Cdc2-mediated Inhibition of Epidermal Growth Factor Activation of the Extracellular Signal-regulated Kinase Pathway during Mitosis*

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Surabhi Dangi and Paul Shapiro‡
From the Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, Baltimore, Maryland 21201

Inhibition of general transcription and translation occurs during mitosis to preserve the high energy requirements needed for the dynamic structural changes that are occurring at this time of the cell cycle. Although the mitotic kinase Cdc2 appears to directly phosphorylate and inhibit key proteins directly involved in transcription and translation, the role of Cdc2 in regulating upstream growth factor receptor-mediated signal transduction pathways is limited. In the present study, we examined mechanisms involved in uncoupling receptor-mediated activation of the extracellular signal-regulated (ERK) signaling pathway in mitotic cells. Treatment with epidermal growth factor (EGF) failed to activate the ERK pathway in mitotic cells, although partial activation of ERK could be achieved in mitotic cells treated with phorbol 12-myristate 13-acetate (PMA). The discrepancy between EGF and PMA-mediated ERK activation suggested that multiple events in the ERK pathway were regulated during mitosis. We show that Cdc2 inhibits EGF-mediated ERK activation through direct interaction and phosphorylation of several ERK pathway proteins, including the guanine nucleotide exchange factor, Sos-1, and Raf-1 kinase. Inhibition of Cdc2 activity with roscovitine in mitotic cells restored ERK activation by EGF and PMA. Similarly, mitotic inhibition of ERK activity in cells expressing active mutants of H-Ras and Raf-1 kinase could also be reversed following Cdc2 inhibition. In contrast, ERK activation in cells expressing active MEK1 was not inhibited during mitosis or affected by roscovitine. These data suggest that Cdc2 inhibits growth factor receptor-mediated ERK activation during mitosis by primarily targeting signaling proteins that are upstream of MEK1.

Cdc2 is the major cyclin-dependent kinase that promotes cell cycle progression through the G2 phase and mitosis (1). Through direct phosphorylation, Cdc2 regulates important mitotic structural changes including nuclear envelope breakdown (2, 3), centrosome assembly (4), and Golgi complex fragmentation (5). In addition, Cdc2 also functions to inhibit other cellular processes that would utilize energy stores needed for the dynamic structural changes that are occurring at this time of the cell cycle. For example, the role of Cdc2 is well established in inhibiting transcription during mitosis (6) through direct phosphorylation of proteins such as RNA polymerase II and TFIIB (7–9). Moreover, evidence suggests that Cdc2 may directly cause inhibition of transcription during mitosis by targeting other unknown kinases (6).

Other metabolic processes such as the high energy requirements of ribosome biogenesis and protein translation are also inhibited during mitosis through Cdc2-mediated phosphorylation (10, 11). Recently, Cdc2 was shown to inhibit the p90 S6 ribosomal kinase-1 through direct phosphorylation providing an additional level of regulation of mitotic inhibition of translation (12). Thus, mitotic cells coordinate the inhibition of biological processes that might interfere with the energetically demanding structural changes that are necessary to ensure the equal segregation of genetic material into each daughter cell.

Mitotic cells are also less responsive to extracellular growth factor stimulation as compared with interphase cells. For example, epidermal growth factor (EGF)1 receptor activity is reduced during mitosis following stimulation, reportedly because of reduced EGF binding and inhibition of EGF receptor dimerization (13–15), possibly through a mechanism involving direct phosphorylation by Cdc2 (16). Inhibition of EGF receptor activity would also be beneficial for preventing the activation of signal transduction pathways that promote gene expression to preserve energy needs that are required for mitotic structural changes. The ability for EGF to activate the extracellular signal-regulated kinase (ERK) signal transduction pathway has been shown to be inhibited in mitotic cells as compared with interphase cells (17). EGF receptor-mediated activation of the ERK proteins occurs sequentially through Ras G-proteins, Raf kinases, and direct phosphorylation by the mitogen activated protein (MAP) or ERK kinases (MEKs) (18, 19). The coupling between the EGF receptor and the Ras G-protein occurs primarily through the Src homology 2 domain containing adapter proteins Shc and Grb2 interacting with phosphorylated tyrosines on the EGF receptor and GTP loading on Ras by the guanine nucleotide exchange factor Sos-1 (20). It is not known whether Cdc2 inhibits EGF receptor signaling during mitosis by targeting these proteins that link the activated EGR receptor with ERK activation.

