The dynamic balance between polymerization and depolymerization of microtubules is critical for cells to enter and exit mitosis, and drugs that disrupt this balance, such as taxol, colchicine, and nocodazole, arrest the cell cycle in mitosis. Although the Raf/MEK/MAPK pathway can be activated by these drugs, its role in mitosis has not been addressed. Here, we characterize activation of Raf/MEK/MAPK by nocodazole when mitosis is induced. We find that at early time points (up to 3 h) in nocodazole induction, Raf/MEK/MAPK is activated, and inhibition of MAPK activation by a MEK inhibitor, PD98059 or U0126, reduces the number of cells entering mitosis by creating a block at G2. At later time points and in mitosis, activation of MEK/MAPK is severely inhibited, even though Raf-1 activity remains high and can be further increased by growth factor. This inhibition is reversed when cells are released from metaphase and enter G1 phase. In addition, we find that binding of Raf-1 to 14-3-3 is necessary to maintain mitotic Raf-1 activity. Our present study indicates that activation of the Raf/MEK/MAPK pathway is necessary for the G2/M progression.

Mitogens such as growth factors, cytokines, and hormones initiate complex signaling cascades that interact to regulate cell proliferation. Activation of the Ras/Raf/MAPK pathway is an important component of this regulation (1). Previous work has shown that growth factors can trigger entry into G1 phase of the cell cycle via activation of the Ras signaling pathway (2–5). In addition, MAPK has been implicated in the regulation of the cell cycle via activation of the Ras signaling pathway (22, 23). Recent reports show that in nocodazole-arrested mitosis, Raf-1 is phosphorylated and exhibits increased binding to 14-3-3. Rebinding of Raf-1 to 14-3-3 is necessary for mitotic activation of Raf-1 (6). After fertilization, inactivation of MAPK is essential for the first G2 to M progression (7, 8). MAPK is activated in early mitosis and inactivated before exit from M phase (9–12). Inappropriate activation of MAPK or its downstream kinase, RSK, causes cell arrest at the corresponding stages (9–14). In Saccharomyces cerevisiae, triple loss-of-function mutant strains lacking Ras1, Ras2, and Ras1 (9–14) and translocation of MEK1 into nuclei during G2/M phase (19) and of active MAPK to kinetochores in mitosis (20).

Microtubules play a central role in chromosome movement by forming the mitotic spindle (21). The dynamic balance between polymerization and depolymerization of microtubules is critical for cells to enter and exit mitosis, and drugs that disrupt this dynamic, such as colchicine, nocodazole, and taxol, interfere with chromosome movement and arrest the cell cycle in mitosis. The mechanism by which these drugs block mitosis appears to be more complex than a simple disruption of microtubule structure, as treatment with these drugs leads to activation of the Raf/MEK/MAPK pathway (22, 23). Recent reports show that in nocodazole-arrested mitosis, Raf-1 is hyperphosphorylated and activated, but MAPK is inactive (24–27). The biological significance and mechanisms of Raf-1 activation and uncoupling from its downstream kinase MAPK in mitosis of mammalian cells has not been addressed. In the present study, we characterize the activation of the Raf/MEK/MAPK pathway in mitosis induced by nocodazole. We show that activation of Raf-1 and MAPK are coupled at early time points of nocodazole treatment and became uncoupled at later time points. Inhibition of MAPK activation by MEK inhibitor PD98059 or U0126 blocks the G2/M transition. During mitosis itself, Raf-1 is highly phosphorylated and exhibits increased binding to 14-3-3. Removal of 14-3-3 from mitotic or EGF-stimulated Raf-1 results in the inhibition of its kinase activity, suggesting that 14-3-3 plays an essential role in keeping Raf kinase active in mitosis.

