B-Raf Is Critical For MAPK Activation during Mitosis and Is Regulated in an M Phase-dependent Manner in Xenopus Egg Extracts*§

Received for publication, February 14, 2006, and in revised form, May 24, 2006. Published, JBC Papers in Press, June 8, 2006, DOI 10.1074/jbc.M601432200

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Activation of the MAPK cascade during mitosis is critical for spindle assembly and normal mitotic progression. The underlying regulatory mechanisms that control activation of the MEK/MAPK cascade during mitosis are poorly understood. Here we purified and characterized the MEK kinase activity present in Xenopus M phase-arrested egg extracts. Our results show that B-Raf was the critical MEK kinase required for M phase activation of the MAPK pathway. Consistent with this, B-Raf was activated and underwent hyperphosphorylation in an M phase-dependent manner. Interestingly B-Raf hyperphosphorylation at mitosis occurred, at least in part, as a consequence of a feedback loop involving MAPK-mediated phosphorylation within a conserved C-terminal SPKTP motif. The kinase activity of a B-Raf mutant defective at both phosphorylation sites was substantially greater than its wild type counterpart when incubated in Xenopus M phase egg extracts. Furthermore suppression of MAPK feedback at mitosis enhanced B-Raf activity, whereas constitutive activation of MAPK at mitosis strongly suppressed B-Raf activity. These results suggest that feedback phosphorylation by MAPK negatively regulates B-Raf activity at mitosis. Collectively our data demonstrate for the first time a role for B-Raf at mitosis and provide new insights into understanding the regulation and function of B-Raf during cell proliferation.

The MAPK pathway plays a key role in regulating cell proliferation. Activation of the Ras/Raf/MEK/ERK (MAPK) pathway by mitogen stimulation promotes entry into the cell cycle and G1/S cell cycle progression (for reviews, see Refs. 1 and 2). MAPK signaling is also important for the G2/M transition (3, 4) and normal M phase progression (5, 6). During mitosis, active phosphorylated forms of MEK1/2 and ERK1/2 localize to discrete structures of the mitotic spindle apparatus (7, 8). Previously we reported that MAPK activation during mitosis is absolutely required for the assembly and stability of the mitotic spindle in both Xenopus egg extracts and mammalian cells (9). Inhibition or depletion of MAPK from Xenopus egg extracts results in defective spindle structures and an alteration to microtubule dynamics (9). Therefore, regulation of cell proliferation by the MAPK pathway extends to the key events of M phase.

The underlying biochemical mechanisms that control transient activation of the MEK/MAPK cascade during mitosis are poorly understood. Raf-1 has been implicated at mitosis because it is activated in nocodazole-arrested fibroblast cells (10–12), and its expression is important for promoting the G2/M phase cell cycle transition (3). However, mitotic activated Raf-1 was shown to be uncoupled to MEK/ERK activation (10, 12–14), suggesting that perhaps another MEK kinase is required for activation of the MAPK pathway at mitosis. A study by Yue and Ferrell (15) detected small amounts of germ cell-specific MEK kinase, c-Mos, in Xenopus egg extracts and provided evidence that c-Mos was important for activating the MAPK pathway at mitosis. However, c-Mos protein is in general undetectable in most somatic tissues, and earlier studies show that c-Mos protein is efficiently degraded and undetectable after egg fertilization (16, 17). Thus, the MEK kinase responsible for activating MEK during mitosis remains uncertain.

In this study we purified a MEK kinase activity from Xenopus M phase-arrested egg extracts and identified B-Raf as its major component. We demonstrated for the first time that B-Raf is essential for activating the MAPK pathway at M phase. Further analysis showed that B-Raf becomes activated during mitosis and undergoes negative feedback regulation via phosphorylation at its C-terminal SPKTP motif by MAPK.

EXPERIMENTAL PROCEDURES

Preparation of Xenopus Egg Extracts—S phase extracts were prepared essentially as described previously (18) except that dejellied Xenopus eggs were parthenogenetically activated with the Ca2+ ionophore A23187 (0.2 μg/ml) for 2.5 min. After Ca2+ ionophore withdrawal, the activated eggs were incubated for
another 50–60 min at room temperature before processing into extracts. This method closely mimics the degradation of c-Mos that is observed following fertilization (see supplemental data S1). M phase-arrested extracts were prepared by supplementing crude S phase egg extracts with recombinant non-degradable sea urchin Δ90 cyclin B1 (75–100 nm final concentration) and incubating at room temperature for 60 min. Expression and purification of GST-Δ90 cyclin B was performed as described previously (9). Note that all preparations of M phase extracts were checked for the activation of MAPK and the absence of c-Mos protein by immunoblot analysis. Cycling extracts were prepared essentially as described previously (18) except that eggs were activated by incubation with calcium ionophore A23187 as described above.