ERK pathway proteins may undergo unique regulation during mitosis. Recently phosphorylated MEK1 has been reported to undergo partial proteolysis at the N terminus during mitosis, which results in the inability for MEK1 to interact with and activate ERK1/2 proteins (21). However, it is unlikely that MEK1 is completely uncoupled from ERK1/2 during mitosis because activation of protein kinase C by treatment with phorbol esters can still activate the Raf-1/MEK/ERK pathway in mitotic cells (14). Moreover, we have reported that the observed proteolysis of MEK1 during mitosis may not be because of

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† To whom correspondence should be addressed. Tel.: 410-706-8522; Fax: 410-706-0346; E-mail: pshapiro@rx.umm.edu.

‡ The abbreviations used are: EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein or extracellular signal-regulated kinases; PMA, phorbol 12-myristate 13-acetate.
inhibiting proteins involved in transcription and translation, by forming complexes with several proteins that couple the negative regulation of receptor-mediated ERK signal transduction (22). The functions of the ERK pathway and mechanisms of regulation during G2 or M phase progression are not well defined and somewhat controversial. Earlier studies that suggested a potential involvement of ERK in mitotic events reported that Raf-1 was activated in cells arrested in mitosis with nocodazole (23). However, Raf-1 activation in nocodazole-induced mitotic cells appeared to occur through a mechanism that is independent of receptor-mediated Ras and did not result in corresponding downstream MEK1 protein activation (24, 25). Similarly, although tyrosine phosphorylation of Raf-1 by Src kinase appears to be partially responsible for mitotic Raf-1 activity (24), a comprehensive analysis of the phosphorylation-dependent mechanisms that regulate mitotic Raf-1 activity has not been done. Thus, the function and targets of active Raf-1 during mitosis remain unknown.

Nonetheless, a requirement for MEK1/2 and ERK1/2 activity during G2 phase and mitosis has been suggested in cell cultures using pharmacological inhibitors of MEK1/2 (26, 27), dominant negative MEK1 mutants (28), MEK2 knock-out cell lines (29), or Raf-1/MEK1/2/ERK1/2 down-regulation using RNA interference (30). Phosphorylated MEK1/2 and ERK1/2 proteins have been shown to localize to the centrosomes and kinetochores, respectively, of mitotic cells and may regulate the function of proteins involved in metaphase to anaphase transitions (31, 32). Raf-1 and MEK1 have also been implicated in regulating Golgi complex fragmentation, which occurs as cells enter mitosis (33–36). Although ERK1/2 proteins are the only known substrates for MEK1 and MEK2, a role for the ERKs in regulating MEKI-induced mitotic Golgi fragmentation was not demonstrated in these studies. The aforementioned studies support a model where non-extracellular signal-mediated ERK pathway activation or phosphorylation occurs in localized intracellular regions and is important for mitotic transitions.

The present study focused on identifying mechanisms involved in the inhibition of receptor-mediated ERK signaling in mitotic cells. Our findings indicate that Cdc2 plays a key role in negative regulation of receptor-mediated ERK signal transduction by forming complexes with several proteins that couple the EGF receptor with ERK activation. Thus, in addition to directly inhibiting proteins involved in transcription and translation, Cdc2 serves an important role in inhibiting extracellular growth factor-regulated signaling pathways during mitosis.