EXPERIMENTAL PROCEDURES

Plasmids, Peptides, and Antibodies—cDNAs encoding wild-type Raf-1, K375M, or S259A/S621A were tagged with a Myc epitope at the amino terminus and cloned into pMT2 (28). cDNAs for wild-type Raf-1 and MEK1 were cloned into pE8 vector (38). Peptides (LPRKINRAS*EPLSRLH) according to the Raf-1 sequence around Ser-
Role of Raf/MEK/MAPK in G2/M Transition

621 (marked as *) were synthesized with or without a phosphate on Ser-621 (29) by Quality Controlled Biochemicals, Inc. Antibodies against Raf-1, cyclin B1, and 14-3-3 were purchased from Santa Cruz Biotechnology, MEK1/2 from Transduction Laboratories, and antibodies against Erk1/2 and phospho-MEK1/2, -Erk1/2 from New England Biolabs.

Cell Culture and Transfection—COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM, from Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS, from Life Technologies, Inc.). Transfections were performed according to the protocol provided by Life Technologies, Inc., with some modifications. Cells from confluent 10-cm plates were split 1:4 and allowed to grow to 50% coverage, then washed with phosphate-buffered saline (PBS) and overlaid with 2 ml of serum-free DMEM. 2 μg of plasmid DNA and 20 μl of Lipofectamine (Life Technologies, Inc.) were combined in 500 μl of serum-free DMEM at room temperature for 30 min and incubated with the cells for 3 h. The transfection mixture was then aspirated and the cells washed and allowed to recover overnight in DMEM with 10% FBS. The cells were then split 1:3 and grown in DMEM with 10% FBS for 1 to 2 days before harvest.

Cell Synchronization, Counting, and FACS Analysis—Induction of mitotic block and FACS analysis were performed according to Ziogas et al. (26). 100 ng/ml nocodazole (Sigma) and 10 ng/ml EGF were applied to cultured COS7 cells transiently transfected with or without expression plasmids for the indicated times. Mitotic samples were collected by incubating cells with nocodazole for 12 h, washing with 5 ml of PBS, adding 2 ml of fresh PBS, and gently tapping the plates to dislodge the loosely adherent mitotic cells. Attached fractions were then either trypsinized (0.05% trypsin, Life Technologies, Inc.) or scraped off the plate. For manual cell counting, cell samples collected as described above were brought to a uniform volume, and 10 μl were loaded onto a hemacytometer. The average cell count over five areas was used to compute the overall cell count. For FACS analysis, 5 × 10^5 cells were trypsinized, seeded equally, washed with PBS, resuspended with 9 ml of 70% ethanol, 30% PBS at 4°C for 1 to 5 h. The samples were then washed once with PBS and resuspended in 1 ml of staining solution (10 μg/ml RNase A, 10 μg/ml propidium iodide (Sigma) in PBS). The samples were then incubated at room temperature in the dark for 1 h. FACS analysis was performed using CellQuest software (Becton Dickinson).

14-3-3 and Raf-1 Binding—Recombinant mouse 14-3-3ζ was expressed as a GST fusion protein in JM109 bacterial cells and purified through a glutathione affinity column (28). For binding assays, 5 μg of GST-14-3-3ζ protein were immobilized on glutathione bead and incubated with cell extracts treated with nocodazole for the indicated times. The isolated complex was then subjected to immunoblotting.

