The Raf-1 serine/threonine kinase transmits proliferative and developmental signals to downstream effectors in the mitogen-activated protein kinase cascade. Studies to date have concentrated on its role in growth factor-mediated activation of the cell cycle. We show here that Raf-1 kinase activity is also highly stimulated during mitosis, with an attendant distinctive electrophoretic mobility shift due to hyperphosphorylation. These results suggest that Raf-1 may play a role in traversal of at least two distinct phases of the cell cycle.

The Raf-1 kinase is an integral part of a cascade that transmits mitogenic and developmental signals from activated growth factor (GF) receptor tyrosine kinases to the mitogen-activated protein kinase cascade. Studies to date have concentrated on its role in growth factor-mediated activation of the cell cycle. We show here that Raf-1 kinase activity is also highly stimulated during mitosis, with an attendant distinctive electrophoretic mobility shift due to hyperphosphorylation. These results suggest that Raf-1 may play a role in traversal of at least two distinct phases of the cell cycle.

**EXPERIMENTAL PROCEDURES**

**Immunoprecipitations and Western Blot Analysis—**Cells were lysed in RIPA buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 10% (v/v) glycerol, 1% Nonidet P-40, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 mM sodium orthovanadate, and 25 mM NaF). Immunoprecipitations were performed as described (8, 9) using an anti-Raf-1 monoclonal antibody (Transduction Labs R19210). Whole cell lysates and immunoprecipitates were resolved by SDS-PAGE and subjected to Western blot analysis using the same anti-Raf-1 monoclonal antibody except for Fig. 2C, where a rabbit polyclonal antibody directed against the 14 C-terminal residues of Raf-1 (a gift from D. Morrison) was used. Bands were visualized using the Renaissance chemiluminescence reagent (DuPont NEN). Retroviral Infection—NIH 3T3 cells were infected as described (10) with the retroviral construct pLJ/Raf-1 (a gift from D. Morrison) or the control construct pLJ. Infected cells were grown in mass culture under G418 selection (400 μg/ml) for 2 weeks prior to experiments being performed. Kinase Assays—Raf-1 immunoprecipitations and kinase assays were performed exactly as described (8, 9). Histidine-tagged kinase-defective MEK-1 was bacterially expressed (11) and employed as an exogenous substrate (approximately 500 ng/reaction). 32P bands were quantitated using a Betagen model 603 betascope.

**RESULTS AND DISCUSSION**

We have observed that Raf-1 immunoprecipitated from nocodazole-arrested mitotic NIH 3T3 cells migrates more slowly (74–78 kDa) in SDS-PAGE than Raf-1 from unsynchronized growing cells (72–74 kDa; Fig. 1, lanes 1 and 2). Following potato-acid phosphatase treatment this mobility retardation was virtually eliminated, demonstrating that it stems from hyperphosphorylation (lanes 3 and 4). Pretreatment of potato-acid phosphatase with a mixture of phosphatase inhibitors (sodium fluoride and sodium vanadate) inhibited this effect (data not shown). To further study alterations of Raf-1 in mitosis we used the retroviral expression vector pLJ/Raf-1 to generate NIH 3T3 cell lines (designated NIH(pLJ/Raf-1)) that stably overexpress human Raf-1. NIH(pLJ) cell lines that were infected with the empty vector pLJ were used as controls. Direct comparisons with both the endogenous and overexpressed Raf-1 showed that the mitotic reduction in electrophoretic mobility was similar to that observed following 5 min of stimulation with platelet-derived growth factor (PDGF) (Fig. 2A, cf. lanes 3 and 2 and lanes 7 and 6). Multiple mobility-retarded Raf-1 bands were observed in both PDGF-treated and mitotic cells, but more highly retarded bands were more abundant in mitotic Raf-1. Raf-1 retardation increased following longer treatment with PDGF but never to the extent observed with Raf-1 from mitotic cells (data not shown).