**Materials and Methods**

**Cell Culture and Reagents**—HeLa or MDA-MB-468 cells were cultured in a complete medium consisting of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics (penicillin, 100 units/ml, and streptomycin, 100 μg/ml) from InterGen Life Sciences (Carlebad, CA). EGF, LY294002, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma. The antibodies specific for phosphorylated ERK1/2 (M-8159), α-tubulin (T-0587), and β-actin (A-1978) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific for phosphorylated MEK1/2 (M-8159), α-tubulin (T-0587), and β-actin (A-1978) were purchased from Cell Signaling Technologies (Beverly, MA). Peroxidase-conjugated mouse and rabbit secondary antibodies were purchased from Cell Signaling Technologies (Beverly, MA). The antibodies specific for phosphorylated MEK1/2 (number 9121) and pY1068/EGFR (number 22385) were purchased from Cell Signaling Technologies (Beverly, MA). The antibodies specific for phosphorylated MEK1/2 (number 9121) and pY1068/EGFR (number 22385) were purchased from Cell Signaling Technologies (Beverly, MA). The antibodies specific for phosphorylated MEK1/2 (number 9121) and pY1068/EGFR (number 22385) were purchased from Cell Signaling Technologies (Beverly, MA). The antibodies specific for phosphorylated MEK1/2 (number 9121) and pY1068/EGFR (number 22385) were purchased from Cell Signaling Technologies (Beverly, MA).

**Cell Synchronization**—HeLa cells were synchronized as has been previously described (40, 41). Briefly, cells (50% confluent) were treated with 2 μm thymidine in Dulbecco’s modified Eagle’s medium + fetal bovine serum for about 16 h. Cells were released back into the cell cycle by washing with Hank’s buffered saline solution and incubated for 8 h in 8% serum modified Eagle’s medium + fetal bovine serum for 16 h, which results in greater than 80% of the cells synchronized in the G1 phase of the cell cycle. Synchronized cells were washed with Hank’s buffered saline solution and then released back into the cell cycle and harvested at various times under various treatments. Cells were arrested in prometaphase of mitosis by treating with nocodazole (100 ng/ml), vinblastine (10 μg/ml), or paclitaxel (1 μM), all purchased from Sigma, for 12–14 h. In some cases, cells transfected with constitutively active mutants of MEK1 (kindly provided by Dr. Natalie Ahn, University of Colorado), BXR-Raf-1 (kindly provided by Dr. Ulf Rapp, University of Wurzburg), of V12 H-Ras (kindly provided by Dr. Melanie Cobb, University of Texas-Southwestern) were arrested with nocodazole and treated in the presence or absence of roscovitine during the last hour of nocodazole treatment.

**Mitotic Shakoff Analysis**—Cells were synchronized at the G1/S phase boundary using excess thymidine and then released back into the cell cycle for 9 h, where the peak number of mitotic cells can be obtained (27, 37). Mitotic cells were dislodged by gently tapping the plates and placed in sterile tubes containing complete medium. The remaining attached cells (G1 phase) and the mitotic cells were immediately treated with EGF or PMA for 5 or 30 min followed by harvesting and immunoblot analysis.

**Immunoblotting**—Immunoblotting was done as has been previously described. Briefly, protein lysates were collected from synchronized cells that were washed twice with cold phosphate-buffered saline, lysed with 300 μl of cold tissue lysis buffer (20 μM Tris, pH 7.4, 137 mM NaCl, 2 mM MgCl2, 1% Triton X-100, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide), allowed to incubate on ice for about 10 min, and then centrifuged at 14,000 revolutions per minute to clarify the lysates of insoluble material. The lysates were then diluted with an equal volume of 2× SDS-sample buffer before resolving them by SDS-PAGE, prior to immunoblot analysis. Immunoblots were then detected by enhanced chemiluminescence Western blotting reagents (Amersham Biosciences).

**Co-immunoprecipitations**—Protein interaction studies were examined in co-immunoprecipitation assays using lysates harvested from asynchronous and mitotic cells stimulated with or without EGF or PMA. Lysates (200–300 μg) were incubated with 2 μl of the indicated primary antibody (0.1–0.2 mg/ml) for 2 h on ice, followed by incubation of 25 μl of a 50% slurry of protein-Sepharose A/G 4 Fast Flow (Amersham Biosciences) at 4 °C for 4 h under constant mixing. The immune complexes were washed twice (0.5 ml each) with 25 mM Hepes, pH 7.4, 25 mM MgCl2, and 1 mM dithiothreitol or with tissue lysis buffer before reconstitution in SDS-PAGE sample buffer and immunoblot analysis of immunoprecipitated and co-immunoprecipitating proteins.