**Immunoblot Analysis**—Primary antibodies used include the following. Mouse monoclonal anti-phospho (Thr-202/Tyr-204)-ERK (1:2000), rabbit polyclonal anti-phospho (Ser-217/Thr-221)-MEK (1:1000), and mouse monoclonal myc tag (1:1000) were purchased from Cell Signaling Technology, Inc.; rabbit polyclonal anti-phospho Ser-217/Ser-221 MEK (1:1000), and mouse monoclonal myc tag (1:2000), rabbit polyclonal anti-phospho (Ser-217/Ser-221)-MEK (1:1000), and mouse monoclonal myc tag (1:1000) were purchased from Cell Signaling Technology, Inc.; and rabbit polyclonal MEK peptide antiserum (1:1000) was prepared by Zymed Laboratories Inc. (South San Francisco, CA) against an N-terminal 16-amino acid sequence of Xenopus MEK1 plus a cysteine residue coupled to bovine serum albumin (CPKKKPTPIQLPNPPEG). Secondary antibodies included species-specific alkaline phosphatase-conjugated anti-mouse (Jackson Immunoresearch Laboratories) and anti-rabbit (Sigma) IgG that were detected with the CDP-Star chemiluminescence substrate (Roche Diagnostics).

**Purification of Mitotic MEK Kinase Activity from Xenopus Egg Extracts**—All purification steps were performed at 4 °C and assayed for MEK kinase activity. Mature Xenopus oocytes laid from 16 female frogs were processed into crude M phase-arrested extracts as described above. 24 ml of crude M phase-arrested extracts (~1.2 g of protein) were ultracentrifuged twice at 100,000 × g for 1.5 h to isolate the cytosolic fraction. MEK kinase activity was precipitated by 20% ammonium sulfate saturation. The pellet was dissolved in buffer A (50 mM HEPES, pH 7.5, 10 mM MgCl$_2$) supplemented with 25 mM NaF, 1 mM Na$_2$VO$_4$, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 10 μg/ml chymostatin and applied to a 10-ml HiTrap Q Sepharose high performance column (GE Healthcare). The proteins were eluted with a stepwise NaCl gradient in buffer A: 0–0.3 M (40 ml) and 0.3–1 M (10 ml). Fractions that contained MEK kinase activity (0.17–0.25 M NaCl) were pooled and applied to a Mono Q high resolution 5/5 column (GE Healthcare). The proteins were eluted with a stepwise NaCl gradient in buffer A: 0–0.35 M (3 ml), 0.35–0.50 M (15 ml), and 0.5–1 M (3 ml). Fractions containing MEK kinase activity (0.37–0.42 M NaCl) were collected and analyzed for protein composition by Western analysis and silver staining with GelCode SilverSNAP stain kit (Pierce).

**MEK Kinase Assays**—To measure MEK kinase activity, samples were incubated in 30 μl of kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl$_2$, 0.1 mM ATP, 25 mM NaF, 1 mM Na$_2$VO$_4$, 1 mM DTT) containing 0.5 μg of recombinant unactive GST-MEK1 protein (Upstate) for 20 min at 25 °C. The kinase reaction was stopped with SDS sample buffer, and reaction products were separated by SDS-PAGE. Phosphorylation of recombinant GST-MEK1 at serine residues 217 and 221 was analyzed by immunoblotting with phospho-Ser-217/Ser-221 MEK antibodies (Cell Signaling Technology, Inc.). Alternatively MEK kinase activity was measured in an in vitro linked kinase assay as described previously (19). Briefly, samples were incubated with 0.5 μg of recombinant unactive MEK1 and 2 μg of recombinant inactive ERK2 (Upstate) for 30 min in 30 μl of kinase buffer at 25 °C. Then the reaction was incubated an additional 10 min in the presence of 5 μCi of [γ-32P]ATP and 20 μg of myelin basic protein (MBP) as a substrate for ERK. The kinase reaction was stopped with SDS sample buffer, separated by 15% SDS-PAGE, and transferred to a poly(vinylidene difluoride) membrane. Radioactivity incorporated into MBP was detected by autoradiography and quantified by ImageQuant software.

**Immunodepletion and Immunoprecipitation**—To immunodeplete B-Raf from S phase extracts, 50 μl of extracts were incubated under gently rotation for 1–1.5 h at 4 °C with 15 μg of rabbit polyclonal B-Raf antibodies (sc9002) prebound to 5 μl of protein A-Sepharose 4B Fast Flow beads (Sigma). B-Raf immunocomplexes were pelleted by a quick centrifugation, and a second round of immunodepletion was performed. As a control, extracts were mock-depleted in parallel using an equivalent amount of rabbit IgG (Sigma). In a similar procedure, Raf-1 was immunodepleted from S phase extracts with 5 μg of rabbit polyclonal Raf-1 antibodies (Santa Cruz Biotechnology, Inc.). B-Raf immunoprecipitations (IPs) were performed by adding 0.5–2 μg of rabbit polyclonal B-Raf antibodies (Santa Cruz Biotechnology, Inc.) to 1–10 μl of Xenopus egg extracts diluted in 10–50 μl of IP buffer (50 mM HEPES, pH 7.5, 10 mM MgCl$_2$, 25 mM NaF, 1 mM Na$_2$VO$_4$). Following a 1.5-h incubation on ice, B-Raf immunocomplexes were recovered on protein A-agarose beads (Sigma), washed twice with IP buffer containing 0.1% Triton X-100, and washed three times with IP buffer alone. Washed B-Raf immune complexes were used directly in an in vitro linked kinase assay or resuspended in SDS sample buffer and analyzed by immunoblotting.

