A Functional Role for p38 MAPK in Modulating Mitotic Transit in the Absence of Stress

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Although p38 MAPK is known to be activated in response to various environmental stresses and to have inhibitory roles in cell proliferation and tumor progression, its role in cell cycle progression in the absence of stress is unknown in most cell types. In the case of G2/M cell cycle control, p38 activation has been shown to trigger a rapid G2/M cell cycle checkpoint after DNA damage stress and to have inhibitory roles in mitotic entry. In addition, long term exposure of the inhibitor disrupts cell cycle progression. A closer examination reveals that p38 inhibition by pharmacologic inhibitors significantly accelerated the timing of mitotic entry. In addition, long term exposure of the inhibitor enhanced Cdc2 activity. These results indicate that p38 activity during G2/M may be involved in a mechanism for fine tuning the initiation of mitosis and perhaps transit of mitosis. Consistent with our previous findings, Cdc25B was phosphorylated on serine 309 at the centrosome during G2/M when p38 was active at this site; Cdc25B phosphorylation inhibits Cdc25B activity, and this phosphorylation was found to be p38-dependent. Taken together, our findings suggest that p38 regulates the timing of mitotic entry via modulation of Cdc25B activity under normal nonstress conditions.

Conventionally, after most stimuli, p38 activation is mediated by the upstream kinases MKK3/6 through dual phosphorylation on threonine and tyrosine located within the activating loop (2). However, two different MKK-independent mechanisms have also been reported for p38 activation either through TAB1 binding or through T cell-specific ZAP-70 phosphorylation (3, 4).

Due to the intrinsic role of p38 in stress signaling, an antiproliferative effect for p38 might be expected and has been demonstrated in a number of reports. For example, in G1 to S cell cycle transition, stress-mediated p38 signaling negatively regulates cyclin D1 transcription and phosphorylates cyclin D1, facilitating its degradation, which in turn contributes to G1/S arrest (5, 6). Furthermore, in the G2 to M phase transition, activated p38 after UV irradiation is required for a G2/M checkpoint involving Cdc25B and Cdc25C, which play pivotal roles in controlling Cdc2-cyclin B1 complex activity during mitosis (7). Active p38 directly phosphorylates serine 216 on Cdc25C and serines 309 and 361 on Cdc25B after UV irradiation, in turn promoting 14-3-3 binding to Cdc25B and Cdc25C (7). In addition, Manke et al. (8) demonstrated that MAPKAP kinase 2 (MAPK-activating protein kinase 2), a direct downstream target of p38, can directly phosphorylate Cdc25B and Cdc25C. Similarly, Skr1 (Sty1-related kinase), a downstream kinase of yeast p38 MAPK, has been shown to control mitotic entry by directly phosphorylating and inhibiting Cdc25 during osmotic stress (9). Taken together, there is strong evidence that the p38 MAPK signaling pathway can play a critical role(s) in controlling mitotic entry through Cdc25 inhibitory phosphorylation in response to a variety of stresses. Checkpoint proteins often have tumor suppressor properties and results from a variety of laboratories have demonstrated this for p38 (10). Recently, Engel et al. have reported that p38 inhibition, either by pharmacological inhibitors or by a genetic approach via disruption of the p38α gene, up-regulated critical mitosis related genes and enhanced proliferation in cultured cardiomyocytes on the basis of an increased fraction of mitotic cells (11); this suggests that endogenous p38 activity even in the absence of stress can regulate cardiomyocyte proliferation and mitosis.