**Densitometry Analysis**—The relative amount of immunoprecipitated protein was measured by densitometry analysis of the immunoblots using NIH imager software. The relative density of the protein bands was calculated in the area encompassing the immunoreactive protein band and subtracting the background of an adjacent non-reactive area in the same lane of the protein of interest. To account for any variations in the amount of protein that was immunoprecipitated, the ratio of the co-immunoprecipitated protein to immunoprecipitated protein was calculated.

**RESULTS**

**ERK Activity in Mitotic Cells Treated with EGF or PMA**—Previously, it has been shown that activation of the EGF receptor is inhibited during mitosis (13–15, 17). Similarly, EGF receptor-mediated activation of the ERK pathway is inhibited in mitotic cells (17). In contrast, phorbol ester induction of the EGF receptor during mitosis has been suggested to be intact in mitotic cells and result in a G2 arrest (14, 38, 39). To explore the mechanisms involved in inactivation of receptor-mediated signaling in mitotic cells, the activation of ERK was compared in cells arrested in mitosis by nocodazole treatment or asynchronous cells following treatment with EGF or PMA. In contrast to previous studies, which claimed that PMA-induced signaling was largely independent of the cell...
cycle (14); in our hands PMA-treated cells showed a delayed ERK activation in mitotic cells compared with asynchronous cells (Fig. 1A). As expected, ERK activation in response to EGF was almost completely inhibited in mitotic cells (Fig. 1B). Increased cyclin B1 expression (Fig. 1A) as well as analysis of mitotic chromosomes and fluorescence-activated cell sorting confirmed that nocodazole-treated cells were arrested in mitosis (data not shown).

The effect of EGF activation of ERK was also tested in the context of other pharmacological agents that induce mitotic arrest. As shown, EGF stimulation of ERK was inhibited in cells arrested in mitosis using vinblastine (Fig. 2A) or paclitaxel (Fig. 2B, B and C). Similar to nocodazole-arrested cells, the PMA-mediated activation of ERK was delayed in cells arrested in mitosis with vinblastine or paclitaxel (Fig. 2A, A and C). We have also demonstrated that mitotic cells isolated by gently dislodging (shake off) from synchronized cells showed a similar decreased ERK activation in response to EGF or PMA as compared with the remaining adherent cells (data not shown). These data indicate that mitotic inhibition of ERK signaling is not related to the pharmacological agents used to induce mitosis. The differences in ERK activation observed following PMA or EGF treatment in mitotic cells prompted us to examine mechanisms responsible for PMA-mediated ERK activity and down-regulation of growth factor receptor-mediated ERK signaling at this time of the cell cycle.

PMA-induced ERK Activation Does Not Require EGF Receptor Tyrosine Kinase Activity—PMA-induced protein kinase C activation has been linked to transactivation of the EGF receptor (40–42). To determine whether the PMA-induced ERK activation observed during mitosis involves the EGF receptor, asynchronous or nocodazole-arrested cells were pretreated with AG1517, which is a potent and specific inhibitor of EGF receptor tyrosine kinase activity (43). Cells were then stimulated with either PMA or EGF and analyzed for ERK activation by immunoblotting. AG1517 had no effect on PMA-induced ERK activation in mitotic or asynchronous cells (Fig. 3A). As a control, pre-treatment with AG1517 effectively blocked EGF activation of ERK in asynchronous cells (Fig. 3B). Thus, PMA-induced activation of ERK during mitosis in HeLa cells does not appear to involve transactivation of the EGF receptor.

