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RAF Inhibitors Activate the MAPK Pathway by Relieving Inhibitory Autophosphorylation

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SUMMARY

ATP competitive inhibitors of the $BRAF^{V600E}$ oncogene paradoxically activate downstream signaling in cells bearing wild-type BRAF ($BRAF^{WT}$). In this study, we investigate the biochemical mechanism of wild-type RAF (RAF^{WT}) activation by multiple catalytic inhibitors using kinetic analysis of purified $BRAF^{V600E}$ and RAF^{WT} enzymes. We show that activation of RAF^{WT} is ATP dependent and directly linked to RAF kinase activity. These data support a mechanism involving inhibitory autophosphorylation of RAF's phosphate-binding loop that, when disrupted either through pharmacologic or genetic alterations, results in activation of RAF and the mitogen-activated protein kinase (MAPK) pathway. This mechanism accounts not only for compound-mediated activation of the MAPK pathway in $BRAF^{WT}$ cells but also offers a biochemical mechanism for BRAF oncogenesis.

INTRODUCTION

Gain-of-function mutations that lead to constitutive activation of the mitogen-activated protein kinase (MAPK) pathway are among the most common in human cancers. BRAF is mutated in ~50% of melanomas (Davies et al., 2002), and ATP-competitive small molecule inhibitors regress $BRAF^{V600E}$ melanomas, but not wild-type RAF (RAF^{WT}) tumors (Flaherty et al., 2010). Not only are RAF^{WT} cancers refractory, but RAF inhibitors paradoxically increase phosphorylation of downstream effectors MEK and ERK and induce proliferation in cells with upstream RAS mutations, which is thought to occur through an allosteric mechanism involving RAF dimerization (Carnahan et al., 2010; Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010). Potentially through a related mechanism, mutations in the phosphate-binding loop (P loop) of BRAF have been characterized, which render BRAF catalytically impaired yet stimulate phosphorylation of downstream targets (Wan et al., 2004). However, the underlying biochemistry causing these phenomena has yet to be fully understood.

RESULTS

To explore the mechanism of paradoxical activation in RAF^{WT} cells, a panel of selective tool compounds (Cmpds A–G) with a wide range of potencies against $BRAF^{V600E}$ (Figure 1A; Figure S1A available online) was tested for activity in both A375 ($BRAF^{V600E}$ mutated) and SW620 ($BRAF^{WT}$) cell lines. As expected, all compounds inhibited phosphorylation of the downstream MAPK pathway target (pERK) and proliferation in A375 cells (Figure 1B). However, in SW620 cells, each compound activated ERK in a biphasic pattern with maximal pathway activation occurring at low compound concentrations and modest pathway inhibition at the highest concentrations. This correlated closely with cell proliferation (also shown previously by others; Carnahan et al., 2010; Hatzivassiliou et al., 2010; Poulikakos et al., 2010).

Significance

Data from clinical trials with RAF inhibitors demonstrate dramatic regression of melanomas carrying the $BRAF^{V600E}$ oncogene but not in $BRAF^{WT}$ tumors. Preclinical studies show that $BRAF^{WT}$ cell lines are not only refractory but paradoxically activate the mitogen-activated protein kinase (MAPK) pathway and proliferate when treated with ATP-competitive RAF inhibitors. This phenomenon is thought to explain the lack of efficacy in $BRAF^{WT}$ cancers, as well as emergence of spontaneous cutaneous tumors in patients treated with RAF inhibitors. In this study, we uncover an autoregulatory mechanism of RAF kinases that is modulated by catalytic RAF inhibitors and bypassed by BRAF oncogenes. This work has significant implications for the development and therapeutic use of RAF inhibitors for targeting the MAPK pathway.

A

Compound	$BRAF^{V600E}$ IC_{50} (nM)	$BRAF^{WT}$ IC_{50} (nM)	CRAF IC_{50} (nM)	A375 pERK EC_{50} (nM)	A375 Cell Proliferation EC_{50} (nM)
Cmpd A	0.05	0.04	0.10	9	17
Cmpd B	0.06	0.07	0.09	3	25
Cmpd C	0.13	0.20	0.15	14	44
Cmpd D	0.18	0.16	0.54	34	411
Cmpd E	0.33	0.18	0.86	76	376
Cmpd F	0.34	0.29	0.73	118	623
Cmpd G	0.58	0.38	2.02	159	1186

