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Wild-Type and Mutant B-RAF Activate C-RAF through Distinct Mechanisms Involving Heterodimerization

Short Article

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Summary

The protein kinase B-RAF is mutated in approximately 7% of human cancers. Most mutations are activating, but, surprisingly, a small number have reduced kinase activity. However, the latter can still stimulate cellular signaling through the MEK-ERK pathway because they activate the related family member C-RAF. We examine the mechanism underlying C-RAF activation by B-RAF. We show that C-RAF is activated in the cytosol in a RAS-independent manner that requires activation segment phosphorylation and binding of 14-3-3 to C-RAF. We show that wild-type B-RAF forms a complex with C-RAF in a RAS-dependent manner, whereas the mutants bind independently of RAS. Importantly, we show that wild-type B-RAF can also activate C-RAF. Our data suggest that B-RAF activates C-RAF through a mechanism involving 14-3-3 mediated heterooligomerization and C-RAF transphosphorylation. Thus, we have identified a B-RAF–C-RAF–MEK–ERK cascade that signals not only in cancer but also in normal cells.

Introduction

The RAS–RAF–MEK–ERK pathway regulates cell proliferation, survival, and senescence in response to extracellular signals (Robinson and Cobb, 1997; Wellbrock et al., 2004). RAS is a small G protein that is attached to the inner surface of the plasma membrane, whereas RAF, MEK, and ERK are cytosolic protein kinases that sequentially activate each other, forming a three-tiered signaling cascade. There are three RAF isoforms in mammals, A-RAF, B-RAF, and C-RAF, and recent data has shown that B-RAF is mutated in approximately 7% of human cancers (Davies et al., 2002). Over 50 distinct point mutations have been described in B-RAF, the majority clustering to two regions of the kinase domain called the glycine-rich loop and the activation segment (see Garnett and Marais, 2004). Importantly, these regions interact under basal conditions, maintaining B-RAF in an inactive conformation, and the mutations are thought to disrupt this interaction and induce the active conformation (Wan et al., 2004).

The most common mutation in B-RAF is a glutamic acid for valine substitution at position 600, producing a 500-fold activated protein that signals to MEK–ERK constitutively, stimulating cell proliferation and survival (see Wellbrock et al., 2004). Most of the other mutants are also activating but, surprisingly, at least three (G466E, G466V, and G596R) have impaired MEK kinase activity in vitro (Houben et al., 2004; Ikenoue et al., 2004, 2003; Wan et al., 2004). G466 is positioned in the glycine-rich loop, and G596 is in the activation segment, so these mutations are also likely to induce the active conformation. However, they are two of the most conserved residues in the kinase family and are functionally important (Hanks and Hunter, 1995), so their mutation is likely to have additional negative effects that account for their lack of activity. Nevertheless, G466E, G466V, and G596R still activate MEK–ERK in cells because they activate C-RAF, which then signals to MEK (Wan et al., 2004).

C-RAF activation is a complex process (see Avruch et al., 2001; Wellbrock et al., 2004) that is initiated after its recruitment to the plasma membrane by RAS. At the membrane, C-RAF then requires conformation changes, regulatory phosphorylation and dephosphorylation events, and binding to other proteins and lipids for activation. RAF oligomerization may also play a role, because C-RAF is activated by artificial homodimer formation (see Avruch et al., 2001; Wellbrock et al., 2004) and C-RAF:B-RAF heterodimerization has been observed in cells (Tzivion et al., 1998; Weber et al., 2001; Wan et al., 2004).

For activation, C-RAF requires phosphorylation of five residues within its kinase domain. S338 and Y341 are within a region called the negative charge regulatory-region or N-region and this phosphorylation is thought to relieve a negative function of the N terminus on the kinase domain (see Avruch et al., 2001; Wellbrock et al., 2004). It may also stabilize the small lobe of the kinase domain (Wan et al., 2004). Two of the other sites, T491 and S494, are in the activation segment (Chong et al., 2001). By analogy to B-RAF, T491 phosphorylation is thought to promote activity by disrupting the interaction between the glycine-rich loop and activation segment (Wan et al., 2004). It may also form a salt bridge to R469 to correctly position D470, the catalytic aspartate. Finally, phosphorylation of S621 in the C terminus of C-RAF allows binding of 14-3-3 adaptor/ scaffold proteins, and this is also required for activation (see Avruch et al., 2001; Wellbrock et al., 2004). 14-3-3 binding to this site appears to mediate the formation of C-RAF:B-RAF heterodimers (Weber et al., 2001), and, in B-RAF, this conserved site is required to couple signals to MEK (MacNicol et al., 2000). Finally, the binding of 14-3-3 to these conserved sites in the different RAF isoforms appears to be differentially and dynamically regulated (Hekman et al., 2004).