Herein, we have found that under normal cell culture conditions, p38 activity was slightly increased during the G2/M phase of the cell cycle, and active p38 was localized at centrosomes, where its upstream kinase MKK3/6 was found to be active at the same time. Furthermore, we also demonstrate that centrosomal Cdc25B was phosphorylated on serine 309 by p38 during mitosis. Inhibition of p38 activity by pharmacological inhibition increased the mitotic population significantly at this time, sug-
sugest that p38 activity during mitosis controls the timing of mitotic initiation through inhibitory phosphorylation of Cdc25B.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The phospho-p38 antibody (Thr180/Tyr182; catalog number 9211), p38 antibody (catalog number 9218), phospho-MKK1/2 antibody (catalog number 9121), phospho-MK2/3/6 antibody (catalog number 9231), phosphohistone H3 (catalog number 9706), and phospho-ATF-2 antibody (catalog number 9221) used in these studies were obtained from Cell Signaling Technology. Blocking peptide for phospho-p38 antibody (catalog number 1170) was from Cell Signaling Technology. The Cdc25B antibody (catalog number 610527) was from BD Transduction Laboratories. The γ-tubulin antibody (T-6557) used for centrosome detection was from Sigma. The other antibodies, such as cyclin B1 (GNS1), PLK (H-152), α-tubulin (B-7), Cdc2 (17), p38 (C-20), and nucleophosmin (NPM) (H-106) were obtained from Santa Cruz Biotechnology.

**Cell Culture**—HeLa, HCT116, RKO, or mouse embryonic fibroblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum with penicillin (100 units/ml) and streptomycin (100 μg/ml). In some experiments, cell synchronization was performed using double thymidine block as previously described (12). Synchronized cells were released back into the cell cycle by removal of thymidine. Wild type and p38α−/− mouse embryonic stem (ES) cells were maintained as described previously (13).

**Immunoblotting**—For harvesting, cells were washed twice with cold PBS, lysed with 300 μl of tissue lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, and protease inhibitor mixture), and centrifuged at 14,000 rpm to clarify lysates. Approximately 20 μg of total protein were separated by SDS-PAGE electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane, blocked for 1–2 h with 5% nonfat dry milk in PBS-T (PBS containing 0.1% Tween 20), and incubated with various primary antibodies in PBS-T containing 1% bovine serum albumin solution for 1–16 h. Membranes were washed several times in PBS-T solution and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (0.1 μg/ml; Jackson ImmunoResearch Laboratories). Immunoreactivity was detected by enhanced chemiluminescence (Amersham Biosciences).

**Immunofluorescence**—Cells grown on round glass coverslips (number 1; VWR) were fixed with 4% paraformaldehyde for 8 min and permeabilized with 0.1% Triton X-100 in PBS for 2 min. Fixed cells were incubated for an additional 1–2 h in PBS-T containing 3% bovine serum albumin for blocking, followed by a 1-h incubation with various antibodies described above. Cells were washed several times with PBS-T solution and incubated with Cy3-conjugated anti-mouse or anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories) or Alexa 488 Fluor-conjugated anti-mouse or anti-rabbit secondary antibodies (Invitrogen) and counterstained for cellular DNA with 4′,6-diamidino-2-phenylindole (DAPI; 0.2 μg/ml in PBS). For infrared imaging, infrared fluorescence conjugated mouse and rabbit antibodies were from Invitrogen (catalog number A21058) and Rockland (catalog number 611-132-122), respectively. Staining for infrared imaging was performed as recommended by the manufacturer (LI-COR Biosciences).

**In Vitro Kinase Assay**—Cdc2 was isolated from G1/S synchronized or mitotic arrested cells lysates by immunoprecipitation. In vitro kinase assays were performed in a reaction buffer of 25 mM Hepes (pH 7.4), 15 mM MgCl2, 1 mM dithiothreitol, 20 mM unlabeled ATP, and 10 μCi/μl [γ-32P]ATP in the presence of isolated kinase from G1/S or G2/M lysates, including histone H1 (Upstate Biotechnology) as substrate. Phosphate incorporation into substrates was determined by PhosphorImager analysis (Amersham Biosciences).

**Mitotic Index Assay**—HCT116 cells grown on coverslips were synchronized at the G1/S-phase boundary as described above and released in the presence or absence of SB203580. At varying times after release, coverslips were fixed, and cellular DNA was stained with phosphohistone H3 antibody and DAPI. Mitotic cells identified by positive phosphohistone H3 staining were counted and expressed as a fraction of the total cells counted to determine the mitotic index (7). Within each exper-
iment, 300–350 cells were counted for each condition and time point.