NIH(pLJ)/Raf-1 mitotic and unsynchronized cell cultures were metabolically labeled with inorganic 32P and immunoprecipitated Raf-1 was analyzed by phosphoamino acid analysis (Fig. 2B). Raf-1 isolated from both mitotic and unsynchronized cells was radiolabeled predominantly on serine residues, but low levels of threonine phosphorylation were also detected. This is similar to results observed following PDGF stimulation of NIH 3T3 cells (12).

Raf-1 Is Activated during Mitosis*

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**FIG. 1. The electrophoretic mobility of Raf-1 is retarded in mitotic NIH 3T3 cells due to hyperphosphorylation.** Lysates were harvested from exponentially growing unsynchronized (U) and nocodazole-arrested (M) cells. Nocodazole-arrested cells were prepared as described previously (13). Immunoprecipitated Raf-1 was incubated without (−) or with (+) potato-acid phosphatase (PAP) as described previously (35), subjected to 8% SDS-PAGE, Western blotted, and probed with Raf-1 antibody. Bands were visualized by chemiluminescence. The broad Raf-1 band actually consists of at least six electrophoretically distinct species. The positions of molecular weight standards (in kDa) are shown.

30 min after replating (data not shown and Ref. 13). Similar results for Raf-1 were obtained using HeLa cells.

Two different assays were used to determine if Raf-1 kinase activity is stimulated during mitosis: 1) an autokinase assay and 2) phosphorylation of a relevant physiological substrate, i.e. purified recombinant MEK-1. Autophosphorylation was assayed using Raf-1 immunoprecipitated from quiescent (serum-starved), PDGF-stimulated, mitotic, and unsynchronized NIH(pLJ/Raf-1) cells (Fig. 3A). As anticipated (3), Raf-1 immunoprecipitated from quiescent (lane 1) or unsynchronized (lane 4) cultures exhibited negligible autokinase activity. Importantly, autokinase activity was stimulated in mitotic cells to a level comparable with that observed following PDGF treatment (cf. lanes 2 and 3). Phosphoamino acid analysis revealed that both PDGF-stimulated and mitotic Raf-1 were autophosphorylated on both serine and threonine (Fig. 3B). The MEK kinase activity of Raf-1 was also stimulated in mitotic cells (Fig. 3C). The activity of Raf-1 from mitotic cells was 10 × over-2 (n = 4) times higher than the activity of Raf-1 from unsynchronized cells. The level of mitotic activity was very similar to that observed following PDGF stimulation (data not shown). Definitive investigation of the role of the mitotic hyperphosphorylation of Raf-1 in its activation must await mapping and mutational analysis of the mitotic phosphorylation sites.

To ensure that Raf-1 hyperphosphorylation and activation were not drug-induced artifacts, we performed similar experiments using mitotic cells collected by mechanical shake-off alone (Fig. 3D). Western blots of immunoprecipitates revealed that Raf-1 mobility was retarded, although to a lesser extent that of Raf-1 isolated from nocodazole-treated cells (Fig. 3D, lower panel). Similarly, Raf-1 kinase activity was stimulated about 3-fold in mitotic cells isolated by shake-off alone (Fig. 3D, upper panel, lane 6) compared with Raf-1 in immunoprecipitates from the adherent monolayer following shake-off (lane 5) or from unsynchronized cells (lane 7). This stimulation was somewhat less than that observed in nocodazole-treated cultures (lane 8), but for reasons discussed elsewhere (13) we believe that this merely reflects heterogeneity of the shake-off population and conclude that the observed mitotic hyperphosphorylation and stimulation of Raf-1 kinase activity are authentic.

We cannot exclude the possibility that the observed enhancement of Raf-1 kinase activity comes from the mitosis-specific association or activation of another serine/threonine kinase that coprecipitates with Raf-1. We believe this is unlikely since 1) MEK-1 appears to be a target for a very restricted set of kinases (1) and 2) the lysis buffer contains 0.1% SDS and 0.5% sodium deoxycholate, which should dissociate any such activity from Raf-1 prior to Raf-1 immunoprecipitation (8). In any case, if such an association/activation occurs, it is specific to mitosis and would therefore itself be of great interest.

