Checkpoint signals in grasshopper meiosis are sensitive to microtubule attachment, but tension is still essential

R. Bruce Nicklas[1,*], Jennifer C. Waters[2,3], E. D. Salmon[2] and Suzanne C. Ward[1]

[1]Department of Biology, Duke University, Durham, NC 27708-1000, USA
[2]Department of Biology, University of North Carolina, Chapel Hill, NC 27599-3280, USA
[3]Department of Biology, Wake Forest University, Winston-Salem, NC 27109, USA
*Author for correspondence (e-mail: bnicklas@duke.edu)

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SUMMARY

The spindle checkpoint detects errors in kinetochore attachment to microtubules and delays anaphase if attachment is improper. The checkpoint is activated by attachment-sensitive components including Mad2 and certain phosphorylated proteins detected by the 3F3/2 antibody. We have studied Mad2 and 3F3/2 immunofluorescence in grasshopper spermatocytes. As in other cells, unattached kinetochores are loaded with Mad2 and are highly phosphorylated, whereas after proper attachment, Mad2 is lost and kinetochores are dephosphorylated. What is it about proper attachment that produces these changes – is it microtubule attachment itself or is it the tension from mitotic forces that follows proper attachment? Using micromanipulation, we created an intermediate state, weak attachment, that provides an answer. Weakly attached kinetochores are not under tension and have few kinetochore microtubules. Despite the absence of tension, many weakly attached kinetochores lose their Mad2 and become dephosphorylated. Therefore we conclude that microtubule attachment determines both Mad2 binding and phosphorylation. Nevertheless, tension plays an absolutely essential role. Tension elevates the number of kinetochore microtubules to the level necessary for the complete loss of Mad2 and dephosphorylation from all kinetochores. This gives a reliable 'all clear' signal to the checkpoint, allowing the cell to progress to anaphase.

Key words: Spindle checkpoint, Mad2, Microtubules, Kinetochore, Micromanipulation

INTRODUCTION

Errors in chromosome attachment to the spindle can lead to cells with missing or extra chromosomes and to organisms with birth defects or cancer. Attachment errors are detected by a checkpoint that delays the onset of anaphase, allowing time for error correction (Nicklas, 1997). Checkpoint action depends on attachment-sensitive proteins, including Mad2, Bub1 and Bub3. All these proteins are bound to unattached kinetochores and are lost as kinetochores become properly attached to spindle microtubules (Amon, 1999). Mad2 is a central player in the checkpoint (Shah and Cleveland, 2000). Mad2 binds to Mad1 at unattached kinetochores (Chen et al., 1999; Chen et al., 1998) and then activates the checkpoint, most likely by the catalytic production and release of a complex of Mad2 and cdc20 that prevents activation of the anaphase promoting complex (Chen et al., 1998; Gorbsky et al., 1998; Howell et al., 2000; Rieder and Salmon, 1998). After a kinetochore becomes properly attached to microtubules, Mad2 is lost from the kinetochore and anaphase follows some minutes after the last kinetochore attaches. In addition to transitory proteins, the phosphorylation of one or more proteins detected by the 3F3/2 antibody is sensitive to attachment: they are phosphorylated at unattached kinetochores and become dephosphorylated after proper attachment (Gorbsky and Ricketts, 1993; Nicklas et al., 1995). The phosphorylation detected by 3F3/2 is likely a component of the spindle checkpoint, since preventing dephosphorylation with injected antibodies prevents anaphase onset (Campbell and Gorbsky, 1995). Phosphorylation may provide the link between attachment and Mad2 action, since in vitro Mad2 binds only to kinetochores that have phosphorylated proteins (Waters et al., 1999). We speak of gaining or losing Mad2 and phosphorylation as seen by antibody immunofluorescence because there is direct evidence for gain and loss as opposed to other alternatives such as masking the epitope by microtubules (Campbell et al., 2000; Howell et al., 2000; Nicklas et al., 1998; Waters et al., 1998).

What is it about microtubule attachment that produces changes in Mad2 and phosphorylation at kinetochores? Proper attachment is quickly followed by tension from pulling forces toward opposite poles. Therefore, checkpoint proteins might be sensitive to the presence or absence of tension and/or kinetochore microtubules. There is direct experimental evidence in insect cells in meiosis that tension is monitored (Li and Nicklas, 1995), while experiments in mammalian cells in mitosis support the idea that microtubule occupancy is what is monitored (Rieder et al., 1995). Clear evidence from taxol-treated cells shows that what is monitored depends on the kinetochore change in question, with phosphorylation sensitive to tension and Mad2 binding sensitive to microtubule

occupancy (Waters et al., 1998). However, there is new evidence that what is monitored may be different in mitosis and meiosis. In budding yeast meiosis, but not mitosis, a tension-sensitive checkpoint plays a critical role in preventing errors (Shonn et al., 2000). In maize meiosis there is a correlation between the chromosome stretching caused by tension and kinetochore dephosphorylation and loss of Mad2, whereas in mitosis in the same plant, attachment itself appears to suffice (Yu et al., 1999).

But there is an alternative to the either/or models, tension or occupancy: it might be microtubule number all the way, all the time. The apparent effect of tension might be due to an increase in the number of kinetochore microtubules when tension is present (Wells, 1996), as has recently been demonstrated in grasshopper spermatocytes (King and Nicklas, 2000). When tension at a kinetochore is removed by micromanipulation, the number of kinetochore microtubules at metaphase drops by 60% (e.g. from an average of 32 to only 13 kinetochore microtubules). We describe this state as 'weak' attachment not only because there are so few kinetochore microtubules but also because it is unstable. A weak attachment is frequently lost and replaced by a new one to the opposite spindle pole (Nicklas and Ward, 1994). In mammalian PtK_1 cells, kinetochores whose sisters are not attached also have a reduced number of kinetochore microtubules (McEwen et al., 1997). In this instance, however, the effect of tension is not clear, because such chromosomes are under some tension from anti-poleward forces (Rieder and Salmon, 1994). In newt cells, the tension on the attached kinetochore from antagonistic poleward and antipoleward forces is approximately equal to the tension from opposed poleward forces after proper attachment of sisters to opposite poles (Waters et al., 1996b).

Three states of kinetochore attachment to the spindle are shown in Fig. 1 and characterized in Table 1. Unattached kinetochores both in mitosis and meiosis (Fig. 1A,B, 'u') have no kinetochore microtubules, are not under tension and send a 'wait' signal to the checkpoint. Strong attachments are formed after attachment of partner chromosomes to opposite poles (Fig. 1A,B, 's'); in both mitosis and meiosis, the maximum number of kinetochore microtubules is present, the kinetochores are under tension, and the 'wait' signal ceases.

