Microinjection of Antibody to Mad2 Protein into Mammalian Cells in Mitosis Induces Premature Anaphase

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Abstract. In yeast, the Mad2 protein is required for the M phase arrest induced by microtubule inhibitors, but the protein is not essential under normal culture conditions. We tested whether the Mad2 protein participates in regulating the timing of anaphase onset in mammalian cells in the absence of microtubule drugs. When microinjected into living prophase or prometaphase PtK1 cells, anti-Mad2 antibody induced the onset of anaphase prematurely during prometaphase, before the chromosomes had assembled at the metaphase plate. Anti-Mad2 antibody-injected cells completed all aspects of anaphase including chromatid movement to the spindle poles and pole–pole separation. Identical results were obtained when primary human keratinocytes were injected with anti-Mad2 antibody. These studies suggest that Mad2 protein function is essential for the timing of anaphase onset in somatic cells at each mitosis. Thus, in mammalian somatic cells, the spindle checkpoint appears to be a component of the timing mechanism for normal mitosis, blocking anaphase onset until all chromosomes are aligned at the metaphase plate.

In cell division, the gain or loss of a single chromosome is catastrophic, causing genetic imbalances that can lead to cell death or unrestrained cell growth. Cells have evolved mechanisms to promote equal partitioning of the chromosomes to the two daughter cells. In mitosis, the two chromatids of each chromosome first attach to microtubules emanating from opposite poles. In vertebrate cells, the chromosomes assemble at the metaphase plate. Subsequently, a simultaneous splitting of all the chromosomes within a cell occurs, and the chromatids move to the poles while the poles separate from each other. To ensure that each daughter cell receives one set of chromatids, anaphase must not initiate prematurely, before each chromosome has stable bipolar attachment to the two spindle poles. Theoretically, anaphase onset might be simply be timed to occur after some prior event, such as nuclear envelope breakdown. Alternatively, surveillance mechanisms, called cell cycle checkpoints, might be used to assess readiness for anaphase onset by monitoring aspects of chromosome assembly on the mitotic spindle.

Substantial evidence suggests that a cell cycle checkpoint does play an important role in regulating anaphase onset. This spindle checkpoint (which has been called the spindle assembly checkpoint, the metaphase checkpoint, the chromosome distribution checkpoint, and the kinetochore attachment checkpoint) appears to monitor the interaction of chromosomes with the spindle microtubules. Early studies in plants and newt cells suggested that chromosomes that lagged in their attachment to the spindle caused a delay in anaphase onset (Bajer and Mole-Bajer, 1956; Zirkle, 1970). Thus, chromosomes that fail to attach properly to the spindle appear to inhibit, at least temporarily, the segregation of the chromatids at anaphase. This delay provides a longer opportunity for misplaced or monooriented chromosomes to achieve bipolar attachment and align on the mitotic spindle. Genetic studies in yeast (Spencer and Hieter, 1992; Neff and Burke, 1993; Wells and Murray, 1996) micromanipulation experiments in insect spermatocytes in meiosis (Li and Nicklas, 1995; Zhang and Nicklas, 1996) and laser ablation studies in mammalian cells (Rieder et al., 1995) have led to the conclusion that the inhibitory checkpoint signal blocking cell cycle progression before anaphase originates at kinetochores that are unattached or improperly attached to mitotic spindle microtubules. Immunolabeling studies with the 3F3/2 monoclonal antibody demonstrated that the kinetochores of noncongressed chromosomes were biochemically distinct, containing phosphoepitopes that were dephosphorylated as the chromosomes aligned at the metaphase plate (Gorbsky and Ricketts, 1993). This same antibody used for microinjection studies in mammalian cells (Camp-

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bell and Gorbsky, 1995) and micromanipulation experiments in insect cells (Nicklas et al., 1995; Li and Nicklas, 1997) showed that dephosphorylation of this kinetochore phosphoepitope correlated with a shut-down of the checkpoint signal.

The function of the spindle checkpoint is most evident when cells are treated with drugs that induce microtubule disassembly, thus disrupting the mitotic spindle. In response to microtubule drugs, most cells arrest in M phase, at least temporarily. To identify genes that might code for the signaling components involved, yeast mutants were screened for those that failed to delay in M phase in response to microtubule inhibitors. Li and Murray (1991) identified three genes called *MAD* for "mitotic arrest deficient,"[1] and Hoyt et al. (1991) identified three distinct genes termed *BUB* for "budding uninhibited by benzimidazole." A mouse homologue of the yeast gene *BUB1* was recently identified (Taylor and McKeon, 1997). Immunolabeling showed that the murine Bub1 protein localized to kinetochores of chromosomes before congression. When the NH_2-terminal region of the Bub1 protein was expressed in HeLa cells, it localized to kinetochores and diminished the ability of the cells to arrest in M phase when treated with nocodazole. This evidence suggests that the NH_2-terminal Bub1 protein fragment acted as a partial dominant negative and that mammalian Bub1 protein plays a role in cell cycle arrest in response to spindle disruption. In other experiments, cells were released from a drug-induced S phase block and examined at subsequent time points with fluorescence-activated cell sorting. In the absence of microtubule drugs, cells expressing high levels of the NH_2-terminal fragment of Bub1 were found to enter the next G1 period ~25 min earlier than controls. This result is consistent with the hypothesis that mammalian Bub1 protein participates in regulating the timing of normal mitosis. Vertebrate homologues of one of the yeast *MAD* genes, *MAD2*, were identified in *Xenopus* and human (Chen et al., 1996; Li and Benezra, 1996). In tissue culture cells, a portion of cellular Mad2 protein associated with the kinetochores of chromosomes in early prometaphase and in cells treated with antimicrotubule drugs. Anti-Mad2 antibody, electroporated into HeLa cells, caused cells arrested in M phase with microtubule drugs to decondense their chromosomes and reform interphase nuclei. Similarly, the addition of anti-Mad2 antibody also overcame the M phase arrest induced by nocodazole treatment of *Xenopus* egg extracts containing a high concentration of sperm nuclei.

The target of the spindle checkpoint appears to be a ubiquitin ligase called the cyclosome or anaphase-promoting complex (APC) (Irniger et al., 1995; King et al., 1995; Sudakin et al., 1995; Yamada et al., 1997). In M phase, the APC targets proteins whose destruction by ubiquitin-mediated proteolysis is necessary for anaphase onset and for exit from mitosis. Abrogating APC function in mammalian cells by microinjection of antibody to the Cdc27 component protein caused cells to arrest at metaphase (Tugendreich et al., 1995). In mammalian cell extracts, Mad2 protein coimmunoprecipitated with the APC, and the addition of exogenous Mad2 protein to cycling *Xenopus* extracts inhibited its ability to ubiquitinylate cyclin B substrate (Li et al, 1997). In *Schizosaccharomyces pombe*, overexpression of the Mad2 protein also arrests cells at metaphase (He et al., 1997). These studies show that the Mad2 protein is important in the M phase arrest of budding yeast, fission yeast, and mammalian cells in response to microtubule inhibitors. In both budding yeast and fission yeast, strains deleted of the *MAD2* gene died if treated with microtubule drugs. However, although they exhibited increased frequency of chromosome loss, yeast mutants were viable under normal culture conditions. The finding that the *MAD2* gene is not essential in budding and fission yeast for successful chromosome segregation suggests that both yeasts and perhaps other organisms rely more heavily on simple timing mechanisms, rather than checkpoints, to regulate anaphase onset under normal circumstances, in the absence of challenge with microtubule drugs or other conditions causing spindle defects (for review see Cohen-Fix and Koshland, 1997).

In this study we tested directly whether the Mad2 protein is or is not important in regulating anaphase onset in the absence of spindle defects in mammalian cells, both with cultured cell lines and primary cell cultures. Microinjection of anti-Mad2 antibody into mammalian cells, in the absence of drug treatment, led to premature anaphase and mitotic catastrophe. This evidence suggests that the Mad2 protein, and the checkpoint signaling pathway of which it is a component, are invoked in each cell cycle in mammalian somatic cells to regulate the proper segregation of the chromosomes in mitosis.

Materials and Methods

Cell Culture

Ptk1 (rat kangaroo kidney) cells were cultured in Minimal Essential Medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 20 mM Hepes buffer, 1× nonessential amino acids, 1 mM sodium pyruvate, 60 μg/ml penicillin, and 100 μg/ml streptomycin. LLC-Pk (porcine kidney) cells were grown in Dulbecco's Modified Minimal Essential Medium with 20 mM Hepes, 10% fetal bovine serum, 60 μg/ml penicillin, and 100 μg/ml streptomycin. Normal human epidermal keratinocyte cells were obtained from Clonectics Corp. (San Diego, CA) as frozen primary cultures from foreskin. Keratinocytes were grown in Defined Keratinocyte-SFM medium (GIBCO BRL) with 60 μg/ml penicillin and 100 μg/ml streptomycin.