RESULTS

Active p38 during G2/M—G1/S-synchronized HeLa or RKO cells by double thymidine block were generally in G2 at 5 h and in mitosis at 7 h after release (supplemental Fig. S1A). As shown in Fig. 1A, relatively distinct immunoreactivity toward phospho-p38 antibody was observed from 5 h and was increased at 7 h when cells are in the G2 and G2/M phase of cell cycle, respectively. This indicates that p38 MAPK becomes active during the G2/M phase of cell cycle in HeLa cells. Immunoblotting for cyclin B1, which is a widely used mitotic marker protein, was performed to confirm the G2/M cell cycle phase.

To determine if p38 MAPK during G2/M contains enzymatic activity, in vitro kinase assays were carried out using ATF-2 fusion protein as a substrate. p38 MAPK isolated from G2/M-synchronized HeLa cells exhibited higher activity toward ATF-2 protein than p38 from G1/S-synchronized cells on the basis of a modest but significant increase of the phospho-ATF-2 signal (Fig. 1B, top). Based on the pixel intensity of phospho-ATF-2 determined by NIH Image software, the activity during G2/M appeared to show a 5–6-fold increase compared with that of G1/S (Fig. 1B, bottom).

Detection of Phospho-p38 at the Centrosomes during Mitosis—Previously, active p38 has been found in the nonstressed developing retina, and positive cells appeared to be in mitosis by immunohistochemistry (14). In order to determine the localization for active p38, immunocytochemistry using a phosphospecific antibody for activated p38 was performed in growing HeLa cells. Interestingly, a distinct immunoreactivity was found, shown as two dotlike foci that appear to be centrosomes from late G2 and throughout mitosis, from prophase to telophase (data not shown and Fig. 2). The same results were obtained in growing RKO cells (data not shown) as well as wild type ES but not p38−/− ES cells (supplemental Fig. S2). To confirm that these signals come from the centrosome, γ-tubulin was co-immunostained along with a phospho-p38 antibody. As shown in Fig. 3A, phospho-p38 immunoreactivity perfectly matched with the signal for γ-tubulin, indicating that phosphorylated p38 MAPK is located at the centrosome during the G2/M phase of the cell cycle. Preincubation with a blocking peptide completely eliminated centrosomal immunoreactivity from mitotic cells, which excludes the possibility of nonspecific signal involvement (Fig. 3B). As noted above, the absence of this signal in p38-deficient cells also demonstrates that this is bona fide p38 staining at the centrosome. As shown in Fig. 3C, Western blotting with phospho-p38 antibody did not display any additional signal from the G2/M sample except in one band, which corresponded to the phospho-p38 signal from the positive control (anisomycin). Ultracentrifugation from the cytoplasmic fraction can precipitate most of the subcellular organelles, such as the Golgi apparatus (15) and centrosomes (16). Thus, in agreement with cell immunostaining data, the increased level of phospho-p38 in the cytoplasmic membrane fraction obtained from G2/M synchronized cells is also demonstrated by this approach, and this is consistent with localization of active p38 at the centrosome during G2/M (Fig. 3D).
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FIGURE 3. Localization of phosphorylated p38 at the centrosomes during mitosis. A, HeLa cells were immunostained with phospho-p38 antibody (top left) and γ-tubulin (top right) as a centrosome marker. The arrows indicate centrosomal signal for phospho-p38. Phospho-p38 and DAPI image were overlapped and shown at the bottom (Merged). B, phospho-p38 antibody was preincubated with its specific blocking peptide (lower two panels) for 30 min prior to immunostaining. C, HeLa cells were incubated with 100 μM anisomycin for 30 min prior to harvest. G1/S and G2/M phase-synchronized cells were obtained by double-thymidine block and 9 h post G1/S release, respectively. Cyclin B1 immunoblot was used to determine the mitotic population (middle). Immunoblot for p38 served as loading control (bottom). D, cells synchronized at G1/S or G2/M phase were separated into cytoplasmic or nuclear protein fraction. The cytoplasmic fraction was further separated into soluble (C) or insoluble (M) proteins by ultracentrifugation. The protein fractions were immunoblotted with phospho-p38 (top), total p38 (middle), and cyclin B1 (bottom) antibodies.

