

ORIGINAL ARTICLE

B-Raf and C-Raf are required for Ras-stimulated p42 MAP kinase activation in *Xenopus* egg extractsJ Yue, W Xiong¹ and JE Ferrell Jr

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During mitosis, a select pool of MEK1 and p42/p44 MAPK becomes activated at the kinetochores and spindle poles, without substantial activation of the bulk of the cytoplasmic p42/p44 MAPK. Recently, we set out to identify the MAP kinase kinase kinase (MAPKKK) responsible for this mitotic activation, using cyclin-treated *Xenopus* egg extracts as a model system, and presented evidence that Mos was the relevant MAPKKK. However, a second MAPKKK distinct from Mos was readily detectable as well. Here, we partially purify this second MAPKKK and identify it as B-Raf. No changes in the activity of B-Raf were detectable during progesterone-induced oocyte maturation, after egg fertilization, or during the early embryonic cell cycle, arguing against a role for B-Raf in the mitotic activation of MEK1 and p42 MAPK. Ras proteins can bring about activation of MEK1 and p42 MAPK in extracts, and Ras may contribute to signaling from the classical progesterone receptor during oocyte maturation and from receptor tyrosine kinases during early embryogenesis. We found that both B-Raf and C-Raf, but not Mos, are required for Ras-induced MEK1 and p42 MAPK activation. These data indicate that two upstream stimuli, active Ras and active Cdc2, utilize different MAPKKKs to activate MEK1 and p42 MAPK.

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Introduction

ERK1 and ERK2 (extracellular signal-regulated kinases 1 and 2) are probably the best studied and best understood of the mitogen-activated protein kinases (MAPKs). ERK1 and ERK2 respond to diverse stimuli, and play important roles in cell proliferation, differ-

entiation, and survival (Ferrell, 1996a; Robinson and Cobb, 1997; Lewis *et al.*, 1998; Ferrell, 1999b; Widmann *et al.*, 1999). Their immediate upstream activators are the kinases MEK1 and MEK2 (for MAPK kinase or ERK kinase), which phosphorylate ERK1 and ERK2 at a threonine and a tyrosine residue in the kinase domain activation loop. MEK1 and MEK2, in turn, are activated by phosphorylation of one or two serine sites in their activation loops (Alessi *et al.*, 1994). Three families of MEK1/2-activating MAP kinase kinase kinases (MAPKKKs) have now been identified: the Raf family, consisting of the A-Raf, B-Raf, and Raf-1 or C-Raf proteins; the Ste11/Byr1-related MEKK family, consisting of MEKK1, MEKK2, MEKK3, and MEKK4; and the Mos oncoprotein. The diversity of the MAPKKKs may allow MEK1/2 and ERK1/2 proteins to plug into different upstream receptors and regulators.

The serine/threonine protein kinases of the Raf family have long been implicated in relaying mitogenic signals to MAPKs (Morrison and Cutler, 1997; Kolch, 2000; Murakami and Morrison, 2001; Chong *et al.*, 2003). For example, the gene for the best studied Raf protein, C-Raf, was initially identified as the cellular homolog of the retroviral oncogenes, v-raf and v-mil, underscoring the potential of C-Raf to be a mitogenic regulator (Jansen *et al.*, 1984). More recently, B-Raf has been shown to be mutated in more than 60% of melanomas and a substantial percentage of many other human cancers (Davies *et al.*, 2002; Pollock and Meltzer, 2002; Rajagopalan *et al.*, 2002; Chong *et al.*, 2003; Pollock *et al.*, 2003; Wan *et al.*, 2004), suggesting that this Raf family member is of particular importance in human disease. All three of the Raf proteins can be activated by Ras proteins, and all can phosphorylate and activate MEK1; C-Raf can activate MEK2 as well (Pritchard *et al.*, 1995; Wu *et al.*, 1996; Marais *et al.*, 1997). The three Raf proteins are expressed in overlapping but distinct patterns, with A-Raf exhibiting the most restricted distribution. Likewise, studies of knockout mice suggest that there are both overlapping and isoform-specific functions of the three Raf proteins, with the B-Raf and C-Raf single knockouts producing embryonic lethality (showing that the Raf proteins are probably not completely redundant functionally), but with cells from the single knockouts showing relatively normal p42 MAPK responses (suggesting some redundancy between

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the Rafs or between Rafs and other MAPKKKs) (Murakami and Morrison, 2001).

We have been studying the regulation of the MEK1 and ERK2 (or p42 MAPK) protein kinases in *Xenopus* oocytes, eggs, and egg extracts. The best characterized initiator of oocyte maturation is progesterone, and it brings about the activation of MEK1 and p42 MAPK through the intermediacy of the Mos MAPKKK (Ferrell, 1999b; Nebreda and Ferby, 2000; Maller, 2003). There may be other routes from progesterone to p42 MAPK activation as well; for example, progesterone binding to the classical progesterone receptor can relay nontranscriptional signals via the Src protein kinases, which may then couple to MEK1 and p42 MAPK via C-Raf (Boonyaratanakornkit *et al.*, 2001). In addition, recent work has shown that in mouse ovarian follicles, gonadotropin signaling results in the production of a succession of EGF receptor ligands, which may also couple to MEK1 and p42 MAPK via C-Raf (Park *et al.*, 2004). These various triggering stimuli culminate in the all-or-none activation of p42 MAPK and Cdc2, which reinforce each other's activation through positive feedback. After eggs are fertilized, the positive feedback loop is broken, the metaphase arrest is released, and p42 MAPK is inactivated (Ferrell, 1999a, b). Thereafter, during embryogenesis a small proportion of the cell's p42 MAPK becomes transiently activated when the embryo enters mitosis (Guadagno and Ferrell, 1998; Yue and Ferrell, 2004). Mitotic activation of ERK1/2 is also seen in several mammalian cell lines, and may play a role in regulating the timing of anaphase onset and mitotic exit (Shapiro *et al.*, 1998; Zecevic *et al.*, 1998).