Changes in Sos-1, Raf-1, and EGF Receptor Phosphorylation during Mitosis—We next examined the phosphorylation status of the guanine nucleotide exchange factor Sos-1 and the Raf-1 kinase during mitosis to determine whether this post-translational modification could account for the inhibition of EGF or PMA-mediated ERK activation. Phosphorylation decreases the mobility of Sos-1 and Raf-1 in a polyacrylamide gel and this can be easily examined by immunoblot analysis. First, Sos-1, which is important for activating Ras, and the Ras effector kinase Raf-1 were examined. Sos-1 has been shown to be phosphorylated by MAP kinases following EGF receptor activation and this phosphorylation is thought to act as a negative feedback
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A. 

B. 

C. 

Fig. 4. Phosphorylation of Sos-1, Raf-1, and the EGF receptor during mitosis. A, asynchronous or nocodazole-treated cells arrested in mitosis were pre-treated with or without AG1517 and then stimulated for the indicated times with PMA or EGF. Cellular lysates were then analyzed by immunoblotting for Sos-1 and Raf-1. B, mitotic cells generated by a 14-h exposure to nocodazole were left untreated or treated during the last hour of incubation with roscovitine (60 μM), staurosporine (2 μM), LY294002 (1 μM), or U0126 (10 μM). Lysates were immunoblotted for Sos-1 and Raf-1. C, asynchronous and nocodazole-arrested cells were stimulated with EGF (5 min) or PMA (20 min) and immunoblotted with antibodies against pY1068 of the EGF receptor (EGFPR) or α-tubulin. Data are representative of at least three independent experiments.

To examine potential classes of kinases that may be responsible for hyperphosphorylated Sos-1 and Raf-1 during mitosis, cells arrested in mitosis with nocodazole were treated during the last 1 h prior to harvesting with the Cdc2 inhibitor roscovitine, the general kinase inhibitor staurosporine, the PI3K inhibitor LY294002, or the MEK1/2 inhibitor U0126. Both roscovitine and staurosporine inhibited the hyperphosphorylated forms of Sos-1 and Raf-1 as suggested by the increase in gel mobility (Fig. 4B). The decrease in Sos-1 phosphorylation was similar when using roscovitine or staurosporine indicating that Cdc2 was the primary kinase that directly or indirectly caused phosphorylation of Sos-1 during mitosis (Fig. 4B). In contrast, greater inhibition of Raf-1 gel mobility occurred in cells treated with staurosporine compared with roscovitine (Fig. 4B). This supports previous studies that suggest Cdc2 and other kinases directly or indirectly mediate Raf-1 phosphorylation during mitosis (25). Cells treated with U0126 or LY294002 had little effect on the mitotic Raf-1 gel mobility indicating that MEK1/2 and PI3K-mediated signaling does not affect Raf-1 phosphorylation events during mitosis (Fig. 4B).

The previous data suggested that Cdc2 might regulate multiple proteins involved in the activation of the ERK pathway. Previously, it was suggested that ligand-induced autophosphorylation of tyrosine residues within the cytoplasmic domain of the EGF receptor is inhibited in mitotic cells (13–15, 46). However, the specific tyrosine residues that may have reduced phosphorylation in response to EGF have not been defined. To extend our examination of proteins involved in ERK regulation, specific phosphorylation of tyrosine on residue 1068 (Tyr1068) of the EGF receptor, a site required for Grb2 adaptor protein interactions (47), was examined. Grb2 recruited to the EGF receptor interacts with and localizes Sos-1 to the plasma membrane where it facilitates GTP coupling to Ras and activation of the ERK signaling cascade (48, 49). Phosphorylation at Tyr1068 on the EGF receptor in asynchronous and mitotic cells was examined using a phospho-specific EGF receptor antibody. Asynchronous and mitotic HeLa cells were stimulated with EGF or PMA and lysates were immunoblotted for phospho-Tyr1068 on the EGF receptor. As expected, EGF stimulation results in an increased phosphorylation of Tyr1068 in asynchronous cells, however, phosphorylation at this site following EGF treatment in mitotic cells was significantly reduced (Fig. 4C). PMA treatment of asynchronous or mitotic cells had no effect on EGF receptor phosphorylation of Tyr1068 (Fig. 4C). Thus, mitotic cells have reduced phosphorylation of the Grb2 interacting site on Tyr1068 of the EGF receptor in response to ligand stimulation as compared with asynchronous cells.

