Microtubule Dependency of p34^{cdc2} Inactivation and Mitotic Exit in Mammalian Cells

Paul R. Andreassen and Robert L. Margolis

Institut de Biologie Structurale, 38027 Grenoble, France

Abstract. The protein kinase inhibitor 2-aminopurine induces checkpoint override and mitotic exit in BHK cells which have been arrested in mitosis by inhibitors of microtubule function (Andreassen, P. R., and R. L. Margolis. 1991. *J. Cell Sci.* 100:299–310). Mitotic exit is monitored by loss of MPM-2 antigen, by the reformation of nuclei, and by the extinction of p34^{cdc2}-dependent H1 kinase activity. 2-AP–induced inactivation of p34^{cdc2} and mitotic exit depend on the assembly state of microtubules. During mitotic arrest generated by the microtubule assembly inhibitor nocodazole, the rate of mitotic exit induced by 2-AP decreases proportionally with increasing nocodazole concentrations. At nocodazole concentrations of 0.12 μg/ml or greater, 2-AP induces no apparent exit through 75 min of treatment. In contrast, 2-AP brings about a rapid exit ($t_{1/2}$ = 20 min) from mitotic arrest by taxol, a drug which causes inappropriate overassembly of microtubules. In control mitotic cells, p34^{cdc2} localizes to kinetochores, centrosomes, and spindle microtubules. We find that efficient exit from mitosis occurs under conditions where p34^{cdc2} remains associated with centrosomal microtubules, suggesting it must be present on these microtubules in order to be inactivated. Mitotic slippage, the natural reentry of cells into G_1 during prolonged mitotic block, is also microtubule dependent. At high nocodazole concentrations slippage is prevented and mitotic arrest approaches 100%. We conclude that essential components of the machinery for exit from mitosis are present on the mitotic spindle, and that normal mitotic exit thereby may be regulated by the microtubule assembly state.

MITOTIC checkpoints block progression through, and exit from, mitosis if a functional mitotic spindle is not present (Hartwell and Weinert, 1989; Murray, 1992). Such checkpoints, whose function is to assure that the cell does not exit mitosis until the genome has been properly segregated, can be subverted. *Saccharomyces cerevisiae* mutants have been isolated that fail to maintain mitotic arrest during exposure to benomyl (Hoyt et al., 1991; Li and Murray, 1991), an inhibitor of microtubule assembly. Under restrictive conditions, these mutants inactivate the p34^{cdc2} kinase and progress forward into G_1 without division. Similarly, various cell types blocked in mitosis for a prolonged period with microtubule inhibitors undergo "mitotic slippage", which is a failure to maintain their arrested state (Chamla et al., 1980; Kung et al., 1990; Rieder and Palazzo, 1992). During mitotic slippage, CHO cells degrade cyclin B and downregulate p34^{cdc2} kinase activity, and resume interphase and DNA replication without first completing chromosome segregation or cell division (Kung et al., 1990).

We have previously shown that mammalian cells can also be induced to evade mitotic checkpoints and exit mitosis prematurely when exposed to the protein kinase inhibitor 2-aminopurine (2-AP)[1] (Andreassen and Margolis, 1991, 1992). Exposure of mammalian cells to 2-AP during mitotic arrest induces the cells to exit mitosis without the completion of late mitotic events such as chromosome segregation or cytokinesis (Andreassen and Margolis, 1991).

The p34^{cdc2} kinase is universally required as the master control enzyme during mitosis in eukaryotes (Nurse, 1990; Norbury and Nurse, 1992). Its activity is regulated during the cell cycle, and peaks at mitosis (Draetta and Beach, 1988; Gautier et al., 1989). Exit from mitosis requires the inactivation of p34^{cdc2} by the degradation of the cyclin B regulatory subunit (Murray et al., 1989; Ghiara et al., 1991; Gallant and Nigg, 1992) and the dephosphorylation of p34^{cdc2} at thr161 (Lorca et al., 1992). A subpopulation of p34^{cdc2} is localized to the mitotic apparatus during mitosis (Bailly et al., 1989; Riabowol et al., 1989; Alfa et al., 1990; Rattner et al., 1990). The localization of p34^{cdc2} is probably important to its function (Pines and Hunter, 1991), and may also be important to its regulation (Alfa et al., 1990; Kubiak et al., 1993; Edgar et al., 1994). Likewise, in the process of mitotic exit, localization of p34^{cdc2} to the mitotic spindle could be important to its inactivation.

Address all correspondence to R. L. Margolis, Institut de Biologie Structurale, 41 avenue des Martyrs, 38027 Grenoble, France. Ph.: (33) 76 88 96 24. Fax: (33) 76 88 54 94.

1. *Abbreviation used in this paper*: 2-AP, 2-aminopurine.

Here, we report that p34^{cdc2} is inactivated during mitotic checkpoint override induced by 2-AP. Further, we find that the level of microtubule assembly determines the capacity of BHK cells to evade mitotic checkpoints and exit from mitosis inappropriately during treatment with 2-AP, and during natural mitotic slippage. Our results show that the major distinction between 2-AP induced exit and mitotic slippage is that 2-AP greatly accelerates the escape from mitotic arrest. These results suggest that critical elements of the machinery for the inactivation of p34^{cdc2} are dependent on microtubules in these cells. The linkage of the microtubule assembly state to the regulation of p34^{cdc2} kinase activity may constitute an inherent checkpoint mechanism that couples the loss of p34^{cdc2} activity at the onset of anaphase with the capacity for accurate microtubule-dependent chromosome segregation.

Materials and Methods

Cell Culture and Synchronization

BHK cells were cultured as monolayers in Dulbecco's Modified Eagle's Medium (GIBCO BRL, Paisley, UK) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Cells were maintained in a humid incubator at 37°C in a 5% CO_2 environment.

Taxol and nocodazole were both obtained from Sigma Chemical Co. (St. Louis, MO) and were prepared as stock solutions in DMSO (5 mg/ml taxol, 1 mg/ml nocodazole) which were kept frozen until used.

Immunofluorescence Microscopy

BHK cells were grown on poly-lysine-coated coverslips for a minimum of 16 h before drug treatment. Except for immunolocalization of p34^{cdc2}, cells were fixed with 2% paraformaldehyde-PBS, permeabilized with 0.2% Triton X-100 in PBS for 3 min, and then processed with primary and secondary antibodies as described previously (Andreassen and Margolis, 1991). Secondary antibodies, all from Cappel Laboratories (West Chester, PA), included FITC-conjugated affinity purified goat anti-human IgG, anti-mouse IgG, and anti-rabbit IgG antibodies, and Texas Red sheep anti-mouse IgG antibodies applied at 2.5 μg/ml.

For immunolocalization of p34^{cdc2}, cells were first extracted in microtubule stabilizing buffer: 45 mM Pipes, 45 mM Hepes, 10 mM EGTA, 5 mM $MgCl_2$, pH 6.9, also including 0.5% Triton X-100, for 90 s at 37°C (Bailley et al., 1989). Cells were then fixed 5 min in methanol at −20°C, washed with PBS, and then incubated with primary and secondary antibodies.

The following antibodies were used for indirect immmunofluorescence microscopy, MPM-2 mouse monoclonal antibody, a gift from Dr. P. N. Rao (University of Texas, M. D. Anderson Cancer Center, Houston, TX), was used as a 750-fold dilution of the ascites fluid. Anti-β-tubulin ascites antibody from Sigma Chemical Co. (TUB 2.1) was used at a 400-fold dilution. Anti-centromere serum from a human patient with CREST Scleroderma, provided by Dr. Barbara Hamkalo (University of California at Irvine, Irvine, CA), was used at a 500-fold dilution. Antibodies generated in rabbits against amino acids 290-297 of human p34^{cdc2} (1419 and 1524) were supplied by Dr. David Litchfield (Manitoba Institute of Cell Biology, Winnipeg, Canada). These anti-p34 sera were used at a 1,000-fold dilution for the crude sera and at 0.5 μg/ml for antibody affinity-purified against the antigen peptide (Litchfield et al., 1991).

For microscopy, coverslips were mounted as previously described (Andreassen and Margolis, 1991). Samples were observed using a MRC-600 Laser Scanning confocal apparatus (Bio-Rad Microscience Division, Herts, England). Photographs were taken on TMAX-100 film with a Polaroid CI-3000 film recorder.

Histone H1 Kinase Assay

BHK cells grown in tissue culture dishes were treated with taxol or nocodazole for 10 h, and a mitotic index of ~60% was obtained. Cells were then placed in medium containing either taxol or nocodazole alone, or in taxol or nocodazole and 10 mM 2-AP. At the indicated timepoints cell extracts were prepared in lysis buffer, which was composed of: 50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 5 mM EGTA, 0.1% NP-40 (Draetta and Beach, 1988), and also containing 0.1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 60 mM β-glycerophosphate, 50 mM NaF, and 0.5 mM sodium vanadate. Cells were transferred to an Eppendorf tube (Brinkman Instruments, Westbury, NY) and kept on ice for 30 min. Lysate supernatants were then collected by centrifugation at 13,000 *g* for 5 min at 4°C.