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Here, we investigate the mechanism of C-RAF activation by B-RAF. We show that B-RAF activates C-RAF in the cytosol in a RAS-independent manner. B-RAF activates the C-RAF kinase domain and requires its residual kinase activity. N-region phosphorylation on C-RAF is not essential, but activation segment phosphorylation and 14-3-3 binding to the C terminus of C-RAF are required. Importantly, we show that wild-type B-RAF can also activate C-RAF and that wild-type B-RAF and C-RAF form a complex that is RAS-induced, whereas mutant forms of B-RAF bind to C-RAF constitutively. Our data suggest that B-RAF activates C-RAF through 14-3-3 mediated heterooligomerization and transphosphorylation and that this pathway signals both in cancer cells and in normal cells.

Results

B-RAF Activates C-RAF in the Cytosol in a RAS-Independent Manner

To study C-RAF activation by B-RAF, we transiently expressed myc-tagged B-RAF and HA-tagged C-RAF in COS cells, immunoprecipitated the HA-C-RAF, and measured its activity in a kinase cascade assay. The impaired activity mutants, ^{G466E}B-RAF, ^{G466V}B-RAF, and ^{G596R}B-RAF all activate C-RAF, but not kinase-dead C-RAF (see Figure S1 in the Supplemental Data available with this article online), demonstrating the specificity of this assay. We previously demonstrated that these B-RAF mutants retain 30%–80% of their MEK kinase activity (Wan et al., 2004). To test if this is required for C-RAF activation, methionine was substituted for the catalytic lysine (K483) in B-RAF. Notably, ^{K483M,G466V}B-RAF and ^{K483M,G596R}B-RAF do not activate C-RAF (Figure 1A), indicating that residual B-RAF kinase activity is required for C-RAF activation.

We next tested if B-RAF activated C-RAF by inducing autocrine growth factors downstream of ERK. Cells expressing ^{G466V}B-RAF were treated with the synthetic small-molecule MEK inhibitor U0126 (Favata et al., 1998), which causes rapid and sustained ERK inhibition (Figure 1B). Significantly, U0126 does not block C-RAF activation by ^{G466V}B-RAF (Figure 1B), and similar results were obtained with ^{G596R}B-RAF (data not shown). Thus, C-RAF is not activated by ERK-induced autocrine growth factor production.

As RAS can directly activate C-RAF, we examined its role in C-RAF activation by B-RAF. For this, we used a RAS capture assay to measure its activation in cells (de Rooij and Bos, 1997). We show that epidermal growth factor (EGF) activates RAS, but RAS is not activated by the impaired activity mutants or by ^{V600E}B-RAF (Figure 2A). Furthermore, whereas EGF induces complex formation between RAS and C-RAF, and ^{G12V}RAS binds directly to C-RAF, a RAS:C-RAF complex is not induced by the impaired activity mutants or by ^{V600E}B-RAF (Figure 2B).

This suggests RAS is not required for C-RAF activation and, because RAS recruits C-RAF to the plasma membrane, we tested whether the B-RAF mutants stimulate membrane recruitment. By using a micro-injection approach, we have shown (Marais et al., 1997) that C-RAF is cytosolic under basal conditions but is recruited to the plasma membrane by ^{G12V}RAS (Figure 2C). In contrast, ^{WT}B-RAF and the impaired activity mutants do not recruit C-RAF to the plasma membrane (Figure 2C). Similar results were obtained in cell-fractionation studies. C-RAF is cytosolic under basal conditions and is recruited to the membrane fraction by ^{G12V}RAS (Figure 2D, lanes 1 and 7) but is not recruited by ^{G466E}BRAF, ^{G466V}BRAF, ^{G596R}BRAF, or ^{V600E}B-RAF (Figure 2D, lanes 3–6). These studies also reveal that B-RAF is cytosolic (Figures 2C and 2D), and the results suggest that the impaired activity mutants activate C-RAF in the cytosol and not at the plasma membrane. To further test this model, we examined whether B-RAF could activate the isolated catalytic domain of C-RAF (ΔC-RAF). This construct lacks the N-terminal domains required for plasma membrane recruitment and so cannot be activated by ^{G12V}RAS (Figure 2E). ^{G466V}B-RAF still activates ΔC-RAF, albeit less efficiently than full-length C-RAF (Figure 2E). Furthermore, the isolated kinase domain of ^{G466V}B-RAF (Δ^{G466V}B-RAF) still activates full-length C-RAF (Figure 2F). Together, these data show that B-RAF targets the kinase domain of C-RAF, activating it in the cytosol in a RAS-independent manner.