The 'weak' attachment state arises from different errors in mitosis and meiosis. In mitosis in vertebrate cells, the common attachment error is a chromosome that has one attached kinetochore and one unattached kinetochore (Fig. 1A, bottom). The unattached kinetochore is not under tension, but the attached one is, because antipoleward forces on the chromosome arms (Fig. 1A, red arrowheads) antagonize the poleward kinetochore force (Fig. 1A, green arrow). In PtK_1 cells, the weakly attached kinetochore has about 60% of the number of kinetochore microtubules found in strong attachment (McEwen et al., 1997). Weak attachment in mitosis leads to a greatly reduced amount of Mad2 compared with that in the unattached state (Chen et al., 1996; Howell et al., 2000; Waters et al., 1998), and phosphorylation detected by the 3F3/2 antibody is also reduced (Gorbsky and Ricketts, 1993). As might be expected, such a weakly attached kinetochore does not send an effective 'wait' signal to the checkpoint (Rieder et al., 1995). Error correction is possible, however, because the unattached kinetochore sends a 'wait' signal (Rieder et al., 1995).

The situation in meiosis is different. The common error in meiosis is the weak attachment of both partner kinetochores to the same pole (Fig. 1B, bottom). In grasshopper spermatocytes, tension is absent in the weak attachment state because antipoleward forces are absent: granules never move away from the pole (Nicklas and Koch, 1972) and neither do chromosome fragments (R.B.N. and S.C.W., unpublished). The behavior of chromosomes with one kinetochore attached to one pole is also revealing. Unlike their counterparts in vertebrate cell mitosis, such chromosomes in insect spermatocytes do not oscillate back and forth at some distance from a pole; instead they just move close to a pole and stay there (Fig. 6A-C) (Wise, 1978). Kinetochore microtubule number is dramatically lower than in strong attachment – less than 40% (King and Nicklas, 2000) (this study).

We have studied Mad2 binding and kinetochore protein phosphorylation in naturally occurring as well as experimentally induced attachments in grasshopper spermatocytes. Surprisingly, we find that kinetochore protein phosphorylation, like the Mad2 level, is regulated by microtubule attachment and not by tension. However, in the absence of tension, phosphorylation, Mad2 levels and the operation of the checkpoint are erratic, evidently because a full set of kinetochore microtubules is not present. Reliable checkpoint operation therefore depends on tension, even though it is microtubule number that the checkpoint monitors.

Table 1. Weak attachment in mitosis (PtK_1 mammalian cells) and meiosis (grasshopper spermatocytes, division I)

	Tension?	Kinetochore microtubules (% occupancy)	3F3/2 Protein phosphorylation	Mad2 level	Checkpoint signal
Mitosis	Yes*	60%§	Little**	Little to none§§	'Proceed'***
Meiosis	No‡	26-38%¶	Maximal‡‡	A lot¶¶	'Wait'¶¶

*Rieder and Salmon, 1998.
‡See text.
§McEwen et al., 1997.
¶King and Nicklas, 2000; this report.
**Gorbsky and Ricketts, 1993.
‡‡Nicklas et al., 1995; this report. Later in prometaphase, phosphorylation is variable.
§§Chen et al., 1996; Howell et al., 2000; Waters et al., 1998.
¶¶This report. Later in prometaphase, Mad2 amounts are variable.
***Rieder et al., 1995.

MATERIALS AND METHODS

Cell culture and manipulations

Spermatocytes from laboratory colonies of the grasshopper *Melanoplus sanguinipes* were cultured at 23-25°C under oil as previously described (Nicklas and Ward, 1994). Cells in meiosis I were visualized using phase microscopy and chromosomes were manipulated as previously described (Nicklas and Ward, 1994). In the experiments on weak attachment, the existence of a real attachment was always verified just before fixation for immunostaining by gently pulling the chromosome toward the opposite pole; if the chromosome came away from the pole without resistance, it was not attached, and we proceeded no further with that cell (this is necessary because weak attachments sometimes lapse) (Nicklas and Ward, 1994).

Immunoblot

Grasshopper testes were collected and homogenized into electrophoresis buffer containing DTT (Waters et al., 1998). The sample was centrifuged, and equal amounts of the supernatant were loaded onto several lanes of a 10% SDS polyacrylamide gel. For a control, bacterially expressed Xenopus Mad2 protein (Waters et al., 1998) was also run. After electrophoresis, the gel was cut in half. Lanes containing MW markers, Mad2 protein, and homogenized grasshopper testes supernatant (GH) were stained with Coomassie blue, while other lanes containing GH were electroblotted onto nitrocellulose paper. Blots were labeled with affinity-purified polyclonal rabbit antibodies raised against recombinant Xenopus Mad2 and alkaline phosphatase secondary antibodies as previously described (Waters et al., 1998).

Immunofluorescence

Cells were lysed and fixed as previously described (Nicklas et al., 1995). For cells that were to be labeled with antibodies to the 3F3/2 phosphoepitope, 10 μM LR microcystin was added to the lysis/fixation buffer to preserve 3F3/2 phosphorylation (Gorbsky and Ricketts, 1993). For immunofluorescence labeling, lysed and fixed cells were first blocked with 5% normal donkey serum (NDS) for 45-60 minutes at room temperature. Cells were then treated with either the same polyclonal Mad2 antibodies used for immunoblotting (as described above) (Waters et al., 1998), or monoclonal mouse 3F3/2 antibodies, diluted into 5% NDS, for 45 minutes at room temperature. Next, cells were rinsed for 20 minutes in MBS (10 mM Mops at pH 7.4 and 150 mM NaCl), replacing the buffer every 5 minutes. Cells were then incubated in LSRC-conjugated donkey anti-rabbit or anti-mouse IgG secondary antibodies (Jackson ImmunoResearch) diluted 1:50 into 5% NDS. Cells were rinsed again for 20 minutes in MBS, replacing the buffer every 5 minutes, then mounted in 1:3 PBS:glycerol with n-propyl gallate for viewing.

To visualize microtubules, the cells were stained at 5°C for 24 hours with anti-α-tubulin and anti-β-tubulin antibodies (Amersham) diluted 1:50 in BSA/PBS and processed as previously described (King and Nicklas, 2000).

Fluorescence microscopy

Z-series optical sections of cells were collected using a Nikon Microphot FX-A microscope equipped with a 60× 1.4 N.A. Plan Apochromat lens, a Hamamatsu C4880 cooled-CCD camera, and MetaMorph (Universal Imaging Corp.) imaging software as previously described (Waters et al., 1996a). The brightness of kinetochore Mad2 and 3F3/2 immunofluorescence was measured in unprocessed images by the 'concentric circles' method previously described (King et al., 2000).

The brightness of kinetochore microtubule immunofluorescence was measured in unprocessed images as previously described (King and Nicklas, 2000) for a 1 μm length of the microtubule bundle, starting 0.5 μm from the kinetochore.

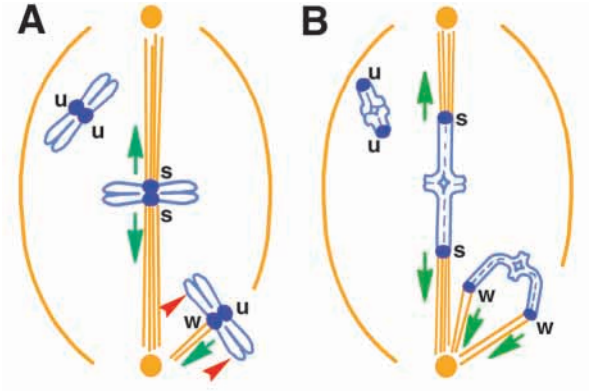

Fig. 1. Three states of kinetochore attachment to the spindle. Chromosomes in blue, kinetochore microtubules, spindle outlines and poles in gold. (A) Mitosis in vertebrate cells. Top to bottom: a chromosome with both kinetochores (blue ovals) unattached (u), a chromosome with both kinetochores strongly attached (s), under tension from oppositely directed poleward forces (green arrows), and a misattached chromosome with one kinetochore unattached (u) and one weakly attached (w); tension from antagonistic antipoleward forces (red arrowheads) and poleward forces (green arrow) is present. (B) First division of meiosis in grasshopper spermatocytes. As for A, except the misattached chromosome at the bottom has both kinetochores weakly attached (w) to the same pole and tension is absent – only poleward forces are present.

