changes in lysed cell models is provided in Fig. 4.

While the examples provided in this article concentrate on serine/threonine phosphorylations recognized by the 3F3/2 antibody, the general approaches are applicable to a wide range of kinases and phosphatases, using phosphoepitopes-specific antibodies. For example replacing Microcystin-LR with the tyrosine phosphatase inhibitor sodium vanadate allows use of anti-phosphotyrosine antibodies to study the regulation of that moiety. More generally the approaches are applicable to any post-translational modification for which a specific reporter probe is available. Continued innovation in fluorescent reporters, in the development of antibodies and other probes to recognize protein post translational modifications, and in the technology of imaging will lead to increasingly better model systems for dissecting signaling pathways in reconstituted systems in vitro.

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Figure 1.

Pathways for in vitro manipulation of kinetochore protein phosphorylations recognized by anti-phosphoepitope antibodies. Filled circles represent a phosphorylated kinetochore epitope. Empty circles depict the dephosphorylated state. Pathway A - Detergent lysis of mitotic cells in the presence of potent phosphatase inhibitors preserves the phosphorylation of kinetochore phosphoepitopes. Pathway B - Detergent lysis of mitotic cells in the absence of phosphatase inhibitors results in dephosphorylation of kinetochore phosphoepitopes. Pathway C - If dephosphorylated lysed cells are rinsed free of all cytoplasm and then treated with buffer containing ATP, phosphoepitopes are regenerated at kinetochores. Pathway D - Treatment of dephosphorylated detergent insoluble cytoskeletons with N-ethylmaleimide inactivates endogenous kinetochore kinases. Providing ATP to NEM-treated cytoskeletons does not generate kinetochore phosphoepitopes. Pathway E - If NEM-treated cytoskeletons are

incubated with ATP in the presence of diluted mitotic cell extract, phosphoepitopes are regenerated at kinetochores.

Figure 2.

Endogenous kinetochore kinases can regenerate the kinetochore phosphoepitope recognized by the 3F3/2 antibody. A. Cells in early prometaphase were lysed in the absence of phosphatase inhibitor to allow dephosphorylation of kinetochore phosphoepitopes. They were rinsed free of all soluble cytoplasm and incubated in buffer containing the phosphatase inhibitor Microcystin-LR in the presence or absence of ATP. The cytoskeletons were then fixed and examined by triple label immunofluorescence. DNA was labeled with Dapi (DNA). Kinetochores were identified by labeling with a human autoimmune serum (Kinetochores). Cytoskeletons incubated in the presence of ATP but not in its absence showed regeneration of kinetochore 3F3/2 phosphoepitopes (3F3/2). B. Differential kinase activity is retained at kinetochores in vitro. Dephosphorylated cytoskeletons were treated with ATP for a short time in buffer containing Microcystin-LR. In the mid prometaphase cell depicted, kinetochores of fully aligned chromosomes (chromosomes at the metaphase plate) showed little or no regeneration of the 3F3/2 phosphoepitope (aligned). Chromosomes near the poles

(monooriented chromosomes) show strong rephosphorylation of the 3F3/2 phosphoepitope on both sister kinetochores. Chromosomes, near the metaphase plate that were likely in the process of moving toward alignment at the spindle equator at the time of cell lysis (moving chromosomes) show greater regeneration of the phosphoepitope on the kinetochore leading the movement. Note that the spindle poles (poles) also express the 3F3/2 phosphoepitope. Bars = 10 μ m.

Figure 3.

Isolated chromosomes contain kinases that catalyze the phosphorylation of the 3F3/2 phosphoepitope on human DNA topoisomerase II α . Chromosomes isolated from HeLa cells in the presence of the phosphatase inhibitor Microcystin-LR show a strong band at 170kD when immunoblotted with 3F3/2 antibody (lane 1). According to evidence detailed elsewhere, this band is human topoisomerase II α (12). If chromosomes are isolated in the absence of the phosphatase inhibitor, labeling of the topoisomerase II α band with 3F3/2 antibody is greatly reduced (lane 2). When chromosomes isolated without phosphatase inhibitor are then incubated in the presence of ATP, then the topoisomerase II α band again becomes reactive with the 3F3/2 antibody (lane 4). This rephosphorylation of topoisomerase II α does not occur in the absence of ATP (lane 3) and is enhanced if Microcystin-LR is added during the in vitro phosphorylation to inhibit phosphatase activity also associated with isolated chromosomes (lane 5). (Modified from (12)).

Figure 4.

Real time imaging of kinetochore phosphoepitope changes. The 3F3/2 antibody was directly conjugated with the fluorophore, Cy3. A. Ptk1 cells attached to a coverslip were lysed in the absence of phosphatase inhibitor to induce dephosphorylation of the 3F3/2 phosphoepitope. The coverslip was incubated on a slide on the stage of a microscope at room temperature in Phosphorylation Buffer containing Cy3-conjugated 3F3/2 antibody. A suitable mitotic cell was located and imaged at successive time points (indicated as min:sec after the start of the in vitro phosphorylation). With time the concentration of 3F3/2 antibody at kinetochores increases. Increases also occur at the poles and in other regions of the cell. Images for each time point reflect a maximum projection of a through focus series. The intensities of the kinetochores were measured for each time point and plotted on the graph.