Immunoblotting

For immunoblotting, cultured Ptk1 and LLC-Pk cells were resuspended from tissue culture plates, washed in PBS, counted, and pelleted by centrifugation. Cell pellets were suspended in denaturing sample buffer (125 mM Tris-HCl, pH 6.8, 1% SDS, 20% glycerol, 5% 2-mercaptoethanol, 100 mg/liter Pyronin Y) and heated at 95°C for 5 min. Proteins were separated by electrophoresis in 5–20% gradient SDS–polyacrylamide gels and transferred to 0.45-μm Immobilon-P membranes (Millipore Corp., Bedford, MA). Blots were blocked in 5% BSA (Amresco, Solon, OH) in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20 (TBST) for 30 min. Blots were incubated with affinity-purified rabbit anti-Mad2 antibody (Chen et al., 1996) at 100 ng/ml in TBST for 1.5 h. After washing with TBST, blots were incubated with HRP-conjugated goat anti–rabbit antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in TBST for 1 h and then washed again with TBST. Blots were incubated with chemiluminescence substrate (Renaissance Kit; Dupont-NEN, Boston,

1. *Abbreviations used in this paper*: APC, anaphase-promoting complex; *BUB*, budding uninhibited by benzimidazole; *MAD*, mitotic arrest deficient.

MA), and immunolabeled proteins were visualized after exposure to autoradiography film.

Immunofluorescence

For immunofluorescence, cells were grown on 22-mm^2 No. 1.5 coverslips. When grown to appropriate density, cells on coverslips were rinsed twice at room temperature with 60 mM Pipes, 25 mM Hepes, pH 6.9, 10 mM EGTA, 4 mM $MgSO_4$ (PHEM) and lysed with 0.5% CHAPS detergent in PHEM containing 100 nM microcystin-LR. After 5 min, the cells were fixed for 15 min with 1.0% freshly prepared formaldehyde in PHEM. Cells were rinsed three times with 10 mM MOPS, pH 7.2, 150 mM NaCl containing 0.05% Tween-20 (MBST). For comparison, some cells were fixed before extraction with detergent. Before labeling with primary antibody, cells were blocked with 20% boiled normal goat serum in 10 mM MOPS, pH 7.2, 150 mM NaCl (MBS). Cells were labeled for 45 min at room temperature with affinity-purified rabbit anti-Mad2 antibody at 0.4 μg/ml in 5% boiled normal goat serum. Some cells were colabeled with human scleroderma autoimmune sera (gifts from Dr. J.B. Rattner, University of Calgary, Calgary, Canada, and Dr. B. Brinkley, Baylor College of Medicine, Houston, TX) at a 1:500 dilution. Cells were then rinsed in MBST and washed for 15 min in the same buffer. Secondary fluorescent antibodies were applied at room temperature for 45 min, and then the coverslips were washed once more in MBST. Cells were labeled with the DNA dye 4,6-diamidino-2-phenylindole (DAPI) at 0.1 μg/ml in water for 5 min. The coverslips were rinsed three times with distilled H_2O and mounted on slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) that had been supplemented with $MgSO_4$ to a final concentration of 10 mM. The coverslips were then sealed with nail polish. Cells were imaged with a Nikon Diaphot microscope (Melville, NY) using a 60×, 1.4 NA planapochromat objective and recorded with a Dage Genisys image intensifier and Dage CCD72 video camera (Dage-MTI, Michigan City, IN) on a microcomputer by means of Image 1 video imaging software (Universal Imaging, Media, PA). An out-of-focus background image was subtracted from each fluorescent image to remove imperfections in the illumination pathway. Some images were captured with a Sensys cooled digital CCD camera (Photometrics, Tucson, AZ) using Metamorph software (Universal Imaging). Images were adjusted for contrast and brightness with Photoshop 4.0 (Adobe Systems Inc., San Jose, CA). Images were imported to Coreldraw 6.0 (Ottawa, Ontario) for annotation and printed with a dye sublimation printer (model 8600; Eastman Kodak Corp., Rochester, NY).

Microinjection

To prepare culture chambers, 60-mm plastic tissue culture dishes were drilled with 18-mm holes. A 22-mm glass coverslip was sealed to the outside of the dish with a ring of silicone grease. A pattern was inscribed on the coverslip to serve as a reference in relocating observed cells. The chambers and lids were sterilized by inversion for 5 min on an ultraviolet transilluminator (UVP Inc, Upland, CA). Ptk1 cells or keratinocytes were plated on the chambers and grown until the coverslips were ~50% confluent. The calcium concentration in the medium of the keratinocytes was raised to 1.5 mM 1–3 h before use. This treatment caused the keratinocytes to remain more flattened during mitosis. Before microinjection, the medium was overlaid with light mineral oil to prevent evaporation and retard pH changes. Culture dishes were placed on the stage of a Nikon Diaphot and microinjected with anti-Mad2 antibody (at 1–5 mg/ml in the injection pipette) in microinjection buffer (10 mM Na_2PO_4, pH 7.4, 100 mM KCl, 1 mM $MgCl_2$). Control cells were injected with preimmune IgG isolated from the rabbit used to raise the immune sera. Cells were microinjected using phase optics with a 40 × 0.55 NA long working distance objective and ultra-long working distance condenser (0.3 NA). After microinjection, a high-resolution phase contrast objective and condenser were substituted for video analysis of mitotic progression. Stage temperature was regulated with an air curtain incubator (Sage, Boston, MA) and was monitored with a digital thermometer (model HH-25TC; Omega Engineering Inc., Stamford, CT) connected to a microprobe attached to the stage. The stage temperature was maintained at 33 ± 2°C.

Microinjections were performed using glass microneedles (1.5 mm with capillary fibers; World Precision Instruments, Sarasota, FL) pulled with a Kopf vertical puller (David Kopf Instruments, Tujunga, CA). Microneedles were back-loaded with antibody solution and placed in a pipette holder made from chromatography fittings. The holder was loaded onto a Narishige micromanipulator (Narishige, Inc., Sea Cliff, NY). Air pressure from a 50 ml air tight syringe was used to pressure the microinjection needle for continuous flow of antibody from the microneedle. Cells were injected to a maximum of ~5% of their volume. The progression of cells through mitosis was monitored by video microscopy and images were recorded with a Dage CCD-72 video camera and ED-Beta video recorder (Sony, Cypress CA). Sequential images from the video tape were captured with the Image 1 system and exported to Coreldraw 6.0 to prepare montages and add annotation. Some sequences were obtained by time lapse imaging with the Sensys digital camera. At varying times after injection, cells were fixed (either before or after detergent treatment) and labeled for immunofluorescence with fluorescent anti–rabbit secondary antibodies to track the distribution of the injected antibody. For some experiments, cells were treated with the microtubule inhibitor nocodazole at 1 μg/ml for 30 min in a 37°C incubator. The cells were then injected at room temperature with anti-Mad2 antibody and returned to the incubator. At various times, the cells were then extracted with detergent and fixed for immunolabeling. In some experiments, cells were also labeled with 3F3/2 antiphosphoepitope antibody.

Results

Antibody Characterization and Immunofluorescence

Affinity-purified anti-Mad2 antibody, which recognizes both amphibian and mammalian Mad2 protein, was prepared to bacterially expressed, *Xenopus* Mad2 protein as previously reported (Chen et al., 1996). This antibody recognized an appropriately sized 24-kD protein in cell extracts prepared from Ptk1 cells and LLC-Pk cells (Fig. 1). In LLC-Pk cells, as reported previously with other cultured cells, high concentrations of Mad2 protein were found at the kinetochores of cells in early prometaphase (Fig. 2 *A*). In addition, we detected significant amounts of Mad2 elsewhere. Some label appeared on variably sized granules that did not colocalize with kinetochores (see merged image in Fig. 2 *A*) and were often concentrated in the region of the spindle poles (Fig. 2 *A*, *arrows*). Labeling with the anti-Mad2 antibody was inhibited if the antibody was preincubated with bacterially expressed Mad2 protein before application to the cells (Fig. 2 *B*). The distribution of Mad2 protein changed through the cell cycle. In interphase LLC-Pk cells (Fig. 3 *A*), a large proportion of the Mad2 protein is nuclear as previously described in *Xenopus* XTC tissue culture cells and mammalian HeLa cells. In addition, we observed considerable amounts of cytoplasmic Mad2 protein, diffusely distributed and concentrated in variably sized granules. Some of the cytoplasmic label appeared to be concentrated near the centrosome (Fig. 3, *arrows*). In prophase cells (Fig. 3 *B*), the Mad2 protein distribution in the nuclei was similar to that seen in interphase except that the label of the nucleus became less homogeneous. Just after nuclear envelope breakdown in early prometaphase, the concentration of Mad2 protein at the kinetochores was most pronounced (Fig. 3 *C, arrowheads*). At the same time, considerable labeling could also be detected in the cytoplasm with a concentration in the region of the spindle poles. In late prometaphase (Fig. 3 *D*), some Mad2 remained associated with the kinetochores and also persisted in the cytoplasm and in the region of the spindle poles. As previously described by others (Chen et al., 1996), chromosomes showed differential labeling with the anti-Mad2 antibody. Chromosomes located between the spindle pole and the metaphase plate often exhibited asymmetric labeling of sister kinetochores (Fig. 3 *D*, *arrowheads*). At metaphase (Fig. 3 *E*), the kinetochore regions of chromosomes were unlabeled or weakly labeled