For illustrations in this report, fluorescence and phase contrast images of the cells were processed digitally, using commercial software, 'PhotoShop' (Adobe Systems Inc.) and 'PowerPoint' (Microsoft Corp.). Often, the images are montages of two focal levels, so that several kinetochores can be compared in a single view. False color was added to the fluorescence images.

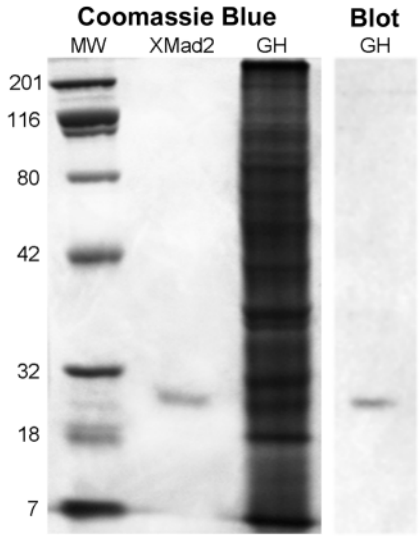

Fig. 2. Mad2 antibodies recognize a protein of approximately 24 kDa on an immunoblot of grasshopper testes. Coomassie blue-stained SDS-PAGE of molecular weight markers (MW), bacterially expressed *Xenopus* Mad2 protein (XMad2), and homogenized grasshopper testes (GH) is shown on the left. A blot of homogenized grasshopper testes labeled with Mad2 antibodies is shown on the right. The blot lane was run on the same gel shown stained on the left (see Materials and Methods).

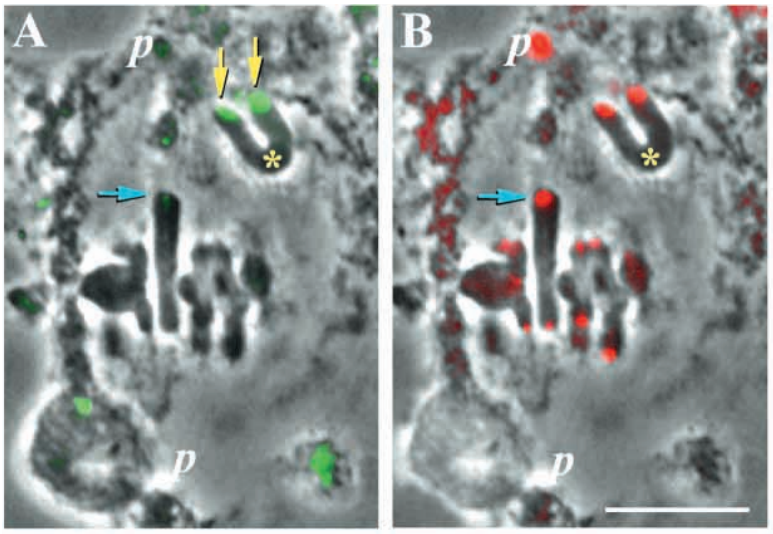

Fig. 3. Mad2 and 3F3/2 phosphoprotein double immunolabeling of an early grasshopper spermatocyte. (A) Mad2 immunofluorescence (green) superimposed on a phase contrast image. (B) 3F3/2 immunofluorescence (red) superimposed on the phase contrast image. In this early cell, one chromosome (asterisk) is improperly attached – both kinetochores (yellow arrows) are attached to the same (upper) pole and other chromosomes have only recently attached as shown by high 3F3/2 brightness of some kinetochores (blue arrow). The upper spindle pole (p) is labeled with 3F3/2 and the approximate position of the lower pole is indicated (p). Bar, 10 μm.

RESULTS

Affinity-purified polyclonal antibodies against Mad2 recognize one protein in grasshopper testes

To determine the specificity and immunoreactivity of our Mad2 antibody (Waters et al., 1998) in meiotic grasshopper cells, we performed western blot analyses of whole grasshopper testes. We found that the antibody recognized one band of similar molecular weight to *Xenopus* Mad2 (Fig. 2).

Mad2 and 3F3/2 during kinetochore attachment to the spindle

Three cells were fixed at about 10 minutes into prometaphase, as kinetochores were attaching to the spindle. They were double labeled with anti-Mad2 and 3F3/2. The fluorescence at the kinetochores was measured and was standardized for each cell by dividing each value by the value for the brightest Mad2 or 3F3/2 labeled kinetochore in that cell. In all, Mad2 and 3F3/2 brightness was measured in 59 kinetochores in the three cells. As in other cell types (Chen et al., 1996; Gorbsky et al., 1998; Howell et al., 2000; Li and Benezra, 1996; Waters et al., 1998; Yu et al., 1999), Mad2 appears on kinetochores as the nuclear envelope breaks down (data not shown) and is lost as chromosomes attach to opposite spindle poles (Fig. 3A). Chromosomes with both kinetochores attached to the same pole ('weak attachment') show very bright Mad2 kinetochore