To preclear cell extracts of proteins nonspecifically binding to Sepharose beads, 50 μl of each extract was incubated for 30 min at 4°C, with agitation, in a 1:1 slurry with protein A–Sepharose 4B beads (Sigma Chemical Co.) in lysis buffer. 40 μg of each cleared extract was then incubated with 4.0 μg affinity-purified antibody raised against the COOH terminus of p34^{cdc2} (Koff et al., 1991) for 1 h at 4°C with agitation. 50 μl of protein A–Sepharose slurry was then added and the extract was further incubated for 1 h at 4°C. The resulting immune complex was washed three times in lysis buffer and one time with kinase buffer, composed of 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT and 0.1 mg/ml BSA. The pellet was resuspended in 50 μl of kinase buffer containing 1 μg histone H1 (Boehringer Mannheim Biochemicals, Indianapolis, IN) 30 μM ATP, and 5 μCi of [γ-^{32}P] ATP (Koff et al., 1991). The H1 kinase reaction was carried out for 30 min at 37°C and was terminated by the addition of sample buffer. Samples were then resolved by SDS-PAGE using 2% polyacrylamide gels (19:1 ratio of acrylamide to *bis*-acrylamide). Autoradiographs were prepared by exposure to Hyperfilm-MP (Amersham Corp.).

Immunoblotting

1.0 μg of BHK lysate, prepared as above, was resolved on 8% polyacrylamide gels (30:0.8 ratio of acrylamide to *bis*-acrylamide) using a mini-gel apparatus (Bio-Rad Laboratories).; Electrophoretic transfer to nitrocellulose was performed according to Khyse-Andersen (1984). After transfer, nitrocellulose sheets (Amersham Corp.) were blocked for 60 min with 5% nonfat milk in 25 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20 (TNT buffer). Blots were then washed twice for 10 min each, and then incubated overnight at RT with anti-p34 (1419) antibody diluted 5,000-fold in TNT buffer. Blots were further processed by washing twice for 5 min with TNT buffer, 5 min with TNT buffer containing 0.5% Tween 20, and again for 5 min with TNT buffer (0.05% Tween 20). HRP-conjugated goat anti-rabbit IgG secondary antibody (Tago, Burlingame, CA), diluted 7,500-fold in TNT buffer (0.05% Tween 20), was applied for 1 h at room temperature. Blots were then washed in the same manner as for primary antibody incubation. Protein-antibody complex was detected by enhanced chemiluminescence (Amersham Corp.).

Flow Cytometric Analysis

Cells were collected by trypsinization, pooled with nonattached cells, resuspended in PBS, and fixed by addition of 90% methanol at −20°C (Schimenti and Jacobberger, 1992). After 10 min fixation, cells were pelleted, and then resuspended and stored in PBS with 0.04% sodium azide. Mitotic cells were determined using MPM-2 antibody, by incubation for 1 h at 37°C with MPM-2 antibody diluted 750-fold in PBS/0.05% Tween 20/3% BSA. After exposure to the antibody, cells were washed twice for 5 min each with PBS, and incubated with FITC-conjugated goat anti-mouse IgG secondary antibody 30 min at 37°C. Cells were then washed twice with PBS and resuspended in 4 mM sodium citrate, also containing 30 U/ml RNase A, 0.1% Triton X-100, and 50 μg/ml propidium iodide for 10 min at 37°C. Sodium chloride was then added at 138 mM, and samples were kept on ice until analyzed (Andreassen and Margolis, 1992).

Data were collected using a FACScan (Becton Dickinson & Co., San Jose, CA) and results analyzed with LYSYS II software. For each sample, 10,000 events were collected and aggregated cells were gated out.

Results

The Mitotic Exit Induced by 2-AP Is Microtubule Dependent

We have previously shown that BHK cells, arrested in mitosis with either 5 μg/ml taxol or 0.04 μg/ml nocodazole, can be induced by 2-AP to reenter interphase without completion of cell division (Andreassen and Margolis, 1992). In our previous work, the parameters followed to determine reentry

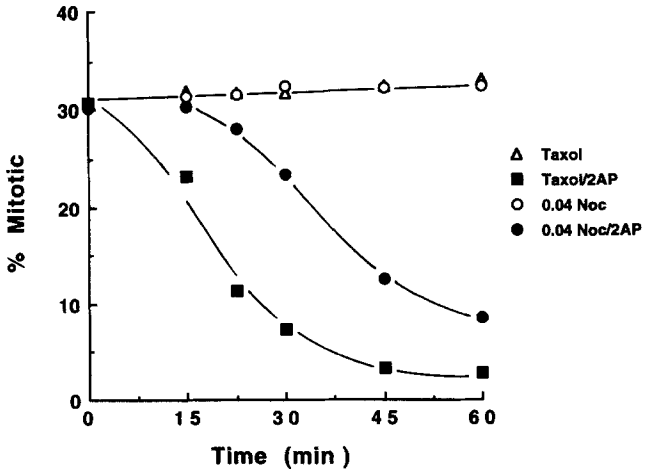

Figure 1. Exit from mitosis induced by 2-AP is more rapid for taxol-arrested cells than for nocodazole-arrested cells. BHK cells were synchronized in mitosis by treatment with either 5 μg/ml taxol or 0.04 μg/ml nocodazole. At $t = 0$ cells were given 10 mM 2-AP in addition to taxol or nocodazole, while controls were maintained in either taxol or nocodazole alone. Cells were fixed at timepoints and processed for immunofluorescence microscopy to determine the mitotic index. Cells were determined to be mitotic by the presence of MPM-2 signal, which detects mitosis-specific phosphorylation epitopes (Davis et al., 1983). The mitotic index was further confirmed by a second assay of chromosome condensation, using propidium iodide stain. All points represent the average of three independent counts of greater than 150 cells each. Standard deviation was less than 2.5% of the ordinate value for all data points.

into interphase were reformation of the nuclear lamin B border, and reconstitution of interphase nuclei as detected by propidium iodide staining (Andreassen and Margolis, 1991). In the present study we further verify exit from mitosis by loss of reactivity with MPM-2 antibodies, which recognize mitosis-specific epitopes (Davis et al., 1983), and by the extinction of $p34^{cdc2}$ H1 kinase activity.

We have determined that the rate of exit is conditional on the method of mitotic arrest. For example, the rate of exit is more rapid for taxol-treated cells than for nocodazole-arrested cells (Fig. 1). Taxol arrests cells in mitosis with an inappropriately high level of microtubule assembly (Schiff and Horwitz, 1980), while nocodazole arrests cells by interfering with microtubule assembly (Jordan et al., 1992).

The rate of blockage in mitosis is not affected by the nature of the mitotic block. Thus, the mitotic indices resulting from arrest with taxol or nocodazole are indistinguishable after 7.5 h of treatment. However, after addition of 2-AP, differences in the mitotic indices rapidly become apparent. Mitotic exit is first detected in taxol-arrested cells by 15 min after the addition of 10 mM 2-AP, and half of the cells exit mitosis by 20 min. In contrast, cells arrested with 0.04 μg/ml nocodazole first display evidence of mitotic exit by 25 min after the addition of 2-AP, and half the cells exit by 45 min. Control cells in the continuous presence of taxol or nocodazole alone do not show evidence of mitotic exit and the mitotic index for these populations continues to increase during the course of the experiment.

Data for analysis of the rate of mitotic exit were obtained by immunofluorescence microscopy, using MPM-2 antibodies. Mitotic cells, and not interphase cells, exhibit a bright and distinct MPM-2 signal, as shown for a taxol-treated population of BHK cells (Fig. 2). Because of the brightness of the mitotic signal, one can rapidly discriminate between mitotic and interphase cells using this marker. The MPM-2 signal disappears upon the addition of 2-AP to cells in taxol induced mitotic arrest. By this criterion, after 60 min of 2-AP treatment taxol-treated cells have reentered interphase,

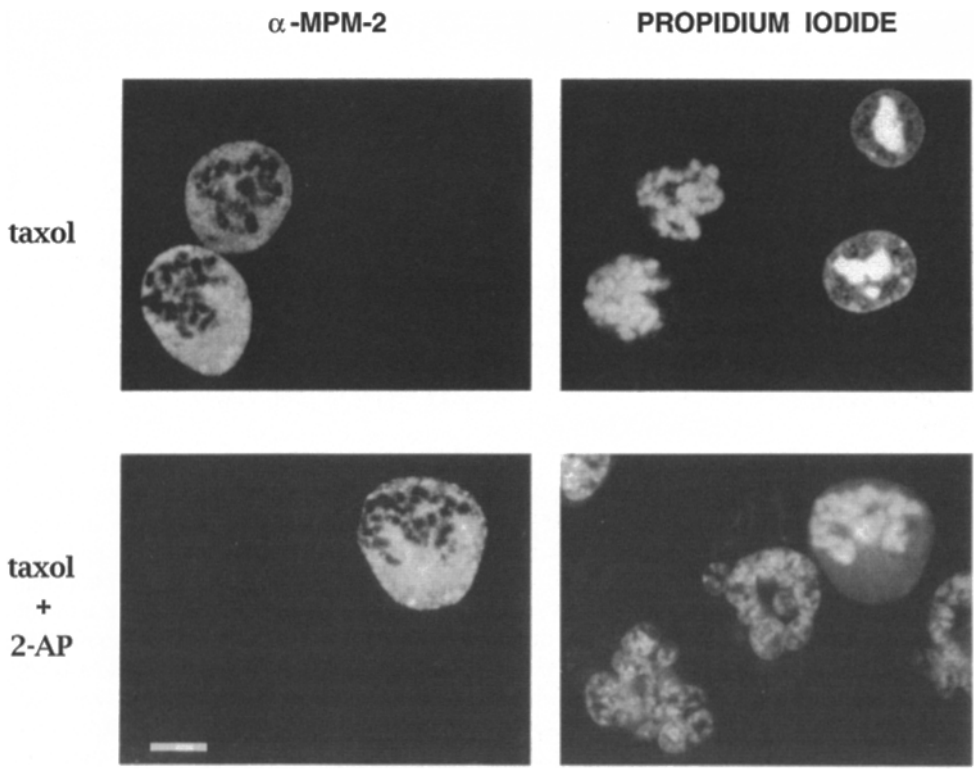

Figure 2. MPM-2 signal is down regulated and nuclei reform during mitotic checkpoint override induced by 2-AP. Mitotic cells that accumulate during treatment with 5 μg/ml taxol react strongly with MPM-2 antibodies (*top left*) and have condensed chromosomes (*top right*), in contrast to interphase cells (as determined by propidium iodide staining at right) which display minimal MPM-2 signal. 60 min after addition of 10 mM 2-AP to taxol-treated cells, exit from mitosis is apparent, as is made evident by the loss of MPM-2 signal (*bottom left*) and the appearance of micronuclei (*bottom right*). An exceptional cell that has remained in mitosis in the presence of taxol + 2-AP is included in the field to demonstrate that MPM-2 signal does not diminish with this treatment until there is actual exit from mitosis. Bar, 10 μm.