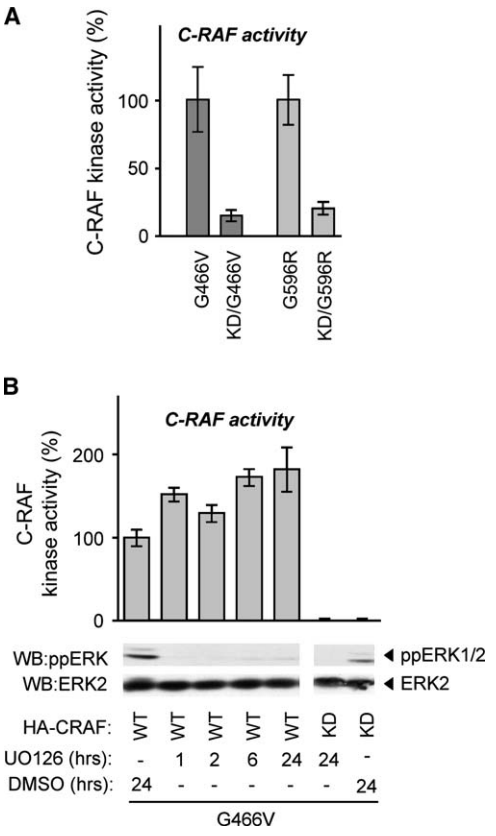

Figure 1. C-RAF Is Not Activated by Autocrine Growth Factors

(A) C-RAF kinase activity in cells coexpressing ^{G466V}B-RAF (G466V), ^{G596R}B-RAF (G596R), or kinase-dead (KD) versions of these mutants. Results are from one experiment, assayed in triplicate with error bars to represent standard deviation from the mean.

(B) C-RAF kinase activity in cells expressing C-RAF (WT), ^{KD}C-RAF (KD), or ^{G466V}B-RAF (G466V) in the presence of UO126 (10 μM) or DMSO for the indicated time. Results are from one experiment, assayed in triplicate with error bars to represent standard deviation from the mean. Western blots for ppERK and total ERK2 in the same extracts are also presented.