**Phosphatase Treatment**—B-Raf IPs from S and M phase-arrested extracts were incubated with 50 units of recombinant A protein phosphatase (Upstate) in 50 μl of phosphatase buffer (50 mM HEPES, pH 7.5, 0.1% bovine serum albumin, 100 μM EDTA, 2 mM MnCl$_2$, 5 mM DTT) for 30 min at 37 °C. To stop reactions, precipitates were washed with a copious amount of buffer containing phosphatase inhibitors (50 mM HEPES, pH 7.5, 10 mM MgCl$_2$, 25 mM NaF, 1 mM Na$_2$VO$_4$).

**In Vitro ERK2 Kinase Assay**—B-Raf IPs were incubated with 20 units of recombinant active ERK2 (New England Biolabs) in 30 μl of kinase buffer (50 mM Tris-HCl, 10 mM MgCl$_2$, 1 mM EGTA, 2 mM DTT, 0.1 mM ATP, 0.01% Brij 35, pH 7.5) with or without radioactive [γ-32P]ATP (5 μCi/reaction) for 30 min at 30 °C. Reactions were stopped by the addition of SDS sample buffer and heating at 95 °C for 5 min.

**In Vitro H1 Histone Kinase Assay**—To measure Cdk1 activity, 1-μl aliquots of Xenopus egg extracts were incubated in 30 μl of kinase buffer (50 mM Tris-HCl, 10 mM MgCl$_2$, 1 mM EGTA, 2 mM DTT, 0.1 mM ATP, 10 μM cAMP-dependent pro-
tein kinase inhibitor, 0.01% Brij 35, pH 7.5) containing 20 μg of H1 protein (Upstate) and 2 μCi of [γ-32P]ATP for 20 min at 30 °C. Reactions were stopped by the addition of SDS sample buffer and heating at 95 °C for 5 min. Reaction products were separated by 10% SDS-PAGE, and levels of histone H1 phosphorylation were determined by autoradiography.

**Generation of Wild Type (WT) and C-terminal APKAP Mutant Xenopus B-Raf Constructs**—A Xenopus B-Raf cDNA clone was obtained from ATCC (Image Clone I.D. 6860469). DNA sequencing analysis confirmed a full-length Xenopus cDNA containing an open reading frame of 803 amino acids (see supplemental Fig. S2). The sequence data of Xenopus B-Raf have been submitted to GenBankTM under accession number DQ097958. The B-Raf coding region was excised at HindIII sites and subcloned into a modified pGEM transcription vector (kindly provided by Dr. Rey-Huei Chen from the University of Cornell) downstream of an N-terminal myc tag. The C-terminal APKAP mutant was generated by changing both serine 784 and threonine 787 to alanines in the conserved C-terminal MAPK phosphorylation site using a QuikChange site-directed mutagenesis kit (Stratagene).

**Expression of WT and C-terminal APKAP Mutant myc-B-Raf Proteins**—Expression of WT and C-terminal APKAP mutant B-Raf proteins was performed similarly to that described previously (20). Briefly wild type and mutant myc-B-raf transcripts were generated using the mMESSAGE mMACHINE T7 transcription kit (Ambion). 4 μg of purified mRNA transcripts were introduced into 21 μl of Xenopus CSF-arrested egg extracts that were supplemented with 2.5 μl of rabbit reticulocyte lysate (Ambion). Protein expression was carried out for 5 h with gentle mixing every 15 min at 23 °C. Expression of recombinant proteins was confirmed by Western blotting with myc epitope antibodies. To reproduce the S phase phosphorylation status of the recombinant B-Raf proteins, aliquots of translated WT or mutant myc-B-Raf proteins were introduced into S phase-arrested extracts in a 1:10 ratio and incubated for 30 min at room temperature. To induce M phase hyperphosphorylation of the recombinant proteins, aliquots of S phase extracts containing myc-B-Raf protein were driven into M phase by adding recombinant non-degradable cyclin B. Western blot analysis revealed that recombinant myc-B-Raf proteins undergo dephosphorylation during incubation in S phase extracts and acquire hyperphosphorylation during incubation in M phase extracts.

**Purification of Recombinant myc-B-Raf Proteins from Xenopus Egg Extracts**—myc-B-Raf recombinant proteins were purified by immunoprecipitation using myc tag antibodies. Aliquots of egg extracts containing myc-B-Raf were diluted 1:10 in EB buffer (80 mM β-glycerol phosphate, pH 7.3, 20 mM EGTA, 15 mM MgCl$_2$, 25 mM NaF, 1 mM Na$_2$VO$_4$, 0.1% Triton X-100) and incubated on ice with myc tag monoclonal antibodies (Cell Signaling Technology, Inc.) for 1 h. Protein A-Sepharose beads were added, and incubation was continued for 12–16 h with gentle inversion at 4 °C. The IP complexes were washed twice with EB buffer and twice with kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 1 mM EGTA, 2 mM DTT, 0.01% Brij 35 or Brij 58) before applying to in vitro kinase assays.