Protein Kinase Assays—Raf-1 kinase assays were performed as described by Luo et al. (28). Cell extracts were prepared in lysis buffer (20 mM Tris-HCl, pH 8.0, 25 mM β-glycerophosphate, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 1% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin) and spun in a microcentrifuge at 4°C at maximum speed for 10 min. Protein concentration was assayed using the Bio-Rad protein assay kit. For immunoprecipitation, 10–20 μl of protein A/G beads (Santa Cruz) were incubated with 2 μg of the indicated antibody for 1 h at 4°C. The beads were then washed thoroughly with lysis buffer, cell extract was added, and incubation at 4°C for 4°C was continued for at least 2 h. For GST immobilizations, 20 μl of glutathione-Sepharose beads (Amersham Pharmacia Biotech) were incubated at 4°C with cell extract for at least 1 h. The precipitated beads were then washed once with lysis buffer, twice with lysis buffer plus 1 mM NaCl, and twice with kinase buffer (25 mM Tris-Cl, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol). Raf kinase activity was assayed by a total of 50 μl of kinase buffer containing 100 μM ATP, 10 μCi of [γ-32P]ATP, 5 mM MgCl2, 100 μg/ml bovine serum albumin, and 4 μg/ml MEK1 at 30°C for 20 min and then supplemented with 40 μg/ml kinase dead ERK2 after which incubation continued for 30 min. Reactions were stopped with Laemmli sample buffer and boiled. MEK kinase activity was assayed by sequential incubation with 2 μg/ml Erk1 and 50 μg/ml myelin basic protein; otherwise, this assay was the same as for Raf kinase. All samples were subjected to SDS-PAGE and transferred to Immobilon-P (Millipore). The radioactively labeled membranes were exposed to an autoradiograph film at −80°C. For MEK activity assays, the labeled Erk2 bands were excised and subjected to scintillation counting after autoradiography.

Western Blotting—Samples were resolved on either 8 or 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P) for 3 to 4 h at 500 mA constant current. The transferred proteins were detected by specific antibodies according to manufacturer's protocols using enhanced chemiluminescence.

RESULTS

Activation of Raf/MEK/Mapk Is Required for Mitotic Arrest Induced by Nocodazole—Several recent studies have demonstrated that Raf-1 is activated and hyperphosphorylated on its serine/threonine residues in cells arrested in metaphase by nocodazole (24–27). To evaluate the role of Raf/MEK activation in nocodazole-induced mitosis, we transiently transfected GST-Raf-1 in COS7 cells, treated the cells with nocodazole, and collected samples at various time points to examine activation of recombinant GST-Raf-1 using a MEK1- and Erk2-coupled assay (28). Raf-1 was found to be activated in a time-dependent manner (Fig. 1a). Raf kinase activity peaked between 1 and 3 h at a level comparable with EGF-stimulated kinase activity and declined as the nocodazole incubation continued. Raf-1 from mitotic cells collected by shake-off after a 12-h exposure to nocodazole showed activity higher than basal levels of kinase activity, although this activity was not as high as during the 1–3-h peak. Since Raf-1 lies upstream of MEK and MAPK in higher eukaryotes, we examined the activation profile of these downstream kinases. We found that MAPK had a similar activation time course as Raf-1, as measured by anti-phospho-antibodies against its active forms. Upon further incubation with nocodazole, the activity of MAPK declined and became lower than basal by 12 h, when more than 60% of the cells were arrested in metaphase, and in mitotic cells isolated by shake-off (Fig. 1b). The diminished activity of MAPK

![FIG. 1. Activation of Raf/MEK/Mapk by Nocodazole](image-url)
was not the result of decreased expression of the protein.

In these studies, maximal activation of MAPK appeared to occur 30 min earlier than Raf activation. This could be explained if nocodazole penetration into the cells was not maximal until 1 h, but the level of Raf-1 kinase activity at 30 min was sufficient to give apparent maximal activation of MEK and MAPK. A second possibility is that nocodazole simultaneously activates MEK/MAPK and triggers events that later inactivate this pathway, so that maximal activation of MEK/MAPK may mirror that of Raf-1 but be masked by the action of the induced inactivation machinery.

To study the relationship between Raf/MAPK activation and mitosis, we examined expression of mitosis-specific cyclin B1 and showed that cyclin B1 was up-regulated in a time-dependent manner. This up-regulation occurred after the activation of MAPK and was correlated with increases in the number of mitotic cells during mitotic induction (Fig. 1b). To further explore the role of Raf/MAPK in mitosis, we treated COS7 cells with a MEK inhibitor, PD98059 or U0126, or with nocodazole and 1 μM wortmannin (Wort.). Cells in the attached and mitotic fractions were counted using a hemacytometer. b, FACS analysis representing the ratio of 2N/4N DNA from the mitotic and attached fractions of cells treated for 12 h with nocodazole plus or minus PD98059 (+ or −PD).