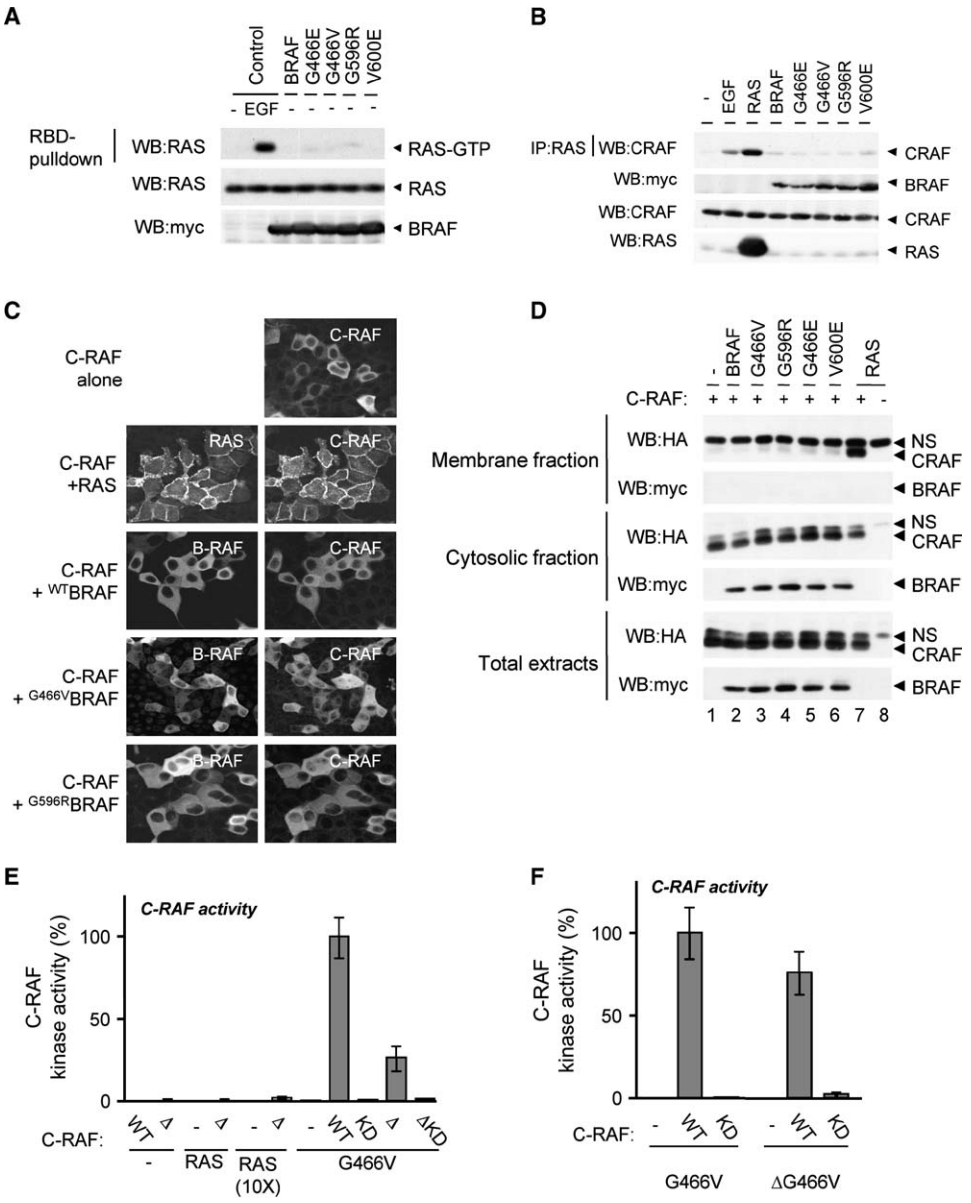

Figure 2. B-RAF Activates C-RAF in the Cytosol in a RAS-Independent Manner

(A) Endogenous RAS activation in cells expressing ^{WT}B-RAF (B-RAF) or the mutants indicated. Western blots of activated RAS (top), total RAS (middle), and B-RAF (lower) are shown. Where indicated, cells were treated with EGF (10 ng/mL, 10 min).

(B) RAS:C-RAF complex formation in cells expressing ^{G12V}RAS (RAS), ^{WT}B-RAF (B-RAF), or the mutants indicated. Upper: Western blot for C-RAF in RAS immunoprecipitates. Lower: Western blots for B-RAF, C-RAF, and RAS in total cell lysates. Where indicated, cells were treated with EGF (10 ng/mL, 10 min).

(C) C-RAF subcellular localization in MDCK cells expressing C-RAF with ^{G12V}RAS (RAS), ^{WT}B-RAF, or the mutants indicated. The same fields of cells were stained for C-RAF and RAS or C-RAF and B-RAF as appropriate.

(D) Cytosolic and membrane fractionation of COS cells expressing C-RAF with ^{WT}B-RAF (B-RAF), ^{G12V}RAS (RAS), or the mutants indicated. Western blots for C-RAF and B-RAF in the membrane fraction (top), cytosolic fraction (middle), or total extract (bottom) are shown. Abbreviation is as follows: NS, nonspecific band.

(E) C-RAF kinase activity in cells expressing wild-type C-RAF (WT), kinase dead C-RAF (KD), ΔC-RAF (Δ), or kinase dead ΔC-RAF (ΔKD) together with ^{G12V}RAS (RAS) or ^{G466V}B-RAF (G466V). Where indicated, 10× extract was used for the immunoprecipitation step. Results are from one experiment, assayed in triplicate with error bars to represent standard deviation from the mean.

(F) C-RAF kinase activity in cells expressing wild-type C-RAF (WT) or kinase dead (KD) C-RAF together with ^{G466V}B-RAF (G466V) or Δ^{G466V}B-RAF (ΔG466V). Results are from one experiment, assayed in triplicate with error bars representing standard deviation from the mean.

C-RAF Requires Activation Segment Phosphorylation and 14-3-3 Binding for Activation by B-RAF

We next examined whether C-RAF requires phosphorylation of known sites within its kinase domain for activation by B-RAF. First, we examined the N region sites by substituting S338 or Y341 for alanine in the full-length protein. ^{Y341A}C-RAF is not activated by ^{G12V}RAS but is still activated by ^{G466V}B-RAF and ^{G596R}B-RAF (Figure 3A). Similarly, ^{S338A}C-RAF is not activated by ^{G12V}RAS