Recently, we attempted to identify the MAPKKK responsible for activating MEK1 and p42 MAPK in interphase *Xenopus* egg extracts treated with a non-degradable cyclin, $\Delta 90$ -cyclin B, a system that produces a permanent mitotic state and so provides a good source material for kinases active in M-phase (Yue and Ferrell, 2004). We presented several lines of evidence that Mos was the responsible MAPKKK. First, we found that although much of the egg's Mos is degraded after fertilization, enough Mos persists in early embryos and interphase egg extracts to be functionally significant. Second, we found that Mos is active in $\Delta 90$ -cyclin B-treated extracts, and is inactive in mock-treated interphase extracts, as would be expected of a MAPKKK responsible for the mitotic activation of MEK1 and p42 MAPK. Third, we found that Mos copurified with a $\Delta 90$ -cyclin B-stimulated MAPKKK activity over several column steps. Finally, we found that immunodepleting Mos from *Xenopus* egg extracts renders the extracts unable to activate p42 MAPK in response to $\Delta 90$ -cyclin B, and that adding back recombinant Mos partially restores p42 MAPK activation (Yue and Ferrell, 2004). Together these results argue that the Mos protein is largely responsible for $\Delta 90$ -cyclin B-induced activation of p42 MAPK.

However, we also noted that Mos was not the only MAPKKK activity present in $\Delta 90$ -cyclin B-treated extracts. A second MAPKKK, which was comparable

in activity to Mos in $\Delta 90$ -cyclin B-treated extracts, and was the main MAPKKK activity present in interphase extracts, was also detectable. Here, we set out to identify this second MAPKKK, to test the hypothesis that it collaborates with Mos in the mitotic activation of p42 MAPK, and to identify under what circumstances its activity is regulated. We have identified this MAPKKK as B-Raf, and have cloned and sequenced a *Xenopus* B-Raf cDNA. Furthermore, we have shown that B-Raf is neither activated by $\Delta 90$ -cyclin B/Cdc2 nor required for $\Delta 90$ -cyclin B/Cdc2-induced p42 MAPK activation; B-Raf appears not to be involved in the mitotic activation of MEK1 and p42 MAPK. However, we have also shown that B-Raf and C-Raf together are responsible for the Ras-induced activation of MEK1 and p42 MAPK in extracts. B-Raf and C-Raf may therefore function together as key intermediaries in tyrosine kinase-stimulated MAPK activation in *Xenopus* oocytes, eggs, and embryos.

Results

Activation of the p42 MAPK cascade by Cdc2-cyclin B in Xenopus egg extracts

We prepared cycloheximide-treated interphase egg extracts, and drove them into M-phase by adding nondegradable $\Delta 90$ -cyclin B. In agreement with previous studies (Roy *et al.*, 1991; VanRenterghem *et al.*, 1993; Fukuda *et al.*, 1994; Minshull *et al.*, 1994; Guadagno and Ferrell, 1998; Yue and Ferrell, 2004), this resulted in high levels of MEK1 and p42 MAPK activity (Figure 1a). We fractionated these extracts by chromatography on Q-Sepharose, hydroxapatite, and heparin Sepharose, in each case eluting with a salt gradient. Western blots of the heparin Sepharose fractions were probed with antibodies raised against various mammalian MAPKKKs, including A-Raf, B-Raf, C-Raf, Mos, and MEK1. Of these five MAPKKKs, we detected appropriately sized bands with B-Raf, C-Raf, and Mos antibodies, and of the three detected, the B-Raf band best correlated with MAPKKK activity, suggesting that B-Raf (or perhaps a subset of the B-Raf molecules, since the correspondence between B-Raf protein levels and MAPKKK activity was not perfect) might be the relevant MAPKKK (Figure 1b and data not shown).

To test the possibility that the partially purified MAPKKK activity was due to *Xenopus* B-Raf, pooled heparin Sepharose column fractions were subjected to immunoprecipitation with mammalian B-Raf antibodies. As shown in Figure 1c, MAPKKK activity was quantitatively brought down by B-Raf antibodies, but not by control IgG. Thus, the partially purified MAPKKK activity was either associated with or related to B-Raf. We also subjected cruder MAPKKK-containing fractions from the Q-Sepharose chromatography step to immunoprecipitation with B-Raf and control IgG antibodies. Again, the B-Raf antibody brought down most of the MAPKKK activity, and the control