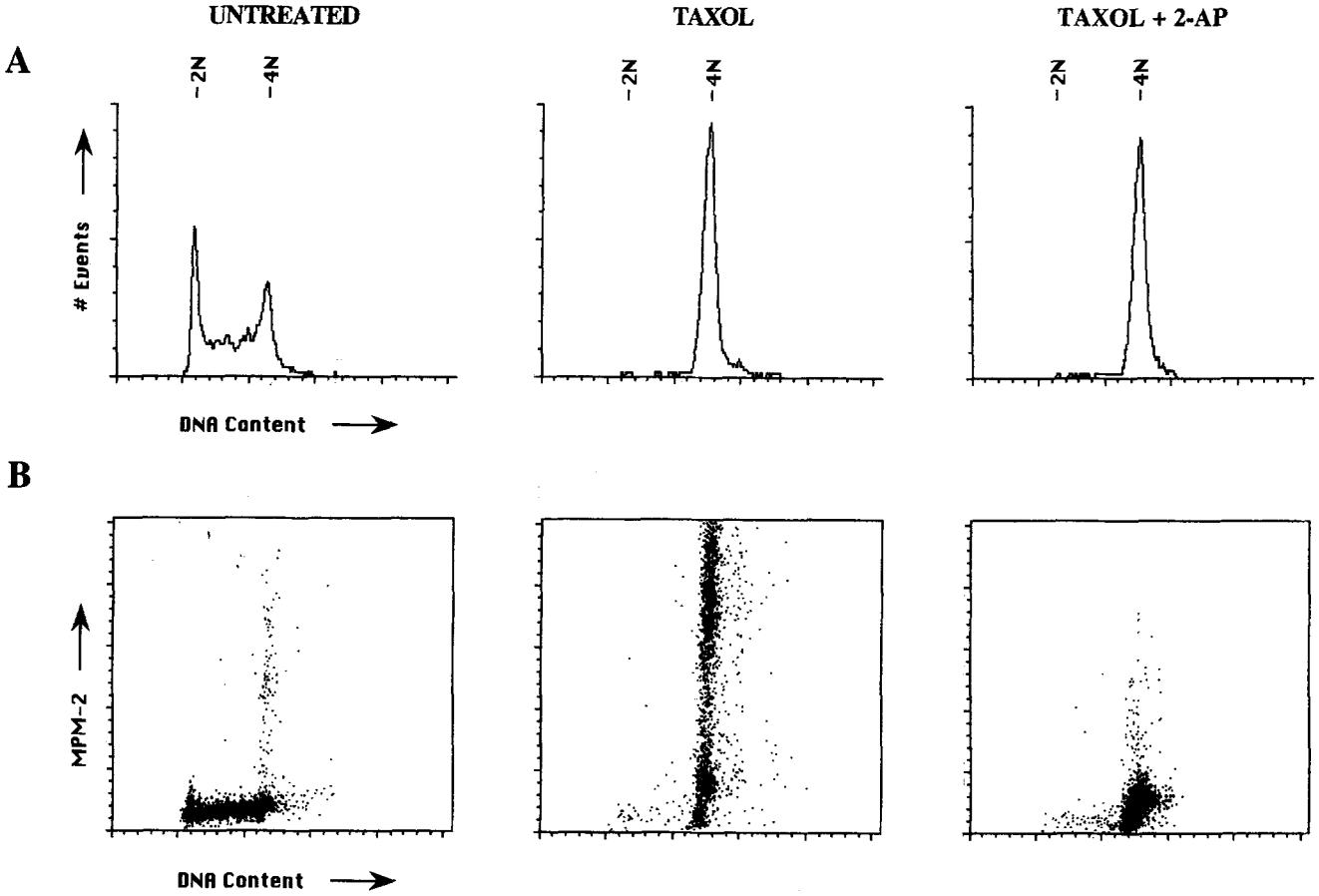

Figure 3. Exit from mitosis induced by 2-AP occurs without cell division and results in quantitative down regulation of MPM-2 signal. Cell populations were analyzed for DNA content and MPM-2 antigen abundance by flow cytometry. Cells subjected to analysis included untreated cycling BHK cells (*left*), a population of mitotic cells obtained by 8-h treatment with 5 μg/ml taxol and harvested by selective detachment (*middle*) and a population of cells exposed to taxol + 10 mM 2-AP for 60 min after selective detachment of taxol treated mitotic cells (*right*). All cell populations were analyzed for DNA content distributions, with data displayed in histogram form (*A*). Mitotic cells in taxol (*middle*) and post-mitotic cells following exposure to taxol + 2-AP (*right*) both have 4N DNA content. Corresponding dot plots of MPM-2 signal vs. DNA content (*B*) show that the MPM-2 signal returns to levels present in untreated interphase cells following treatment with taxol + 2-AP.

and they can also be distinguished from premitotic cells by the presence of micronuclei (Fig. 2). The decrease in MPM-2 signal is tightly coupled to exit from mitosis, and cells that remain in mitosis, as determined by other criteria, fully retain the MPM-2 signal until the time of mitotic exit (Fig. 2).

The decrease in MPM-2 signal induced by 2-AP treatment can be quantitatively assessed for a population of cells using two-dimensional flow cytometry (Fig. 3). Before drug treatment, most BHK cells are in G_1 or S phase (<4N DNA content) and have a correspondingly low MPM-2 signal. High MPM-2 signal is restricted to the mitotic (4N) subpopulation (Fig. 3, *A* and *B*, *left*). A mitotic population of cells, obtained by 5 μg/ml taxol arrest and selective detachment, has a 4N DNA content and displays a strongly elevated MPM-2 signal (data not shown). This profile is unchanged in the continued presence of taxol over a time course of 60 min (Fig. 3, *A* and *B*, *middle*). In contrast, when 2-AP is added to taxol arrested cells after selective detachment, the MPM-2 signal has returned to interphase levels by 60 min (Fig. 3 *B*, *right*). The DNA content of the 2-AP–treated cells remains 4N (Fig. 3 *A*, *right*), indicating that checkpoint override and exit from mitosis have occurred without cell division.

The observation that taxol-treated cells exhibit a greater rate of mitotic exit than nocodazole-treated cells suggests that mitotic exit is dependent upon the microtubule assembly state, since taxol causes nearly complete microtubule assembly (Schiff and Horwitz, 1980), whereas nocodazole inhibits microtubule assembly (Jordan et al., 1992). To further test the relationship between the microtubule assembly state and the ability of a cell to exit mitosis, populations of BHK cells were arrested in mitosis over a range of nocodazole concentrations (Fig. 4). From the data it is clear that 2-AP induced exit from mitosis is most rapid and complete for cells arrested with the lowest concentration (0.04 μg/ml) of nocodazole. A partial exit is obtained after 75 min of 2-AP treatment in cells arrested with 0.08 μg/ml nocodazole, and no mitotic exit is detected through 75 min of 2-AP treatment of cells arrested with nocodazole at concentrations ranging between 0.12–1.0 μg/ml. We conclude that 2-AP–induced mitotic exit is highly dependent on the microtubule assembly state.

We next made an analysis of the extent and sites of micro-

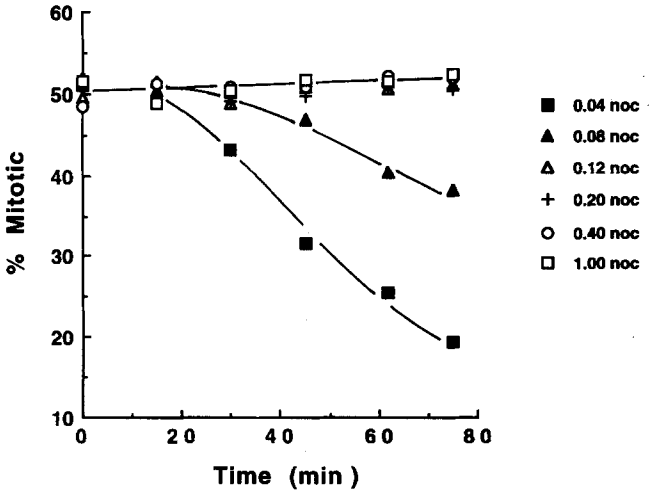

Figure 4. Nocodazole concentration influences the ability of 2-AP to induce override of mitotic arrest. BHK cells were accumulated in mitosis by 10 h treatment with nocodazole at concentrations ranging from 0.04–1.00 μg/ml. The resulting mitotic indices of the different populations were indistinguishable. At $t = 0$, 10 mM 2-AP was added to each cell population, while cells continued to be maintained in nocodazole at the same concentration as during the accumulation phase of the experiment. The mitotic index was determined by MPM-2 immunofluorescence assay and by the state of DNA condensation (propidium iodide stain). All points represent an average of three counts of greater than 150 cells each, and all standard deviations were less than 2.5% of the ordinate value.

tubule assembly in mitotic cells during the various drug treatments, as determined by immunofluorescence microscopy with anti-tubulin antibodies (Fig. 5). Mitotic cells treated with 5 μg/ml taxol have multiple dense, highly fluorescent microtubule asters, around which chromosomes are clustered. An interphase cell (Fig. 5, *inset*) displays peripheral bundles of microtubules, giving evidence of the abnormal taxol-induced microtubule bundles typically present in interphase cells.