**RESULTS**

**Purification of B-Raf as an M Phase MEK Kinase from Xenopus Egg Extracts**—Xenopus egg extracts represent an ideal model system for purifying and identifying the MEK kinase responsible for mitotic activation of the MEK/MAPK cascade. First, Xenopus eggs provide a rich source of components of the MAPK cascade. Second, activation of the MAPK cascade is restricted to mitosis in Xenopus egg extracts that undergo synchronous cell cycles of S and M phases (5, 21, 22). Finally the Xenopus egg extract system is amenable to biochemical manipulations not feasible with tissue culture cells.

We used an in vitro kinase assay to detect MEK kinase activity in Xenopus egg extracts. Phosphorylation of recombinant GST-MEK at Ser-217/Ser-221 was detected strongly in M phase egg extracts compared with S phase egg extracts (Fig. 1A). Several MEK kinases have been shown to directly activate MEK1/2 including Raf family members (23), mixed lineage kinase 3 (24), MEKKs (MAPK/extracellular signal-regulated kinase kinases) 1–3, c-Mos, and Tpl-2 (25). To ascertain the MEK kinase associated with the kinase activity in Xenopus M phase egg extracts, we developed a four-step purification scheme (Fig. 1B). Crude S phase extracts prepared from parthenogenetically activated Xenopus eggs were cycled into a stable M phase by the addition of non-degradable Δ90 cyclin B. By Western analysis we showed that the extracts were devoid of c-Mos (see “Experimental Procedures”). The crude M phase extracts were separated into cytosolic and membrane fractions by ultracentrifugation. The cytosolic fraction, containing nearly all of the MEK kinase activity (Table 1), was separated by ammonium sulfate precipitation. Approximately 90% of the MEK kinase activity was precipitated in the 0–20% ammonium sulfate cut and used for further purification by anion exchange chromatography on HiTrap Q Sepharose and Mono Q columns. Collectively these purification steps enriched MEK kinase activity by ~260-fold (Table 1). At the final purification step, a single protein peak eluted from the Mono Q column (Fig. 1C) that contained MEK kinase activity as assessed by both phosphorylation of recombinant MEK (Fig. 1D) and activation of the MAPK cascade in an in vitro linked kinase assay (data not shown). Therefore, our results suggest that the kinase activity purified from M phase-arrested Xenopus egg extracts represents a bona fide MEK kinase.

Silver staining analysis of the final MEK kinase active fractions revealed several protein bands. An example of one of the active fractions is shown (Fig. 1E, lane 1). A prominent protein band between 90 and 100 kDa correlated with M phase MEK kinase activity and migrated at a molecular mass range similar to that of B-Raf. Therefore, we used a polyclonal antibody raised against a highly conserved N-terminal domain of human B-Raf (supplemental Fig. S2) that was able to detect Xenopus B-Raf in crude S and M phase egg extracts as a 95-kDa doublet (Fig. 1E, lanes 5 and 6). Indeed when a purified fraction containing mitotic MEK kinase activity was immunoblotted with the B-Raf antibody, B-Raf was strongly detected (Fig. 1E, lane 2 versus lane 6). In contrast, neither c-Mos nor Raf-1 were detected in the MEK kinase active fraction (lanes 3 and 4). Similar results were observed for other fractions containing par-
FIGURE 1. Purification of B-Raf as an M phase MEK kinase from Xenopus egg extracts. A, M phase Xenopus egg extracts contain MEK kinase activity and active MAPK. Equal amounts of S and M phase Xenopus egg extracts were subjected to an in vitro MEK kinase assay with recombinant GST-MEK as a substrate. Phospho-MEK antibodies were used to analyze phosphorylation of GST-MEK. Activation of endogenous MAPK was analyzed by phospho-MAPK Western blotting. Equal sample loading was confirmed by immunoblotting for MAPK protein (data not shown). B, scheme for purification of an M phase MEK kinase activity. C, Mono Q elution profile over a three-step 0–1.0 M NaCl gradient. D, Mono Q fractions 10–16 contain an M phase MEK kinase activity. MEK kinase activity of Mono Q fractions 4–60 was measured in an in vitro MEK kinase assay. MEK kinase activity was not detected in the flow-through volume. E, B-Raf is enriched in the final purification step of MEK kinase activity. Mono Q fraction 12 was separated by 8% SDS-PAGE and analyzed by either silver staining (lane 1) or Western blotting with anti-B-Raf (lane 2), anti-Raf-1 (lane 3), or anti-c-Mos (lane 4) antibodies. Similar results were obtained with fraction 11. Equal amounts of total protein from crude S phase (lane 5), M phase (lanes 6—9), and CSF extracts (lane 9) were immunoblotted for B-Raf (lanes 5 and 6), Raf-1 (lane 7), and c-Mos (lanes 8 and 9). Note that the asterisk indicates recombinant GST-cyclin B recognized by Santa Cruz Biotechnology B-Raf polyclonal antibodies raised against a GST-conjugated B-Raf peptide. mAUI, milliarbitrary units; MEK-P, phospho-MEK.
MAPK Activation by B-Raf at Mitosis