We performed FACS analysis to determine the cell cycle stage of the attached (non-mitotic) cells after treatment with nocodazole and a MEK inhibitor, PD98059. More than 90% of the attached cells were at the G2 phase with 4N DNA. This was a similar 2 N/4 N DNA ratio to cells incubated with nocodazole but no MEK inhibitor (Fig. 2b). More than 95% of the mitotic shake-off cells contained 4N DNA in every treatment. Based on these findings, we concluded that activation of MAPK is essential for the G2 to M transition induced by nocodazole.

14-3-3 Participates in the Regulation of Raf-1 Kinase Activity in Mitosis—To investigate whether 14-3-3 is involved in the regulation of Raf-1 activity in mitosis, we examined the ability of Raf-1 to bind to 14-3-3. Raf-1 kinase was immunoprecipitated from both unsynchronized and mitotic cells and the relative content of associated 14-3-3 assessed by immunoblotting against 14-3-3. As shown in Fig. 3a, 14-3-3 binding to Raf-1 is enhanced in mitosis, whereas the levels of 14-3-3 and Raf-1 are almost constant between unsynchronized and mitotic cells. The increased ability of Raf-1 to bind to 14-3-3 was verified by an in vitro assay in which recombinant GST-14-3-3 was immobilized on glutathione beads and incubated with extracts of unsynchronized and mitotic cells in the presence of a non-phospho-peptide containing a 14-3-3 binding site. Raf binding was examined by Western blot with a Raf-1 antibody. c, COS7 cells were incubated with nocodazole as described in Fig. 1. Cell extracts were incubated with the immobilized GST-14-3-3 and blotted against 14-3-3. As shown in Fig. 3b, binding of Raf-1 to 14-3-3 is stronger than that of Raf from unsynchronized cells and that it was inhibited by the phospho-peptide but not by the non-phospho-peptide containing the 14-3-3 binding site (Fig. 3b). We then tested whether the binding of Raf-1 to 14-3-3 could be induced by nocodazole and was associated with the G2/M progression. As shown in Fig. 3c, the binding of Raf-1 to

Fig. 2. MAPK-dependent entry into mitosis. a, COS7 cells were stimulated for 12 h with nocodazole alone, with nocodazole and 50 μM PD98059 or 20 μM U0126, or with nocodazole and 1 μM wortmannin (Wort.). Cells in the attached and mitotic fractions were counted using a hemacytometer. b, FACS analysis representing the ratio of 2N/4N DNA from the mitotic and attached fractions of cells treated for 12 h with nocodazole plus or minus PD98059 (+ or −PD).

Fig. 3. Increased binding of 14-3-3 to Raf during induction of mitosis by nocodazole. a, 5-mg extracts from unsynchronized (N) and mitotic cells (M) were immunoprecipitated (IP) with anti-Raf-1 antibody (C12) and immunoblotted for either Raf-1 or 14-3-3. 10 μg of crude extracts were used for immunoblotting with an anti-14-3-3 antibody. b, 10 μg of bacterially expressed recombinant GST-14-3-3 were immobilized on glutathione beads and incubated with 500 μg of extract from unsynchronized and mitotic cells in the presence of 0.2 mM synthetic non-phospho (P)-, or phospho(PS)-peptide containing a 14-3-3 binding site. Raf binding was examined by Western blot with a Raf-1 antibody. c, COS7 cells were incubated with nocodazole as described in Fig. 1. Cell extracts were incubated with the immobilized GST-14-3-3 and blotted against 14-3-3 as antibody as in panel b. d, mitotic cells were replated in DMEM + 10% FBS at 37 °C and collected 2 or 4 h later; otherwise, the procedure was as described in b and c.
14-3-3 was progressively enhanced as the retarded mobility shift of Raf-1 was induced with prolonged incubation of cells with nocodazole. When mitotic cells were replated in nocodazole-free medium, cells progressed into G2/M phase, and Raf/14-3-3 binding gradually decreased. By 4 h, Raf-1/14-3-3 binding decreased to a level equal to that of unsynchronized cells, and Raf-1 molecular weight decreased to the same level as in unsynchronized cells (Fig. 3c). We previously showed that, in contrast to the present results, the binding of 14-3-3 to Raf-1 was not influenced by growth factors (28), suggesting that EGF and nocodazole activate Raf-1 through distinct mechanisms.