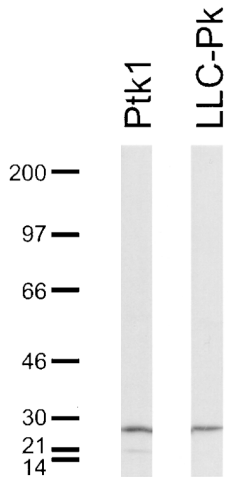

Figure 1. Anti-Mad2 antibody recognizes a 24-kD band in whole cell extracts prepared from Ptk1 cells and LLC-Pk cells. Cells were dissolved in SDS sample buffer, electrophoresed on polyacrylamide gradient gels under denaturing conditions, transferred to Immobilon-P paper, and probed with anti-Mad2 antibody.

though labeling persisted in the cytoplasm and concentrated in the region of the poles. During anaphase (Fig. 3 *F*), cytoplasmic labeling waned somewhat, and in telophase (Fig. 3 *G*), Mad2 protein appeared to accumulate at the reforming nuclei of the daughter cells. Similar distributions of Mad2 protein were also revealed by immunofluorescence with Ptk1 cells (Fig. 4). The Ptk1 cells, which contain fewer and larger chromosomes, also demonstrated more clearly the stronger association of Mad2 protein with kinetochores (Fig. 4, *arrowheads*) of chromosomes that had not yet congressed to the metaphase plate. In both cell types, the different pools of Mad2 protein, at the kinetochores, the spindle poles, and in the cytoplasm, could be detected whether cells were fixed before permeabilization (Figs. 2 *A* and 4 *A*) or were first lysed with detergent before fixation (Figs. 3 and 4 *B*).

Microinjection of anti-Mad2 Antibody into Cells from Permanent Lines or Primary Cultures Induces Premature Anaphase

For microinjection studies, Ptk1 cells in prophase or prometaphase were selected. Cells in prophase, before breakdown of the nuclear envelope, or in prometaphase, after breakdown of the nuclear envelope, were microinjected with anti-Mad2 antibody. Injection in either the cytoplasm or the nucleus had no discernible effect on the normal progression of prophase. Prometaphase events including attachment of chromosomes to spindle microtubules and congression toward the metaphase plate appeared unaffected. However, anti-Mad2 antibody injected in prophase or prometaphase caused premature initiation of chromatid separation during prometaphase before all chromosomes had congressed to the metaphase plate. An example of a cell injected in mid-prometaphase is shown in Fig. 5. At the time of injection, some chromosomes in this cell were at the metaphase plate while others remained at some distance from it (Fig. 5 *A*). Normal chromosome movements continued for several minutes after injection (Fig. 5, *B* and *C*). Approximately 8 min after injection, anaphase initiated, before all chromosomes had congressed to the metaphase plate (Fig. 5 *D*). All chromatids underwent simultaneous separation. Some chromosomes, presumably those with normal bipolar attachment to the spindle microtubules, segregated their chromatids normally. However, some chromosomes apparently lacked stable bipolar attachment of both kinetochores. Thus, one

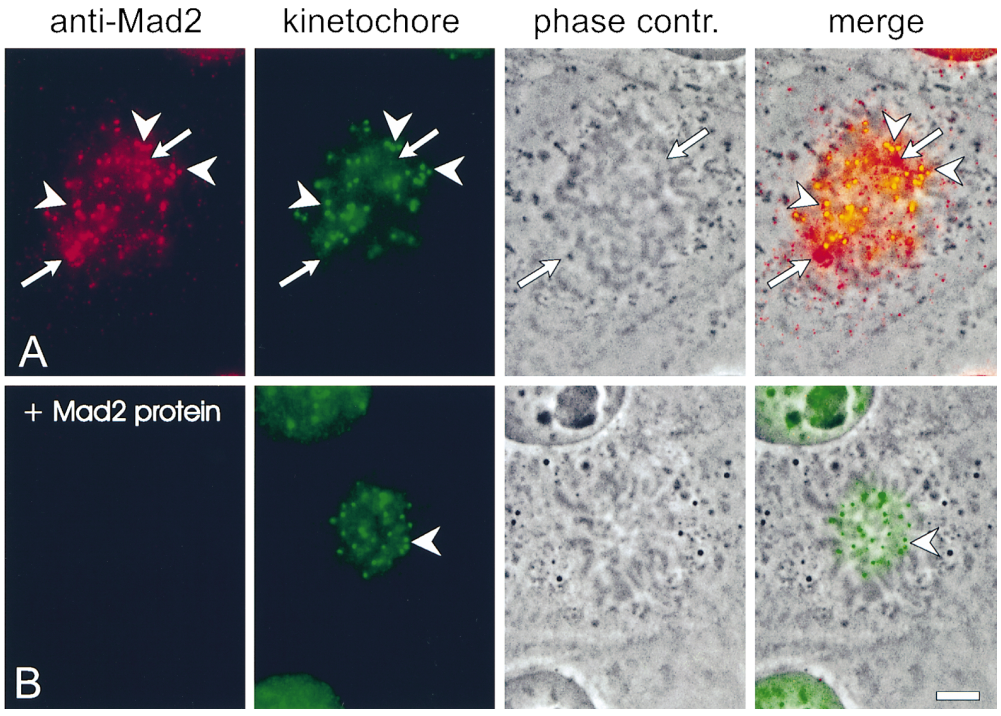

Figure 2. Mad2 protein is distributed on kinetochores, spindle poles, and in the cytoplasm of early prometaphase LLC-Pk cells. Cells were fixed with formaldehyde and then permeabilized with detergent. In early prometaphase cells, immunofluorescence with anti-Mad2 antibody reveals a high concentration of label at the spindle poles (*arrows*) and at the kinetochores (*arrowheads*) with some granular label in the cytoplasm. Kinetochore labeling with a human autoimmune serum is shown in the second column. A phase contrast image is shown in the third column, and a merged fluorescence and phase contrast image is shown in the fourth column. The kinetochores appear yellow because of superimposition of the red anti-Mad2 antibody label and the green antikinetochore label. When anti-Mad2 antibody was blocked by coincubation with bacterially expressed Mad2 protein, no anti-Mad2 fluorescence is detected (*B*), though kinetochores were still labeled with the antikinetochore antiserum. Bar, 5 μm.