TABLE 1
Purification of MEK kinase activity

| Total protein | Total activitya | Specific activity | Yield | -Fold purification |
|---------------|-----------------|-------------------|-------|-------------------|
| mg            | a.u.            | a.u./mg           |       |                   |
| Crude M phase extract | 1156.8 | 246.2 | 0.21 | 1 |
| Cytoxol fraction | 302.0 | 260.0 | 0.86 | 100 |
| 20% ASa cut | 28.0 | 236.8 | 8.46 | 100.1 |
| HiTrap Q Sepharose | 3.8 | 105.75 | 27.83 | 132.5 |
| Mono Q | 0.405 | 22.08 | 54.44 | 259.2 |
| a.a., arbitrary units. Fractions from each purification step were subjected to an in vitro MEK kinase assay; the levels of GST-MEK phosphorylation were determined by Western blotting and quantified by using ImageQuant software.
| Ammonium sulfate. |

![FIGURE 2. B-Raf, but not Raf-1, is required for activation of the MAPK cascade at mitosis in Xenopus egg extracts. A, B-Raf protein levels in S phase extracts after mock and B-Raf depletions and adding back recombinant His-tagged B-Raf. B, MAPK activation at mitosis is blocked in B-Raf-depleted extracts and restored after addition of recombinant B-Raf. Mock- and B-Raf-depleted extracts were driven into mitosis with non-degradable cyclin B and assessed for MAPK activation by phospho-MAPK Western blotting at the indicated times. Results are representative of six independent experiments. C, Raf-1 protein levels in S phase extracts after mock and Raf-1 immunodepletion. D, Raf-1 is not required for activation of MAPK during mitosis. Mock- and Raf-1-depleted extracts were driven into mitosis with non-degradable cyclin B. Aliquots of the egg extract were collected at the indicated times to monitor MAPK activation by phospho-MAPK immunoblotting. Results are representative of four independent experiments. Equal loading of samples (A–D) was confirmed by Ponceau S staining. depl, depleted; MAPK-P, phospho-MAPK.](image)

![tially purified MEK kinase activity. Thus, we conclude that B-Raf, not Raf-1 or c-Mos, is enriched during purification of the mitotic MEK kinase activity.

B-Raf, but Not Raf-1, Is Required for Activation of the MAPK Cascade at Mitosis in Xenopus Egg Extracts—Based on the purification results, we postulated that B-Raf might be required for activation of the MEK/MAPK pathway at mitosis. To test this directly, endogenous B-Raf was quantitatively removed (~99%) from S phase extracts by two rounds of immunodepletion (Fig. 2A), and the depleted extracts were cycled into mitosis by the addition of recombinant non-degradable cyclin B. At the indicated times, aliquots of extract were collected to assess MAPK activation. Entry into mitosis was monitored by nuclear envelope breakdown and chromatin condensation, which in control and B-Raf-depleted extracts occurred at 20 min after cyclin B addition. Following mitotic entry, MAPK became activated at 30 min in control extracts (Fig. 2B). In contrast, mitotic MAPK activation was strongly inhibited in B-Raf-depleted extracts (Fig. 2B). The addition of recombinant B-Raf protein to B-Raf-depleted extracts was sufficient to restore MAPK activation at levels similar to that in control extracts (Fig. 2B). Thus, we conclude that B-Raf is essential for activation of the MAPK pathway during mitosis in Xenopus egg extracts.

Studies in mammalian cells have implicated a possible role for Raf-1 in G2/M progression (3, 11, 14). However, depletion of endogenous Raf-1 protein had no effect on mitotic activation of MAPK in Xenopus egg extracts (Fig. 2, C and D). Taken together, our data show that B-Raf, but not Raf-1, is required for mitotic activation of the MAPK cascade in Xenopus egg extracts.