Raf-1 activity is already known to participate in the G0/G1 transition. The data presented here suggest that it may also function in the transition into or passage through mitosis. A reduction in the electrophoretic mobility of Raf-1 in mitotic murine fibroblasts has been previously reported, but no changes in Raf-1 activity were described (14). In future experiments it will be important to determine 1) if Raf-1 is first activated before or during mitosis, 2) if the mechanism of its mitotic activation is related to the mechanism employed following GF stimulation (i.e. via Ras (2)), and 3) its mitotic targets. Interestingly, there is already evidence indicating that a Raf-1-mediated pathway(s) participates in entry into meiosis. For example, activated GF receptor tyrosine kinases stimulate Xenopus meiotic maturation in a Raf-1-dependent manner (15), and progesterone-initiated maturation is partially Raf-1-dependent (15, 16). Furthermore, ERK1 (which lies downstream from Raf-1) regulates a meiotic checkpoint.

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2 S. Taylor and D. Shalloway, unpublished observations.
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The recent finding that Raf-1 phosphorylates and activates Cdc25A, an activator of cyclin-dependent kinases, during cell cycle activation further indicates that it may play a significant role in cell cycle regulation (29).

We do not yet know if the mitotic hyperphosphorylation of Raf-1 is a cause or effect of its activation. It will be important to test the possibility that Src (or other tyrosine kinases) plays a role in the mitotic activation of Raf-1. Microinjection of anti-Ras inhibitory antibodies reverts the transformed phenotypes of v-Src-transformed cells, demonstrating that v-Src acts upstream of the Ras/Raf-1 pathway (30). Moreover, oncogenic Src can activate Raf-1 in insect and mammalian cells (9, 31, 32). We did not detect increased tyrosine phosphorylation of Raf-1 during mitosis (Fig. 2B). However, it should be noted that enhanced tyrosine phosphorylation is confined to a small fraction of Raf-1 molecules following activation by coexpression with oncogenic Ras and Src in mammalian cells, even though tyrosine phosphorylation has been implicated in Raf-1 activation in this situation (32). In vivo mitotic hyperphosphorylation was observed overwhelmingly at serine (Fig. 2B) while Raf-1 autophosphorylates in vitro at both serine and threonine (Fig. 3B and Refs. 31 and 33). MAPKs are probably responsible for at least some of the Raf-1 hyperphosphorylation seen in GF-stimulated cells (34) and might therefore also contribute to Raf-1 mitotic hyperphosphorylation in a feedback loop.

The results presented here indicate that Raf-1 must be added to the list of proto-oncoproteins whose activities become modulated in mitosis and suggest that deregulated Raf-1 activity could perturb control mechanisms at multiple points in the cell cycle.

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FIG. 3. Raf-1 kinase activity is stimulated during mitosis. A, autokinase assay. Lysates were prepared from serum-starved (−), GF-stimulated (+), mitotic (M), and unsynchronized (U) cultures of NIH 3T3 cells. Immune complex kinase assays were performed using anti-Raf-1 immunoprecipitates from 200 µg of total cell protein and 20 µCi of [γ-32P]ATP. Reaction products were analyzed by 9% SDS-PAGE and autoradiography (upper panel). All products of the immunoprecipitates were subjected to Western blot analysis to confirm that Raf-1 loading was comparable for each sample (lower panel). For control immunoprecipitations the anti-Raf-1 primary antibody was omitted (lanes 5–8). B, autophosphorylated PDGF-stimulated (+) and mitotic (M) Raf-1 bands were excised from kinase gels and subjected to phosphoamino acid analysis as described for Fig. 2B. C, Raf-1 bands were combed from the same blot and subjected to phosphoamino acid analysis as described for Fig. 2B (lanes 5–8, upper panel). The position of the autophosphorylated PDGF-stimulated Raf-1 is indicated (lanes 1–4). D, Raf-1 in Xenopus oocytes. Cells were collected by shake-off (S) without nocodazole treatment as described previously (13). Lysates were also prepared from the adherent cells (A) that remained on the same plates. Raf-1 MEK kinase activity (upper panel) was assayed for these cells as well as for nocodazole-arrested mitotic (M) and unsynchronized (U) cells as described in C.
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