To study the functional role of 14-3-3 binding of mitotic Raf-1, COS7 cells were transfected with Myc-tagged Raf-1 variants including Raf wild-type, Raf 259A/621A (mutated at two 14-3-3 binding sites), and Raf K375M (a kinase dead mutant). The kinase activity of recombinant Raf-1 was assayed after immunoprecipitation from extracts of mitotic, EGF-stimulated, or unsynchronized cells. As shown in Fig. 4a, wild-type Raf-1 was moderately activated in mitosis, as compared with EGF treatment, whereas neither Raf 259A/621A nor the kinase dead mutant Raf-1 could be activated by EGF stimulation or nocodazole-induced mitosis. Another approach was necessary to ascertain that loss of kinase activity of Raf 621A is not due to misfolding of the Raf kinase catalytic domain. A logical experiment would be to immunoprecipitate 14-3-3 and compare the kinase activity of Raf associated with immunoprecipitated 14-3-3 and Raf remaining in the 14-3-3 immunodepleted extract. Two practical considerations prevented this approach. First, there is no antibody available that can deplete all isoforms of 14-3-3. Second, 14-3-3 associates with other MEK kinases besides Raf-1 (data not shown), which makes interpretation impossible. We devised an alternative experiment to demonstrate that 14-3-3 is necessary for the activity of mitotic Raf. Wild-type Myc-Raf was cotransfected with full-length 14-3-3 or with amino-terminally truncated 14-3-3, both expressed as GST fusion proteins and known to bind to Raf-1 equally well (28). After induction of mitosis, wild-type and truncated 14-3-3 were purified by glutathione affinity beads, eluted with free glutathione, and subjected to anti-Myc (Raf-1) immunoprecipitation and Raf kinase assay. As shown in Fig. 4b, mitotic Raf associating with full-length 14-3-3 displays much higher activity and can be further stimulated by EGF, whereas Raf kinase associated with truncated 14-3-3 is totally inactive and unresponsive to EGF stimulation.

To further determine whether 14-3-3 binding is necessary to maintain the active conformation of mitotic Raf-1 as it is for EGF-stimulated Raf-1 (29, 31), the kinase activity of recombinant GST-Raf-1 isolated by glutathione immobilization from unsynchronized and mitotic cells was assayed in the absence or presence of the phospho-peptide containing the 14-3-3 binding site (Fig. 4b). We observed that Raf-1 from mitotic and attached cells had higher than basal activity and that EGF could further stimulate this activity. Under all of these conditions, removal of 14-3-3 by the phospho-peptide diminished Raf-1 kinase activity. Thus, binding of 14-3-3 to Raf-1 is augmented during nocodazole-induced G2/M arrest, and such binding is necessary to maintain Raf-1 kinase activity.