B-Raf Activity Is Cell Cycle-regulated in Xenopus Egg Extracts—Next we characterized B-Raf activity in Xenopus egg extracts. We predicted that B-Raf activity would be highest in M phase egg extracts based on its requirement for activating the MAPK cascade during mitosis. To measure B-Raf activity, equivalent amounts of B-Raf were immunoprecipitated from S and M phase-arrested egg extracts and subjected to an in vitro linked kinase assay. Our data show that B-Raf activity was markedly elevated (4–6-fold) in M phase-arrested extracts compared with S phase extracts (Fig. 3A). A modest amount of B-Raf activity detected in S phase extracts likely represents its high basal activity due to both constitutive phosphorylation and the presence of a phospho-mimicking aspartic acid residue in the positive regulatory N-region (26). Controls that either lack recombinant MEK and ERK or measure background kinase activity associated with rabbit IgG complexes demonstrated the specificity of the in vitro cascade reaction. To rule out the possibility that an MEK kinase associated to B-Raf might contribute to the activation of MAPK in the linked kinase assay reaction, we immunoprecipitated a catalytically inactive B-Raf mutant from M phase egg extracts. Our results show that the catalytically inactive B-Raf mutant contained no MEK kinase activity in a linked kinase assay compared with wild type B-Raf (supplemental Fig. S3) demonstrating that the activation of the MAPK cascade in the in vitro linked assay reflects B-Raf activity. Finally, we examined B-Raf activity in Xenopus cycling egg extracts that naturally oscillate between S and M phases. Our
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In a recent report, high amounts of B-Raf activity were detected in Xenopus egg extracts, but the authors were unable to measure differences between S and M phase egg extracts (27). We speculate that the negative data could be caused by functional limitations of the two-component in vitro MEK kinase assay used to measure B-Raf activity. In our studies, we utilized an in vitro B-Raf/MEK/ERK/MBP-linked kinase assay, which allows greater signal amplification, and used at least 5-fold more MEK. Thus, saturation of the reaction by purified active B-Raf complexes is less likely to occur under our assay conditions. Therefore, we conclude that our cascade kinase reaction is more sensitive and reliable for detecting changes in B-Raf activity.

Next we examined whether M phase activation of B-Raf correlated with its ability to associate with MEK, the direct target of B-Raf. B-Raf or MEK complexes were immunoprecipitated from S and M phase Xenopus egg extracts and subjected to Western analysis for detection of MEK or B-Raf, respectively. As shown in Fig. 3C, MEK and B-Raf were found in a complex together in both S and M phase egg extracts suggesting that their association with each other is independent of B-Raf activation at mitosis.

M Phase Hypershift of B-Raf Is Due to Phosphorylation—As revealed by Western blotting, B-Raf, particularly the upper band of the doublet, undergoes a prominent electrophoretic shift at mitosis in M phase-arrested egg extracts (Fig. 4A and 4B, 60–70 and 140 min). Therefore, we tested whether the electrophoretic shift was due to phosphorylation of B-Raf. B-Raf IP complexes isolated from S and M phase egg extracts were treated with λ protein phosphatase, separated by SDS-PAGE, and subjected to immunoblot analysis with anti-B-Raf antibodies. The results show that phosphorylation treatment eliminated the electrophoretic mobility shift of B-Raf isolated from M phase extracts (Fig. 4C) demonstrating that it stemmed from phosphorylation. B-Raf isolated from S phase egg extracts is also sensitive to phosphorylation treatment because it is constitutively phosphorylated in the positive regulatory N-region (22). Therefore, we conclude that Xenopus B-Raf is con-
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If MAPK directly phosphorylates B-Raf at mitosis, then it might be possible to detect the association between these two proteins. To test this possibility, equivalent amounts of MAPK were immunoprecipitated from S and M phase egg extracts and subjected to Western analysis with B-Raf antibodies. B-Raf was mainly detected in the MAPK complexes isolated from Xenopus M phase egg extracts compared with S phase egg extracts and was not detected in mock IPs using rabbit IgG (Fig. 5C). Thus, MAPK associates in a complex with B-Raf in M phase egg extracts.

The association of MAPK with B-Raf suggests that it might contribute directly to B-Raf hyperphosphorylation. Therefore, we performed an in vitro kinase assay using endogenous Xenopus B-Raf isolated from S phase extracts as a substrate in the presence of both active ERK2 and radiolabeled ATP. The results show that active ERK2 readily phosphorylated B-Raf in vitro (Fig. 5D). Moreover, phosphorylation of B-Raf by ERK2 caused a reduction in the electrophoretic mobility of B-Raf (Fig. 5E, lanes 1 and 5). Therefore, we conclude that MAPK contributes to the mitotic shift of B-Raf by direct phosphorylation.

MAPK Phosphorylates B-Raf at the C-terminal SPKTP Motif—Human B-Raf is phosphorylated directly by ERK2 at both MAPK phosphorylation sites (S/T)P sites within the C-terminal SPKTP motif (28). However, the significance of these phosphorylations on B-Raf activity has not been described. Because the SPKTP motif is conserved between human and Xenopus B-Raf (Fig. 6A), we introduced alanine residues at the two phosphoacceptor sites (serine and threonine) of Xenopus B-Raf to generate a phosphorylation-defective mutant (APKAP). mRNAs of WT and mutant myc-tagged B-Raf were translated in CSF-arrested extracts (see “Experimental Procedures” for details). Then the recombinant myc-tagged B-Raf proteins were reisolated by immunoprecipitation with myc tag antibodies and used as a substrate for in vitro kinase assays in the presence of recombinant active ERK2 and [γ-32P]ATP. As shown in Fig. 6B, radiolabeling of the Xenopus myc-tagged B-Raf mutant in the presence of active ERK2 was strongly suppressed compared with wild type myc-B-Raf suggesting that these conserved sites are phosphorylated by ERK2. Furthermore, the electrophoretic hyperphosphorylation at mitosis was markedly reduced in the B-Raf (APKAP) mutant compared with wild type B-Raf (Fig. 6C, lanes 3 and 4).
suggested that these sites are indeed phosphorylated in *Xenopus* M phase egg extracts. Interestingly the B-Raf (APKAP) mutant ran lower after treating M phase egg extracts with the MEK inhibitor U0126. This indicates that perhaps another site(s) of B-Raf may be phosphorylated by MAPK feedback during mitosis.