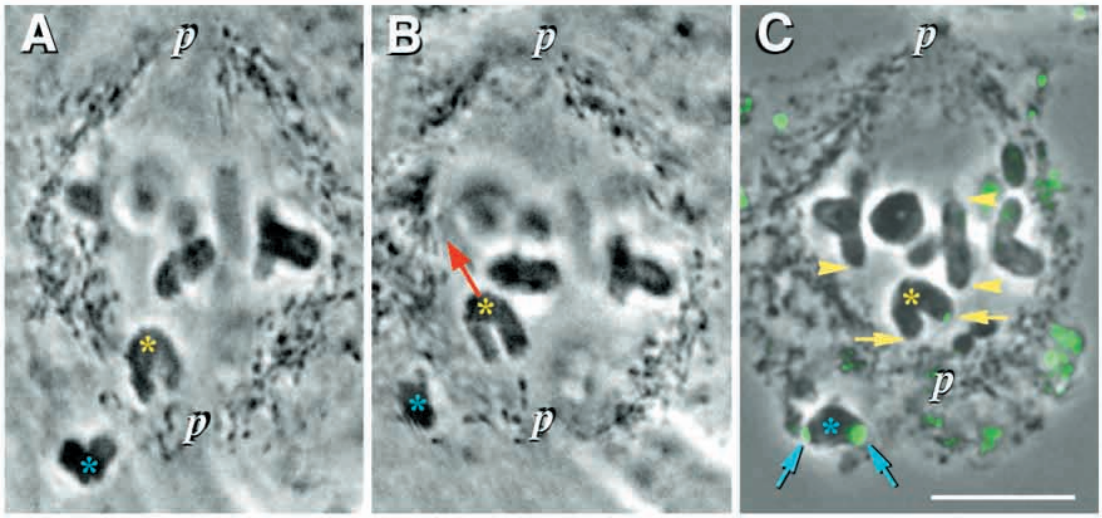

Fig. 4. Detaching kinetochores from microtubules leads to Mad2 binding, and tension from a microneedle leads to Mad2 loss. (A,B) Phase contrast images of the cell in life. The chromosomes labeled with blue and yellow asterisks were detached from the spindle with a micromanipulation needle, moved to the cytoplasm, and kept detached for 10 minutes. The chromosome labeled with the yellow asterisk was then moved so that both kinetochores pointed to the lower pole. After 3 minutes, its kinetochores had attached and the chromosome was pulled away from the pole (red arrow), imposing tension; tension was greater on the left kinetochore than the other one (note that the left arm of the chromosome is more greatly stretched and is thinner). The chromosome was kept under tension for 7 minutes and then the cell was fixed and immunostained. (C) Mad2 immunostaining (green) superimposed on a phase contrast image. The kinetochores of the detached chromosome are brightly labeled (blue arrows), but the kinetochores of the chromosome under tension from the microneedle (yellow arrows) are unlabeled (left) or very lightly labeled (right). The kinetochores of unmanipulated chromosomes, which had been under tension from mitotic forces, are unlabeled (yellow arrowheads). The approximate positions of the spindle poles are labeled 'p'. Bar, 10 μm.

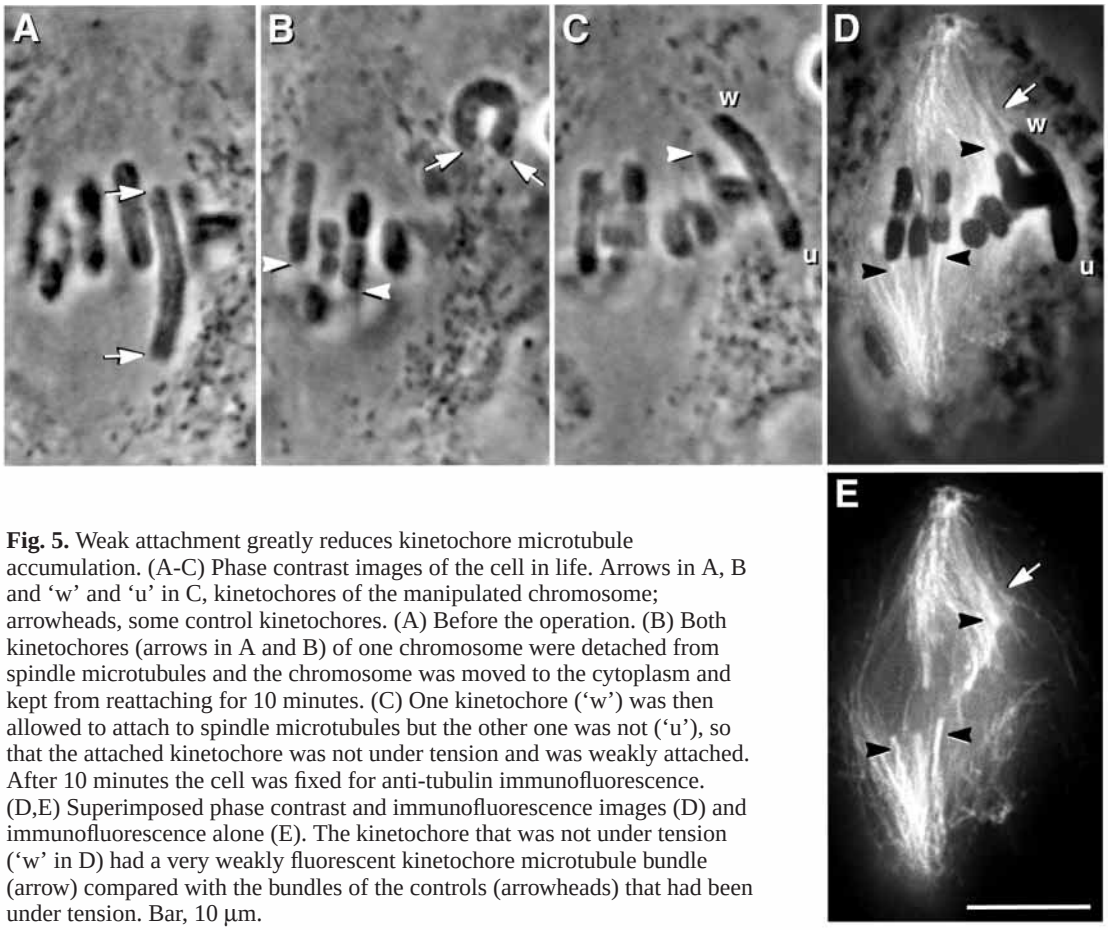

Fig. 5. Weak attachment greatly reduces kinetochore microtubule accumulation. (A-C) Phase contrast images of the cell in life. Arrows in A, B and 'w' and 'u' in C, kinetochores of the manipulated chromosome; arrowheads, some control kinetochores. (A) Before the operation. (B) Both kinetochores (arrows in A and B) of one chromosome were detached from spindle microtubules and the chromosome was moved to the cytoplasm and kept from reattaching for 10 minutes. (C) One kinetochore ('w') was then allowed to attach to spindle microtubules but the other one was not ('u'), so that the attached kinetochore was not under tension and was weakly attached. After 10 minutes the cell was fixed for anti-tubulin immunofluorescence. (D,E) Superimposed phase contrast and immunofluorescence images (D) and immunofluorescence alone (E). The kinetochore that was not under tension ('w' in D) had a very weakly fluorescent kinetochore microtubule bundle (arrow) compared with the bundles of the controls (arrowheads) that had been under tension. Bar, 10 μm.

labeling (Fig. 3A, yellow arrows). Mad2 is not seen at properly attached kinetochores at the spindle equator (Fig. 4C; Fig. 6D; Fig. 7E,F) or on anaphase kinetochores (data not shown). The pattern of kinetochore protein phosphorylation detected with 3F3/2 (Fig. 3B) is consistent with previous observations of grasshopper spermatocytes (Nicklas et al., 1995) and follows the same general pattern seen with Mad2 (Fig. 3A,B): 3F3/2 labeling is very bright in kinetochores of weakly attached chromosomes (Fig. 3B, asterisk) and decreases as chromosomes attach to the spindle, although some still remains at metaphase (Fig. 6C; Fig. 7D). Attachment quickly leads to a great loss of Mad2 and of much of the phosphorylation detected by 3F3/2. However, at the early stage shown in Fig. 3, the loss of phosphorylation often trails the loss of Mad2 – some attached kinetochores still have substantial 3F3/2 brightness but have lost most of their Mad2. The kinetochore identified by blue arrows in Fig. 3 is an example; its Mad2 signal is only 13% as bright as the weakly attached kinetochores but its 3F3/2 signal is 71% as bright.