Inhibition of Grb2 Interactions with Sos-1 during Mitosis—Given that Sos-1 is hyperphosphorylated in mitosis (Fig. 4), we next determined whether the interactions between Grb2 and Sos-1 were impaired in mitotic cells. Grb2 was immunoprecipitated from asynchronous or mitotic cells treated with or without EGF and co-immunoprecipitating Sos-1 was examined by immunoblotting. As shown in Fig. 5, A and C, EGFR treatment significantly increased the amount of Sos-1 that interacted with Grb2 in asynchronous cells as compared with mitotic cells. These results suggest that interactions between hyperphosphorylated Sos-1 and Grb2 are impaired in mitotic cells and provide an additional level of regulation to prevent EGF receptor activation of the ERK pathway during mitosis.

Cdc2 Interacts with Sos-1, Grb2, and Raf-1 during Mitosis—Because Cdc2 appeared to be responsible for part or all of the mitotic phosphorylation of Sos-1 and Raf-1 (Fig. 4B), we proposed that Cdc2 interactions with these signaling proteins is involved in mitotic inhibition of receptor-mediated ERK signaling. To test this, Cdc2 interactions with Sos-1 and Grb2 in asynchronous or mitotic cells were examined. Increased Cdc2 co-immunoprecipitation was observed in mitotic cells following Sos-1 or Grb2 immunoprecipitation (Fig. 6, A and B). Similarly, immunoblot analysis showed increased Cdc2 co-immunoprecipitation with Raf-1 in mitotic cells compared with asynchronous cells (Fig. 6, C and E). Semiquantitative analysis suggested a nearly 3-fold increase in Cdc2 interacting with Raf-1 in mitotic cells as compared with asynchronous cells (Fig. 6F). In contrast, MEK1, a downstream target of Raf-1, did not appear to have increased interactions with mitotic Cdc2 in co-immunoprecipitation studies (Fig. 6F). These data provide evidence that Cdc2 may interact with and phosphorylate mul-
multiple signaling proteins that leads to the disruption of EGF receptor activation of the ERK pathway during mitosis.

Inhibition of Cdc2 Restores ERK Activation in Mitotic Cells through De-repression of EGF Receptor, Ras, and Raf-1 but Not MEK-1—To provide more definitive evidence that Cdc2 was linked to the inhibition of the ERK signaling proteins, we tested whether blocking Cdc2 activity would restore ERK activation in mitotic cells treated with PMA, EGF, or expressing constitutively active ERK activating mutants. Asynchronous or mitotic cells were treated during the last hour of incubation with the Cdc2 inhibitor roscovitine and then stimulated with PMA or EGF 5 min prior to harvesting. As was shown previously, ERK activation by PMA or EGF was reduced in mitotic cells compared with asynchronous cells (Fig. 7, A and B). However, the addition of roscovitine restored ERK activation in response to PMA or EGF in mitotic cells to levels comparable with asynchronous cells (Fig. 7, A and B). Roscovitine treatment alone had no effect on ERK activity in asynchronous or mitotic cells (Fig. 7, A and B). These data suggest that Cdc2 activity is necessary to suppress ERK pathway activation in response to EGF signaling during mitosis.