**Inhibition of MEK Accounts for Uncoupling of Raf-1 and MAPK Activation during Mitotic Arrest**—Several recent papers have reported that, during mitosis, MAPK activity is diminished and uncoupled from the increased activity of Raf-1 (24–27). Our present data extend these previous findings by showing that the activations of MAPK and Raf-1 are coupled early in nocodazole treatment and uncoupled at late time points and during mitosis (Fig. 1). To elucidate the mechanism by which the Raf/MAPK pathway is regulated during G2/M progression, we next addressed two questions: 1) is the signaling pathway initiated by extracellular mitogenic signals impacted?; and 2) if it is inhibited, at which level does the uncoupling occur? As shown in Fig. 4b, the activity of Raf-1 from mitotic and attached cells increased further when the cells were stimulated with EGF. However, when MAPK under the same conditions was blotted with an antibody that recognizes the active state of MAPK, we found severely impaired MAPK activation in EGF-stimulated mitotic and attached cells as compared with EGF-stimulated unsynchronized cells (Fig. 5a). This inhibition was reversed with time when mitotic cells were released from nocodazole induction (Fig. 5b) and entered...
G2/G1 phase as indicated by FACS analysis (data not shown).

To determine whether the uncoupling between Raf-1 and MAPK occurs at MEK, we examined MEK activation under the same conditions as described above for MAPK. MEK exhibited a similar activation profile to MAPK as shown by an anti-phospho-MEK1/2 immunoblot (Fig. 6a). At later time points in nocodazole treatment and in mitosis, the anti-phospho MEK1/2 antibody gave barely detectable signals for active MEK, whereas nonspecific signals increased. The low signals were not due to decreased expression of MEK1 and MEK2, as no change was observed by anti-MEK1 or MEK2 immunoblots. In the next experiment, we transiently expressed GST-MEK1 in COS7 cells and assayed its kinase activity. We show here that both EGF and nocodazole activate MEK1 at early time points, but activation of MEK by EGF is severely inhibited in mitotic and attached cells (Fig. 6b). The inhibition of MEK is not due to sequestering of the kinase in different cellular compartments in mitotic versus unsynchronized cells because the majority of MEK is found to be in the cytosol in both mitotic and nonmitotic cells (data not shown). These results indicate that the uncoupling of Raf-1/MAPK activation in nocodazole-induced mitosis is due to inhibition of MEK.

**DISCUSSION**

Previous studies have demonstrated that Raf-1 is hyperphosphorylated on serine/threonine residues and activated in mitosis after a long (12–16 h) induction with nocodazole (24–27). However, questions regarding the mechanism of Raf-1 activation and its role in mitosis remain to be addressed. Our current study extends previous findings by showing that: 1) Raf-1 is activated during the early phase of nocodazole treatment; 2) activation of the Raf-1 pathway is necessary for entry of cells into mitosis; and 3) Raf-1 in mitosis exhibits increased ability to associate with 14-3-3 and binding of 14-3-3 to mitotic Raf-1 is essential to the maintenance of Raf-1 kinase activity.

Regulation of G2/M progression by MAPK appears to occur through multiple mechanisms. 40% of MAPKs are found associated with microtubules, perhaps making them more accessible to their substrates or modulators (32). For example, MAPKs phosphorylate microtubule-associated proteins (MAPs, cellular factors that regulate microtubule dynamics), and this regulation could play a significant role in microtubule polymerization/dem polymerization dynamics (33). MAPK has also been reported to be critical for the microtubule spindle assembly checkpoint (34, 35). Additionally, RSK, an immediate downstream kinase of MAPK, can phosphorylate and inactivate Myt1, a major kinase inhibitor of cyclin B/cdc2 complexes during S and G2 phases in somatic cells (36). Finally, MAPK can phosphorylate cyclin B and facilitate the translocation of cyclin B/cdc2 to the nucleus, where it is dephosphorylated and activated by cdc25C (37).

To our knowledge, the involvement of MAPK in microtubule-destabilizing drug-induced mitotic arrest has not previously been documented. Thus, the present study is the first to demonstrate that nocodazole activates MAPK through Raf-1 and that such activation is necessary for G2/M progression. Our results indicate that incubation with MEK inhibitors and nocodazole substantially decreases the number of mitotic cells compared with incubation with nocodazole alone. The corresponding attached fractions are proportionately increased in number, indicating that a slowing of cell growth and/or increased apoptosis were not responsible for this difference. Also, more than 90% of the attached cells under these conditions contained 4N DNA, indicating that the cells had reached G2 or early M phase and that the point of cell cycle inhibition by the
MEK inhibitor was at the G₂/M transition.