FIGURE 4. Coincidence of spatial and temporal activation of upstream kinase, MKK3/6 with p38 MAPK during G2/M. A, cells synchronized at G1/S or G2/M phase were immunoblotted with phospho-MKK3/6, phospho-p38, phospho-NPM, and p38 antibodies. B, growing HeLa cells were immunostained for phospho-MKK3/6 (left top panel) and α-tubulin (right top panel). A mitotic cell (M) was identified by DAPI staining (left bottom panel) of condensed chromosome. The adjacent interphase cell (I) is displayed for comparison. The arrows indicate centrosomal signal for phospho-MKK3/6.

activation was monitored by immunoblotting and immunostaining using a phospho-specific antibody for MKK3/6. As shown in Fig. 4A, immunoblotting for phospho-MKK3/6 demonstrated an increased level of phospho-MKK3/6 in the G2/M phase of the cell cycle when p38 MAPK has also been shown to be active. Unless p38 activation by MKK3/6 occurs in the cytoplasm and translocates to the centrosome during G2/M, activation of MKK3/6 could also be occurring at the centrosome at these times. In agreement with the latter scenario, we found distinct phospho-MKK3/6 immunoreactivity at the centrosome where the mitotic spindle stained by α-tubulin antibody was nucleated (Fig. 4B). As with previous phosphoimmunoblots (Fig. 3C), immunoblotting results for phospho-MKK3/6 did not show any additional nonspecific band in the G2/M sample, indicating that the centrosomal signal of phospho-MKK3/6 antibody is specific for phospho-MKK3/6 proteins (Fig. 4B). To our knowledge, this is the first demonstration that the MKK3/6 and p38 MAPK signaling pathways become active in a cell cycle-dependent manner under nonstress conditions.

SB203580 Treatment Accelerates Mitotic Initiation—Since many phosphoproteins found at the centrosome during G2/M, such as Chk1 (17), Aurora A (18), and Plk1 (19), have been shown to play important roles in regulating mitosis initiation or progression, either positively or negatively, and since it has been suggested that centrosomes facilitate mitotic entry by condensing critical mitotic regulatory factors, such as cyclin B1, Cdc2, and Plk1 (17), we hypothesized that phosphorylated p38 at the centrosome may have a role(s) in regulating mitotic entry. To address this question, we examined cell cycle progression from G1/S-synchronized HCT116 cells. Taking advantage of phosphohistone H3 antibody (phosphoserine 10) staining, mitotic cells were identified at the given time after G1/S release either in the presence or absence of p38 inhibitor. As expected, at 9 h post-G1/S, when most of the cells have progressed to mitosis, more phosphohistone H3-positive cells were found in the presence of the p38 inhibitor than in the control group (Fig. 5A). The increased mitotic population by p38 inhibitor was confirmed by infrared fluorescence scanning that enabled us to quantify the signal intensity. In experiments similar to Fig. 5A, strong nuclear accumulation of cyclin B1 shown in the G2/M sample verified the fractionation method as well as G2/M synchrony (Fig. 3D, bottom). Immunostaining after a cold methanol fixation also showed the same result (supplemental Fig. S3A). In addition, we also observed the same centrosomal phospho-p38 signal in primary cells, such as early passage mouse embryo fibroblasts and immortalized normal fibroblasts, such as W1-38-hTERT- and BJ-hTERT-transformed cells (supplemental Fig. S3B) (data not shown).

Taken together, these data lead us to conclude that p38 MAPK activity is regulated in a cell cycle-dependent manner and that activated p38 is localized to the centrosome during the G2/M phase of the cell cycle. These observations suggest a novel functional role for p38 that is not related to the stress response during mitosis.