By immunofluorescence microscopy (Fig. 5), it is evident that total microtubule mass decreases with increasing nocodazole concentrations. In populations treated with 0.04 μg/ml nocodazole for 10 h, mitotic cells have a single large centrosomal aster with a distinguishable microtubule array. At 0.08 μg/ml nocodazole, microtubules are distributed in a mixture of centromeric and centrosomal arrays. Among the population of mitotic cells, some have single centrosomal asters and other cells contain multiple small asters with foci at centromeres (see Fig. 6 for confirmation of centromeric microtubule association). The size of the centrosomal asters is diminished at 0.08 μg/ml nocodazole compared with those observed at 0.04 μg/ml nocodazole. At nocodazole concentrations of 0.12 μg/ml and 0.20 μg/ml (not shown), mitotic cells have large numbers of centromere-associated microtubule foci. The foci are larger in cells treated with 0.12 μg/ml than with 0.20 μg/ml nocodazole, having the appearance of small asters. At the highest nocodazole concentrations that were assayed, 0.40 and 1.0 μg/ml (not shown), centrioles are the only structures detected with anti-tubulin antibodies. Note that the centrioles are not apparent at the highest nocodazole concentration in Fig. 5, as confocal microscope settings were optimized to samples treated with 0.04 μg/ml nocodazole and kept constant for collection of all images.

Microtubule fluorescence at the position of chromosomes, and the high number of microtubule foci found in cells treated with 0.08–0.20 μg/ml nocodazole both suggested that microtubule assembly might be nucleated at centromeres under these conditions. This possibility was confirmed using confocal microscopy for analysis of the colocalization of human autoimmune anti-centromere antibodies (patient JD) and anti-tubulin antibodies (Fig. 6). As shown, in cells exposed to these nocodazole concentrations, multiple centromeres are frequently associated with single microtubule foci. The apparent coassociation of centromeres and microtubules was further confirmed by digital image overlay (data not shown). Centromeres appear in small clusters which are independent of the two centrosomes, as confirmed by double-label immunofluorescence microscopy with γ-tubulin antibody (data not shown).

The immunofluorescent analysis, as summarized in Fig. 5, suggested that efficient exit from mitosis might require the presence of a centrosomal aster of microtubules. Exit did not occur after 75 min of 2-AP treatment in those cells which contained only centromeric microtubules (0.12–0.20 μg/ml nocodazole; Fig. 4). Most interestingly, in cells exposed to a condition where partial exit is observed (0.08 μg/ml nocodazole), the initial population consists of a mixture of cells in which there are either centromere or centrosome associated microtubules (Fig. 5), but the cells remaining in mitosis after 75 min of treatment are exclusively those with only centromere-associated microtubules (data not shown). From our evidence, induction of mitotic exit minimally requires a critical mass of microtubules, but it is possible that exit further requires the specific interaction of these microtubules with the centrosomes of the mitotic cell.

Initiation of mitosis requires activation of $p34^{cdc2}$ (Lee and Nurse, 1987; Murray et al., 1989) and $p34^{cdc2}$ is normally inactivated during exit from mitosis (Murray et al., 1989; Hunt et al., 1992). As a further confirmation of the progression of mitotic exit following 2-AP addition, we thus assayed $p34^{cdc2}$ kinase activity during mitotic checkpoint override induced by 2-AP. To determine $p34^{cdc2}$ activity we performed a radiolabel assay of histone H1 phosphorylation by $p34^{cdc2}$ immunoprecipitates. The specificity of the assay was verified by the absence of H1 kinase activity in immunoprecipitate complexes lacking $p34^{cdc2}$ antibody, and by the greater $p34^{cdc2}$ activity in mitotic extracts as compared to extracts from S-phase arrested cells (Fig. 7 *A*).

Our results show that $p34^{cdc2}$ activity dramatically declines within 15 min following 2-AP addition to cells arrested in mitosis with taxol (Fig. 7 *B*). In contrast, cells continually arrested with taxol alone maintain high levels of $p34^{cdc2}$ activity over a period of sixty min (Fig. 7 *B*). Cells arrested with 0.40 μg/ml nocodazole, sufficient to fully depolymerize microtubules (Fig. 5), and then co-treated with 2-AP also show no decline in $p34^{cdc2}$ protein kinase activity over 60 min (Fig. 7 *B*). At the intermediate nocodazole concentration of 0.04 μg/ml, 2-AP addition induces an intermediate decrease in $p34^{cdc2}$ activity (Fig. 7 *B*). These results demonstrate that $p34^{cdc2}$ kinase activity decreases during mitotic checkpoint override induced by 2-AP, as occurs during a normal mitotic exit, and that the down regulation of $p34^{cdc2}$ ac-

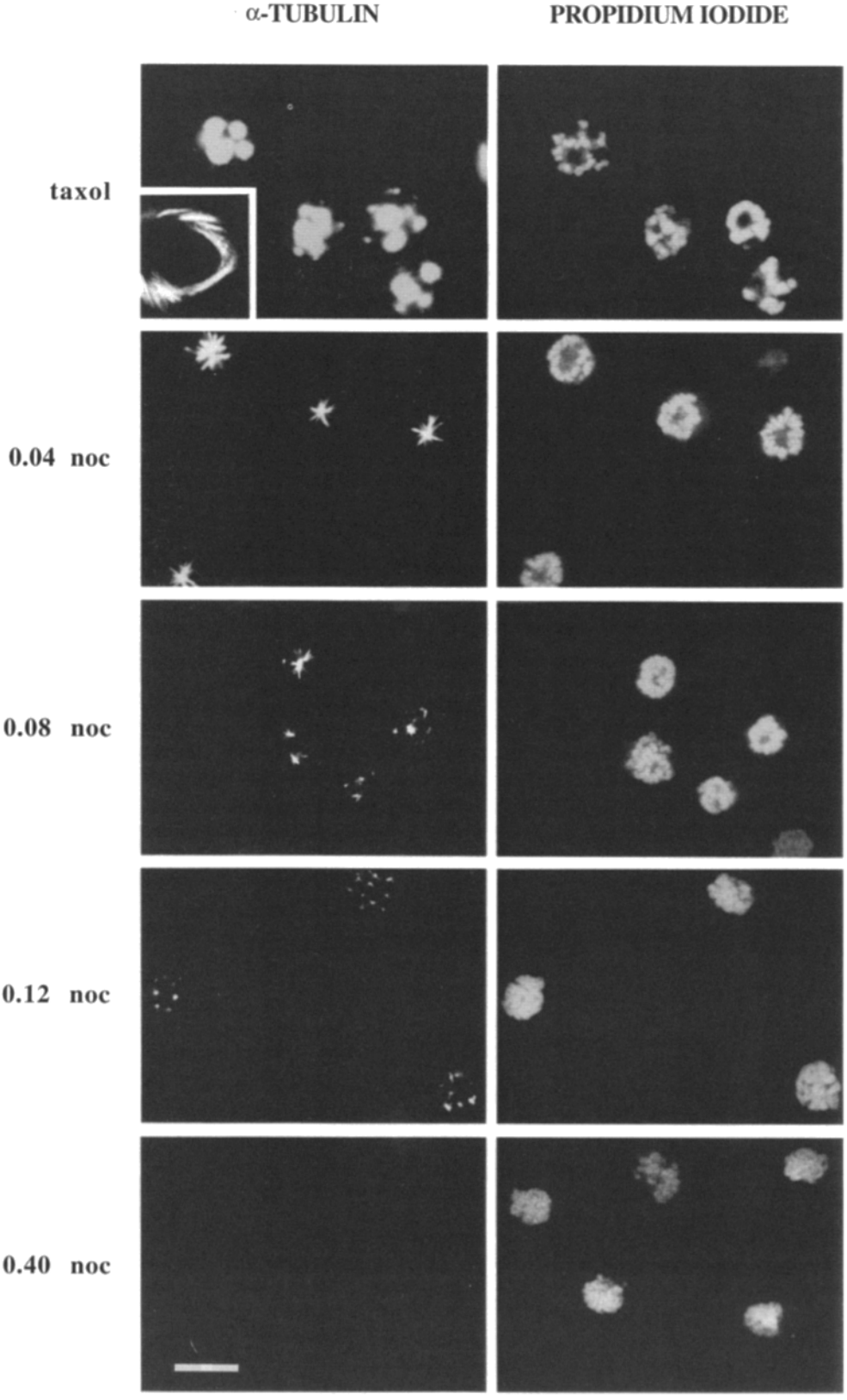

Figure 5. The site and extent of mitotic microtubule assembly varies with the conditions of drug treatment. Microtubule structures were detected by immunofluorescence microscopy using anti-β-tubulin antibodies and FITC-goat anti-mouse IgG secondary antibodies (*left*). Confocal microscope settings were optimized within the linear range for samples exposed to 0.04 μg/ml nocodazole and were kept constant for the collection of all images. Conditions of decreasing microtubule assembly (*taxol*, and then increasing nocodazole concentrations) are shown by descending order in the figure. A taxol treated interphase cell (*taxol, inset*) is included to show the high level of microtubule assembly and bundling induced by this taxol concentration. Chromatin position is shown by propidium iodide staining (*right*). Bar, 20 μm.

tivity is strongly dependent upon the microtubule assembly state.