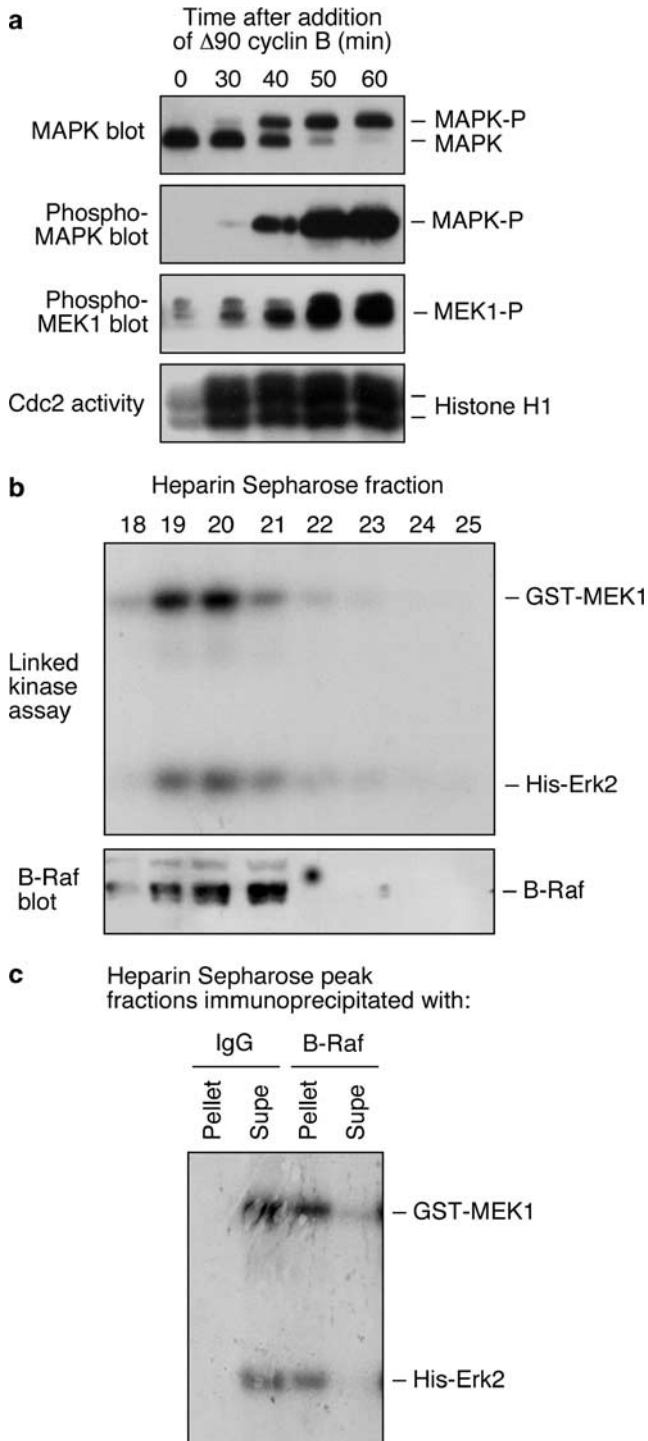


Figure 1 Partial purification of a MEK-activating MAPKKK. (a) Activation of the p42 MAPK cascade by $\Delta 90$ -cyclin B in *Xenopus* egg extracts. (b) Purification of a MAPKKK activity. Interphase egg extracts were treated with 200 nM $\Delta 90$ -cyclin B for 60 min. Lysates were prepared and subjected to chromatography on Q Sepharose followed by hydroxyapatite and heparin Sepharose. MEK-activating MAPKKK activities were detected by a linked kinase assay. (c) Precipitation of the partially purified MAPKKK activity with B-Raf antibodies.

IgG did not (data not shown). Finally, both B-Raf protein and the MAPKKK activity were found to bind quantitatively to a GST-MEK1 affinity column (data not shown). These results demonstrate that a B-Raf-like protein, or conceivably a B-Raf-associated protein, accounted for the MAPKKK activity.

Cloning Xenopus B-Raf

We therefore undertook the cloning, expression, and characterization of *Xenopus* B-Raf. We used *Xenopus* EST sequence data to design B-Raf-specific PCR primers, and obtained the full-length B-Raf open reading frame by RACE. Overall, the predicted *Xenopus* B-Raf protein was 82% identical to human B-Raf, and was less similar to human A-Raf (46% identical) and C-Raf (47% identical). Over their kinase domains, human and *Xenopus* B-Raf were 98% identical, and over the epitope from which the human B-Raf antibodies were generated (amino acids 12–156) the two proteins were 95% identical. This high degree of similarity accounts for the apparent recognition of the *Xenopus* protein by anti-human B-Raf antibodies. Dendrogram analysis showed that the kinase domains of the vertebrate B-Raf proteins are more highly conserved than those of the vertebrate C-Raf and A-Raf proteins, suggesting that there may be more constraints to B-Raf evolution than there are to C-Raf and A-Raf evolution (Figure 2). In addition, the kinase domains of the *Caenorhabditis elegans* and *Drosophila* Raf homologs are slightly more similar to those of the vertebrate B-Raf proteins than to A-Raf or C-Raf proteins (Figure 2).

The full-length *Xenopus* B-Raf cDNA was subcloned into a bacterial expression vector, and the recombinant

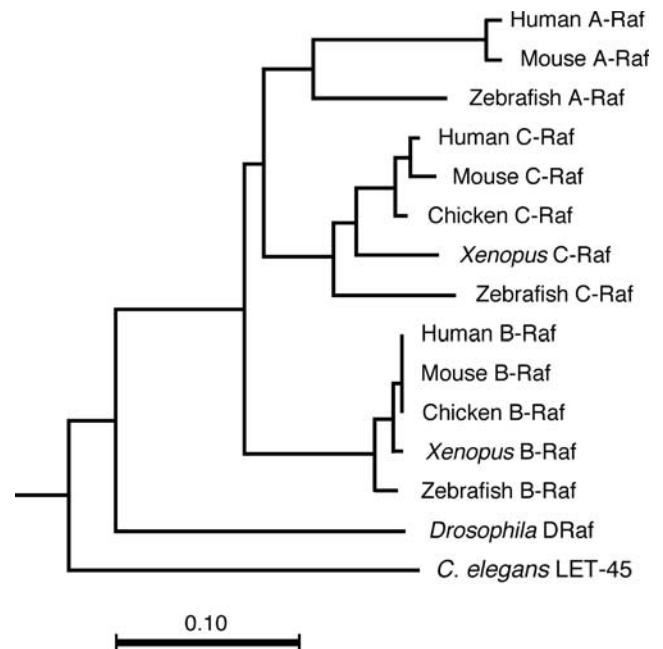


Figure 2 Dendrogram of kinase domain sequences of A-Raf, B-Raf, and C-Raf proteins. Kinase domains were aligned using Clustal X, and a bootstrapped tree was constructed. Scale bar denotes 10% sequence divergence.

Xenopus His₆-B-Raf protein was expressed and purified. The recombinant protein migrated as a single band by immunoblotting, with an apparent molecular mass of 98 kDa (Figure 3a, lane 1), consistent with the expected molecular mass of the tagged recombinant B-Raf protein. We also subjected crude egg extracts (Figure 3a, lane 4) and B-Raf immunoprecipitates (Figure 3a, lane 2) to immunoblotting with the B-Raf-specific antibody. A diffuse doublet was detected, with apparent molecular mass of 97–99 kDa (Figure 3a, lane 4). By comparing the intensity of the B-Raf bands to the intensities of recombinant *Xenopus* B-Raf standards, we estimated B-Raf to be present at a concentration of approximately 450 nM (Figure 3b). By comparison, C-Raf was estimated to be approximately 100 nM (Figure 3c), and Mos is approximately 10 nM (Yew *et al.*, 1992). B-Raf therefore appears to be an unusually abundant MEK kinase, and is comparable in abundance to its downstream targets MEK1 (1300 nM) and p42 MAPK (330 nM) (Ferrell, 1996b).