In the other two early prometaphase cells, all the chromosomes had already attached to both spindle poles. The Mad2 brightness of individual kinetochores has a roughly linear relationship to 3F3/2 brightness in these cells. The correlation coefficient of Mad2 with 3F3/2 is 0.756 for one cell, which is highly significant (P=0.0001), and for the other cell it is 0.542, which is significant at the 1% level (P=0.011). Thus, a majority of kinetochores show roughly equal losses of Mad2 and 3F3/2 phosphorylation. Hence, while the loss of Mad2 may start before dephosphorylation or be faster (as in the kinetochore at the blue arrow in Fig. 3), such an initial period must be soon over.

Mad2 rebinds to kinetochores after they are detached from the spindle and leaves after kinetochores reattach and tension is applied

The effect on Mad2 from the loss of kinetochore microtubules as well as from regaining microtubules and tension can be shown by experiments in a single cell (Fig. 4). Grasshopper spermatocyte chromosomes can be detached from the spindle with a glass microneedle, and their kinetochores lack microtubules (Nicklas and Kubai, 1985). In the cell in Fig. 4, two chromosomes (asterisks) were detached and kept from reattaching for 10 minutes by prodding as necessary with the micromanipulation needle. The repeated prodding does not affect the outcome (King and Nicklas, 2000). One chromosome was still detached when the cell was fixed and immunostained for Mad2 (Fig. 4, blue asterisks). Its kinetochores were brightly stained (Fig. 4C, blue arrows); in the absence of kinetochore microtubules, kinetochores accumulated Mad2. The second detached chromosome (Fig. 4, yellow asterisks) was bent into a U-shape with the microneedle and placed so that both of its kinetochores faced the lower pole (Fig. 4A). The kinetochores were allowed to attach for 3 minutes, and then tension was applied (Fig. 4B, red arrow) by pulling the chromosome away from the spindle pole to which it was attached. By pulling slightly to one side, the kinetochore on the left arm was placed

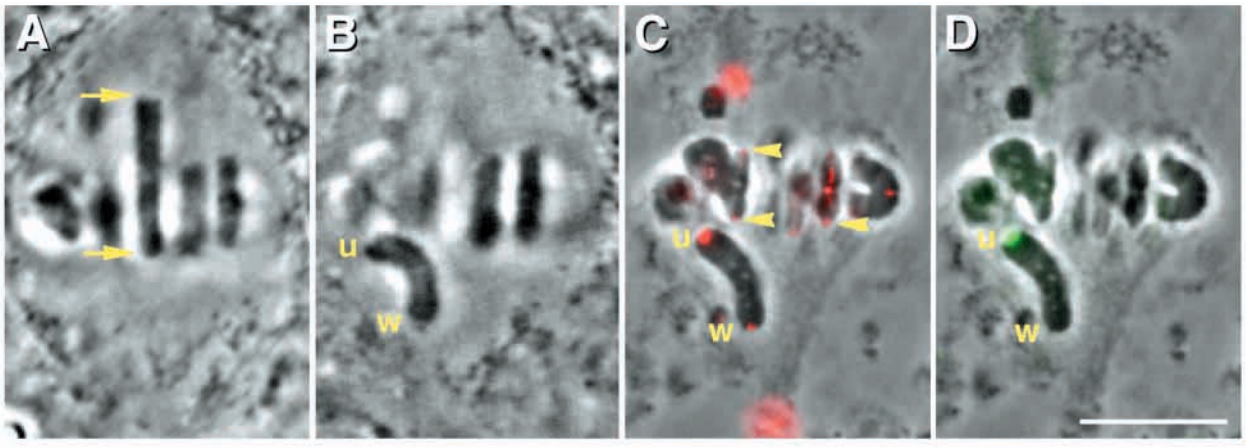

Fig. 6. Weak attachment can inhibit kinetochore phosphorylation and Mad2 binding. (A,B) Phase contrast images of the cell in life. One kinetochore (upper arrow in A, 'u' thereafter) of a metaphase chromosome was detached from the spindle and kept detached for 10 minutes, while the other kinetochore (lower arrow in A and 'w' thereafter) remained weakly attached in the absence of tension. (C,D) The cell after fixation and immunostaining for 3F3/2 (C, red) and Mad2 (D, green). In both C and D the unattached kinetochore (u) is bright – it is highly phosphorylated and loaded with Mad2. The weakly attached kinetochore (w) is dim, as are the kinetochores of unmanipulated chromosomes (yellow arrowheads), which had been strongly attached. Bar, 10 μm.

under greater tension than its partner on the right (Fig. 4B: note that the left arm is thinner, more pulled out). After 7 minutes of tension the cell was fixed and immunostained. The kinetochore under greater tension (Fig. 4C, left yellow arrow) showed no Mad2 label, like the kinetochores of the unmanipulated chromosomes (yellow arrowheads), which were under tension from mitotic forces. The kinetochore under less tension was dimly labeled (Fig. 4C, right yellow arrow), very dim compared with the kinetochores of the detached chromosome (blue arrows).

We examined a total of 54 kinetochores of unattached chromosomes and found that all 54 were labeled with Mad2 antibody. Most (91%) labeled brightly (such as those at the blue arrows in Fig. 4C), while the rest (9%) labeled dimly. Of 15 kinetochores fixed after reattachment and 7 minutes under tension, 60% were dimly labeled (such as the one at the right yellow arrow, Fig. 4C), 27% were unlabeled (Fig. 4C, left yellow arrow), whereas 13% labeled as brightly as unattached, control kinetochores of detached chromosomes.

Clearly, the loss of kinetochore microtubules leads to the rebinding of Mad2. Allowing such kinetochores to reattach to microtubules and then mimicking the effect of mitotic forces by applying tension for 7 minutes leads to loss of most of this Mad2.

Weak attachment: the effects of kinetochore attachment in the absence of tension

Weak attachment delays the onset of anaphase, but not indefinitely

Grasshopper spermatocytes occur in cysts of cells that progress quite synchronously through the cell cycle, so that a delay in anaphase is easily detected (Nicklas et al., 1995). We micromanipulated one cell in a group of cells in late prometaphase or metaphase and used the rest of the cells as controls (the number of control cells varied from 3 to 12, with an average of 7). In the manipulated cell, a chromosome was detached from the spindle with a microneedle and bent so that both kinetochores faced the same pole. The kinetochores of such chromosomes promptly attach to that pole, leading to weak attachment just like the naturally occurring example at the asterisk in Fig. 3. One or the other of the weakly attached kinetochores makes a new attachment to the opposite pole in a few minutes (Nicklas and Ward, 1994). When that happened we detached that kinetochore before its movement led to tension and pointed it back at the original pole, maintaining the weak attachment of both kinetochores. We kept enforcing the weak attachment until anaphase intervened or until we could no longer reach the chromosome (repeated manipulation gradually pushes them deeper into the cell). In seven experiments, anaphase in the manipulated cell was delayed by an average of 117 minutes (range, 36-219 minutes) from the time the last control cell entered anaphase. The delay is not due to general perturbation of the spindle by the micromanipulation needle since even the most vigorous, continuous movements of a needle within a spindle do not delay the onset of anaphase. The delay compared to control cells is highly significant statistically (two tailed *t*-test, paired values: the hypothesis of equal means is rejected, with P=0.002). The delay does not last forever, since three of the cells entered anaphase with the weakly attached chromosome still present.