The Correlation of p34^{cdc2} Localization with Mitotic Exit

The inactivation of p34^{cdc2} is required for exit from mitosis, and p34^{cdc2} has been demonstrated to be partially localized to the mitotic apparatus during mitosis (Bailly et al., 1989; Riabowol et al., 1989; Alfa et al., 1990; Rattner et al., 1990). It was therefore important to test whether p34^{cdc2} localization to the mitotic apparatus correlates with inactiva-

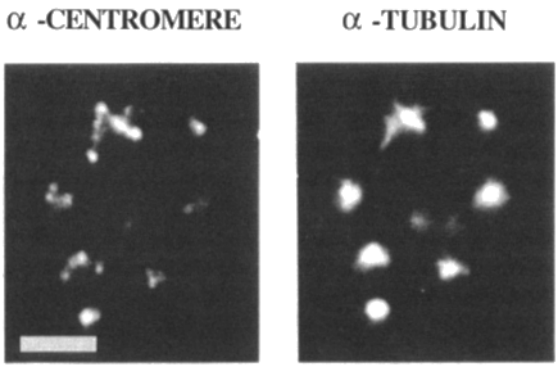

Figure 6. Microtubule structures colocalize with centromeres in cells treated with intermediate concentrations of nocodazole. Mitotic BHK cells arrested with 0.08–0.20 μg/ml nocodazole have multiple microtubule foci, as shown here at 0.12 μg/ml nocodazole. Colocalization with centromeres was tested by digital overlay of double-label immunofluorescent images processed with antibodies recognizing centromeres (human anti-centromere serum) and microtubules (anti-tubulin antibodies) and collected by computer-assisted confocal microscopy. For the purpose of demonstrating colocalization, an optical section including only a subset of the centromeres and microtubule foci is presented here. Primary antibodies were detected with FITC-goat anti-human IgG (anti-centromere) and Texas Red sheep anti-mouse (anti-tubulin) secondary antibodies. Bar, 5 μm.

tion of p34^{cdc2} and exit from mitosis in different microtubule assembly states. We therefore conducted an immunofluorescence analysis of the presence of p34^{cdc2} on microtubules under conditions where cells can or can not be induced to exit mitosis with 2-AP.

The p34^{cdc2} antibody used in this study was raised against a COOH-terminal peptide of p34^{cdc2} (Litchfield et al., 1991). On immunoblots of whole cell extracts, p34^{cdc2} was specifically recognized by the anti-p34^{cdc2} antibody, and preimmune serum gave no reaction (Fig. 8 *A*). In intact mitotic cells p34^{cdc2} is distributed throughout the cytoplasm, but is concentrated on microtubules at drug concentrations where microtubules persist (data not shown). To enhance visualization of p34^{cdc2} within the mitotic apparatus, cells were prepared for immunofluorescence by extraction in microtubule stabilizing buffer before fixation (Fig. 8 *B*).

Immunofluorescence microscopy of taxol-treated cells reveals that p34^{cdc2} is concentrated on microtubule asters and that p34^{cdc2} is also clearly present as an abundant signal at centromeres and at the centrosome (Fig. 8 *B*). In mitotic cells blocked with 0.04 μg/ml nocodazole, p34^{cdc2} is present on microtubules, centromeres, and centrosomes, but has a lower apparent abundance on microtubules. At nocodazole concentrations sufficient to maintain mitotic arrest in 2-AP (0.12 and 1.0 μg/ml), p34^{cdc2} remains associated with centromeres and the centrosome, but, as demonstrated by an anti-tubulin antibody counterstain, one finds minimal microtubule arrays and minimal association of p34^{cdc2} with microtubules at these nocodazole concentrations. Centromeric localization of p34^{cdc2} in mitotic cells was confirmed by correspondence of p34^{cdc2} with centromeric antigens recognized by human autoimmune anti-centromere serum (Fig. 8 *C*). The bright central p34^{cdc2} signal that does not correspond to centromere signal represents p34^{cdc2} localized to the centrosome (see Fig. 8 *B*).

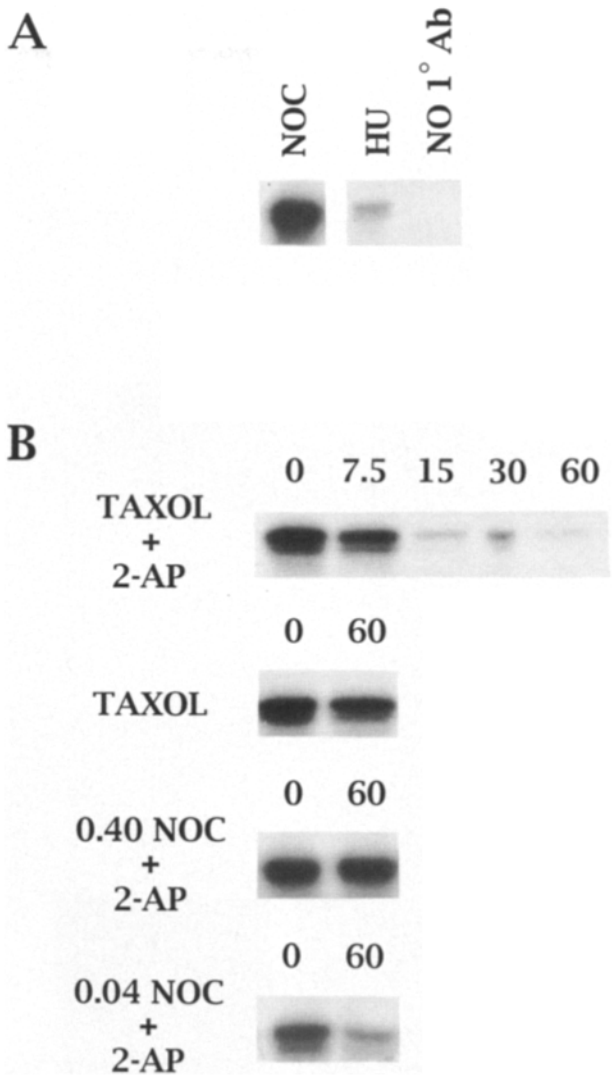

Figure 7. p34^{cdc2} is inactivated during mitotic exit induced by 2-AP. The protein kinase activity of p34^{cdc2} was determined by radiolabel assay of histone H1 phosphorylation. To assure specificity of the kinase activity, the assay used p34^{cdc2} immunoprecipitated from lysed cell extracts. The specificity of the assay is shown in *A*. Activity is greater for mitotic cells (*NOC*) than for S phase cells (arrested with 2 mM hydroxyurea; *HU*). Activity is not detected in protein from nocodazole-synchronized mitotic extracts processed as usual, but without p34^{cdc2} primary antibodies. p34^{cdc2} H1 kinase activity was determined at timepoints for mitotically synchronized cells, either with or without exposure to 10 mM 2-AP (*B*). Cells were treated with either 5 μg/ml taxol, or with 0.04 μg/ml or 0.40 μg/ml nocodazole for 10 h before the addition of 10 mM 2-AP. Time 0 represents the time of addition of 2-AP after 10 h of mitotic arrest. Times shown are elapsed times (in minutes) between 2-AP addition and cell lysis for the preparation of extracts. H1 kinase activity is diminished under conditions of mitotic exit (*TAXOL + 2-AP*; or *0.04 μg/ml NOC + 2-AP*), but is retained during 60 min of continued mitotic arrest (*TAXOL*, or *0.40 μg/ml NOC + 2-AP*).

The p34^{cdc2} localization was cross-checked with a second rabbit anti-p34^{cdc2} serum (designation 1524), which gave an identical localization pattern. The pre-immune serum for