Incubation of B-Raf with λ phosphatase collapsed the B-Raf doublet down to a tighter ~97 kDa band (Figure 3a, lane 3). This finding indicates that the two B-Raf bands represent hyperphosphorylated (upper) and hypophosphorylated (lower) B-Raf protein, rather than two splice variants of B-Raf.

Cdc2/cyclin B does not regulate B-Raf kinase activity

To further explore the interplay between Cdc2/cyclin B and B-Raf, we examined whether the activity of B-Raf is stimulated in Δ 90-cyclin-B-treated egg extracts. As shown in Figure 4b, B-Raf immunoprecipitates from control and Δ 90-cyclin-B-treated egg extracts showed equal activities towards recombinant GST-MEK1. To make sure that the equal activities were not a consequence of saturation of the kinase assay, we assayed the activities of various amounts of B-Raf from control and Δ 90-cyclin B-treated extracts. The amount of activity measured was proportional to the amount of extract from which the B-Raf was precipitated (Figure 4b), arguing against saturation. In contrast, B-Raf was activated by the addition of active H-Ras-V12 (Figure 4a).

Next, we examined whether B-Raf changed in activity in cycling *Xenopus* egg extracts. As shown in Figure 4c, there was no apparent change in B-Raf activity as extracts entered and exited mitosis.

We also examined the activity of B-Raf during progesterone-induced *Xenopus* oocyte maturation (Figure 4d), after parthenogenetic activation of *Xenopus* eggs (Figure 4e), and during the first few embryonic cell cycles (Figure 4f). We detected no significant changes in B-Raf activity during any of these processes.

B-Raf is required for oncogenic Ras-stimulated p42 MAPK activation in *Xenopus* egg extracts

Given the lack of evidence for regulation of B-Raf downstream of Δ 90-cyclin B-Cdc2, and our previous study showing that Mos is required for Δ 90-cyclin B-induced p42 MAPK activation but B-Raf is not

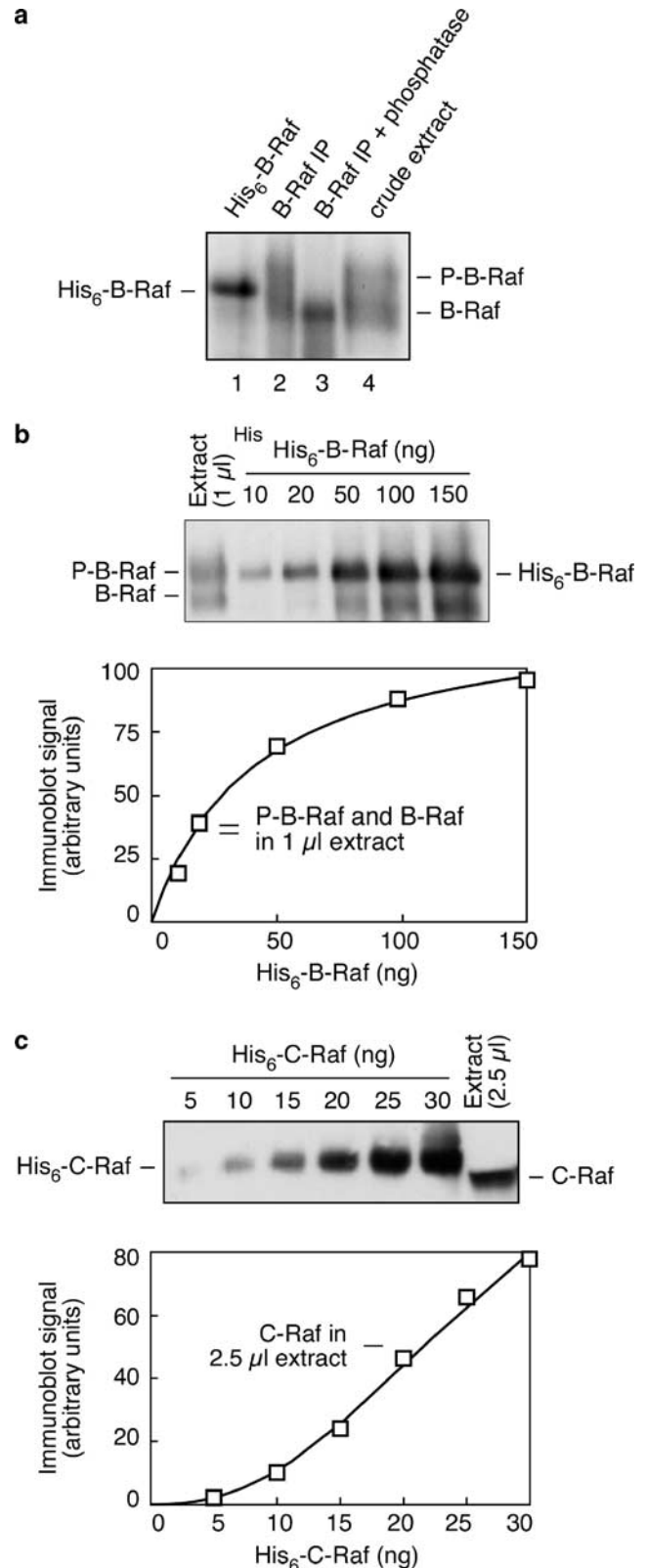


Figure 3 Characterization of B-Raf. (a) Recombinant B-Raf (lane 1), immunoprecipitated B-Raf from *Xenopus* egg extracts (lane 2), λ -phosphatase-treated immunoprecipitated B-Raf (lane 3), and B-Raf in crude extracts (lane 4). (b) Abundance of B-Raf, using partially purified B-Raf as an immunoblotting standard. (c) Abundance of C-Raf, using partially purified C-Raf as an immunoblotting standard.