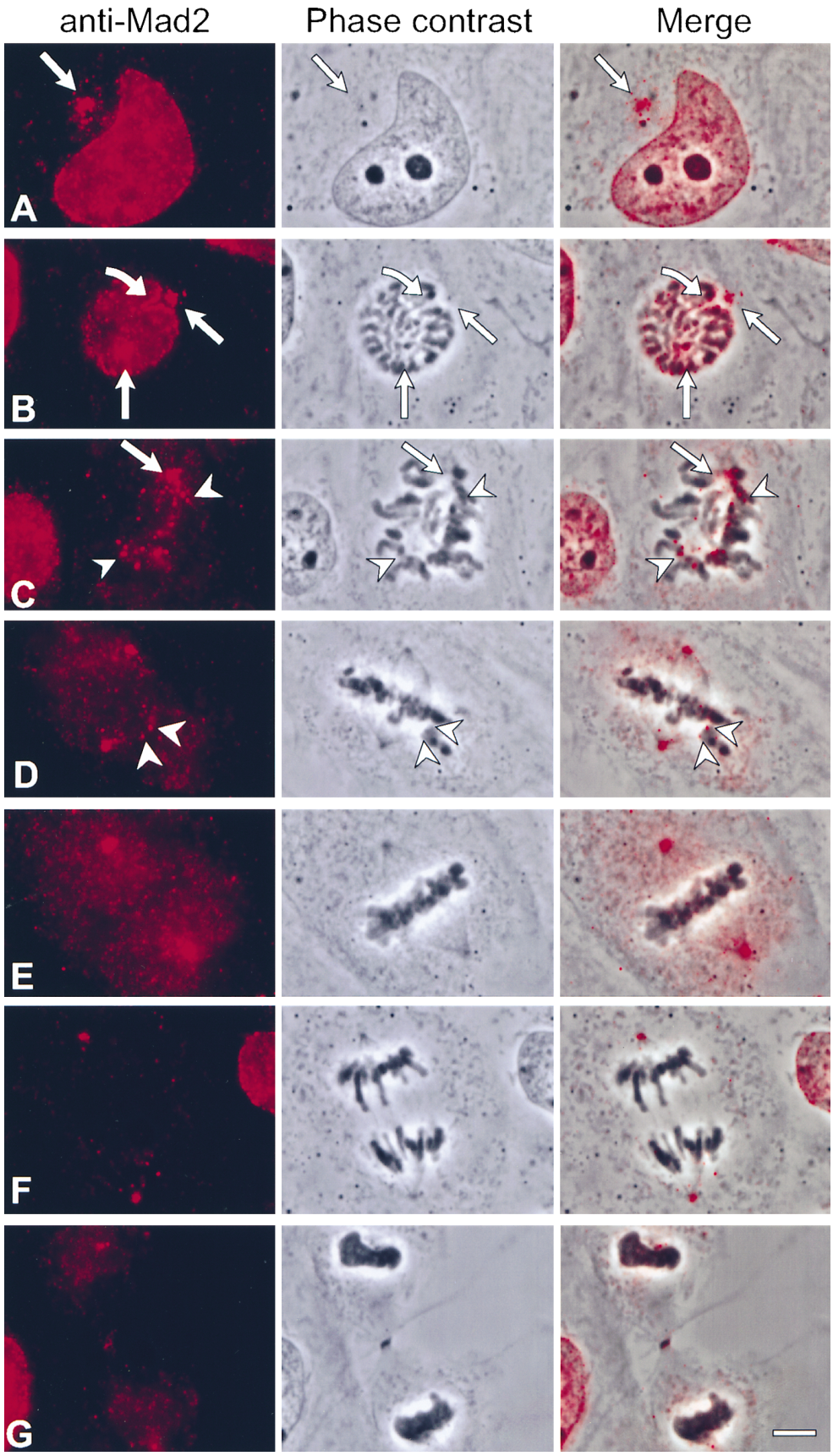

Figure 3. Cell cycle distribution of Mad2 protein in detergent-extracted LLC-Pk cells. Immunofluorescence labeling with anti-Mad2 antibody is shown in the first column, phase contrast of the same field is shown in the second column, and a merged image is shown in the third column. In interphase (*A*), the Mad2 protein is concentrated in the nucleus. In the cytoplasm, Mad2 is present diffusely and is concentrated in small granules, many of which are clustered in the region of the centrosome (*arrow*). In prophase (*B*), the Mad2 protein remains in the nucleus with concentrations of the label continues to be associated with the separating centrosomes (*arrows*). (The lower centrosome is in a different plane of focus.) Note the indentation of the nuclear envelope (*curved arrow*), presumably due to ingrowth of microtubules nucleated from the nearby centrosome. At early prometaphase (*C*), most chromosomes show concentrations of Mad2 at the kinetochores (*arrowheads*). Anti-Mad2 label is also found associated with the centrosomes (*arrow*) and in the cytoplasm. At late prometaphase (*D*), anti-Mad2 labeling is weak or absent at the kinetochores of fully congressed chromosomes. However, noncongressed chromosomes continue to show labeling of their kinetochores. Chromosomes located between the spindle pole and the metaphase plate often exhibit asymmetric labeling of the two sister kinetochores (*arrowheads*). At metaphase (*E*), labeling in the region of the chromosomes is weak, but considerable Mad2 protein remains near the centrosomes and distributed through the cytoplasm. At anaphase (*F*), the overall level of anti-Mad2 labeling appears to diminish. The centrosomes continue to exhibit accumulations of Mad2 protein, and some labeled granules are also present in the cytoplasm. At telophase (*G*), diffuse anti-Mad2 label appears to concentrate in the vicinity of the reforming nuclei. Bar, 5 μm.

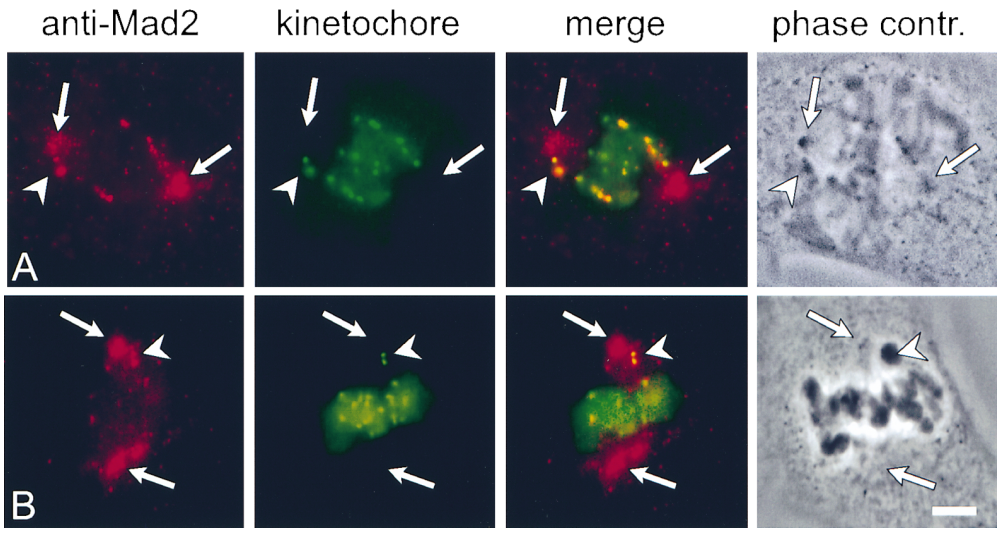

Figure 4. Mad2 protein is found at spindle poles, kinetochores, and in the cytoplasm of Ptk1 cells. Ptk1 cells were fixed in formaldehyde and then permeabilized with detergent (*A*) or preextracted with detergent before fixation (*B*). With both specimen preparations, immunofluorescence of mid-prometaphase cells reveals that the Mad2 protein is concentrated near spindle poles (*arrows*) and at kinetochores (*arrowheads*) of chromosomes before their congression to the metaphase plate. Granular labeling is also detected in the cytoplasm. Bar, 5 μm.

chromatid moved to the pole while its partner appeared to drift free in the cytoplasm (Fig. 5, *E*, *arrow*). Anti-Mad2 antibody injection launched the complete program of anaphase including chromatid movement to the poles (anaphase A) and separation of the poles from each other (anaphase B) (Fig. 5, *F* and *G*).

An example of a Ptk1 cell injected in prophase is shown in Fig. 6. Prophase continued unaffected (Fig. 6 *A*), and nuclear envelope breakdown occurred ~29 min after injection (Fig. 6 *B*). Prometaphase continued for an additional 13 min (Fig. 6, *C–E*). Chromosomes attached to the spindle during prometaphase (Fig. 6, *C–E*), and some moved to the metaphase plate. Others were still in the process of congression between the poles and the metaphase plate. One chromosome moved above the upper pole and remained there (Fig. 6, *C–E*, *arrowhead*). Anaphase onset occurred ~42 min after injection (Fig. 6 *F*), while many chromosomes had still not congressed to the metaphase plate. In some instances, both chromatids of a single chromosome moved toward the same pole (Fig. 6, *F–I*, *curved arrow*). This suggests either that the both sister kinetochores were bound to microtubules from one pole before anaphase onset or that one of the chromatids became attached to microtubules from the nearby pole after chromatid separation.

The anti-Mad2 antibody microinjections in Ptk1 cells suggested that the unperturbed function of the Mad2 protein was essential in preventing abnormal chromatid segregation. This finding implied that mammalian cells, unlike yeast, are dependent on the spindle checkpoint in every mitosis, not only when the cells are treated with microtubule inhibitors. However, Ptk1 cells, while not phenotypically transformed, are an immortalized, permanent cell line. It seemed possible that continuous culture conditions might select for lines more reliant on the spindle checkpoint to ensure proper segregation of their chromosomes. To examine whether our results could be reproduced in a very different and completely nontransformed cell type, we performed injection experiments with cultures of primary human keratinocytes. Because such cells are infrequently studied in mitosis, we show in Fig. 7 images of a human keratinocyte injected with control preimmune antibody. In this control cell, the chromosomes aligned in a discrete metaphase plate (Fig. 7 *E*, *arrowhead*) before anaphase onset (Fig. 7 *F*, *arrowhead*).

In contrast to the keratinocytes injected with preimmune IgG, those injected with anti-Mad2 antibody showed premature initiation of anaphase. Shown in Fig. 8 is a keratinocyte injected with anti-Mad2 antibody during prometaphase. After injection, the chromosomes continued to congress to the metaphase plate (Fig. 8 *A*). Although the separation of individual chromatids could not be resolved, at ~14 min after injection, as revealed by time lapse video, two masses of chromatids began to move in opposite directions signaling the onset of anaphase (Fig. 8 *B*). This onset of anaphase occurred before a complete metaphase plate had been established. The cell underwent anaphase (Fig. 8, *C–F*) and cytokinesis was initiated (Fig. 8, *E* and *F*, *arrowheads*). The culture was later fixed and the injected cell relocated, showing that it had completed cytokinesis, chromosome decondensation, and reformation of the interphase nuclei (Fig. 8 *G*). Shown in Fig. 9 is a human keratinocyte injected with anti-Mad2 antibody near the time of nuclear envelope breakdown (Fig. 9 *A*). Because in this cell the mitotic poles had not yet separated in prophase, a monopolar spindle was formed with the chromosomes arranged in a C-shaped arc around the unseparated poles (Fig. 9 *B*, *arrow*). Ordinarily, in control cells, this monopolar stage would resolve a normal bipolar spindle as the poles separated during prometaphase. However, in the anti-Mad2–injected cell, anaphase onset occurred before pole separation could take place (Fig. 9 *C*). Since no bipolar spindle was present, the chromosomes separated only a short distance. Subsequently the chromosomes began to decondense (Fig. 9 *D*), and an interphase nucleus, encompassing both sets of chromatids, began to reconstitute (Fig. 9 *E*). Accumulated data for antibody microinjection experiments in both Ptk1 cells and keratinocytes are shown in Table I.