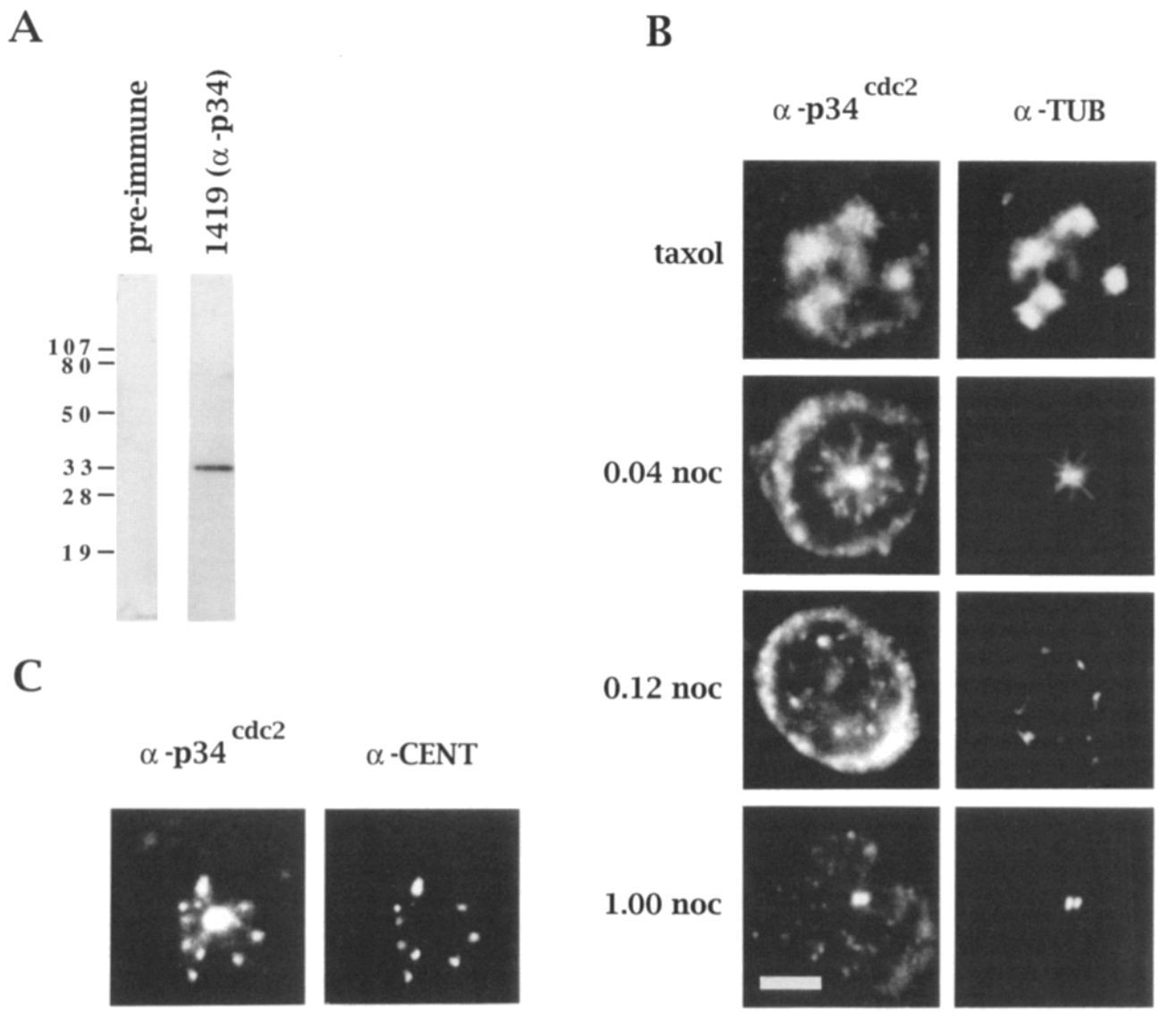

Figure 8. Localization of p34^{cdc2} during mitosis under various conditions of drug treatment. (*A*) p34^{cdc2} is specifically recognized in whole cell lysates by 1419 anti-p34^{cdc2} antibodies (Litchfield et al., 1991), but not by pre-immune serum at the same dilution (*left*), on immunoblots. (*B*) Double-label immunofluorescence for p34^{cdc2} (*left*) and tubulin (*right*). Fluorescence analysis was performed using FITC-goat anti-rabbit IgG and Texas Red sheep anti-mouse IgG secondary antibodies, respectively. p34^{cdc2} is present at centrosomes and centromeres under all conditions shown, and on microtubules when significant microtubule mass is present (taxol and 0.04 μg/ml nocodazole). The p34^{cdc2} staining pattern is not due to signal cross-over, and was obtained whether anti-tubulin antibody was present or not. (*C*) Double label immunofluorescence for p34^{cdc2} (*left*) and anti-centromere antiserum (*right*) is shown for a mitotic cell arrested with 0.04 μg/ml nocodazole. Bar, 5 μm.

both antibodies gave only a background signal (data not shown).

These results show a consistent pattern of correlation between p34^{cdc2} association with centrosomal microtubules and the capacity of cells to exit mitosis. We conclude that efficient exit from mitosis occurs only under conditions where p34^{cdc2} is found associated with centrosomal microtubules.

Mitotic Slippage Is Microtubule Dependent

Mitotic slippage is the natural failure of rodent cells to maintain mitotic arrest during long-term exposure to drugs that interfere with either microtubule dynamics or with formation of the mitotic spindle (Kung et al., 1990). Mitotic slippage and mitotic checkpoint override induced by 2-AP both involve the inactivation of p34^{cdc2} and occur without completion of late events in mitosis, including cytokinesis. The parallels between mitotic slippage and 2-AP-induced checkpoint override led us to consider whether mitotic slippage is also microtubule dependent in BHK cells.

Subconfluent populations of cells were treated with taxol or various concentrations of nocodazole and the mitotic index was determined at timepoints by scoring for the percentage of MPM-2-positive cells (Fig. 9). During the first 9 h of drug treatment, the mitotic index increases equivalently for all cell populations, regardless of whether treatment is with 5 μg/ml taxol, or with 0.04, 0.20, or 1.0 μg/ml nocoda-

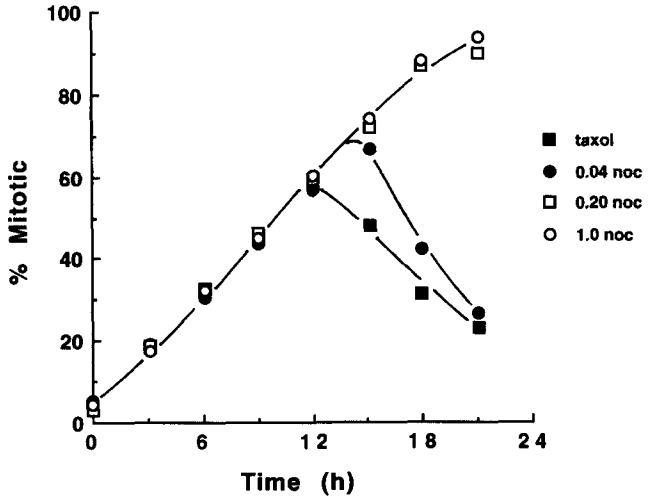

Figure 9. Mitotic slippage is dependent on the level of microtubule assembly. Beginning at $t = 0$, BHK cells were incubated in medium containing either taxol (5 μg/ml) or nocodazole at 0.04, 0.20, or 1.00 μg/ml. Cells were fixed at timepoints and prepared for immunofluorescence labeling with MPM-2 antibody and counterstained with propidium iodide. The mitotic index, scored on the basis of cells positive for MPM-2 and containing condensed chromosomes, rises uniformly for all populations through 12 h of drug treatment, but then declines in populations treated with taxol or 0.04 μg/ml nocodazole. All points represent an average of three counts of greater than 150 cells each, and no point has a standard deviation of more than 3.0%.

zole. No midbodies are detected by immunofluorescence microscopy with anti-tubulin antibodies (data not shown), providing evidence that mitotic progression is blocked at each of the drug conditions.

After treatment for times in excess of nine hours, the number of cells blocked in mitosis begins to diverge in the different drug treatments. Cells treated with 0.20 or 1.0 μg/ml nocodazole continue to accumulate linearly in mitosis, until >90% of the cells are in mitosis after 18 h of treatment. At these high drug concentrations, the mitotic index remains >90% through 21 h of total treatment. In contrast, after 12 h of drug treatment the mitotic index no longer increases linearly for populations arrested with taxol or 0.04 μg/ml nocodazole. The taxol and 0.04 μg/ml treated populations reach maximal mitotic indices of 60 and 70% after 12 and 15 h of treatment, respectively. Their mitotic indices then decline to less than 25% after 21 h of treatment (Fig. 9). Treatment with 0.08 μg/ml nocodazole yields intermediate levels of mitotic slippage, while treatment with 0.12 μg/ml nocodazole yields the same result as with 0.20 or 1.0 μg/ml nocodazole (data not shown). From these results it is evident that, as for 2-AP–induced exit from mitosis, mitotic slippage is microtubule dependent and occurs more readily under conditions of full microtubule assembly or in the presence of centrosomal microtubule asters.

The mitotic index curves were generated by immunofluorescence microscopy of the mitosis specific antigen MPM-2. The microscopic images themselves clearly demonstrate that over the first 12 h a striking increase in MPM-2 reactivity correlates with duration of either taxol or 1.0 μg/ml nocodazole drug treatment (Fig. 10). The mitotic index is low in both populations before drug treatment, and cells are mononucleate. After 12 h of treatment, an increase in the incidence of MPM-2–positive cells is evident in both populations. Similar to the distribution of $p34^{cdc2}$, MPM-2 reactivity is present at the centrosomes, centromeres, and microtubules. Association of MPM-2 epitopes with microtubules is most apparent in taxol treated cells. However, after 21 h of drug arrest the population treated with 1.0 μg/ml nocodazole is predominantly MPM-2 positive, whereas the population treated with taxol is predominantly negative for MPM-2. Those cells treated with high nocodazole that are MPM-2 positive also contain condensed chromosomes, while most of the taxol treated cells are highly multinucleate after 21 h of treatment. Micronucleation is a reliable marker for aberrant exit from mitotic arrest by mitotic slippage during taxol treatment, just as for 2-AP–induced checkpoint override and mitotic exit (Fig. 2).

We have also used two-dimensional flow cytometry to confirm the microtubule dependency of mitotic slippage (Fig. 11), comparing DNA content and MPM-2 signals at timepoints for cells treated with either 0.04 or 1.00 μg/ml nocodazole. Before drug treatment, the cell population predominantly has a 2N DNA content. By flow cytometric assay, the mitotic population is distinguished by its 4N DNA content and elevated MPM-2 signal. After 7 h of drug treatment, about one-half of the treated population has 4N DNA content and a corresponding elevation of MPM-2 signal. By 12 h, most of the cells have 4N DNA content and display high MPM-2 levels. Populations treated with 0.04 or 1.0 μg/ml nocodazole are indistinguishable by these cytometry parameters after 7 and 12 h of drug treatment, indicating that significant mitotic slippage does not occur during the first 12 h of drug treatment. As also demonstrated by immunofluorescence analysis, after 21 h of treatment with 1.0 μg/ml nocodazole the cell population is almost entirely mitotic, exhibiting a 4N DNA content and an elevated MPM-2 signal. It is interesting that, even at high nocodazole levels, the average MPM-2 signal declines during prolonged mitotic arrest. In contrast, cells treated with 0.04 μg/ml nocodazole have background levels of MPM-2 signal by 21 h of treatment, consistent with exit from mitosis.