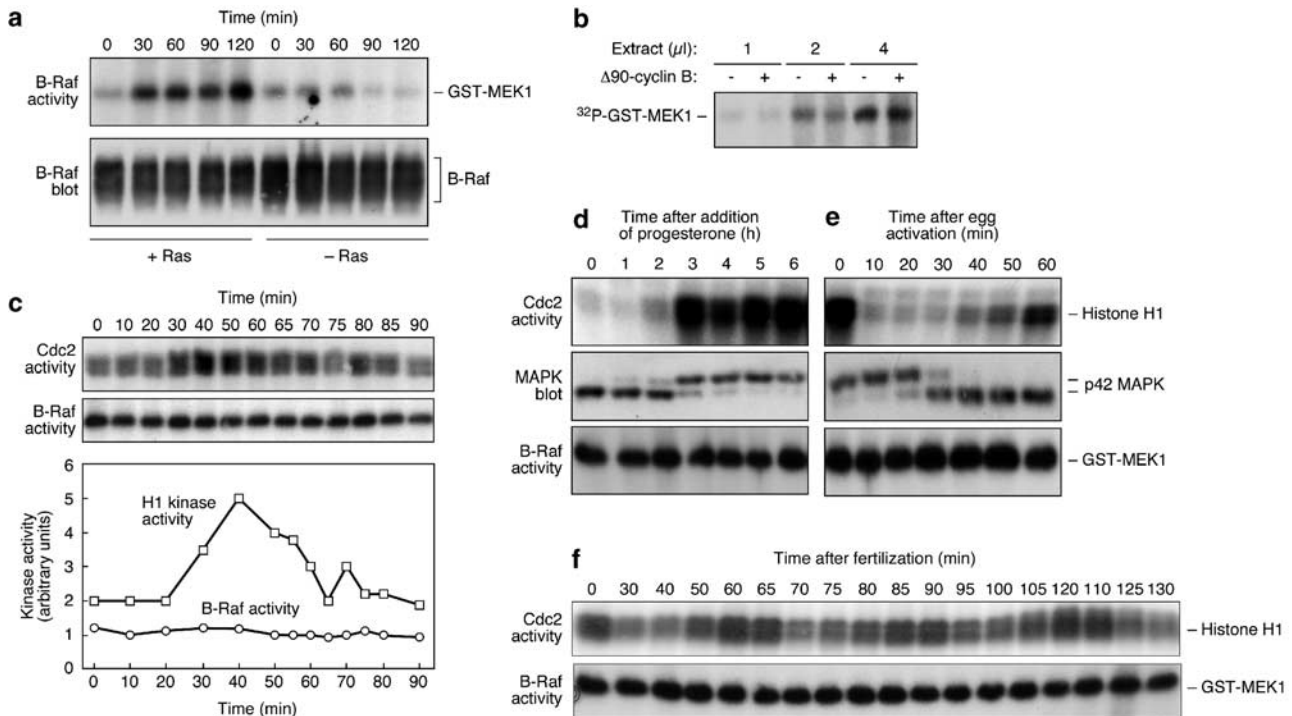


Figure 4 Cdc2-cyclin B does not regulate B-Raf activity. (a) Effect of H-Ras-V12 on B-Raf activity. Extracts were treated with 1.6 μ M H-Ras-V12 for the lengths of time indicated. B-Raf kinase activity was assessed by immune complex kinase assay with GST-MEK1 as substrate, and the amount of B-Raf present was assessed by immunoblotting. (b) Effect of Δ 90-cyclin B on B-Raf activity. Interphase extracts were treated with or without Δ 90-cyclin B. B-Raf kinase activity was assessed using GST-MEK1 as substrate. (c) B-Raf kinase activity in cycling extracts. A cycling extract was prepared and warmed to room temperature to initiate cycling. The times indicated are times after warming. B-Raf activity was assessed by immune complex kinase assay with GST-MEK1 as substrate. Cdc2 activity was assessed by histone H1 kinase assay. Assays were quantified by PhosphorImager analysis (Molecular Dynamics). (d) B-Raf activity during oocyte maturation. Stage VI oocytes were stimulated with progesterone (6 μ M) and frozen at the times indicated. B-Raf kinase assay using GST-MEK1 as substrate (bottom panel). A p42 MAPK immunoblot (middle panel) and H1 kinase assays (top panel) were also performed. (e) B-Raf activity after egg activation. Eggs were parthenogenetically activated by electric shock and lysed at the indicated time points. B-Raf assays, histone H1 kinase assays, and a p42 MAPK immunoblot were performed as in (a). (f) B-Raf activity during early embryogenesis. Eggs were fertilized *in vitro* and lysed at the indicated time points. Histone H1 kinase assays and B-Raf kinase assays using GST-MEK1 as substrate were then performed.

(Yue and Ferrell, 2004), we turned our attention to other MAPK-activating stimuli that might be mediated by B-Raf. Activated forms of Ras can bring about p42 MAPK activation in *Xenopus* oocytes and *Xenopus* egg extracts (Barrett *et al.*, 1990; Sadler *et al.*, 1990; Hattori *et al.*, 1992; Shibuya *et al.*, 1992; VanRenterghem *et al.*, 1993), and, as mentioned above, this may be physiologically important for transmitting signals from the classical progesterone receptor through Src to p42 MAPK (Bayaa *et al.*, 2000; Tian *et al.*, 2000; Boonyaratankornkit *et al.*, 2001), or from receptor tyrosine kinases to p42 MAPK (Kimelman *et al.*, 1988; Amaya *et al.*, 1991; Park *et al.*, 2004). We therefore examined whether B-Raf and/or C-Raf (the other Raf family member detectable in *Xenopus* eggs) were required for Ras-mediated p42 MAPK activation in egg extracts.