In the absence of a bipolar spindle in the cell shown in Fig. 9, cytokinesis did not occur. Lack of cytokinesis after anaphase onset in cells with unseparated spindle poles was

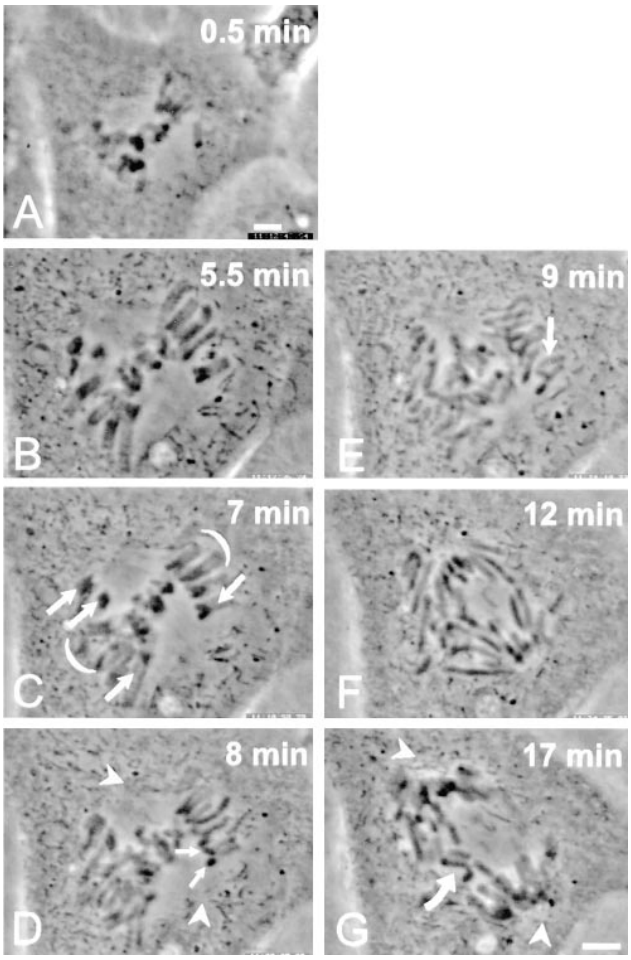

Figure 5. Injection of anti-Mad2 antibody induces premature anaphase. A Ptk1 cell in mid-prometaphase that had undergone partial congression of the chromosomes was injected with anti-Mad2 antibody (time of injection = time 0). At 0.5 min, a low-resolution image was recorded (*A*). High-resolution phase contrast optics were used for subsequent images. Congression of chromosomes in prometaphase continued at 5.5 min (*B*). By 7 min after injection (*C*), many chromosomes were at the metaphase plate (*brackets*), but others were only partially congressed (*arrows*). The cell entered premature anaphase between 7 and 8 min. At 8 min (*D*), separation of chromatids can be clearly visualized (*small arrows*). The positions of the spindle poles (centrosomes) are indicated by arrowheads. Anaphase A movements occur with many chromatids undergoing normal poleward movement to the poles, but others, presumably those that lacked stable attachment to the pole, drift in the cytoplasm with little evidence of directed movement (*arrow*) (*E*). Anaphase A chromatid separation continues with chromosomes nearing the poles (*F*). By 17 min (*G*), anaphase A movements have finished, but anaphase B, the separation of the spindle poles, continues. (Compare distance between poles [*arrowheads*] in *D* versus *G*). Some chromatids, which presumably lacked stable attachment to microtubules at anaphase onset, remained at the metaphase plate (*curved arrow*). Bar, 5 μm.

also noted in two anti-Mad2 antibody-injected Ptk1 cells (data not shown). Although not a primary focus of this study, successful cytokinesis in anti-Mad2–injected cells occurred in most but not all cells if they contained bipolar spindles at the time anaphase was initiated. Excluding the monopolar cells, of 19 Ptk1 cells and 5 keratinocytes for which we have information, 13 Ptk1 cells and 3 keratinocytes accomplished cytokinesis. The others became bi- or multinucleate.

Microinjection of anti-Mad2 Antibody Causes Cytoplasmic Aggregation of Mad2 Protein and 3F3/2 Phosphoepitopes

Attempting to assess the consequences of anti-Mad2 antibody microinjection, we fixed cells at various times after injection and labeled with secondary antibodies to track the microinjected antibody. In cells fixed after injection without preextraction, labeling with fluorescent secondary antibody resulted in intense fluorescence throughout the cytoplasm (not shown). However, if microinjected cells were extracted with detergent before fixation, most of the diffuse, injected antibody was removed, leaving antibody bound to components that were detergent resistant. In some instances, to assess the effects of antibody injection on another element of the spindle checkpoint pathway, we also labeled the injected cells with the 3F3/2 antiphosphoepitope antibody. In cells injected with anti-Mad2 antibody and detergent lysed before fixation, aggregates of injected antibody were detected in the cytoplasm. These same aggregates colabeled with the 3F3/2 antibody. Fig. 10 presents an example of a Ptk1 cell, microinjected with anti-Mad2 antibody in prometaphase. At 12 min after injection, the cell was still in prometaphase (Fig. 10 *A*). At 14 min, the chromatids began to separate (Fig. 10 *B*, *arrowhead*) and move toward the spindle poles (*arrows*) signaling the onset of anaphase. The cell was observed through the early stages of anaphase (Fig. 10 *D*). Upon reaching mid-anaphase (Fig. 10 *E*), the cell was detergent lysed and fixed. The fixed cell (Fig. 10 *F*) maintained the morphology and chromosome distribution of the live cell. Aggregates of the injected anti-Mad2 antibody, which had resisted detergent extraction, were detected in the cytoplasm. These same aggregates were found to colabel with the 3F3/2 antiphosphoepitope antibody. These aggregates were larger and fewer in number than the granules detected by standard anti-Mad2 immunofluorescence in mitotic cells. In another experiment presented in Fig. 11, we injected anti-Mad2 antibody into cells whose spindle checkpoint had been fully activated by treatment with the microtubule inhibitor nocodazole. Consistent with previous results for HeLa cells (Li and Benezra, 1996), injection of anti-Mad2 antibody into drug-arrested Ptk1 cells induced reentry into interphase as revealed by decondensation of the chromosomes and reformation of the nuclear envelope (Fig. 11 *B*, *arrow*). Upon immunolabeling, the cells showed aggregates of anti-Mad2 antibody in the cytoplasm that colabeled with the 3F3/2 antiphosphoepitope antibody (Fig. 11, *C* and *D*, *curved arrows*).

Discussion

The spindle checkpoint involves three components of signaling: first, activation of the checkpoint by one or more chromosomes that lack stable, bipolar attachment to the spindle microtubules; second, broadcast of the checkpoint signal to the entire mitotic apparatus; and third, inhibition of the machinery for anaphase onset. The checkpoint may