Attachment in the absence of tension results in kinetochores with few microtubules

We can induce weak kinetochore attachments in two ways, by starting with a kinetochore that has many microtubules and relieving the tension or by starting with a kinetochore that has no kinetochore microtubules and preventing tension from occurring. It is already known that kinetochores that start with many microtubules suffer a dramatic decrease after tension is relieved (King and Nicklas, 2000), but how an absence of tension affects the acquisition of microtubules by bare kinetochores was not known. We determined this by detaching a chromosome from the spindle and keeping it detached for 10 minutes (Fig. 5A,B) so that neither partner kinetochore had any microtubules. We then allowed one of the kinetochores to attach to the spindle weakly (Fig. 5C, 'w') while the other

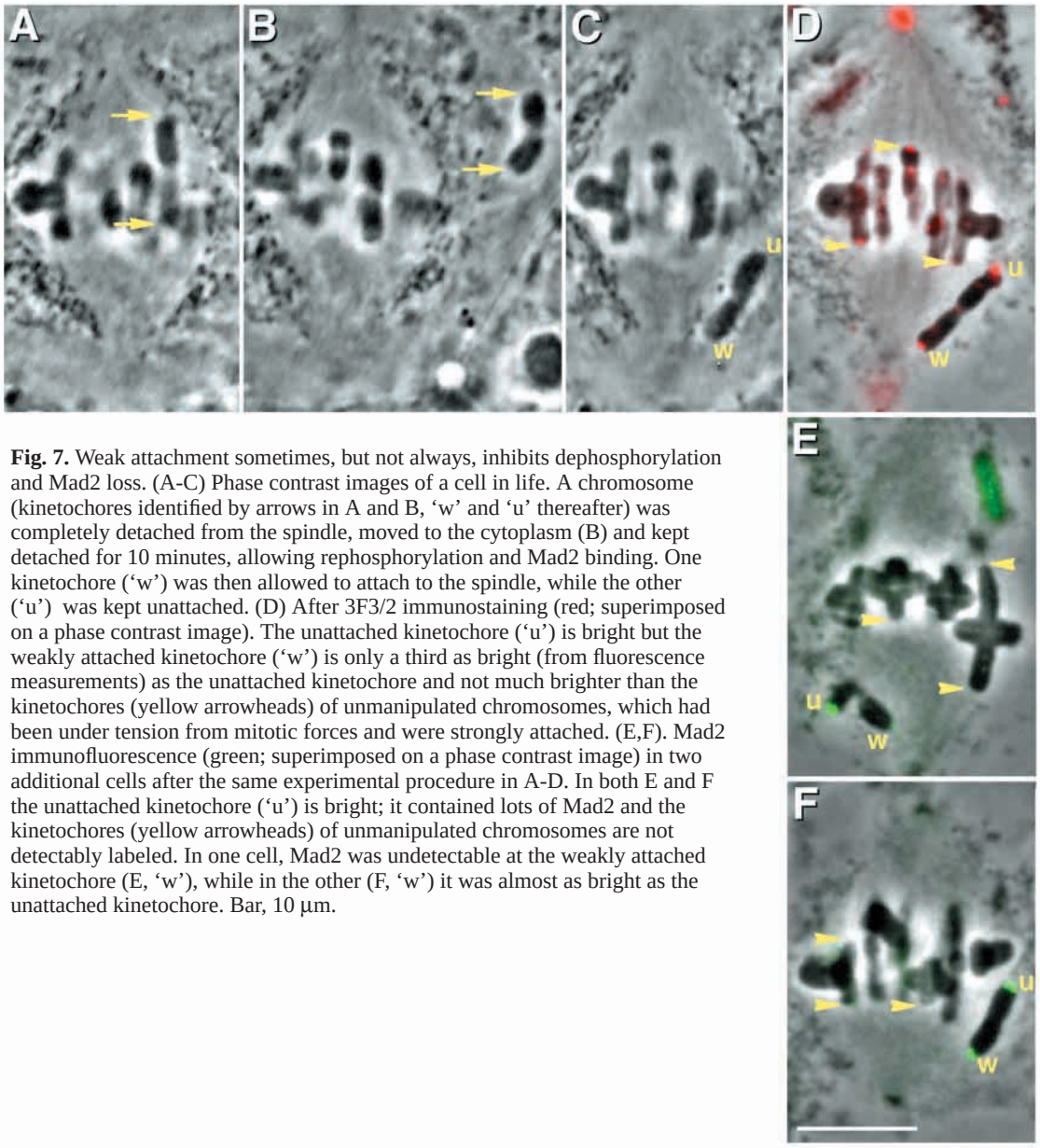

Fig. 7. Weak attachment sometimes, but not always, inhibits dephosphorylation and Mad2 loss. (A-C) Phase contrast images of a cell in life. A chromosome (kinetochores identified by arrows in A and B, 'w' and 'u' thereafter) was completely detached from the spindle, moved to the cytoplasm (B) and kept detached for 10 minutes, allowing rephosphorylation and Mad2 binding. One kinetochore ('w') was then allowed to attach to the spindle, while the other ('u') was kept unattached. (D) After 3F3/2 immunostaining (red; superimposed on a phase contrast image). The unattached kinetochore ('u') is bright but the weakly attached kinetochore ('w') is only a third as bright (from fluorescence measurements) as the unattached kinetochore and not much brighter than the kinetochores (yellow arrowheads) of unmanipulated chromosomes, which had been under tension from mitotic forces and were strongly attached. (E,F). Mad2 immunofluorescence (green; superimposed on a phase contrast image) in two additional cells after the same experimental procedure in A-D. In both E and F the unattached kinetochore ('u') is bright; it contained lots of Mad2 and the kinetochores (yellow arrowheads) of unmanipulated chromosomes are not detectably labeled. In one cell, Mad2 was undetectable at the weakly attached kinetochore (E, 'w'), while in the other (F, 'w') it was almost as bright as the unattached kinetochore. Bar, 10 μm.

kinetochore remained unattached (Fig. 5C, 'u'), so that no tension was present. After 10 minutes, the cell was fixed and immunostained for microtubules. Thick, brightly fluorescent bundles of microtubules were seen at the control kinetochores of chromosomes that were attached to opposite poles and had been under tension (arrowheads, Fig. 5D,E), but the kinetochore that attached in the absence of tension had only a thin, wispy kinetochore microtubule bundle (Fig. 5D,E, arrow). The fluorescence of a 1 μm length of the weakly attached kinetochore's microtubule bundle was 32% of the average fluorescence of five control kinetochores. Similar results were obtained in a total of six experiments. The fluorescence of the weakly attached kinetochore's microtubules averaged only 26% as great as the controls (range: 8-36%) and the reduction is highly significant statistically (two tailed *t*-test, paired values: the hypothesis of equal means is rejected, with P=0.005). From direct comparisons with kinetochore microtubule counts by electron microscopy, we know that the measured fluorescence faithfully reflects kinetochore microtubule number (King and Nicklas, 2000). We therefore conclude that whether the starting point is many kinetochore microtubules or no kinetochore microtubules, an absence of tension results in dramatically fewer kinetochore microtubules compared to kinetochores under tension.