We found that the addition of V12 H-Ras to interphase *Xenopus* egg extracts brought about activation of MEK1 and p42 MAPK (Figure 5a) as well as B-Raf (Figure 4a), consistent with previous reports (Shibuya *et al.*, 1992; Shibuya and Ruderman, 1993; VanRenterghem *et al.*, 1993; Fukuda *et al.*, 1994). Immunodepletion of MEK1 blocked Ras-dependent

p42 MAPK activation (Figure 5c), indicating that MEK1 is required for this process, as expected. MEK1 antibodies also precipitated a substantial fraction of the B-Raf and C-Raf (Figure 5b), suggesting that MEK1 physically associates with B-Raf and C-Raf in extracts. Depletion of either B-Raf or C-Raf from egg extracts partially inhibited Ras-induced p42 MAPK activation, and codepletion of both B-Raf and C-Raf completely blocked Ras-induced p42 MAPK activation without depleting MEK1 (Figure 5c). In contrast, depletion of Mos from egg extracts had little effect on Ras-induced p42 MAPK activation (Figure 5c). These data indicate that both B-Raf and C-Raf contribute to Ras-induced p42 MAPK activation in *Xenopus* egg extracts, and that Mos is not required.

Discussion

Here, we report the purification and characterization of *Xenopus* B-Raf from egg extracts. We have shown that B-Raf is an abundant MAPKKK (~450 nM in

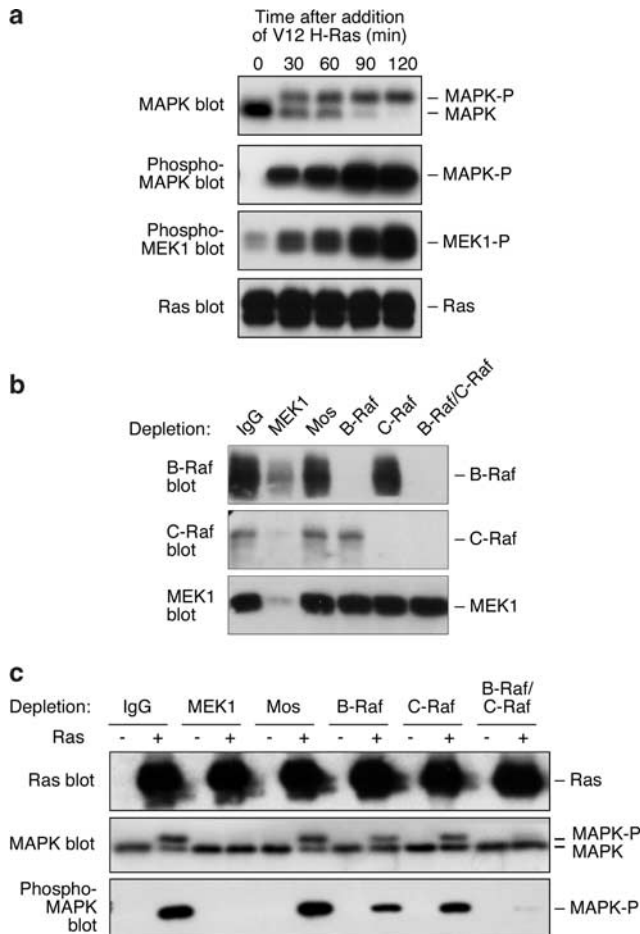


Figure 5 Ras-induced p42 MAPK activation depends upon B-Raf and C-Raf, but not Mos. (a) Time course of V12 H-Ras-induced activation of p42 MAPK and MEK1. Ras blots are shown as a control. (b) Immunodepletion of B-Raf, C-Raf, and MEK1. (c) Effect of MEK1, Mos, B-Raf, and C-Raf depletion on Ras-induced activation.

concentration, compared to ~ 10 nM for Mos and ~ 100 nM for C-Raf; Figure 3), and has a substantial but unchanging activity throughout oocyte maturation and early embryogenesis (Figure 4). We have also demonstrated that both Cdc2-cyclin B (Figure 1a) and the oncogenic V12 H-Ras protein (Figure 5a) can activate the p42 MAPK cascade in egg extracts, in agreement with previous reports (Hattori *et al.*, 1992; Shibuya *et al.*, 1992; Itoh *et al.*, 1993; VanRenterghem *et al.*, 1993; Fukuda *et al.*, 1994). However, Cdc2-cyclin B does not regulate B-Raf kinase activity or its interaction with MEK1 (Figure 4 and data not shown). Immunodepletion of B-Raf, C-Raf, or both from egg extracts does not block the ability of Cdc2-cyclin B to activate the p42 MAPK cascade, whereas depletion of Mos does (Yue and Ferrell, 2004). In contrast, depletion of B-Raf and C-Raf from extracts inhibits the activation of p42 MAPK by V12-H-Ras, whereas depletion of Mos has no effect (Figure 5). Collectively, our results indicate that B-Raf and C-Raf act as

partially redundant mediators of Ras signaling in *Xenopus* egg extracts, and there is little if any overlap between the signals mediated by the Raf proteins and those mediated by Mos.

Two groups previously reported the fractionation of MAPKKK activities from *Xenopus* egg extracts. Both groups found that their main MAPKKK activities bound to anion exchange resins and eluted at about the same salt concentration as B-Raf does from Q-Sepharose (Itoh *et al.*, 1993; Matsuda *et al.*, 1993). This suggests that the Nishida group's MAPKKK activity (Matsuda *et al.*, 1993) and the Takai group's Ras-activated Erk kinase stimulator (REKS) (Itoh *et al.*, 1993) were, in fact, B-Raf. Indeed, the Takai and Weber groups later showed that the main MEK activator protein in bovine brain was B-Raf (Catling *et al.*, 1994; Yamamori *et al.*, 1995), again consistent with our findings here for *Xenopus* eggs. The high abundance of B-Raf in *Xenopus* eggs rationalizes its identification in this system. Perhaps B-Raf is similarly high in abundance in brain.