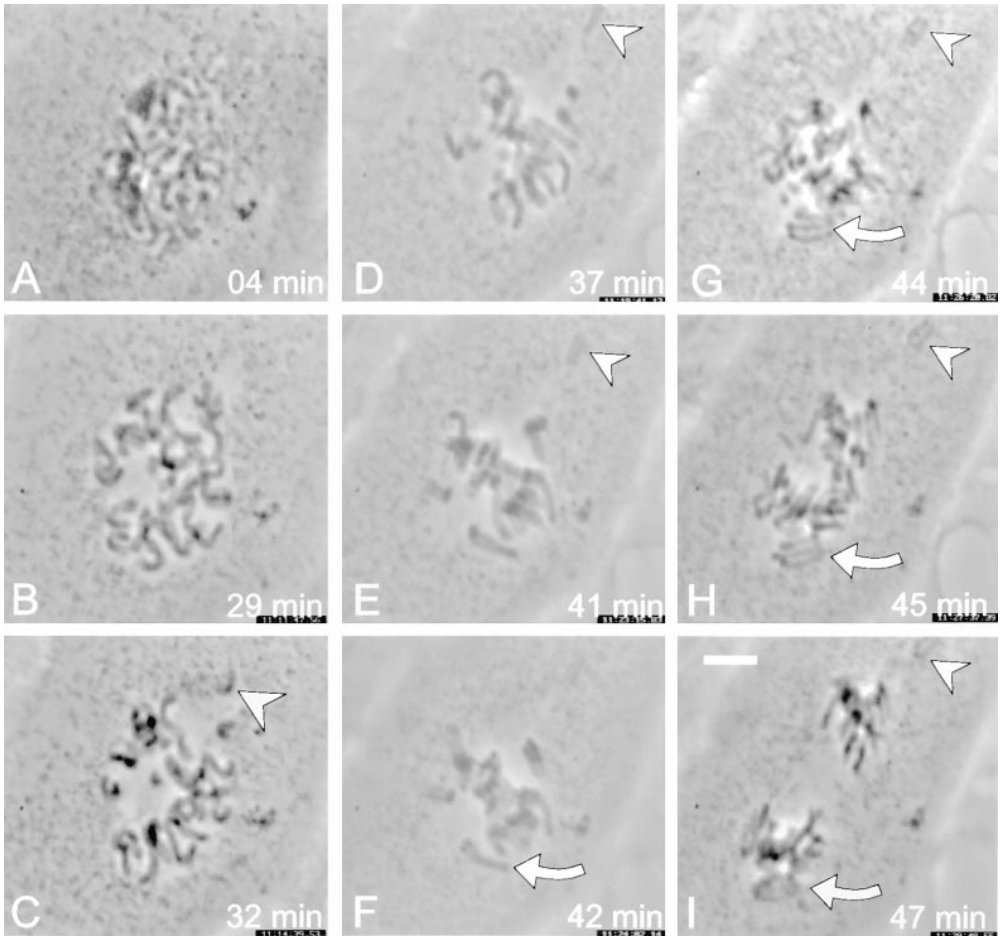

Figure 6. Injection of anti-Mad2 antibody in prophase induces premature anaphase onset. A Ptk1 cell in mid-prophase was injected in the cytoplasm with anti-Mad2 antibody at time 0. Prophase continued unaffected (*A*), and nuclear envelope breakdown occurred ~29 min after injection (*B*). Chromosomes attached to the spindle during prometaphase (*C–E*), and some moved to the metaphase plate. One chromosome (*arrowhead*) moved above the upper spindle pole and remained monopolarly oriented. Anaphase onset occurred ~42 min after injection (*F*) while many chromosomes were still far from the metaphase plate. During anaphase (*G–I*) chromatids moved to the spindle poles, and the spindle poles moved apart. In some cases (*curved arrow*), both sister chromatids from a single chromosome moved to the same spindle pole. Bar, 5 μm.

be initially activated by a lack of mechanical tension at the kinetochore (Li and Nicklas, 1995; Nicklas et al., 1995) or by lack of full occupancy of kinetochores by dynamic microtubules (Rieder et al., 1994; Waters et al., 1998). How mechanical tension or kinetochore occupancy is transmuted to biochemical signals broadcast through the cell is yet unclear, but evidence suggests that the products of the *MAD*, *BUB*, and *MPS1* genes are likely involved (Hoyt et al., 1991; Li and Murray, 1991; Hardwick et al., 1996). Anaphase onset is timed by activation of the APC which ubiquitinylates proteins destined for proteolysis (Holloway et al., 1993; Hershko et al., 1994; Irniger et al., 1995; King et al., 1995; Tugendreich et al., 1995). Candidate target proteins, whose proteolysis is involved in chromatid separation, include Pds1 in budding yeast and Cut2 in fission yeast (Cohen-Fix et al., 1996; Funabiki et al., 1996; Yamamoto et al., 1996). The spindle checkpoint pathway appears to inhibit ubiquitinylation of these target proteins by the APC, thus blocking anaphase onset. While expression of the Mad2 protein was found not essential for chromosome segregation in yeast under normal culture conditions, the significant differences in the details of mitosis in yeast and mammals may require that the Mad2 protein have broader functions in mammalian cell mitosis.

Mitosis in Mammalian Somatic Cells Requires the Spindle Checkpoint

The spindle checkpoint, induced with microtubule inhibitors, is abrogated in mammalian cells and in cycling *Xenopus* extracts by introducing anti-Mad2 antibody (Chen et al., 1996; Li and Benezra, 1996). We sought to perturb the function of Mad2 protein in non–drug-treated mammalian cells by antibody microinjection. Functionally, we found that the microinjection of anti-Mad2 antibody into Ptk1 cells in M phase led to premature anaphase onset under conditions where congression had been proceeding normally. This result suggests that the Mad2 protein participates in a pathway that is used in each mitosis to ensure that anaphase onset occurs only after the chromosomes have congressed to the metaphase plate. Thus, in this cell line the spindle checkpoint pathway involving the Mad2 protein is required for normal chromosome segregation. We also found that antigens recognized by the 3F3/2 antibody were coaggregated with injected antibody. Since the 3F3/2 antibody does not bind directly to Mad2 protein (data not shown), it is likely that Mad2 is complexed with other proteins that are recognized by the 3F3/2 antibody. Thus, the anti-Mad2 antibody injections may also perturb the function of Mad2-associated proteins. One conclusion of these observations is that the antibody microinjection experiments in mammalian cells cannot be equated with the disruption of the gene and failure to produce the protein in yeast or in mammals.

Careful observations of mitosis in primary cultures from plants led Bajer and Mole-Bajer (1956) to propose that chromosomes slow in moving to the metaphase plate caused a delay in anaphase onset. In primary cells cultured

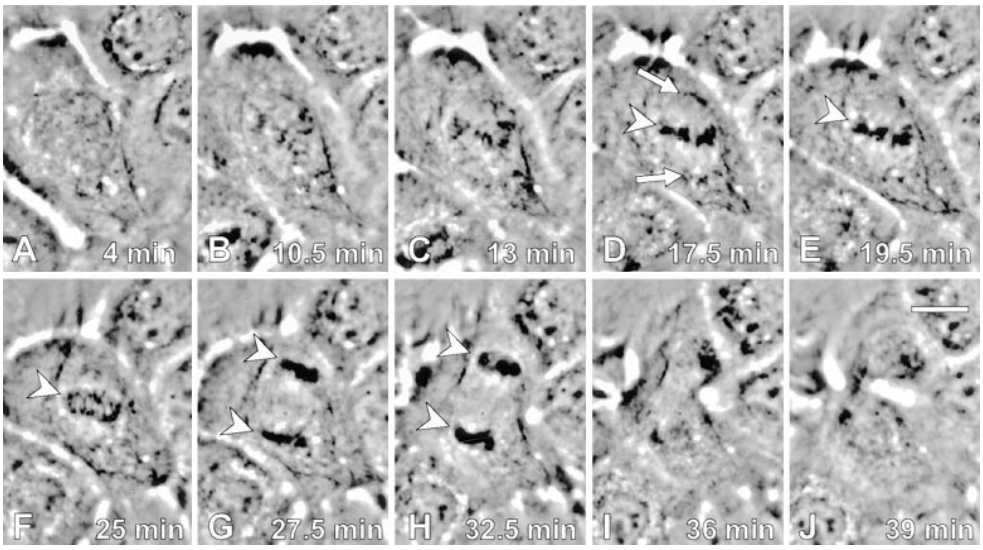

Figure 7. Normal progression of mitosis in a human keratinocyte injected with control, preimmune IgG. A keratinocyte in prophase was injected with preimmune IgG at time 0. The cell progressed through prophase (*A*) and underwent nuclear envelope breakdown. During prometaphase (*B–D*), a mitotic spindle formed with discrete spindle poles (*D*, *arrows*). During prometaphase, the chromosomes congressed to the spindle equator assemble the metaphase plate (*D*, *arrowhead*). At metaphase (*E*), the chromosomes were assembled into a tight plate (*E*, *arrowhead*). Anaphase onset (*F*) ensued with chromatid separation (*F*, *arrowhead*). The chromatids (*arrowheads*) separated normally as the cell progressed through anaphase, and cytokinesis was initiated (*G* and *H*). During telophase (*I* and *J*), cytokinesis was completed. Bar, 5 μm.

from newts, Zirkle (1970) felt that cells never entered anaphase while a chromosome remained off the spindle equator. However, Rieder and Alexander (1989) reported that it was "not uncommon" for primary cells from newts to initiate anaphase with one or more chromosomes remaining near the spindle poles. With mammalian cells, both permanent cell lines and primary cultures, it has long been known that microtubule drugs cause cells to arrest in a prometaphase-like state with chromatids still joined. However, observations on the role of lagging chromosomes in regulating anaphase onset in primary cultures of mammalian cells in the absence of drugs have not been performed.