Currently it is unknown whether feedback phosphorylation of the conserved C-terminal SPKTP motif of B-Raf plays a role in regulating its kinase activity. Therefore, we analyzed the kinase activity of the myc-tagged B-Raf (APKAP) mutant in *Xenopus* egg extracts. To do this, myc-tagged B-Raf wild type and (APKAP) mutant proteins translated in CSF-arrested egg extracts were diluted in S phase and then cycled into a stable M phase by the addition of non-degradable cyclin B. myc-tagged B-Raf proteins in S or M phase egg extracts were reisolated by means of myc tag antibodies and subjected to an *in vitro* linked kinase assay. As expected, wild type B-Raf kinase activity was low in S phase extracts and increased 2–4-fold more in M phase egg extracts (Fig. 7A, lanes 1 and 3). Interestingly the B-Raf (APKAP) mutant protein was strongly activated at mitosis such that its kinase activity was ~4-fold higher than that of wild type B-Raf (Fig. 7A). These results suggest that phosphorylation of the C-terminal SPKTP motif renders a negative effect on B-Raf activity. The B-Raf (APKAP) mutant showed higher basal activity in S phase extracts compared with wild type B-Raf possibly because of it being more resistant to inactivation due to the absence of the MAPK phosphorylation sites. To examine this feedback further, we measured B-Raf activity in M phase egg extracts treated with the MEK specific inhibitor U0126. Our results showed that B-Raf became more active as MAPK feedback was reduced compared with non-treated extracts (Fig. 7B). Likewise we tested whether B-Raf activity would decrease under conditions of constant MAPK feedback. The addition of a constitutively active MEK mutant (29) to M phase egg extracts produced higher levels of MAPK activity that resulted in a strong inhibition of B-Raf (Fig. 7C). Together these results implicate MAPK in a feedback loop that negatively regulates B-Raf activity, at least in part, through phosphorylation of its C-terminal SPKTP motif.

**DISCUSSION**

In this study we describe for the first time an M phase role for B-Raf in promoting the activation of the MAPK pathway. Consistent with this role, we show that B-Raf is activated in an M phase-dependent manner in *Xenopus* cycling egg extracts. Furthermore we provide evidence that B-Raf undergoes negative regulation by a feedback loop involving inhibitory phosphorylation of a conserved C-terminal SPKTP motif by MAPK. Thus, we propose that B-Raf is a critical regulator of the MAPK pathway at mitosis.

*B-Raf, but Not Raf-1, Regulates MAPK Activation at Mitosis in Xenopus Egg Extracts*—The primary aim of this study was to identify the MEK kinase responsible for mitotic activation of the MEK/MAPK pathway in *Xenopus* egg extracts. Fractionation of *Xenopus* M phase-arrested egg extracts led to the purification of a single peak of MEK kinase activity (Fig. 1, C and D) that was identified by Western analysis as B-Raf (Fig. 1E). Neither c-Mos nor Raf-1 was detected in the purified MEK kinase activity. The results from our immunodepletion experiments definitively show that B-Raf is critical for activating the MAPK pathway during mitosis in *Xenopus* egg extracts (Fig. 2B). In contrast, the related Raf family member Raf-1 was not required for M phase activation of the MAPK pathway (Fig. 2D). In addition, we did not detect any evidence for Raf-1 forming heterodimers with B-Raf in *Xenopus* mitotic egg extracts, suggesting that the role of Raf-1 is not to promote B-Raf activation at mitosis.

Our results implicating B-Raf at mitosis conflict with a previous study suggesting that c-Mos might regulate M phase activation of the MAPK cascade in *Xenopus* egg extracts (15). We speculate that the main reason for these conflicting results stem from differences in how the egg extracts were prepared. In contrast to study by Yue and Ferrell (15), we did not detect any c-Mos protein in our egg extract preparations (see “Experimental Procedures”). Moreover we did not detect any MEK kinase activity associated with anti-Mos antibody complexes isolated from *Xenopus* M phase egg extracts (see supplemental Fig. S1, panel B). Therefore, we were unable to assess any biological significance for c-Mos in our egg extract system. We suggest.

3 S. I. Borysov and T. M. Guadagno, unpublished observations.
that the absence of c-Mos in our egg extracts closely mimics its disappearance shortly following fertilization that was demonstrated in earlier studies (16, 17) and in this study (supplemental Fig. S1, panel A). Furthermore the proposal that c-Mos could trigger the transient activation of the MAPK pathway at mitosis does not comply with its well established role as a cytostatic factor during oocyte maturation or from studies showing that microinjection of even small amounts of c-Mos can mediate a metaphase arrest in cleaving *Xenopus* embryos (30). Finally gene knock-out studies in mice argue against an essential role for c-Mos at mitosis in somatic tissues because mos−/− mice are viable without any tissue abnormalities (31, 32). On the other hand, homozygous knock-outs for B-Raf are embryonic lethal (33, 34). Thus, we propose that B-Raf rather than c-Mos plays an essential role during mitosis in transiently activating the MAPK cascade.