Coincidence of Spatial and Temporal Activation of Upstream Kinase, MKK3/6, with p38 MAPK during G2/M—Because in most cases p38 activation is mediated by MKK3/6, the activation of MKK3/6 should coincide with p38 activation temporally and spatially unless active p38 is translocated to the centrosome after cytoplasmic activation. To examine this process, MKK3/6 activation was monitored by immunoblotting and immunostaining using a phospho-specific antibody for MKK3/6. As shown in Fig. 4A, immunoblotting for phospho-MKK3/6 demonstrated an increased level of phospho-MKK3/6 in the G2/M phase of the cell cycle when p38 MAPK has also been shown to be active. Unless p38 activation by MKK3/6 occurs in the cytoplasm and translocates to the centrosome during G2/M, activation of MKK3/6 could also be occurring at the centrosome at these times. In agreement with the latter scenario, we found distinct phospho-MKK3/6 immunoreactivity at the centrosome where the mitotic spindle stained by α-tubulin antibody was nucleated (Fig. 4B). As with previous phosphoimmunoblots (Fig. 3C), immunoblotting results for phospho-MKK3/6 did not show any additional nonspecific band in the G2/M sample, indicating that the centrosomal signal of phospho-MKK3/6 antibody is specific for phospho-MKK3/6 proteins (Fig. 4B). To our knowledge, this is the first demonstration that the MKK3/6 and p38 MAPK signaling pathways become active in a cell cycle-dependent manner under nonstress conditions.

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mitotic cells were identified and then determined by phospho-histone H3 antibody. The antibody against Ki-67, proliferating antigen, was used for normalizing signal from the total number of cells. Consistently, the mitotic population was significantly increased at 8 h post-G1/S by p38 inhibitor compared with control (Fig. 5, B and C). There was an approximately 2-fold increase of pH3/Ki-67 signal in SB203580-treated cells at 8 h over control. Next, to exclude possible side effects of the p38 inhibitor itself in cell cycle and cell proliferation, we made use of p38−/− ES cells to compare cell growth to wild type ES cells. As shown in Fig. 5D, p38−/− ES cells grew significantly faster than wild type ES cells. Taken together, these data indicate that p38α plays an important role in negatively controlling mitotic initiation and consequently modulating cellular proliferation, even in the absence of exogenous stress.

p53 and p38 MAPK during Mitosis—Regarding downstream effectors of p38, p53 is a strong candidate for the following reasons. First, p53 has been reported to be a target of p38 after UV radiation stress at various sites (20, 21). Second, p53 has been shown to locate to the centrosome during G2/M in an ATM-dependent manner (22). And third, p53 plays a pivotal role in G2 arrest after DNA damage (23). Therefore, we speculated that p38 may control the timing of mitotic initiation through p53 at the centrosome. Thus, we compared the mitotic index in the presence or absence of SB203580 between HCT116 wild type and HCT116 p53−/− cells. Interestingly, SB203580 increased the mitotic population in both HCT116 wild type and HCT116 p53−/− cells to a similar degree (supplemental Fig. S4, yellow triangles), whereas UV irradiation (10 J/m²) expectedly failed to arrest cells at G2 in HCT116 p53−/− cells, (supplemental Fig. S4, red squares). The UV-induced mitotic delay was prevented by SB203580 pretreatment, which is consistent with our previous report that p38 has one or more roles in G2/M arrest after DNA damage induced by UV irradiation (7). These data indicate that p38 regulates mitotic entry in a p53-independent manner in the absence of stress.

Cdc25B and p38 MAPK during Mitosis—As a next candidate, we investigated Cdc25B for the following reasons. First, Cdc25B is reportedly phosphorylated and thus inhibited by active p38 after UV irradiation with resultant G2/M checkpoint activation (7). Second, centrosomal Cdc25B has been shown to contribute to mitotic initiation in cooperation with Cdc25A (24, 25). Interestingly, by immunostaining, Cdc25B was found to co-localize with phospho-p38 at the centrosome during mitosis (Fig. 6A). Therefore, we hypothesized that centrosomal Cdc25B at G2/M might be regulated by transiently increased p38 MAPK activity at the same time and might control the timing of mitotic initiation. We next examined whether the phosphorylated form of Cdc25B, which has been reported to be phosphorylated on ser-