Because the evidence from Rieder and Alexander (1989) indicated that primary cultures of newt cells would undergo anaphase in the presence of chromosomes not aligned at the metaphase plate, and because the Mad2 protein is not ordinarily essential for equal chromosome segregation in both budding and fission yeast, we were concerned that the apparent reliance of Ptk1 cells on Mad2 function and the spindle checkpoint may have developed from selection in tissue culture. In other words, perhaps cell culture conditions are suboptimal for spindle function even in the absence of added drugs, and thus cells with better developed spindle checkpoint pathways are selected. To test whether our findings on the effect of anti-Mad2 antibody injections were also applicable to primary mammalian cells, we microinjected early cell cultures derived from human foreskin keratinocytes. Control cells injected with preimmune IgG assembled normal metaphase plates and never appeared to undergo anaphase onset in the presence of unaligned chromosomes. However, like

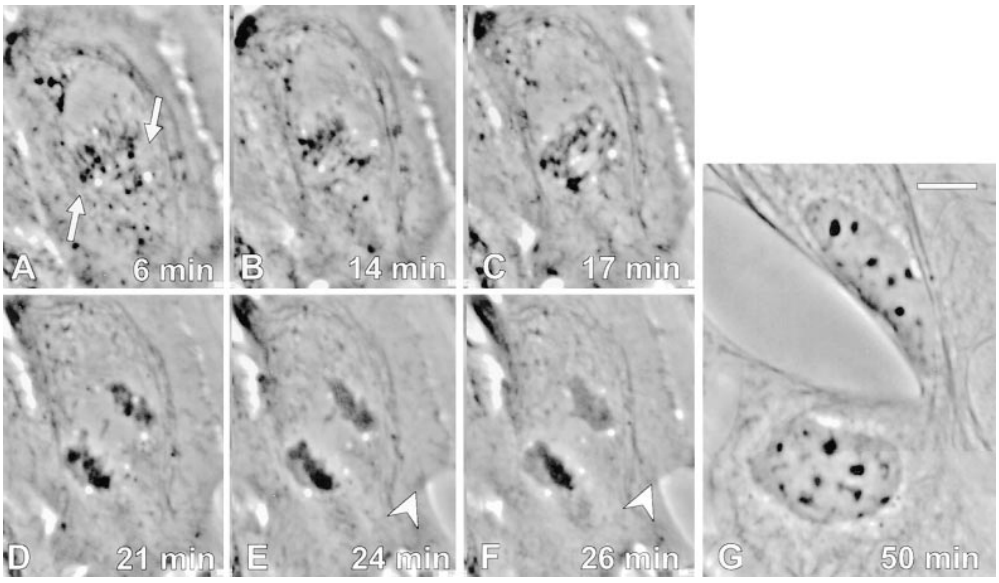

Figure 8. Injection of a keratinocyte with anti-Mad2 antibody induces premature anaphase. A keratinocyte was injected in prometaphase at time 0. At 6 min after injection (*A*), the cell had assembled a bipolar spindle, and the chromosomes began to congress. The approximate locations of the spindle poles are indicated by the arrows. At 14 min (*B*), the cell had initiated anaphase before congression was complete. Anaphase continued (*C* and *D*). The cleavage furrow (*arrowhead*) appeared in late anaphase (*E*). At 50 min, the culture was fixed (*G*). Images of the fixed cells showed that cytokinesis had been completed. Bar, 5 μm.

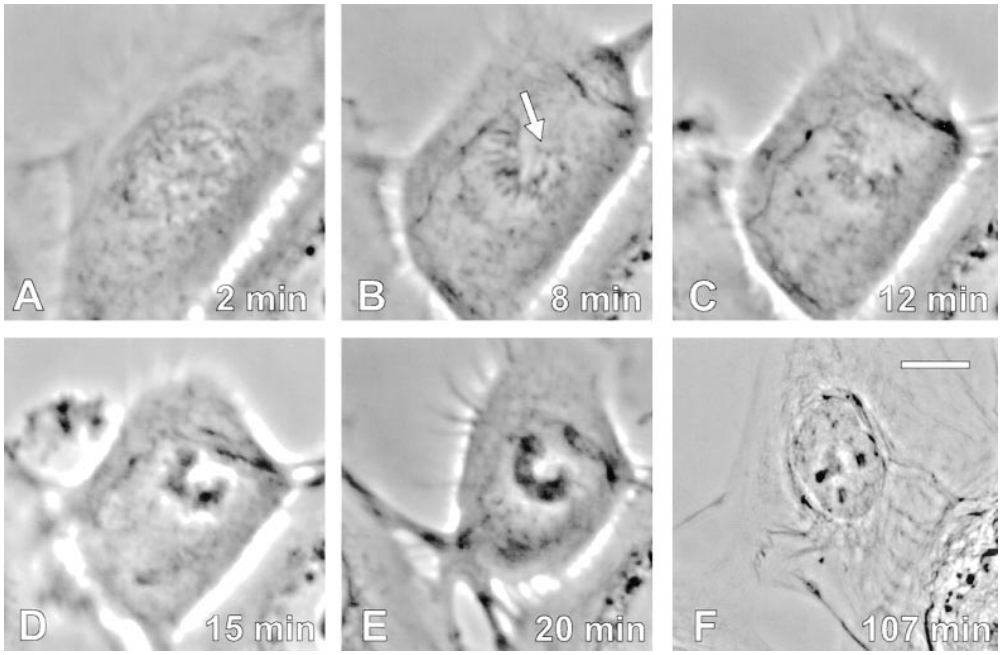

Figure 9. Injection of a keratinocyte with anti-Mad2 antibody induces premature anaphase before assembly of a bipolar spindle. A keratinocyte was injected with anti-Mad2 antibody just before nuclear envelope breakdown (*A*). As happens spontaneously in these cells, the spindle poles had not separated appreciably before nuclear envelope breakdown. The chromosomes clustered around the unseparated spindle poles (*arrow*) (*B*). Anaphase initiated while the chromosomes were still clustered around the unseparated spindle poles (*C*). The chromatids separated but moved very little during "anaphase" (*D*). The chromosomes then decondensed and the nucleus reformed (*E*). The culture was later fixed. The cell was relocated, showing that it had progressed into interphase. In the absence of a bipolar spindle, the cell did not undergo cytokinesis. Bar, 5 μm.

Ptk1 cells, keratinocytes injected with anti-Mad2 antibody underwent premature anaphase leading to mitotic aneuploidy. Thus, dependence on the Mad2 pathway for the timing of normal mitosis is not merely an adaptation of permanent cell lines to tissue culture conditions.

The injection of anti-Mad2 antibody did not induce chromatid separation alone. Rather, all aspects of anaphase and exit from mitosis were initiated. Cells exhibited both chromatid migration to the spindle poles (anaphase A) and pole–pole separation (anaphase B). Most cells underwent cytokinesis, and all cells observed for a long enough period decondensed their chromosomes and reformed interphase nuclei.

Table I. Microinjection or Ptk1 Cells or Keratinocytes with Anti-Mad2 Antibody Induces Premature Anaphase

Cell type	Antibody	Number of cells injected	Phase injected prophase/prometaphase	Premature anaphase	Normal anaphase
Ptk1	anti-Mad2	37	12/25	36	1
	preimmune	11	6/5	1	10
Keratinocyte	anti-Mad2	11	5/6	9	2*
	preimmune	5	2/3	0	5

*Injection of keratinocytes was technically more difficult than injection of Ptk1 cells. After observation, the injected keratinocytes were fixed and labeled with fluorescent secondary antibody. Of the 11 keratinocytes injected with anti-Mad2 antibody, the nine that underwent premature anaphase showed strong labeling. In the two that underwent normal anaphase, one cell showed no evidence of injected antibody, and the other showed only a faint trace of label. We conclude that the two cells that underwent normal anaphase failed to receive sufficient anti-Mad2 antibody to affect anaphase onset. All five control-injected cells were strongly fluorescent after labeling with secondary antibody.

The Regulation of Anaphase Onset in Mammalian Cells

Our evidence suggests that the spindle checkpoint plays an essential role in timing anaphase onset in somatic cell mitosis. However, in embryonic cell divisions it appears that the spindle checkpoint may be inactive or may play a lesser role. *Xenopus* embryos treated with microtubule inhibitors, though unable to form mitotic spindles, do not undergo M phase arrest though unable to form mitotic spindles (Clute and Masui, 1995). Although amphibian embryos are developmentally regulative and may eliminate abnormal cells during embryogenesis, there is no evidence of wide-scale chromosome imbalances occurring during early mitotic divisions. This suggests that mechanisms other than checkpoints can support proper timing of anaphase onset in different organisms and in different stages in the life of one organism. These noncheckpoint mechanisms could potentially play a partial role in regulating the timing of somatic cell mitosis. In prophase cells injected with anti-Mad2 antibody, anaphase was never initiated until several minutes after nuclear envelope breakdown. One explanation for this lag is that a refractory period just after nuclear envelope breakdown exists during which cells acquire the competence to undergo anaphase.