The Ras/MAPK pathway has been best characterized previously in response to growth factor stimulation. These studies have shown that sequential activation of Ras, Raf, MEK, and MAPK leads to various cellular responses (38). In contrast, a number of recent studies have shown that as the cell cycle progresses, some components of this pathway can become uncoupled from their usual upstream or downstream partners and that they become activated without growth factor stimulation. Taylor and Shalloway (39) showed that Ras was activated in mid G₁ in the absence of Shc tyrosine phosphorylation or Grb2 binding and that MAPK was not activated under the same conditions. MAPK also appears to be universally inactivated in mitosis, even though Raf is active and hyperphosphorylated at that time. In the present study, we found that not only was MEK/MEK inactive in mitotic cells, but it did not respond to EGF stimulation, despite a further increase in mitotic Raf activity.

The uncoupling of Raf-1 and MEK activation by growth factors can be achieved when cell growth conditions are switched from adherence to suspension (40). Loosely adherent mitotic cells may possess some features similar to suspended cells. However, the mechanism of uncoupling is probably different, because inhibition of MEK/MAPK is also observed in the attached cells after 12 h of nocodazole incubation. Therefore, several mechanisms may be considered for such inhibition. First, MEK1 could be phosphorylated on Thr-286 and Thr-292 by cyclin B/cdc2, resulting in its inactivation, as shown by Rossomando et al. (41). This is an unlikely explanation of our current results, however, because we did not observe a mobility shift of endogenous MEK1 in mitotic cells (41), and MEK2, which is at least as abundant as MEK1 in COS7 cells, lacks these two phosphorylation sites. Second, redistribution of MEK1/2 could sequester Raf and MEKs in different subcellular compartments. This is also not likely to be the reason for MEK inhibition, as we did not observe a significant difference in MEK subcellular localization between unsynchronized and mitotic cells. Third, a specific phosphatase could dephosphorylate and inactivate MEKs. Thus far, no such phosphatase has been found. Fourth, induction of an inhibitory cellular factor, such as a polypeptide, could specifically bind to MEK to inhibit the interaction between MEK and Raf-1. Fifth, an inhibitory phosphorylation of MEKs might occur in mitosis. One intriguing model meriting further study is the possible inhibition of p42/44 MAPK by p38 MAPK. This protein kinase was recently shown to be activated in mitosis and to be necessary for mitotic progression, as opposed to p44/p42 MAPK, which is inactive in mitosis (42). Activation of p38 MAPK by MEKK3 could suppress Ras-induced transformation and cause G₂ arrest (43).

Raf-1 contains two 14-3-3 binding sites, one of which is located around Ser-621, at the carboxyl terminus, and appears to be essential for Raf kinase activity. Displacement of 14-3-3 from the carboxyl-terminal binding site by the phosphorylated 14-3-3 binding peptide or mutation of Ser-621 to Ala causes inactivation of Raf-1 (29, 31). The other 14-3-3 binding site is around Ser-259, at the amino terminus; it is dispensable for Raf-1 kinase activity but plays an inhibitory role in Raf kinase activation, as mutation of this site results in high basal kinase activity and enhanced activation by growth factors. Furthermore, phosphorylation of this site by AKT/protein kinase B inhibits Raf kinase activation in vivo (29, 44–46). Hence, it has been suggested that binding of 14-3-3 to the amino-terminal site of Raf-1 holds the kinase in an inactive conformation; when stimulated by growth factor, Ras-GTP then displaces 14-3-3 from the amino-terminal site on Raf-1, thus allowing additional modification, such as phosphorylation, to secure its active state (47).