The mitotic activation of p42 MAPK occurs not only in *Xenopus* egg extracts, but also at the kinetochores in several mammalian cell lines (Shapiro *et al.*, 1998; Zecevic *et al.*, 1998), and microinjected Mos localizes to kinetochores in PtK1 cells (Wang *et al.*, 1994). In contrast, immunostaining with a specific anti-B-Raf antibody in HeLa cells shows that B-Raf is located in cytoplasm, with no apparent kinetochore staining (Yue and Ferrell, unpublished results). Hence, it is possible that endogenous Mos localizes to kinetochores, which could put Mos in the appropriate location to be responsible for the mitotic activation of kinetochore-associated MEK and ERKs.

Recently, C-Raf was shown to be required for MEK1 activation on the Golgi apparatus in late prophase and to be required for Golgi complex fragmentation in mitosis in mammalian cells (Acharya *et al.*, 1998; Colanzi *et al.*, 2003). *Xenopus* egg extracts may provide a good system for assessing the roles of C-Raf and B-Raf in mitotic Golgi regulation.

B-Raf, like other Raf family members, is regulated by multiple mechanisms involving phosphorylation/dephosphorylation, protein-protein interaction, and intracellular translocation (Chong *et al.*, 2003). In addition, alternative splicing can modulate the kinase activity and oncogenic properties of B-Raf (Barnier *et al.*, 1995; Eychene *et al.*, 1995; Papin *et al.*, 1998). In *Xenopus* egg extracts and oocytes, B-Raf appears to be expressed as a single spliced form, but migrates as two diffuse bands due to the presence of some B-Raf hyperphosphorylation even in unstimulated extracts and oocytes. Along these lines, S445 in B-Raf has been shown to be constitutively phosphorylated, whereas the homologous site in C-Raf is phosphorylated in a Ras-dependent fashion, which may account for the high basal activity of B-Raf compared to C-Raf (Mason *et al.*, 1999).

In *Xenopus* oocytes, eggs, and embryos, probably the best established function of Ras is in mesoderm induction (Harland and Gerhart, 1997). Clearly B-Raf is a plausible candidate for a critical Ras effector in this

context. In addition, recent studies implicate EGF receptor ligands in the regulation of mouse oocyte maturation (Park *et al.*, 2004), although current evidence suggests that the EGF receptor ligands act on cumulus cells, not directly on oocytes. Other studies of the classical progesterone receptor in oocytes have implicated Src in relaying nontranscriptional signals (Boonyaratankornkit *et al.*, 2001), raising the possibility that Ras and B-Raf may be relevant to *Xenopus* oocyte maturation through this mechanism (although we have not detected any changes in B-Raf activity in progesterone-treated oocytes). Moreover, two previous studies have shown that dominant-negative C-Raf proteins interfere with progesterone-induced p42 MAPK activation in *Xenopus* oocytes (Fabian *et al.*, 1993; Muslin *et al.*, 1993), again supporting the idea that C-Raf or B-Raf or both proteins are relevant to oocyte maturation. Although the present results underscore the separateness of Mos (which is relevant to $\Delta 90$ -cyclin-induced p42 MAPK activation) from B-Raf and C-Raf (which are relevant to Ras-induced p42 MAPK activation) in the responses to some stimuli, it seems possible that in a physiological context, all three MAPKKs contribute to progesterone-induced p42 MAPK activation. It should be interesting to further examine the interplay between Mos, B-Raf, and C-Raf in oocyte maturation.

Materials and methods

Preparation and manipulation of oocytes, eggs, and egg extracts
Xenopus eggs were obtained as described (Murray, 1991). Cycloheximide-treated interphase egg extracts and cycling egg extracts were prepared as described (Murray, 1991; Smythe and Newport, 1991). To drive interphase extracts into a permanent mitotic state, extracts were incubated with 200 nM nondegradable sea urchin $\Delta 90$ -cyclin B for 60 min. Progression into mitosis was monitored by sperm morphology changes, visualized by DAPI staining and fluorescence microscopy, and by histone H1 kinase assay.

Eggs were fertilized *in vitro* as described (Newport and Kirschner, 1982). Collagenase-treated *Xenopus* oocytes were obtained, incubated, and lysed as described previously (Sohaskey and Ferrell, 1999; Bagowski *et al.*, 2001).

Immunodepletions

Immunodepletion of B-Raf, C-Raf, Mos, or MEK1 was accomplished by incubating extracts for 60 min for two rounds at 4°C with anti-B-Raf antibody (SC-9002, Santa Cruz Biotechnology), anti-C-Raf/Raf-1 antibody (SC-133, Santa Cruz Biotechnology), anti-Mos antibody (SC-86, Santa Cruz Biotechnology), or anti-MEK1 antibody (#662, generated in this laboratory) prebound to protein A-Sepharose beads (Sigma). Mock depletions were carried out with rabbit immunoglobulin G (IgG) in place of primary antibody.

In vitro MEK kinase assay

An *in vitro* linked kinase assay using recombinant GST-MEK1 (human) and His₆-p42 MAPK (*Xenopus*) as substrates was performed on column fractions. Briefly, 10 μ l aliquots of column fractions were added to 10 μ l of kinase reaction mixture consisting of 10 μ M cAMP-dependent protein kinase

inhibitor, 0.75 mM Na₃VO₄, 5 mM EGTA, 0.2 mM ATP, 13 mM HEPES-NaOH, pH 7.3, 0.1 μ Ci/ μ l [γ -³²P]ATP, and 64 mM MgCl₂, and incubated at 30°C for 10 min. Next, recombinant GST-MEK1 proteins were added to the reaction and the incubation was continued for 10 min. Recombinant His₆-Erk2 protein was then added to the reaction. After 10 min, the reaction was stopped by the addition of SDS sample buffer. Proteins were subjected to electrophoresis through 12.5% SDS polyacrylamide gels (acrylamide: bisacrylamide, 29:1), and transferred to an Immobilon P (Millipore) blotting membrane. ³²P labeled GST-MEK1 and His₆-Erk2 were visualized by autoradiography.