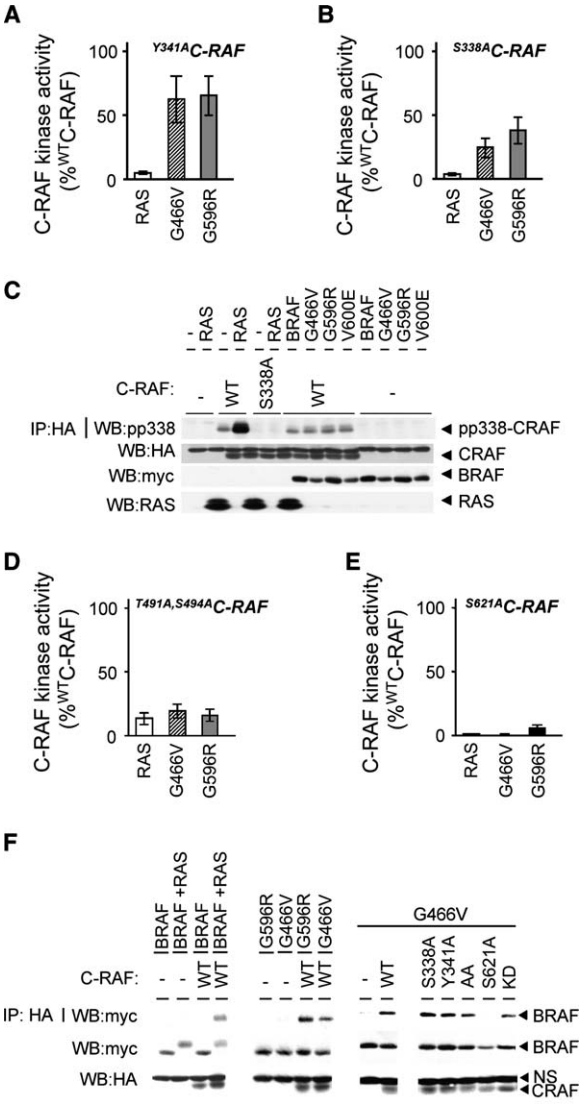

Figure 3. Activation Segment and S621 Phosphorylation Are Required for C-RAF Activation by B-RAF

(A and B) ^{Y341A}C-RAF (A) and ^{S338A}C-RAF (B) kinase activity in cells coexpressing ^{G12V}RAS (RAS), ^{G466V}B-RAF (G466V), or ^{G596R}B-RAF (G596R). Results are expressed as a percentage of ^{WT}C-RAF activity and are from one experiment, assayed in triplicate with error bars representing standard deviation from the mean.

(C) Western blot analysis for S338 phosphorylation on ^{WT}C-RAF (WT) or ^{S338A}C-RAF (S338A) in cells coexpressing ^{G12V}RAS (RAS), ^{WT}B-RAF (B-RAF), or the mutants indicated. As controls, cell extracts were also probed for C-RAF, B-RAF, and RAS.

(D and E) ^{T491A,S494A}C-RAF (D) and ^{S621A}C-RAF (E) kinase activity from cells coexpressing ^{G12V}RAS (RAS), ^{G466V}B-RAF (G466V), or ^{G596R}B-RAF (G596R). Results are expressed as a percentage of ^{WT}C-RAF activity and are from one experiment, assayed in triplicate with error bars to represent standard deviation from the mean.

(F) Binding of C-RAF to B-RAF. COS cells were transfected with C-RAF (WT), ^{S338A}C-RAF (S338A), ^{Y341A}C-RAF (Y341A), ^{T491A,S494A}C-RAF (AA), ^{S621A}C-RAF (S621A), or ^{KD}C-RAF (KD), together with ^{WT}B-RAF (B-RAF), ^{G12V}RAS (RAS), ^{G466V}B-RAF (G466V), or ^{G596R}B-RAF (G596R). The upper panel shows HA-immunoprecipitated C-RAF, followed by myc-epitope Western blots for coprecipitating B-RAF. The lower panels show myc and HA blots to control for protein expression. Note that B-RAF has reduced motility when coexpressed with RAS (Marais et al., 1997).

but retains 20% to 40% of its activation by ^{G466V}B-RAF and ^{G596R}B-RAF (Figure 3B). Consistent with this, we find that whereas ^{G12V}RAS stimulates S338 phosphorylation on C-RAF, ^{G466V}B-RAF and ^{G596R}B-RAF do not induce S338 phosphorylation above the levels seen with ^{WT}B-RAF or ^{V600E}B-RAF (Figure 3C). We also examined the role of the activation segment phosphorylation sites (T491 and S494) and the C-terminal 14-3-3 binding site (S621). $^{T491A/S494A}$C-RAF is poorly activated by ^{G12V}RAS, ^{G466V}B-RAF, and ^{G596R}B-RAF (Figure 3D). Similarly, ^{S621A}C-RAF is not activated by ^{G12V}RAS, ^{G466V}B-RAF, or ^{G596R}B-RAF (Figure 3E). These data suggests that N-region phosphorylation is not essential for C-RAF activation by mutant B-RAF, whereas activation segment phosphorylation and C-terminal 14-3-3 binding are required.