Laird et al. (27) showed that mutation of the amino-terminal 14-3-3 binding site does not impair Raf-1 activation by mitotic induction (29). However, their studies cannot exclude the involvement of the carboxyl-terminal binding site. Our present study indicates that 14-3-3 participates in the regulation of Raf-1 kinase activity in mitosis by showing that: 1) binding of 14-3-3 to Raf-1 is progressively increased during induction of mitosis; 2) mutation of two 14-3-3 binding sites abolishes mitotic activation of Raf-1; 3) mitotic Raf-1 associates with function-deficient, amino-terminally truncated 14-3-3 does not exhibit any kinase activity, whereas Raf-1 associating with the wild-type 14-3-3 contains high kinase activity; and 4) in vitro removal of 14-3-3 strongly inhibits Raf-1 kinase activity. Therefore, the data strongly indicate that 14-3-3 participates in the regulation of Raf-1 kinase activity in mitosis.

Several studies suggest that activation of Raf-1 in mitosis involves a complex phosphorylation event that is distinct from the mechanism of growth factor stimulated Raf-1 activation. First, mitotic Raf is highly phosphorylated on serine and threonine as reflected by slowed mobility on SDS-PAGE. Second, activation of mitotic Raf-1 is dependent upon phosphorylation of the carboxyl-terminal kinase domain (25, 27). Third, activation of mitotic Raf-1 is Ras-independent (26). These observations have prompted us to examine the interaction between Raf-1 and 14-3-3 during mitosis. Previous data demonstrated that Ser-259 and Ser-621 are the major constitutively phosphorylated sites (48); likewise, the binding of Raf-1 to 14-3-3 is not influenced by growth factors (28). Our results point out another difference in Raf kinase activation by growth factor and mitosis induction. The regulation of Raf-1 through 14-3-3 in mitosis appears to be a secondary event following serine/threonine phosphorylation, because the maximal activation of Raf-1 by nocodazole does not mirror maximal stimulation of its ability to associate with 14-3-3. Higher Raf kinase activity in mitotic shake-off and non-mitotic attached cells may result from the enhanced binding of Raf-1 to 14-3-3, as the latter may protect Raf-1 from inactivation, such as through dephosphorylation (49). The increased binding of Raf-1 to 14-3-3 may be caused by increased phosphorylation of the 14-3-3 binding sites or other sites that may interact directly with 14-3-3 or in which phosphorylation induces a conformational change of the 14-3-3 binding site that makes it more accessible to 14-3-3.

One interesting question that arises from our results is whether Raf-1 acts upon other substrates during mitosis, as MEK can no longer be activated by Raf-1 at that stage of the cell cycle. It is also possible that 14-3-3 facilitates the interaction of Raf-1 and mitosis-specific substrates, similar to the way it mediates interactions between Raf-1 and PKCζ (50) and between Raf-1 and Bcr (51).

In the present study, similar changes in Ras/MEK/MAPK activities were observed in the attached, G₂-arrested cells after 12 h of nocodazole induction, despite the fact that Raf-1 was not hyperphosphorylated. We suggest that MAPK had also been activated early in the time course and later inhibited in those cells, because they were exposed to the drug but for some reason were unable to progress to or had delayed entry into mitosis. In fact, cyclin B1 was not induced in these cells. Taken together, our data suggest that activation of MAPK is necessary but not sufficient to induce entry into mitosis. We were not able to show activation of recombinant Raf-1 in mitosis to the same extent as EGF-stimulated Raf-1 as seen in the work of others (26, 27), but this is probably because of the high expression of recombinant Raf-1 overwhelming endogenous activating factors. This explanation is supported by the observation that retarded mobility was easily observed in endogenous Raf-1 but hardly at all in recombinant Raf-1.
Acknowledgment—We thank Dr. Neil Ruderman for helpful discussion and suggestions on our manuscript. We also thank Christine Waelde for technical assistance.

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