To examine the role of Cdc2 in inhibiting the EGF receptor during mitosis, the phosphorylation of Tyr\textsuperscript{1068} following EGF stimulation was examined in nocodazole-arrested cells treated with or without roscovitine during the last hour of arrest. As shown, roscovitine-treated cells restored EGF-induced Tyr\textsuperscript{1068} phosphorylation (Fig. 7C). To further demonstrate that specific ERK pathway signaling proteins are also inhibited by Cdc2 during mitosis, cells were transfected with constitutively active mutants of H-Ras, Raf-1, or MEK1 and then arrested in mitosis with nocodazole. During the last hour of mitotic arrest, transfected cells were treated with roscovitine to inhibit Cdc2 activity. In asynchronous cells, all active mutants stimulated ERK activity as expected (Fig. 7D). However, ERK activity during mitosis was inhibited in cells transfected with active Ras or Raf-1 but not active MEK1 (Fig. 7D). Equal expression of the active mutants was observed under each condition (data not shown). MEK1-induced ERK activity appeared to be slightly elevated in nocodazole-treated cells (Fig. 7D), which argued against the uncoupling of MEK1 and ERK proteins during mitosis as previously suggested (21). Roscovitine inhibition of Cdc2 activity restored H-Ras and Raf-1 induced ERK activity in nocodazole-treated cells but had no effect on MEK-1-induced ERK (Fig. 7D). These data support a mechanism where Cdc2 primarily inhibits receptor-mediated ERK activity by targeting signaling proteins that are upstream of MEK proteins.

DISCUSSION

We demonstrate in the present study that Cdc2 uses multiple mechanisms to inhibit membrane receptor-mediated ERK signal transduction during mitotic transitions (Fig. 8). The inhibition of extracellular stimulated ERK signaling is likely
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important to ensure that general cessation of transcription and translation events occurs to preserve the energy requirements needed for the dynamic structural changes occurring in mitotic cells. We describe enhanced Cdc2 interactions with several ERK pathway signaling proteins including Sos-1, Grb2, and Raf-1 in mitotic cells. In addition, we show hyperphosphorylation of Sos-1 and Raf-1 is, in part, dependent on Cdc2 activity. Importantly, we find that inhibition of Cdc2 activity in mitotic cells restores the ERK activation induced by EGF or expression of constitutively active Ras and Raf-1 mutants.

Although previous findings indicated that EGF receptor and downstream ERK activity is inhibited during mitosis (13–15, 17), inhibition of signaling events that link the EGF receptor with the ERK proteins has not been examined. Cdc2 has been suggested to interact with and inhibit the EGF receptor through phosphorylation (16). Whereas it is possible that inhibition of the EGF receptor alone is sufficient to block all receptor-mediated activation of the ERK pathway during mitosis, other mechanisms may be in place to ensure that growth factor signaling at this time is prevented. Especially in cases where EGF receptor activation is elevated, Cdc2 regulation of other signaling proteins downstream of the EGF receptor may be an additional mechanism to prevent inappropriate signal transduction during mitosis. As an example, the overexpression of EGF and EGF-like receptors, which is accompanied by increased activity of ERK and other signal transduction pathways, is observed in many cancer types (50) and underscores the importance of understanding the regulation of EGF receptor signaling during the cell cycle. As such, inhibition of EGF and EGF-like receptors may be a promising approach for the treatment of a variety of cancers (51–53).

One possible consequence of EGF receptor overactivation and the inability to prevent EGF receptor-mediated signaling pathways during mitosis may be an increase in chromosome segregation defects that lead to chromosome instability characteristic of transformed cells. We have evidence that activation of the ERK pathway by EGF in G_{2} phase cells will cause an increase in chromosome abnormalities that are observed during the next round of the cell cycle. Similarly, changes in other proteins that cause overactivation of the ERK pathway have previously been linked to enhanced chromosome instability. For example, constitutively active mutations in Ras G-proteins, which are found in many human cancers and increase ERK pathway activation, has been shown to increase the characteristics of chromosome instability within one cell cycle (54). Similarly, naturally occurring mutations in B-Raf have been found in several cancer types and may lead to cellular changes that promote cancer cell growth through activation of the ERK pathway (55, 56). In addition, increased signs of chromosome instability, including multinucleated cells and centrosome amplification, have been shown in liver cancer cells expressing the hepatitis B virus X oncoprotein, which also results in elevated ERK activation (57). Importantly, reversal of these chromosomal aberrations with pharmacologic MEK1/2 inhibitors or overexpression of dominant negative MEK2 or Ras mutant proteins provided evidence for the requirement for ERK signaling in this phenotype (57).