Mitotic slippage is evident in the population treated with 0.04 μg/ml nocodazole. The DNA content of this population is 4N or greater, as cells that have exited mitosis have reinitiated DNA synthesis during the subsequent S phase. Few of these cells have less than 4N DNA content, confirming that they have exited mitosis without the completion of cytokinesis. Despite slippage back into interphase without completing any of the normal mitotic processes, these cells are competent to initiate DNA synthesis in the next interphase.

We conclude that both mitotic slippage and 2-AP induced exit from mitosis give evidence of common microtubule-dependent mechanisms by which cells control the inactivation of $p34^{cdc2}$, and thereby reenter interphase.

Discussion

Microtubule Dependency of 2-AP–induced Exit from Mitosis

$p34^{cdc2}$ protein kinase is central to the regulation of mitosis in eukaryotes (Nurse, 1990; Norbury and Nurse, 1992). Inactivation of $p34^{cdc2}$ is required for mitotic exit (Murray et al., 1989; Ghiara et al., 1991; Gallant and Nigg, 1992), and

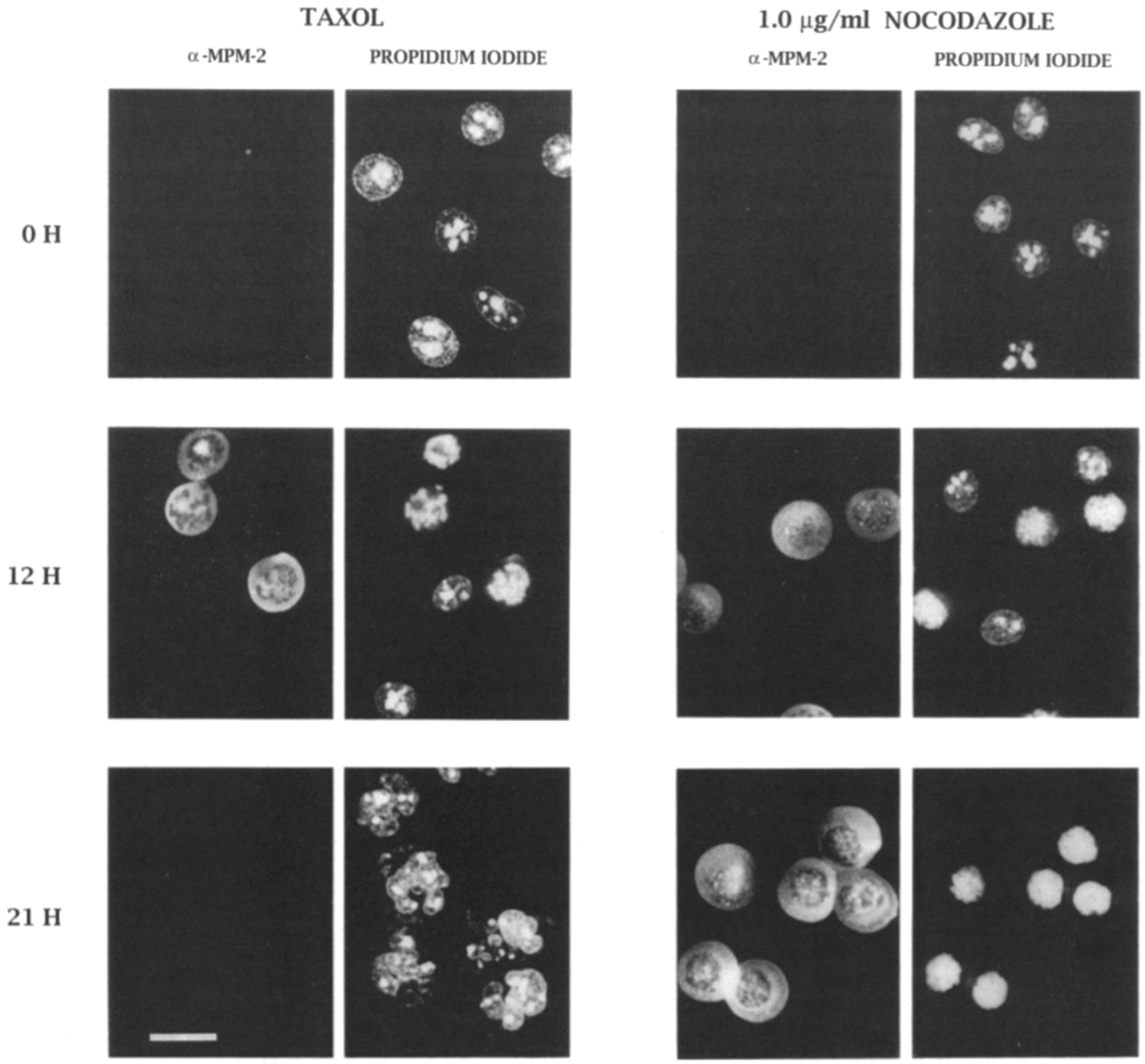

Figure 10. MPM-2 signal is attenuated and nuclei reform during mitotic slippage of cells treated with taxol. Taxol treated cells undergo mitotic slippage. In contrast, cells treated with 1.0 μg/ml nocodazole continue to accumulate in mitosis through 21 h, as demonstrated both by MPM-2 antibody reactivity and by the presence of condensed chromosomes (*PROPIDIUM IODIDE*). BHK cells were fixed prior to drug treatment (*0 H*) or at 12 or 21 h following the addition of 5 μg/ml taxol or 1.0 μg/ml nocodazole to the culture medium. Samples were then prepared for immunofluorescence analysis with MPM-2 antibodies, and were counter-stained with propidium iodide. MPM-2 antibodies were detected with FITC-goat anti-mouse IgG secondary antibodies. Bar, 25 μm.

its continued activity can maintain the cell in the mitotic state for a prolonged period until certain conditions are met for mitotic exit. These conditions, which act as checkpoints to the completion of mitosis, include the assembly of a mitotic spindle (Hoyt et al., 1991; Li and Murray, 1991), microtubule attachment to centromeres (Zirkle, 1970), centromere function (Tomkiel et al., 1994), and the proper alignment of chromosomes at the metaphase position (Zirkle, 1970; Rieder and Alexander, 1990).

We have demonstrated that mammalian cells are induced to inactivate the $p34^{cdc2}$ kinase and to exit mitosis by addition of the protein kinase inhibitor 2-AP, but only if a critical assembly state of microtubules exists in the cell. The rate of 2-AP-induced exit from a mitotic block is most rapid in the presence of taxol, a mitotic inhibitor which induces an inappropriately high level of microtubule assembly. In contrast, increasing nocodazole concentrations decrease the capacity for mitotic exit. 2-AP-induced exit from mitosis thus reveals an intrinsic role for microtubules in control of mitotic checkpoints in BHK cells, independent of their mechanical role in chromosome segregation. These conclusions are also supported by our demonstration that exit from mitosis due to mitotic "slippage" is dependent on the presence of a critical level of microtubule assembly.

Both the microtubule mass and the localization of microtubules in the cell are altered by nocodazole in a concentration