Mad2 and phosphorylation gain and loss at weakly attached kinetochores

Micromanipulation permits the gain and loss of attachment-sensitive kinetochore properties to be cleanly separated and studied independently. In all these experiments, one kinetochore of a chromosome is weakly attached, while the other is unattached, free of kinetochore microtubules. We always have controls of two sorts. The unattached kinetochore provides a standard for the brightness of Mad2 and 3F3/2 immunofluorescence in the absence of kinetochore

Table 2. Weak attachment and the gain or loss of Mad2 and phosphorylation

	Gaining Mad2 or phosphorylation*		Losing Mad2 or phosphorylation‡	
	Mad2	3F3/2	Mad2	3F3/2
Brightly labeled§	0%	67%	30%	45%
Mean±s.d.		0.71±0.16	0.80±0.15	1.09±0.41
Range		0.53-0.93	0.60-0.99	0.51-1.83
Dimly labeled¶	50%	33%	50%	55%
Mean±s.d.	0.26±0.15	0.28±0.05	0.28±0.12	0.41±0.06
Range	0.13-0.45	0.24-0.34	0.15-0.45	0.30-0.49
Not labeled**	50%	0%	20%	0%
n	10	9	20	20

*Percentage of kinetochores labeled compared with unattached control kinetochores. One kinetochore of a bivalent is detached from the spindle - this is the control; its partner remains attached but is not under tension - will it accumulate Mad2 and be rephosphorylated?

‡Percentage of kinetochores labeled compared with unattached control kinetochores. Both kinetochores of a bivalent are detached from the spindle and kept detached for 10 minutes; one kinetochore is then allowed to reattach, the other serves as the control. Will a few kinetochore microtubules at the reattached kinetochore lead to the loss of Mad2 and phosphorylation? More experiments were done in this category because of its special relevance to normal events and to understanding how Mad2 binding and phosphorylation are determined.

§More than half as bright as an unattached kinetochore in the same cell.

¶Less than half as bright as an unattached kinetochore in the same cell.

**Brightness not above background.

microtubules, while the strongly attached kinetochores in the same cell provide a control for the starting condition: kinetochores under tension from mitotic forces that are expected to have large numbers of kinetochore microtubules, no Mad2 and minimal phosphorylation.

The gain of Mad2 and phosphorylation was studied by starting with a properly attached chromosome in late prometaphase or metaphase (Fig. 6A), whose kinetochores would contain no Mad2 and would be minimally phosphorylated. One kinetochore of the chromosome was detached from its kinetochore microtubules (Fig. 6B, ‘u’) and was kept unattached by occasional nudges with the manipulation needle. The partner kinetochore (Fig. 6B, ‘w’) remained attached to the spindle but was no longer under tension because opposed poleward pulling forces are lost when one kinetochore is unattached. The absence of tension produces a visible shortening of the chromosome [see Fig. 2A of (King and Nicklas, 2000)]. After 10 minutes the cell was fixed and immunostained with either the Mad2 antibody, 3F3/2 or both (single or double staining gives the same results). In this example, the weakly attached kinetochore was dimly labeled by 3F3/2 (Fig. 6C, ‘w’); measurements show that it was only 24% as bright as the unattached kinetochore (the unattached kinetochore was 6.3 times as bright as properly attached kinetochores, whereas the weakly attached kinetochore was 1.5 times as bright). The weakly attached kinetochore was not labeled by Mad2 antibodies (Fig. 6D, ‘w’). The unattached kinetochore was brightly labeled for both 3F3/2 and Mad2 (Fig. 6C,D, ‘u’). Thus, weak attachment prevented Mad2 binding and greatly reduced kinetochore phosphorylation, in this instance.

Results from ten experiments show that the rebinding of Mad2 was greatly inhibited by weak attachment: half the kinetochores were dimly labeled, half were unlabeled (Fig. 6D), and no kinetochores were labeled as brightly as unattached controls (Table 2). Rephosphorylation was sometimes strongly affected by weak attachment, as in Fig. 6C, but more often it was less affected: 67% of nine kinetochores were at least half as bright as unattached controls (Table 2). The difference between Mad2 binding and rephosphorylation at weakly attached kinetochores is significant statistically (Mann-Whitney W test; P=0.032 for the null hypothesis of no difference between the medians).

A second experimental design allows the loss of Mad2 and phosphorylation to be studied. Here again, we began with a properly attached late prometaphase or metaphase chromosome, but in this set of experiments we detached both kinetochores and kept them detached for 10 minutes (Fig. 7A,B), allowing them to rebind maximal amounts of Mad2 and to become fully rephosphorylated. Then, one kinetochore of the chromosome was allowed to reattach to the spindle (Fig. 7C, ‘w’) while its partner was kept unattached (Fig. 7C, ‘u’), so that the reattached kinetochore was not under tension. This produced a weakly attached kinetochore plus an unattached, control kinetochore. After 10 minutes the cell was fixed and immunostained with 3F3/2 (Fig. 7D) or with Mad2 antibodies (Fig. 7E,F; different cells) or was double-stained. In this instance, phosphorylation (Fig. 7D) was greatly affected by weak attachment; the weakly attached kinetochore (‘w’) measured only 31% as bright as the unattached kinetochore (‘u’) and not much brighter than the kinetochores of properly attached chromosomes (arrowheads). Sometimes this is also true for Mad2 (Fig. 7E; the brightness of the weakly attached kinetochore ‘w’ is unmeasurably low, like the properly attached kinetochores (arrowheads), while the unattached kinetochore ‘u’ is very bright). Often, however, Mad2 is less affected by weak attachment (Fig. 7F; the weakly attached kinetochore ‘w’ measured 80% as bright as the unattached kinetochore ‘u’).

The loss of Mad2 and phosphorylation after weak attachment is variable. In 20 experiments, a third to a half of the kinetochores were as bright as the unattached kinetochore in the same cell; evidently they lost little label as a consequence of weak attachment (Table 2, right). The majority of kinetochores, however, were affected since they were dimly labeled or unlabeled (Table 2, right). Importantly, almost all weakly attached kinetochores lost some Mad2 or phosphorylation by comparison with the controls: 90% of Mad2-labeled kinetochores and 80% of 3F3/2 kinetochores were at least 10% less bright than the unattached kinetochore. Mad2 loss and dephosphorylation were similarly affected by weak attachment (the difference between the medians is

statistically insignificant, with P=0.074 for the Mann-Whitney W test). The variability is of interest in itself: 10 minutes of weak attachment does not reliably reduce Mad2 and phosphorylation to the levels seen after proper attachment and tension are present.

DISCUSSION

Mad2 is bound to unattached kinetochores and is lost after strong attachment

As others have shown for both mitosis and meiosis, kinetochores in grasshopper meiosis that are not attached to the spindle have abundant Mad2 (Chen et al., 1996; Waters et al., 1998; Yu et al., 1999). We show this experimentally without using drugs by detaching chromosomes from the spindle with a micromanipulation needle (Fig. 4). The spindle checkpoint is activated when unattached kinetochores are present, and Mad2 is an essential player (Rieder and Salmon, 1998; Shah and Cleveland, 2000). As expected, the presence of Mad2 on kinetochores of chromosomes detached by micromanipulation is associated with the inhibition of anaphase; the inhibition lasts as long as the chromosome is kept unattached (Nicklas et al., 1995).