We demonstrated that although EGF-mediated ERK activation was almost completely inhibited in mitotic cells, some ERK activity was retained in mitotic cells treated with PMA (Fig. 1A). It has been shown that PMA can activate the ERK pathway at the level of Raf-1 through a protein kinase C-dependent mechanism (58, 59). Thus, we hypothesized that multiple proteins that regulate ERK activation, including Raf-1 and upstream regulators that respond to the EGF receptor, are inhibited during mitosis. Although we show that Cdc2 interacts with and phosphorylates ERK pathway signaling proteins in a mitosis-specific manner, these findings alone did not conclusively demonstrate that Cdc2 inhibits protein function. To provide further evidence that Cdc2 inhibits ERK activation in mitosis, we showed that Cdc2 inhibition restores the ability for EGF to activate ERK in mitotic cells (Fig. 7B). Because this experiment

\[ S. Dangi and P. Shapiro, unpublished observation. \]
did not define the level of the ERK signaling cascade at which the Cdc2-mediated inhibition might be occurring, we used cells expressing activating mutants of three proteins (H-Ras, Raf-1, or MEK1) that sequentially result in ERK activation. Consistent with the inhibitory effects of mitosin on EGFR-stimulated ERK activation, we show that Ras- or Raf-1-induced ERK activity was also inhibited in mitotic compared with asynchronous cells (Fig. 7D). Moreover, pharmacological inhibition of Cdc2 restored Ras- or Raf-1-induced ERK activity in mitotic cells (Fig. 7D). These findings indicate that mitotic inhibition of EGFR-mediated ERK activation occurs through Cdc2 interaction and phosphorylation of Raf-1 (Figs. 4B and 6C). Identification of the Cdc2 phosphorylation sites on Raf-1 and their role in Raf-1 regulation are the goals of future studies.

In contrast, our observation that MEK1-induced ERK activity was not inhibited in mitotic cells indicates that MEK1 and ERK interactions are not affected in mitotic cells (Fig. 7D). Previous studies suggested that MEK1 activity is inhibited through phosphorylation of Thr286 and Thr292 in the C terminus by Cdc2 (60) and that the major site regulated during mitosis is Thr286 (21). Our data suggest that Cdc2-mediated phosphorylation of MEK1 at Thr286 is insufficient to inhibit the constitutively active MEK1 mutant used in the present study. The active MEK1 mutant was generated by deleting amino acids 44–51 in the N-terminal region and making acidic amino acid substitutions at the activating serine sites (61). It is possible that these mutations on MEK1 will negate any inhibitory effects because of phosphorylation on Thr286. The role of threonine phosphorylation in regulating MEK1 activity during the cell cycle is still unresolved. For example, Thr292 phosphorylation has been shown to be constitutive (62), which argues against a role for this site in cell cycle regulation. To our knowledge, a thorough examination of Thr286 and Thr292 phosphorylation of Raf-1 (Figs. 4B and 6C) indicates that localized ERK pathway activity during mitosis is not known but may likely involve cell cycle-specific changes in localized phosphatase activities.

Another function for the ERK pathway during mitosis may be to regulate the disassembly or fragmentation of the Golgi apparatus, which occurs to evenly segregate Golgi membranes into each daughter cell (63). For example, Raf-1-mediated MEK1 activity during late prophase has been reported to regulate Golgi fragmentation (33–35). However, these studies failed to provide evidence that ERK1/2 activity was involved in this process. As another possibility, MEK1-induced Golgi fragmentation may occur through a mechanism involving ERK proteins that are phosphorylated on the tyrosine residue within the Thr-Glu-Tyr activation site (37). Although both phosphorylations of the Thr and Tyr residues are required for full ERK activation, single phosphorylations at either Thr or Tyr within the activation site may affect ERK protein function or cause partial ERK activity (64). The mechanisms involved in regulating localized ERK activities in mitosis are not known but may possibly involve cell cycle-specific changes in localized phosphatase activities.

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