**B-Raf Is Activated at M Phase in Xenopus Egg Extracts**—In the absence of growth controls, transient activation of the MAPK cascade is restricted to M phase in *Xenopus* egg extracts (5, 9, 22). Likewise B-Raf activity showed a similar M phase-dependent activation pattern in *Xenopus* cycling egg extracts (Fig. 3B) indicating that the B-Raf/MEK/MAPK cascade is coupled to M phase. This begs the question of how B-Raf becomes activated at M phase? Although beyond the scope of this study, we speculate that the mechanism is distinct from mitogen/Ras-mediated mechanisms of B-Raf activation based on the observation that active B-Raf is cytosolic in *Xenopus* M phase egg extracts (Table 1). Interestingly mitotic Raf-1 in fibroblast cells was shown to be activated independently of Ras and plasma membranes (12, 13). Because B-Raf activation is coupled to M phase in *Xenopus* egg extracts, we speculate that Cdk1/cyclin B might be directly or indirectly involved in its activation. In support of this possibility, the addition of non-degradable cyclin B to S phase extracts was sufficient to trigger M phase activation of the B-Raf/MEK/ERK cascade (Figs. 2B and 3A) and Cdk1 activation precedes B-Raf activation at mitosis in cycling egg extracts (Fig. 3B). In addition, other B-Raf regulators such as Rap1 (for a review, see Ref. 1), 14-3-3 proteins (35–37), or scaffolding proteins may also contribute to regulating B-Raf activation at mitosis. Studies are in progress to identify novel protein interactions with B-Raf as well as other phosphorylation events that would positively regulate B-Raf activation at mitosis.

**B-Raf Undergoes Feedback Regulation at Mitosis**—A previous report studying activation of B lymphocytes identified novel ERK-mediated feedback phosphorylation sites at the C terminus of human B-Raf (28) but did not assess the role of these phosphorylations on B-Raf activity. Similarly we show in this study that *Xenopus* B-Raf was phosphorylated at the same conserved C-terminal SPKTP motif by ERK (Fig. 6). Importantly our data suggest that ERK feedback at the C-terminal SPKTP motif plays a role in negatively regulating B-Raf kinase activity at mitosis (Fig. 7). By blocking ERK feedback, the inhibition of B-Raf activity was suppressed (Fig. 7B), and likewise, constant stimulation of ERK feedback further enhanced B-Raf inhibition (Fig. 7C). Because feedback loops play important roles in the regulation of signaling pathways, negative feedback by ERK could be a mechanism to turn down B-Raf activity and to assure transient activation of the MAPK cascade during

FIGURE 7. ERK feedback negatively regulates B-Raf activity in Xenopus M phase egg extracts. A, mutation of the C-terminal MAPK phosphorylation sites in B-Raf elevates an activated kinase activity.*Xenopus* WT (SPKTP) or mutant (APKAP) full-length myc-B-Raf proteins were incubated either in S or M phase extracts, immunopurified by myc epitope antibodies, and subjected to an in vitro linked kinase assay. B, blocking MAPK feedback leads to increased B-Raf activity in M phase extracts. S phase-arrested extracts were driven into M phase with recombinant non-degradable cyclin B in the presence or absence of 50 μM U0126. C, constant MAPK activation leads to a reduction in B-Raf activity at mitosis. M phase egg extracts were treated with a constitutively active (CA) His-tagged MEK mutant (MEK R4F) for 1 h at room temperature to induce constant ERK feedback signaling. To measure endogenous B-Raf activity, B-Raf was immunopurified with B-Raf antibodies and subjected to in vitro linked kinase assay. Quantitation of B-Raf activity was performed by obtaining scintillation counts of excised radiolabeled MBP bands and graphing the counts in arbitrary units (a.u.) relative to immunoprecipitated B-Raf. Levels of phospho-MAPK or myc-B-Raf per reaction were estimated by Western blotting. Results are representative of three independent experiments. Note that addition of U0126 or constitutively active MEK did not affect Cdk1/cyclin B activity (not shown). MT, mutant; MAPK-P, phospho-MAPK.
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In summary, we show that B-Raf is an essential activator of the MAPK pathway at mitosis in Xenopus egg extracts. Our novel findings provide new insight into the central role of B-Raf in promoting cell proliferation.

**Acknowledgments**—We thank the Chellappan laboratory (Moffitt Cancer Center) for Raf antibodies; Dr. Rey-Huei Chen (University of Cornell) for pGEM transcription vector; Chris Marohonic (University of South Florida) for valuable assistance in using the fast protein liquid chromatography system; Omar Hammad for contributions to characterizing the Xenopus B-Raf clone; members of the Guadagno laboratory for contributions; and K. Chellappan, J. Wu, and S. Guadagno for comments on the manuscript.

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