C-RAF Binding to ^{WT}B-RAF Is Stimulated by RAS, Whereas Its Binding to Mutant B-RAF Is Constitutive

We also examined C-RAF binding to B-RAF. ^{WT}C-RAF does not bind to ^{WT}B-RAF unless ^{G12V}RAS is also present (Figure 3F). In contrast, ^{WT}C-RAF binds constitutively to ^{G466V}B-RAF and ^{G596R}B-RAF (Figure 3F). ^{G466V}B-RAF still binds to ^{KD}C-RAF, indicating that C-RAF kinase activity is not required for binding and confirming that ^{KD}C-RAF is a valid control in our kinase assays. Furthermore, ^{G466V}B-RAF still binds to ^{S338A}C-RAF, ^{Y341A}C-RAF, and $^{T491A/S494A}$C-RAF (Figure 3F), demonstrating that phosphorylation of the N region and activation segment is not required for binding, and that the reason that ^{G466V}B-RAF does not activate $^{T491A/S494A}$C-RAF is not because it cannot bind. Importantly however, ^{S621A}C-RAF does not bind to ^{G466V}B-RAF (Figure 3F), suggesting that 14-3-3 may mediate C-RAF binding to B-RAF.

Wild-Type B-RAF Activates C-RAF, but C-RAF Does Not Activate B-RAF

We have demonstrated that cancer-associated mutant forms of B-RAF activate C-RAF. However, an important question is whether ^{WT}B-RAF can also activate C-RAF and whether this occurs in normal cell signaling. To test this, we used ^{Y341A}C-RAF, because it can be activated by ^{G466V}B-RAF and ^{G596R}B-RAF (Figure 3A) and is not activated by either ^{G12V}RAS or ^{WT}B-RAF alone (Figure 4A). Significantly, when ^{G12V}RAS and ^{WT}B-RAF are coexpressed, they strongly activate ^{Y341A}C-RAF (Figure 4A). This shows that once it is activated by RAS, ^{WT}B-RAF can activate C-RAF. To further investigate the role played by ^{WT}B-RAF in C-RAF activation, we used RNA interference to deplete B-RAF in COS cells (Figure 4B). This causes a 40%–50% reduction in endogenous C-RAF activation by EGF (Figure 4B). Thus, B-RAF appears to contribute to growth-factor-mediated C-RAF activation in these cells.

We also performed the inverse experiment, testing whether C-RAF activates B-RAF. We used a version of C-RAF ($^{DD/ED}$C-RAF) in which Y340 and Y341 are substituted with aspartic acids to mimic N-region phosphorylation (Marais et al., 1997), and T491 and S494 are substituted with glutamic and aspartic acid respectively to mimic activation segment phosphorylation (Chong et al., 2001). $^{DD/ED}$C-RAF is highly active against MEK (Figure 4C), so we tested if it could activate B-RAF. We show that ^{WT}B-RAF is activated by ^{G12V}RAS, whereas

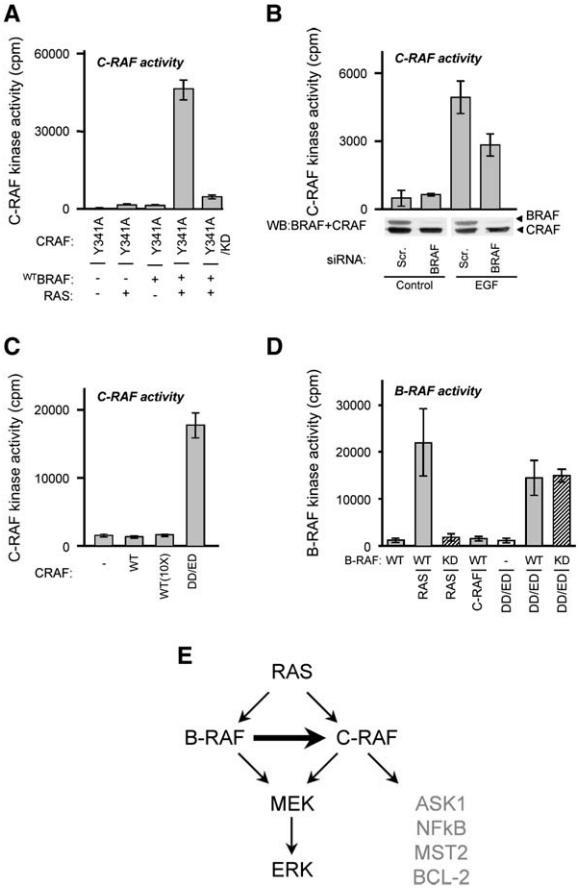

Figure 4. Wild-Type B-RAF Activates C-RAF

(A) ^{Y341A}C-RAF (Y341A) or kinase dead ^{Y341A}C-RAF (Y341A/KD) kinase activity in cells expressing ^{G12V}RAS (RAS) and/or ^{WT}B-RAF (WT). Results are from one experiment, assayed in triplicate with error bars to represent standard deviation from the mean.