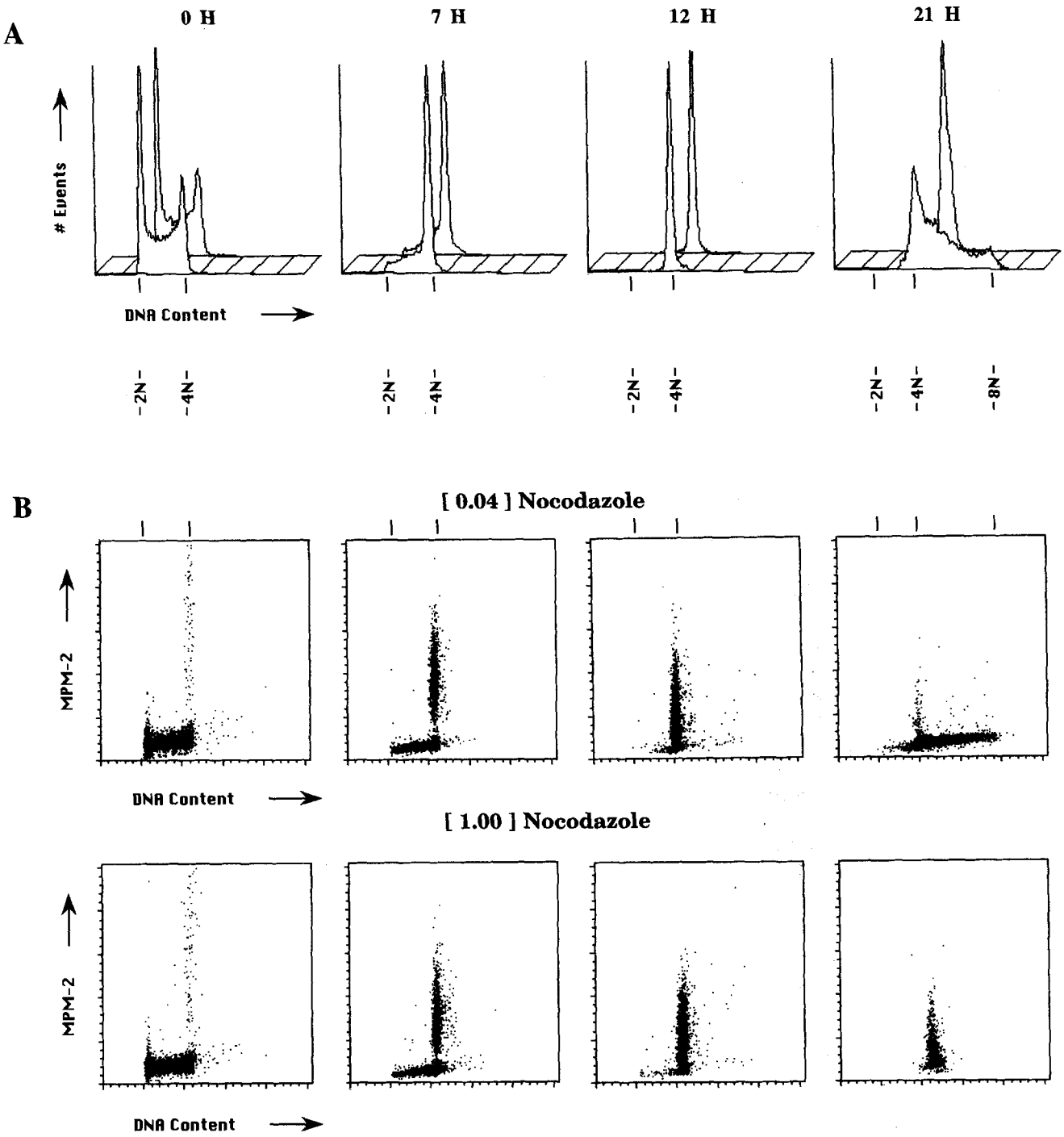

Figure 11. Mitotic slippage analysis by flow cytometry. Flow cytometry was used to assay both the attenuation of MPM-2 signal and the resumption of DNA synthesis during mitotic slippage. DNA content histograms are shown (*A*) for samples before drug addition or following the addition of 0.04 μg/ml (front) or 1.00 μg/ml (back) nocodazole to the culture medium for the times indicated. Dot plots of MPM-2 antibody fluorescence signal vs. DNA content (propidium iodide) are shown in *B*. Accumulation in mitosis is identical through 12 h treatment, but cells treated with 0.04 μg/ml nocodazole undergo mitotic slippage and progress toward 8N DNA content with low MPM-2 signal by 21 h.

dependent manner. At nocodazole concentrations where 2-AP induces mitotic exit (0.04–0.08 μg/ml), microtubules are present as a single aster centered on the centrosome. In contrast, at 0.12–0.20 μg/ml nocodazole, 2-AP cannot induce mitotic exit, and in this case microtubules have no apparent centrosome association but are instead clearly associated with kinetochores. These results suggest that mitotic exit induced in response to 2-AP may involve centrosomal control, mediated through centrosome-associated microtubules.

The microtubule dependency of mitotic exit demonstrates that $p34^{cdc2}$ inactivation must be coupled in some important manner to the mitotic spindle. Such spindle-associated mechanisms might routinely function to assure that a normal mitotic spindle is present, thereby increasing the fidelity of

chromosome segregation and preventing aneuploidy (Hartwell and Weinert, 1989; Li and Murray, 1991).

Our results suggest that efficient checkpoint regulation of $p34^{cdc2}$ might require the localization of $p34^{cdc2}$ and cyclin B to the mitotic spindle. Indeed, we find that a portion of $p34^{cdc2}$ is localized to spindle microtubules, and to the centrosome and kinetochores, and that induction of mitotic exit by 2-AP occurs only under conditions where $p34^{cdc2}$ is associated with astral microtubules. Other recent studies have suggested that the active form of $p34^{cdc2}$ is concentrated at the mitotic spindle (Tombes et al., 1991; Kubiak et al., 1993), and Edgar et al. (1994) have recently suggested that the mitotic spindle may be the site of local degradation of cyclin B during exit from mitosis in early *Drosophila* embryos.

It is important, in this regard, to note that several molecular elements implicated in the control of cyclin B degradation are localized to the mitotic spindle. Calmodulin-dependent protein kinase II, which modulates cyclin B degradation and exit from meiotic arrest in Xenopus oocytes (Lorca et al., 1993) is present on the mitotic spindle (Ohta et al., 1990). Ca^{2+}/calmodulin (Welsh et al., 1979), which regulates calmodulin-dependent protein kinase II, and is critical to the regulation of mitotic exit in yeast, Xenopus, and mammalian cells (Rasmussen and Means, 1989) is also present on the mitotic spindle. Additionally, elements directly involved in cyclin B destruction are concentrated on the mitotic spindle. These include ubiquitin (Murti et al., 1988), which becomes conjugated to cyclin B to signal its destruction (Glotzer et al., 1991), and the 20S proteasome core of the 26S protease complex (Amsterdam et al., 1993) which is implicated in the degradation of ubiquitinated cyclin (Ghislain et al., 1993; Gordon et al., 1993; Hersko and Ciechanover, 1992).

While it is evident that efficient checkpoint override of mitotic arrest in BHK cells requires spindle microtubules, the effect is not absolute. In the experiments reported here, 2-AP induced exit and exit through "slippage" will eventually occur in the absence of detectable microtubules. Further, it appears that various cell types have differences in the mechanism of M phase arrest. At an extreme, drug disassembly of microtubules does not induce cell cycle arrest in Xenopus embryos before the mid-blastula transition (Gerhart et al., 1984).

It has recently been reported that cyclin B degradation is microtubule dependent during the metaphase–anaphase transition in meiotic mouse oocytes (Kubiak et al., 1993). The mouse oocyte observations, together with our results, give evidence that microtubule dependent signals are important to cell cycle progression in both meiotic and mitotic mammalian cells. There is, however, an important difference between meiotic and mitotic control mechanisms. C-mos protein kinase is specifically required to maintain prolonged meiotic arrest in a variety of organisms including Xenopus (Sagata et al., 1989) and mice (O'Keefe et al., 1991), and, under the influence of c-mos, the meiotic cell remains arrested in a metaphase configuration with a fully assembled spindle until the oocyte is fertilized. C-mos is meiosis specific, and is not involved in maintenance of mitotic cells in metaphase (Mutter and Wolgemuth, 1987; Herzog et al., 1988).

Implications for the 2-AP Mechanism of Action

The microtubule dependency of mitotic exit also has important implications for the mechanism of action of 2-AP in inducing checkpoint override. It has been reported that 2-AP acts as a protein kinase inhibitor (Farrell et al., 1977) and that it has a highly selective effect on the pattern of protein phosphorylation in intact cells (Mahadevan et al., 1990). If 2-AP causes checkpoint override and mitotic exit by suppressing a protein kinase activity, it is certain that 2-AP does not act by directly suppressing the activity of $p34^{cdc2}$. If that were the case, all cells arrested in mitosis would exit upon addition of 2-AP, regardless of the microtubule assembly state. This interpretation was also implied in our previous results (Andreassen and Margolis, 1992) which showed that 2-AP dependent override of interphase checkpoints advances cells forward into mitosis.

Microtubule Assembly from Kinetochores

The association of microtubules with kinetochores at intermediate nocodazole concentrations suggests that microtubules are capable of nucleating at kinetochores under appropriate in vivo conditions. Kinetochore nucleation has previously been reported in mammalian tissue culture cells recovering from colcemid treatment (Witt et al., 1980). We have suggested that kinetochore-dependent microtubule assembly may have a part in the maintenance of the normal microtubule steady state, and mitotic spindle function (Margolis and Wilson, 1981; Garel et al., 1987). Furthermore, proximal assembly of microtubules at kinetochores is implicit in the observed metaphase and anaphase poleward flux of kinetochore microtubules (Mitchison, 1989; Mitchison and Salmon, 1992). It is interesting to note that microtubules seeded from kinetochores must have a greater stability than those seeded from centrosomes, since they alone persist at the appropriate nocodazole concentrations. This stability may relate to microtubule stabilizing factors that associate specifically with kinetochore microtubules (Margolis et al., 1990).

Mitotic Slippage Is Microtubule Dependent

During mitotic slippage, cyclin B is degraded and $p34^{cdc2}$ is inactivated as cells escape mitotic blockage and resume interphase (Kung et al., 1990). In BHK cells, we find that the rate of mitotic slippage is highly dependent on the level of microtubule assembly in the arrested cell. We have shown that the rate of entry into mitosis is not influenced by either the nature or concentration of the pharmacological reagent utilized to interfere with microtubule function, but that the rate of mitotic exit through slippage increases with the level of microtubule assembly. Therefore, as with 2-AP–induced exit from mitotic arrest, mitotic slippage appears to depend upon the presence of microtubules for the inactivation of $p34^{cdc2}$.

There are many similarities between mitotic slippage and mitotic checkpoint override induced by 2-AP. Both processes are dependent on the level of microtubule assembly, involve inactivation of $p34^{cdc2}$, and occur without chromosome segregation or cell division. But while 2-AP induces a rapid exit from mitosis, presumably through inhibition of a protein kinase essential to maintenance of the mitotic state (Andreassen and Margolis, 1991), mitotic slippage occurs gradually because of the inability of the cell to indefinitely maintain cyclin B and active $p34^{cdc2}$ (Kung et al., 1990).

The involvement of microtubule assembly state in the con-

trol of mitotic slippage may have implications for tumor chemotherapy. Inhibitors of microtubule dynamics, including vinblastine, vincristine, and taxol, have been used in chemotherapy for a variety of human tumors (Rowinsky and Donehower, 1991). It is unknown if optimal cell killing occurs as a result of sustained mitotic arrest or as a result of mitotic slippage. Such information should make it possible to more rationally determine the most effective doses for pharmacological agents that act on the microtubule assembly state.

We have presented evidence that the ability to override mitotic arrest is sensitive to the level of microtubule assembly in mammalian cells. We conclude from these studies that microtubules play an important role in the control of mitotic exit. Effects of the microtubule assembly state on cell cycle progression during interphase have also been reported. For example, microtubule depolymerization stimulates DNA replication in serum starved fibroblasts (Crossin and Carney, 1981), and can enhance the response of G_0 cells to growth factors (McClain and Edelman, 1980). We are presently undertaking an analysis to determine whether the microtubule assembly state may play a general role in the control of progression at various phases of the cell cycle.

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