Mad2 is lost from grasshopper spermatocyte kinetochores as they become properly attached, as in other cells in mitosis and meiosis (Chen et al., 1996; Waters et al., 1998; Yu et al., 1999). In spermatocytes, this strong attachment state can be induced experimentally at a weakly attached kinetochore by pulling the chromosome with a micromanipulation needle, imposing tension; the result is the loss of Mad2 from most kinetochores (Fig. 4).

Weak attachment: the gain and loss of Mad2 and phosphorylation in the absence of tension

Weak attachment is an important intermediate state in both mitosis and meiosis (Fig. 1). Micromanipulation permitted us to induce weak attachment under controlled conditions, so that the gain and loss of Mad2 and phosphorylation could be separately characterized for the first time. Moreover, since tension is lacking on weakly attached kinetochores in spermatocytes, the effect of microtubule attachment itself, uncomplicated by tension, could be studied.

We studied Mad2 and phosphorylation gain by starting with kinetochores depleted of Mad2 and minimally phosphorylated and then creating a weak attachment (Fig. 6). Such kinetochores have about 40% as many kinetochore microtubules as strongly attached kinetochores (King and Nicklas, 2000). The weak attachment greatly inhibited the recruitment of Mad2 compared with unattached kinetochores (Fig. 6D). Rephosphorylation, however, often was comparable to unattached kinetochores and most kinetochores became brightly labeled (Table 2, left; an exception is shown in Fig. 6C). This result from insect cells in meiosis parallels the findings from taxol-treated mammalian cells in mitosis (Waters et al., 1998), in which the presence of kinetochore microtubules in the absence of tension does not inhibit phosphorylation but generally prevents Mad2 binding.

It should be noted that regaining Mad2 and rephosphorylation at kinetochores that once had achieved proper, strong attachment is very rare in normal, unaltered cells. Properly attached kinetochores are generally under tension (Waters et al., 1996b), and attachment rarely lapses long enough for Mad2 to be recruited or for rephosphorylation to occur. The manipulation and taxol experiments certainly tell us something worth knowing about attachment and kinetochore molecular dynamics, but after kinetochore microtubules have been bound, it is the regulation of loss, not gain, that is important for normal cell life.

We studied the loss of Mad2 and phosphorylation by starting with kinetochores that had much Mad2 and were highly phosphorylated and then creating a weak attachment (Fig. 7). The number of kinetochore microtubules in weak attachments created in this way had not been determined previously. We found that kinetochores in such an attachment have on average only 26% as much kinetochore microtubule immunofluorescence as strongly attached kinetochores (Table 1; Fig. 5). Immunofluorescence measurements correlate perfectly with direct microtubule counts by electron microscopy (King and Nicklas, 2000), so the 26% fluorescence value corresponds to only 6-10 kinetochore microtubules in cells at mid-prometaphase to metaphase, when 23-37 microtubules are found at strongly attached kinetochores (Nicklas and Gordon, 1985; Nicklas and Kubai, 1985). Moreover, these are average microtubule numbers and the number must often be less, even zero, since weak attachments often lapse altogether (Nicklas and Ward, 1994). These few kinetochore microtubules suffice to promote a loss of Mad2 and decreased phosphorylation in more than half of the weakly attached kinetochores (Table 2, right). This result differs from what is seen very early in prometaphase, when naturally occurring weak attachments are associated with high amounts Mad2 and maximal phosphorylation (Fig. 3). The stage of mitosis probably accounts for the difference. Mad2 and phosphorylation may often be lost in later stages because weak attachment then is characterized by a higher number of kinetochore microtubules than at the start of mitosis. During the first 30 minutes of prometaphase, even strongly attached chromosomes have no more than 10 kinetochore microtubules (polarization and immunofluorescence microscopy, observations not shown). A weakly attached kinetochore at that time would have very few microtubules indeed, too few, evidently, to affect Mad2 and phosphorylation.

Mechanisms of Mad2 and phosphorylation dynamics: the tension and microtubule connection

The effect of a few kinetochore microtubules on phosphorylation is surprising in view of earlier results that dephosphorylation requires tension (Nicklas et al., 1995). Two facts still favor tension in the regulation of dephosphorylation: (1) many kinetochores remain highly phosphorylated in the absence of tension (Table 2, right column); and (2) in taxol-treated mammalian cells, kinetochores become rephosphorylated when tension is absent but a complete complement of microtubules is present (Waters et al., 1998). It is easy to imagine how tension could directly regulate phosphorylation by deforming the kinase, the phosphatase or the substrate, all of which are in the kinetochore (Bousbaa et al., 1997; Nicklas et al., 1998). Nevertheless, it is inescapable that over half of the phosphorylated kinetochores in Table 2 (right) became dephosphorylated in the absence of tension and 80% had some reduction in phosphorylation. How can this be

possible if dephosphorylation requires that the substrate or whatever must be deformed or otherwise directly modified by tension? We conclude that dephosphorylation, just like the loss of Mad2 (Waters et al., 1998) (this report), is promoted by kinetochore microtubule occupancy. The conflict with the conclusion from the taxol experiments favoring tension is not serious, since those experiments concern the conditions for gaining phosphorylation, rather than losing it as in the manipulation experiments under discussion.

So microtubule occupancy at the kinetochore promotes dephosphorylation and Mad2 loss. But this does not mean that tension plays no role. Tension is absolutely necessary in grasshopper spermatocytes for a full set of kinetochore microtubules (King and Nicklas, 2000), and the complete loss of Mad2 and the full extent of dephosphorylation are seen only when tension and the higher number of kinetochore microtubules associated with tension are present. Sending a reliable 'all clear' signal to the checkpoint thus depends on tension. Very likely this is also true in mammalian cells in mitosis. It is true that a single weakly attached kinetochore in mitosis (Fig. 1A, lowest chromosome) has greatly diminished Mad2 (Chen et al., 1996; Howell et al., 2000; Waters et al., 1998) and does not send an effective 'wait' signal (Rieder et al., 1995), but that kinetochore in fact is under tension from antipoleward arm forces (Fig. 1A). Significantly, the final, complete loss of Mad2 is associated with bipolar attachment, when kinetochores are under tension from opposed poleward forces and have an increased number of kinetochore microtubules (McEwen et al., 1997). When the tension is relieved by taxol at such kinetochores, they retain their full complement of microtubules but do not bind Mad2, leading to the conclusion that Mad2 localization to kinetochores depends on microtubule attachment, not tension (Waters et al., 1998). That conclusion is certainly valid – tension does not directly affect Mad2. But indirectly it certainly does, if tension is essential to achieve the state examined in the taxol experiments – a kinetochore fully loaded with microtubules.

In conclusion, in grasshopper spermatocytes and probably in many other cells, kinetochore microtubule occupancy determines both phosphorylation status and the binding of such transitory components as Mad2. Nevertheless, tension also plays an essential role because tension from one source or another is necessary for the acquisition of a full set of kinetochore microtubules. This in turn is necessary for the reliable and complete loss of Mad2 and protein dephosphorylation and a dependable 'proceed to anaphase' signal to the checkpoint.

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