(B) Endogenous C-RAF kinase activity in extracts from COS cells treated with scrambled (Scr) or B-RAF-specific siRNA and stimulated with EGF (10 ng/mL, 10 min), where indicated. Western blots for endogenous B-RAF and C-RAF are shown as controls for siRNA specificity. Results are from one experiment, assayed in triplicate with error bars to represent standard deviation from the mean.

(C) C-RAF kinase activity in extracts from cells expressing C-RAF (WT) or $^{DD/ED}$C-RAF (DD/ED). Where indicated, 10× extract was used when measuring C-RAF activity. Results are from one experiment, assayed in triplicate with error bars to represent standard deviation from the mean.

(D) B-RAF kinase activity from cells expressing HA-B-RAF (WT) or kinase dead B-RAF (KD) together with ^{G12V}RAS (RAS), ^{WT}C-RAF (C-RAF), or $^{DD/ED}$C-RAF (DD/ED). Results are from one experiment, assayed in triplicate with error bars to represent standard deviation from the mean.

(E) Cross signaling between RAF isoforms and other effector pathways. See text for details.

^{KD}B-RAF is not (Figure 4D), demonstrating that we are measuring B-RAF kinase activity in this assay. ^{WT}B-RAF is not activated by ^{WT}C-RAF (Figure 4D). We observe strong kinase activity associated with ^{WT}B-RAF when it is coexpressed with $^{DD/ED}$C-RAF, but there are similar levels of activity in the ^{KD}B-RAF controls (Figure 4D). We conclude that $^{DD/ED}$C-RAF does not activate ^{WT}B-RAF and that the activity we see in the ^{WT}B-RAF immunoprecipitates is from coprecipitating $^{DD/ED}$C-RAF. In summary, we show that activated wild-type B-RAF signals to C-RAF, contributing to its growth-factor-mediated activation, whereas C-RAF does not activate B-RAF.

Discussion

We have examined the mechanism underlying C-RAF activation by B-RAF. We show that C-RAF is not activated by feed-forward production of autocrine growth factors, but occurs in the cytosol in a RAS-independent manner. We show that B-RAF targets the C-RAF kinase domain and activates C-RAF in a manner that is partially independent of N-region phosphorylation but still requires activation segment and S621 phosphorylation. We find that C-RAF binds to B-RAF only under activating conditions, whereas mutant B-RAF binds to C-RAF constitutively. Finally, we show that wild-type B-RAF can also activate C-RAF. These data have important implications for our understanding of RAF signaling in cancer and normal cells.

Multistep Activation of C-RAF by B-RAF

In light of our data, we propose the following model for C-RAF activation by B-RAF. Under basal conditions, B-RAF is held in a "closed" conformation, preventing it from binding to C-RAF. When B-RAF is activated, it adopts an "open" conformation, allowing C-RAF to bind in a 14-3-3-dependent manner. B-RAF then activates C-RAF, which signals to MEK. Importantly, the mutations that occur in B-RAF also promote the "open" conformation and this allows constitutive binding to C-RAF in cancer cells, thereby stimulating persistent C-RAF and MEK signaling. Structural studies of B-RAF suggest that activation segment phosphorylation aligns key residues within the kinase domain for catalytic activity (Wan et al., 2004). We show that C-RAF activation segment phosphorylation is required for its activation by B-RAF. Furthermore, B-RAF kinase activity is required for C-RAF activation. Taken together, these results suggest that B-RAF induces activation segment phosphorylation on C-RAF either directly or indirectly through recruitment of another kinase.

Unlike the activation segment, the N region is less important for C-RAF activation by B-RAF than by RAS. N region charge is thought to relieve a negative function of the N terminus on the catalytic domain (Chong and Guan, 2003; Cutler et al., 1998), and may also stabilize the active conformation of the small lobe of the kinase domain (Wan et al., 2004). Our data suggests that B-RAF binding may serve both functions to stimulate C-RAF activation, with B-RAF acting as an allosteric inducer of these conformational changes in C-RAF. An important component of this mechanism is that B-RAF binding to C-RAF is mediated by 14-3-3 as previously established (Weber et al., 2001) and detailed here. Further studies will be required to investigate how the differential and dynamic regulation of 14-3-3 binding to the conserved C-terminal sites in C-RAF and B-RAF (Hekman et al., 2004) affects binding of B-RAF to C-RAF. Finally, as mentioned above, B-RAF may also stimulate activation segment phosphorylation on C-RAF. This multi-step process of C-RAF activation by B-RAF is analogous to the intricate nature of C-RAF activation by RAS. It also has similarities to the processes

underlying receptor tyrosine kinase signaling, which require ligand-induced dimerization and kinase domain transphosphorylation.