FIGURE 5. SB203580 treatment accelerates mitotic initiation. G1/S synchronized HCT116 cells were released into normal cell cycle with or without SB203580 (10 μM). A, the mitotic index was measured as described under “Experimental Procedures.” Data show the average and S.E. from three independent experiments. B, at each time, cells were fixed and stained with phospho-histone H3 and Ki-67 antibodies. Intensity of signal was measured by Odyssey analysis software. C, intensity for phosphohistone H3 versus Ki-67 was obtained and is presented graphically. D, either wild type ES cells or p38α−/− ES cells were grown as previously described (13). An equal number of trypsinized cells was plated and grown one more day. Cells were trypsinized and counted at each of the indicated times. Growth, as determined by cell number per plate, is shown (open square, wild type (WT); closed square, p38α−/− ES cells).
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next we investigated whether failure of inhibitory phosphorylation could promote Cdc2 activity during mitotic initiation. G₁/S synchronized HCT116 were treated at G₂ (5 h after G₁/S), either with or without SB203580, and collected at various times. Cdc2 activity was confirmed by the level of phosphorylation of NPM; NPM has previously been shown to be a Cdc2 substrate at the centrosome (16). As shown in Fig. 8A, the level of NPM phosphorylation was higher in the nucleus during mitosis compared with that of G₁/S (Fig. 8A, top), which is expected. Besides, higher phospho-NPM level in the membrane fraction (M) from SB203580-treated cells than control (Fig. 8A) can indicate higher Cdc2 activity in the presence of p38 inhibitor. Since the centrosomal compartment is the only known subcellular organelle where phosphorylated NPM exists except the nucleus (26), it is plausible that the phospho-NPM signal from cytoplasmic insoluble fraction (M) results from Cdc2-mediated phosphorylation at the centrosome. Thus, increased phosphorylation of NPM in the cytoplasmic insoluble fraction with p38 inhibitor at 8 h after G₁/S indicates that Cdc2 activity at the centrosome was increased transiently at this time compared with the control (Fig. 8A). Next, to examine cell cycle progression more closely, cells were fractionated into cytoplasmic and nuclear fractions at various times after G₁/S. At 6 h after release from G₁/S, when cells usually are in G₂, cells with SB203580 contained a higher level of cyclin B1 in both the cytoplasmic and nuclear fractions than control cells at this point (Fig. 8B). Later, at 8 h after G₁/S, p38 inhibitor-treated cells still showed a significantly higher level of cyclin B1 in the cytoplasmic fraction compared with control cells (Fig. 8B). Nuclear protein toposomerase I has been shown to locate only in the nuclear fraction but not cytoplasmic fraction, ruling out the possibility that increased cyclin B1 in SB203580-treated samples is caused by cross-contamination from the nuclear fraction (Fig. 8B, middle).

For long term exposure, p38 inhibitors SB203580 or PD169636 were incubated with HCT116 cells for 24 h, and the G₂/M population was monitored by determining the level of cyclin B1 and NPM phosphorylation. As expected, levels of NPM phosphorylation and cyclin B1 expression were dramatically increased both in the cytoplasmic insoluble fraction and nuclear fraction by p38 inhibitor treatment, which indicates a higher mitotic population by p38 inhibition (Fig. 8C, top and middle). To confirm increased Cdc2 activity by p38 inhibition, Cdc2 activity in the cytoplasmic insoluble fraction, which is equivalent to centrosomal Cdc2 activity, was measured by an in vitro kinase assay using histone H1 as substrate. The amount of phosphate incorporated into the histone was moderately increased in either SB203580- or PD169636-treated cytoplasmic insoluble fraction, whereas nuclear Cdc2 activity was clearly enhanced by the p38 inhibitor (Fig. 8D). Nocodazole-activating spindle checkpoint arrest stops mitotic exit leading to 80–90% accumulation in mitosis. Since Cdc2 activity by p38 inhibition, either by SB203580 or PD169636, was increased moderately both in the cytoplasmic insoluble (2-fold increase) and nuclear fraction (5–6-fold increase), unlike robust increase of Cdc2 activity by nocodazole treatment (~20-fold increase), it is clear that the moderate Cdc2 activity increase by p38 inhibition is caused by increased mitotic population rather than by mitotic