We speculate that anaphase onset in higher eukaryotes is timed by three events: (*a*) the breakdown of the nuclear envelope, (*b*) a refractory period after nuclear envelope breakdown during which cells acquire competence to initiate anaphase, and (*c*) inhibition of anaphase onset by the spindle checkpoint. Spindle assembly and the manifestation of the spindle checkpoint in higher eukaryotes ap-

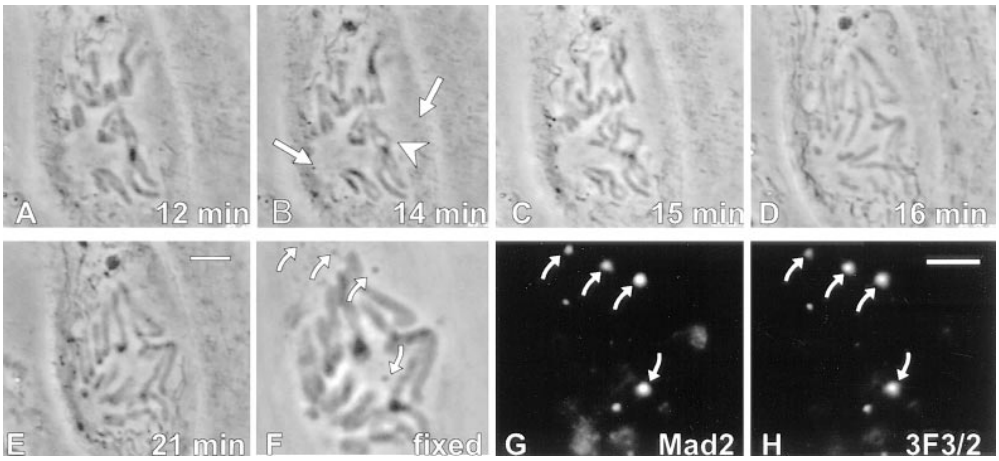

Figure 10. Injected anti-Mad2 antibody collects in cytoplasmic aggregates that also label with 3F3/2 antiphosphoepitope antibody. A Ptk1 cell at mid-prometaphase was microinjected with anti-Mad2 antibody at time 0. At 12 min after microinjection, the cell was still in prometaphase (*A*). The cell entered anaphase at 14 min (*B*) with clear separation of the chromatids (*arrowhead*). The spindle poles are indicated by the straight arrows. Anaphase continued (*C* and *D*). The final image of the live cell was obtained in late anaphase (*E*). The cell was then extracted with detergent and fixed. The phase contrast image of the fixed cell reveals that the chromosomes have swollen somewhat during the extraction and fixation protocol, but morphology is preserved (*F*). The microinjected antibody was localized by applying a fluorescent anti-rabbit IgG secondary antibody (*G*). The 3F3/2 phosphoepitope was immunolocalized in the same cell (*H*). Comparison of the phase and fluorescent images reveals that microinjected anti-Mad2 (*F*) antibody accumulated in aggregates (*curved arrows*) that also labeled with the 3F3/2 phosphoepitope antibody (*G*). These aggregates are also visible in the phase contrast image of the fixed cell, though the phase density may be at least partially due to the primary and secondary antibodies used for immunolabeling. Bars, 5 μm.

pears regulated by nuclear envelope breakdown. In insect spermatocytes, Zhang and Nicklas (1995) found that spindle assembly and mitotic progression were induced by artificially rupturing prophase nuclei. Similarly, spindle assembly did not proceed if prophase nuclei were removed before nuclear envelope breakdown.

We suggest that the acquisition of competence to initiate anaphase onset requires several minutes after nuclear envelope breakdown. We hypothesize that by the end of this period, the spindle checkpoint is being generated at unattached kinetochores. Thus as cells become competent to undergo anaphase, inhibition by the spindle checkpoint takes over to prevent the anaphase onset before bipolar attachment of all the chromosomes. Finally, we suggest that the final component in the timing of anaphase onset is decay of the checkpoint signal after stable attachment of the last chromosome. Using laser ablation of kinetochores in Ptk1 cells, Rieder and colleagues (1995) found that anaphase was initiated on average ~20 min after ablation of the final unattached kinetochore. This slow decay of the checkpoint signal provides necessary time for the last attaching chromosomes to move to the metaphase plate.

Function of the Mad2 Protein

Recent evidence from several systems suggests that, in vivo, the Mad2 protein is part of a larger complex. In *Saccharomyces cerevisiae*, the Mad2 protein interacts with Mad1 protein (Chen, R.-H., K.G. Harwick, and A.W. Murray, unpublished). Mad2 and Mad3 proteins coprecipitate with the Cdc20 protein (Hwang et al., 1998). The *S. pombe* homologue of Mad2 protein also was found to interact with Slp1, a Cdc20 homologue (Kim et al., 1998). Li et al. (1997) reported that mammalian Mad2 protein associates with the APC, and excess Mad2 added to cycling *Xenopus* extracts inhibits ubiquitinylation of cyclin B. In mammals, immunoprecipitation studies showed that Mad2 complexes with the APC by virtue of its ability to bind p55CDC, a mammalian homologue of Cdc20 protein (Kallio et al., 1998). Like the Mad2 protein, p55CDC is found

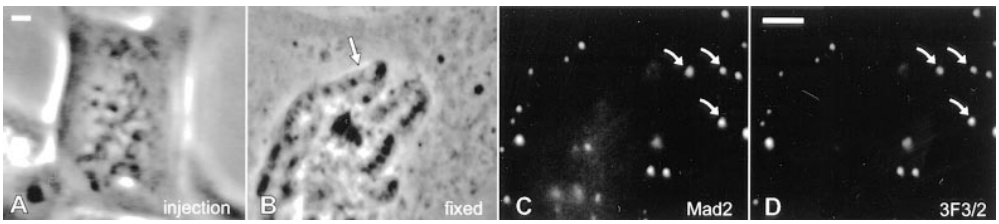

Figure 11. Anti-Mad2 antibody injected in nocodazole-treated Ptk1 cells induces exit from M phase and formation of Mad2 aggregates in the cytoplasm. Anti-Mad2 antibody was injected into a Ptk1 cell arrested in M phase by pretreatment with nocodazole. The cell at low magnification at the time of injection is shown in *A*. After incubation at 37°C for an additional 25 min in the presence of nocodazole, the cell was lysed with detergent and fixed. A phase contrast image at high magnification (*B*) showing a portion of the cell reveals that the chromosomes have partially decondensed and the nuclear envelope has begun to reform (*B*, *arrow*). These indicators show that the cell was exiting M phase at the time of fixation. Immunolabeling with anti–rabbit secondary antibody (*C*) shows that detergent-insoluble anti-Mad2 antibody aggregates are present in the cytoplasm (*C*, *curved arrows*). Colabeling with 3F3/2 antibody (*D*) shows that these same aggregates contain the 3F3/2 phosphoepitope. Bars, 5 μm.

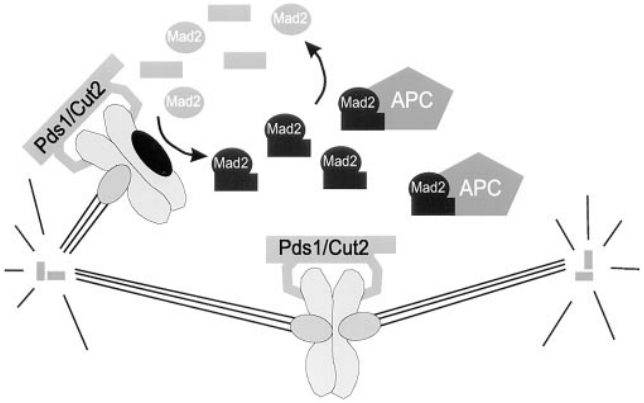

Figure 12. A proposed model for Mad2 protein dynamics and inhibition of anaphase onset. The unattached kinetochore (*black*) catalyzes the assembly and/or activation of a Mad2-containing complex (*black shading* indicates active complex). This complex is released from the kinetochore and blocks the ability of the APC to ubiquitinylate target anaphase inhibitor proteins such as Pds1 in budding yeast and Cut2 in fission yeast. The Mad2-containing complex undergoes spontaneous inactivation (indicated by shading change from *black* to *gray*) perhaps by dephosphorylation or disassembly. However, as long as unattached kinetochores persist, active Mad2-containing inhibitor complex is continuously regenerated, perhaps by recycling inactivated subunits. Attachment of kinetochores halts the assembly/activation of the Mad2-complex (indicated by *gray* color of kinetochores attached to microtubule bundles). Residual inhibitor complex is slowly inactivated, thus allowing time for the final attaching chromosome to move to the metaphase plate. The APC, released from inhibition, then catalyzes ubiquitinylation of the Pds1/Cut2-type anaphase inhibitor proteins. These proteins are then degraded by the proteasome, and anaphase is initiated.

throughout the mitotic cytoplasm but is concentrated at kinetochores. In both budding and fission yeast, expression of dominant alleles of Cdc20 that fail to bind Mad2 or overexpression of the wild-type protein abrogates cell cycle arrest in response to spindle disruption (Hwang et al., 1998; Kim et al., 1998; Schott and Hoyt, 1998).

While other studies have highlighted the association of the Mad2 protein at mitotic kinetochores, our immunofluorescence data suggest that there are significant pools of Mad2 at other locations in the cell. We suggest that these various populations undergo exchange in the living cell. We hypothesize unattached or improperly attached kinetochores are catalytic sites for the assembly and/or activation of Mad2 with other components to form complexes that are released to inhibit APC activity throughout the cell (Fig. 12). We suggest that these inhibitory complexes have a limited lifespan in the cytoplasm and are inactivated after some time by processes such as dephosphorylation or disassembly. The individual components might then diffuse back to the kinetochore to be reassembled and reactivated. As long as unattached kinetochores persist, the inhibitors would be regenerated and the cells would be prevented from entering anaphase. After the last kinetochore attaches, inhibitor production would cease. When the active inhibitor level fell below a threshold, anaphase onset would then take place. Because biochemical components of the spindle checkpoint are evolutionarily conserved, continued integration of studies in several systems should rapidly elucidate the signaling pathways involved.

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