Cross-Signaling between RAF Isoforms

This work confirms the important role played by C-RAF in signaling by the impaired-activity B-RAF mutants. These mutants activate C-RAF 5- to 20-fold more efficiently than ^{G12V}RAS (Figure S1). We also find that the kinase activity of C-RAF is required for this mode of signaling because ^{KD}C-RAF acts as a dominant-negative to suppress impaired-activity mutant signaling to MEK (data not shown). Thus, C-RAF is not simply acting as a scaffold that facilitates signaling, but rather it participates in the signaling process, presumably by directly phosphorylating MEK. We also find that ^{V600E}B-RAF activates C-RAF (Figure S1), but this activity is not required for ERK activation because unlike the impaired-activity mutants, ^{V600E}B-RAF directly activates MEK in cells. These findings are supported by our previous study showing that siRNA-mediated C-RAF ablation blocks ERK activity in cancer cells harboring the impaired-activity B-RAF mutants, but not in cells expressing ^{V600E}B-RAF (Karasarides et al., 2004; Wan et al., 2004).

Our most striking finding is that activated wild-type B-RAF can activate C-RAF, and this appears to contribute to signaling to MEK downstream of EGF. ERK activation by growth factors is severely compromised in *B-raf* null mouse embryo fibroblasts (Pritchard et al., 2004), and this result was interpreted to show that B-RAF is the major RAF isoform that signals to ERK in these cells. However, our data suggest that loss of B-RAF could lead to a loss of C-RAF activation, which could also account for the reduction in ERK signaling. This work therefore identifies a new paradigm of RAF signaling. Previously, RAS was thought to signal to B-RAF and C-RAF, both of which then independently signal to MEK. This would provide redundancy and a mechanism by which different input signals could regulate MEK. We now show that in addition, B-RAF can also activate C-RAF downstream of RAS, but that C-RAF does not activate B-RAF. Thus, we have identified a RAS–B-RAF–C-RAF–MEK–ERK signaling pathway in addition to the RAS–B-RAF–MEK–ERK and RAS–C-RAF–MEK–ERK pathways previously identified (Figure 4E). What is the purpose of this intricate signaling network? One outcome could be that B-RAF can modify MEK activity through C-RAF to provide subtle regulation of signaling intensity or duration. Alternatively, B-RAF may utilize this pathway to signal to other C-RAF effectors such as apoptosis-signal-regulating kinase 1 (ASK1) (Chen et al., 2001), mammalian sterile 20-like kinase 2 (MST2) (O'Neill et al., 2004), the prosurvival factor BCL-2 (Wang et al., 1996), and the transcription factor NF-κB (see Cox and Der, 2003) (Figure 4E). Finally, because B-RAF can activate C-RAF in the cytosol, this may alter the C-RAF substrate spectrum by allowing it to signal to alternative effectors that are not accessible when it is activated at the plasma membrane. In conclusion, our data establish an unexpected facet of RAF signaling that appears to function in cancer cells and also in normal cells.

Experimental Procedures

Mammalian expression vectors for epitope-tagged human B-RAF and C-RAF have been described (Marais et al., 1997). ΔC-RAF consists of amino acids 325–648, and ΔB-RAF consists of amino acids 434–769. COS-7 cells were propagated, transfected, and extracted as described (Marais et al., 1997). RAF kinase activities were measured with GST-MEK, GST-ERK, and myelin basic protein (MBP) as sequential substrates (Marais et al., 1997), with 5 mM ATP for the MEK activation step. RAS-capture assays (Marais et al., 1998), microinjection assay (Marais et al., 1997), and cell-fractionation experiments (Chiloeches et al., 2001) were as described. B-RAF:CRAF complexes were detected using the HA antibody 12CA5 (5 μg) (Wan et al., 2004). Western blotting antibodies were as follows: mouse anti-HA (12CA5), mouse anti-myc (9E10), mouse anti-RAS (Y13-238), and rabbit anti-ERK2 (all from the ICR Hybridoma Unit); rat anti-C-RAF-phosphoS338 (Mason et al., 1999); mouse anti-phospho-ERK1/2 (Sigma); rabbit anti-phospho-MEK1/2 (Cell Signaling); and mouse anti-MEK1, mouse anti-C-RAF, and mouse anti-RAS (all from Transduction Laboratories).

Supplemental Data

Supplemental Data include one figure and can be found with this article online at http://www.molecule.org/cgi/content/full/20/6/963/DC1/.

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