FIGURE 6. Cdc25B and p38 MAPK during mitosis. A, NIH 3T3 cells grown on the coverslips were immunostained with Cdc25B and phospho-p38 antibodies. The arrows and arrowheads indicate the centrosomal signal for Cdc25B and phospho-p38, respectively. B, HeLa cells grown on coverslips were immunostained with phospho-Cdc25B (serine 309-specific) or phospho-p38 antibodies. Mitotic cells were identified by chromosome condensation pattern after DAPI staining. Cells designated as P are in prophase, and interphase cells (I) are shown for comparison. The arrows (top) and arrowheads (bottom) indicate centrosomal signal of phospho-Cdc25B and phospho-p38, respectively.
On the basis of the results showing reduced phospho-Cdc25B immunoreactivity by p38 inhibition in immunostaining as well as immunoblotting followed by increased Cdc2 activity, we reasoned that disruption of inhibitory regulation of Cdc25B by p38 inhibition enhanced Cdc2 activity. To address this, we measured inhibitory phosphorylation of tyrosine 15 on Cdc2, which Cdc25B dephosphorylates, over time in the presence of SB203580. The level of phosphorylation of tyrosine 15 on Cdc2 appeared to be reduced gradually after a 4-h incubation of SB203580 and remarkably weakened after 24 h (Fig. 8E, top). Consistent with reduced inhibitory phosphorylation level of Cdc2 by SB203580 incubation, Cdc2 activity toward histone H1 was increased over time by in vitro kinase assay (Fig. 8E, bottom).

Taken together, these data strongly indicate that p38 inhibition promotes Cdc2 activity at the centrosome (cytoplasmic membrane fraction) promoting mitotic entry. This regulation of Cdc2 by p38 activity, in the absence of stress during mitosis, may be mediated by mitosis-specific Cdc25B-inhibitory phosphorylation.

**DISCUSSION**

Currently, most of the studies for p38 have been done under various exogenous stress conditions, such as UV radiation, osmotic shock, and inflammatory stresses. Under these stressors, p38 activation has been shown to play roles in cell cycle arrest, apoptosis, and inflammation induction. In contrast, under unstressed conditions, the role of p38 MAPK at the cellular level has been overlooked, except for a recent paper where SB203580 treatment induced genes involved in mitotic progression in cardiomyocytes, suggesting that p38 is an important negative regulator for cellular proliferation in this cell type (11). Similarly, Kramer et al. have shown that the G2 checkpoint protein Chk1 protein is active at the centrosome during interphase in the absence of stress and that chemical inhibition of Chk1 could induce premature Cdc2 (also called Cdk1) activation (17). These examples imply that some stress response proteins may serve as important cell-cycle regulators even under nonstress conditions.

Here, we demonstrate evidence that p38 is moderately activated at the centrosome in a cell cycle-dependent manner and may play an appreciable role in regulating mitotic initiation. The inhibition of activated p38 during mitosis appeared to promote Cdc2 activity, resulting in a higher mitotic population over a given time period (Fig. 5, A–C). Because we have found that during G2/M, p38 activity only increased 3–4-fold as compared with p38 activity in the G1/S phase of the cell cycle (Fig. 1B), it is also possible that the p38 protein may mediate a different signal pathway, depending on the signal intensity and duration. This notion has already been suggested and demonstrated in a variety of molecules, such as Raf (27) and ERK1/2 (28). Thus, a 3–4-fold moderate increase of p38 activity at the centrosome may be strong enough to incline the balance of Cdc25B activity toward inhibition in the presence of other Cdc25B-activating factors.
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kinases but not enough to induce G2/M arrest as shown before (7). Similarly, MLK3 (mixed lineage kinase 3), which has been known to mediate a proapoptotic signal once activated, was found to be moderately active at the centrosome during mitosis (29, 30) and to play a positive role in mitosis progression as well as proliferation (30, 31). Therefore, it is not surprising that p38 may have an intrinsic function in cell growth control in the nonstressed condition.

Recently, there has been emerging evidence that p38 MAPK acts as a tumor suppressor, indicating that the reduction of p38 kinase activity is procarcinogenic and that increased p38 activation protects cells from tumorigenesis (10). Additionally, p38 MAPK activates a G2/M checkpoint, phosphorylating Cdc25B phosphatase at serine 309 either directly (7) or indirectly (8) after UV radiation. Similarly, in fission yeast Srl1 kinase, which associates with the stress-activated p38, Styl MAPK reportedly regulates mitosis onset by negatively regulating Cdc25 (9).