In vitro histone H1, p42 MAPK, or MEK1 kinase assays were performed as described previously (Walter *et al.*, 1997; Sohaskey and Ferrell, 1999).

An *in vitro* B-Raf kinase assay was performed on interphase extracts treated with or without cyclin B, or on cycling egg extracts. Briefly, extracts (5 μ l) were diluted in 100 μ l EB buffer, and were incubated with anti-B-Raf antibody prebound to protein A-Sepharose beads (Sigma) with rocking for 1 h at 4°C. After three washes in EB containing 0.1% Nonidet P-40 and one wash in detergent-free EB, 25 μ l of kinase reaction mixture were added containing 100 ng GST-Flag-MEK1. The reaction was stopped, after incubation at 30°C for 10 min, with SDS sample buffer, and the proteins were separated on 10% SDS polyacrylamide gels (acrylamide:bisacrylamide, 29:1) and transferred to an Immobilon P (Millipore) blotting membrane. ³²P labeled GST-MEK1 was visualized by autoradiography.

Purification of B-Raf from *Xenopus* egg extract

All chromatography steps were carried out using a fast protein liquid chromatography (FPLC) station (Pharmacia) at 4°C. After incubation with the nondegradable $\Delta 90$ -cyclin B (200 nM) for 60 min at room temperature, egg extracts (4 ml) were applied to two HiTrap Q columns in tandem (Amersham) equilibrated with buffer A (10 mM Hepes, 1 mM MgCl₂, 0.01 mM CaCl₂, 50 mM Sucrose). The column was washed with 10 column volumes of buffer A and eluted with a linear gradient of 40 ml buffer A to buffer B (buffer A + 1 M NaCl). Fractions of 1 ml were collected and assayed for MEK activity as described above. The active fractions were pooled, mixed with 15% ammonium sulfate for overnight, and centrifuged at 20 000 g for 15 min at 4°C. The resulting pellet was resuspended in 1 ml buffer A and loaded to a 5 ml hydroxyapatite column (Bio-Rad). The hydroxyapatite column was eluted with a linear gradient of 20 ml buffer A to buffer B. Fractions of 1 ml were collected and the active fractions were pooled and applied to 5 ml Heparin column (Amersham) equilibrated with buffer A. The column was then eluted with a linear gradient of 20 ml buffer A to buffer B. The fractions of 0.5 ml were collected and assayed for MEK activity.

Cloning a *Xenopus* B-Raf cDNA

RNA was typically obtained from 20 frozen *Xenopus* eggs using RNAeasy kits (Qiagen) according to the manufacturer's instructions. The cDNA pools were then generated from egg total RNA by RT-PCR (Invitrogen) according to the manufacturer's instructions. Five *Xenopus* B-Raf EST clones were found in Genbank, including one covering the 3' end of *Xenopus* B-Raf. The 5' sequence of *Xenopus* B-Raf was obtained by 5' RACE reactions performed as recommended by the manufacturer (Invitrogen). The primers used in 5' RACE were: GSP1, GGGAAAGGAAGACTCGAAC AAT; GSP2, AGGTGCCAAGGATAGATTCTGA; GSP3, CGAA

GAGGGGGTGTGCAGGGCTGA. Three DNA fragments ranging from 400 to 550 bp were obtained. All three fragments contain the same start codon and some 5' UTR. The full length of *Xenopus* B-Raf was then obtained by PCR from *Xenopus* egg cDNA pool. The primers used in PCR were: 5' primer, CATGCATGCCATGGCGGCGCTAAGTGGGGGATCAG CAGAG; 3' primer, CCCCCAAGCTTTCACAGGGCCCC GATAACAAACCCACCATA. The PCR-amplified fragments were gel-purified and subcloned into pRSET-A (Invitrogen).

Recombinant proteins

His₆-B-Raf was expressed in bacteria and lysed essentially as described (Wang *et al.*, 1997). The clarified cell extracts were loaded to a 5 ml nickel-chelated HiTrap column (Amersham) using FPLC station. The column was washed with 10 volumes of wash buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 20 mM imidazole), and fusion proteins were eluted with 10 ml elution buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 200 mM imidazole).

His₆- and FLAG-tagged recombinant C-Raf was expressed in insect cells and partially purified on FLAG beads. Δ90-cyclin B was expressed in bacteria and purified on a 5 ml GSTrap column (Amersham). p42 MAPK was expressed in bacteria and purified on a 5 ml nickel-chelated HiTrap column (Amersham). GST-tagged V12 H-Ras was purchased from Cytoskeleton (Denver, CO, USA) and was loaded with GTP as described (Shibuya *et al.*, 1992).

Western blot analysis

Aliquots of egg extracts or column fractions were diluted in SDS sample buffer and submitted to electrophoresis on 10 or 12.5% SDS polyacrylamide gels (acrylamide:bisacrylamide,

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- 100:1). Proteins were transferred to an Immobilon P (Millipore, Bedford, MA, USA) blotting membrane, which was then blocked with 3% milk in Tris-buffered saline (20 mM Tris, 150 mM NaCl, pH 7.6), and incubated for 1 h with one of the following primary antibodies: B-Raf (SC-9002, Santa Cruz Biotechnology), 1:500 dilution; C-Raf/Raf-1 (SC-133, Santa Cruz Biotechnology), 1:500 dilution; MEK1 (662, this laboratory), 1:1000 dilution; or p42 MAPK (DC3, this laboratory), 1:1000 dilution. After washing, the blots were probed with a secondary antibody and bands were detected by chemiluminescence.

Abbreviations

ERK, extracellular signal-regulated kinase; FPLC, fast protein liquid chromatography; MEK, MAPK kinase or ERK kinase; MAPK, mitogen-activated protein kinase; MAPKKK, MAP kinase kinase kinase; REKS, Ras-activated ERK kinase stimulator.

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