The essential role of Cdc25B activity for mitotic initiation has been suggested and proven by evidence that overexpressed Cdc25B is more efficient than Cdc25C, which is often considered the main mitotic phosphatase among the three Cdc25 isoforms (Cdc25A, -B, and -C), in leading to premature mitosis (32). In support of this, the active phosphorylated form of Cdc25B was found at the centrosome during the G2 to M phase (32). It has been proposed that both positive and negative signaling for mitotic initiation exist at the centrosome. Activating phosphorylation of cyclin B1 accumulates at the centrosome in early prophase (34), and centrosomal Cdc2-cyclin B1 activation precedes nuclear mitotic events (35). As described earlier, centrosomal Chk1 controls Cdc2 activity under unstimulated conditions, and chemical inhibition of Chk1 activity resulted in accelerated mitotic onset as well as in premature Cdc2 activation and indicates an intrinsic role for Chk1 at the centrosome as a controller of mitotic onset in the unperturbed cell cycle (17).

In agreement, we have found that Cdc25B localizes to the centrosome during G2/M and is phosphorylated on serine 309, an inhibitory site for Cdc25B activity, which is known to involve p38 MAPK signaling (7). Since inhibitory phosphorylation of Cdc25B at the centrosome was found to be dependent on p38 activity early in mitosis, it is plausible that during mitotic initiation, p38 also contributes to the control of the onset of mitosis through negative regulation of Cdc25B at the centrosome. Therefore, p38 activity at the centrosome may prevent premature mitotic onset in a fashion similar to centrosomal Chk1 (17), and thus both control mechanisms converge by inhibiting Cdc25B signaling. Unlike Chk1, which is required for viability as discussed in Ref. 17, deletion of Cdc25B does not prevent viability in mice, so one needs to be cautious in comparing p38 to Chk1.

Checkpoint proteins, such as Chk1 or p38, delay G2/M transit after stress and thus prevent progression into mitosis with resultant mitotic catastrophe (17, 36). Blocking the moderate increase in p38 (or Chk1) activity accelerates progression to mitosis and may accelerate cell growth rate. However, although
progression to and through mitosis is moderately accelerated, we did not observe frequent aberrant premature mitoses; nor were they reported for Chk1 inhibition (17). Obviously, a greatly accelerated cell cycle progression with mitotic entry prior to completion of S phase would probably lead to aberrant mitoses, but this does not appear to be the case in the absence of stress. In summary, our data provide evidence that p38 localizes at the mitotic centrosome and controls Cdc25B activity negatively and that inhibition of mitotic p38 activity accelerates mitotic entry timing even under unstressed culture conditions.

Some questions still remain to be clarified. Although centrosomal phospho-p38 exists throughout cellular mitosis (Fig. 2), cells manage to initiate and complete mitosis in due time. How is Cdc25B activity regulated in the presence of inhibitory phosphorylation? According to previous studies, various positive regulators for Cdc25B during mitosis have been identified, including Aurora A (25), pEG3 (24), and Cdc2 (37). Consistently, we have observed that mitotic Cdc25B in the cytoplasmic membrane (including centrosomes) fraction displayed multiple gel shifts, indicating multiple phosphorylation states (data not shown). Therefore, the balance between activating phosphorylation and inhibitory phosphorylation may determine the activity of Cdc25B toward Cdc2-cyclin B1 at the critical point(s) during transit to and through mitosis. Additionally, MAPKAP kinase-2 was recently shown to phosphorylate Cdc25B after DNA damage at the same site on Cdc25B as p38 (8). Since MAPKAP kinase-2 is strongly associated with p38 in most cases, it is possible that Cdc25B phosphorylation by p38 may be through direct interaction of MAPKAP kinase-2 bound to p38. However, in our studies, we were unable to detect the active form of MAPKAP kinase-2 at the centrosome using phospho-MAPKAP kinase-2 antibody, although we observed slightly increased phospho-MAPKAP kinase-2 signals in the nucleus at early mitosis compared with interphase (data not shown). To our knowledge, there is no evidence to date for MAPKAP kinase-2 localization at the centrosome. It could be argued that trace amounts of MAPKAP kinase-2 bound to p38 under the detection range may be responsible for Cdc25B phosphorylation at the centrosome; alternatively, p38 may be able to directly phosphorylate Cdc25B at the centrosome due to a highly concentrated centrosomal environment that enables enhanced protein-protein interactions.

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