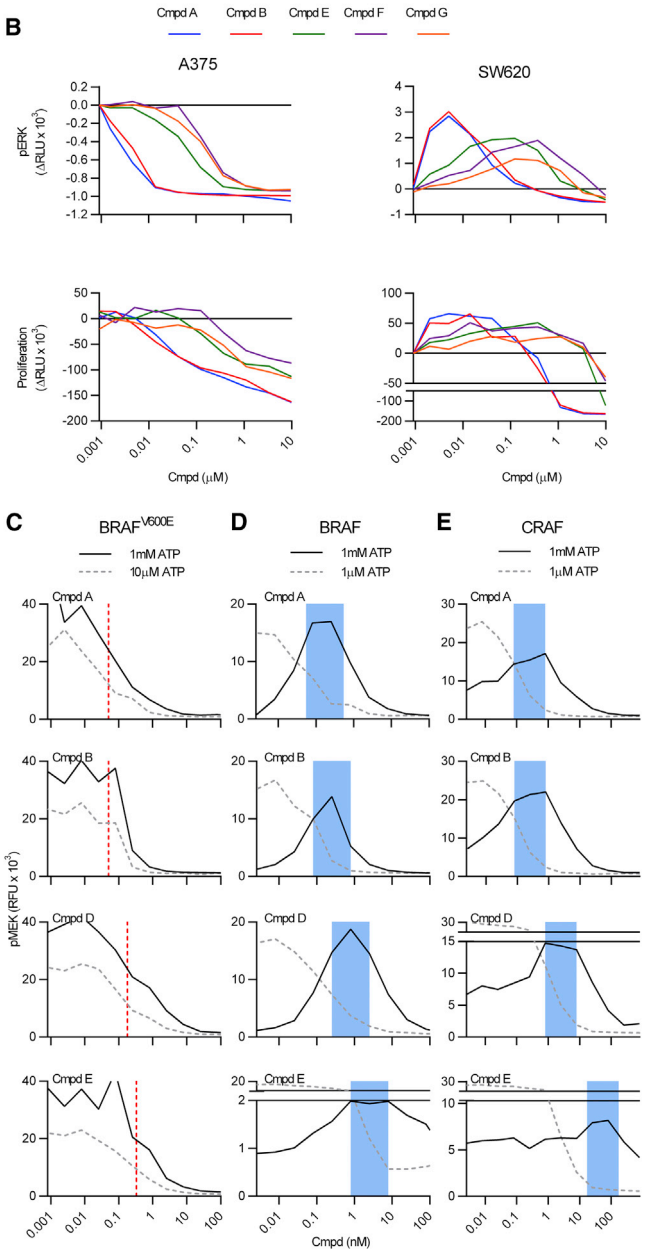

Figure 1. Potent Catalytic $BRAF^{V600E}$ Inhibitors Are Potent RAF^{WT} Activators In Vitro and In Vivo

(A) Biochemical and cellular potencies for compounds.

(B) A375 (*$BRAF^{V600E}$*) and SW620 (*$BRAF^{WT}$* and *$KRAS^{G12V}$*) were treated with increasing compound concentrations (Cmpd). pERK was measured after 3 hr of treatment, and cell viability was measured after 4 days. All values expressed as change from DMSO-treated cells.

(C–E) Purified RAF kinase domains were incubated with inhibitor. MEK phosphorylation was measured after adding ATP. Compounds are shown in order of potency for $BRAF^{V600E}$ (C) inhibition. Averaged IC_{50} values at 10 μM ATP for each compound is depicted by a vertical red dotted line. MEK phosphorylation measured for $BRAF^{WT}$ (D) and CRAF (E) at 1 μM ATP (gray dotted line) and 1 mM ATP (black line). Peak enzyme activities shaded in blue to highlight potency for RAF activation.

See also Figure S1.

Of note, compound potency for MAPK induction in SW620 correlated well with compound inhibition half-maximal effective concentration (EC_{50}) values in A375; the most potent $BRAF^{V600E}$ inhibitors in A375 (Cmpd A and Cmpd B) activate the MAPK pathway at the lowest concentrations in SW620. The observation that catalytic RAF inhibitors block growth of *$BRAF^{V600E}$* cells but stimulate growth in *$BRAF^{WT}$* with the same relative potencies led to the hypothesis that RAF^{WT} may negatively regulate the MAPK pathway through a RAF kinase-dependent mechanism.

Compound Activation of $BRAF^{WT}$ and CRAF Is ATP Dependent

Having identified a correlation between compound potency for $BRAF^{V600E}$ inhibition and potency of RAF^{WT} activation in cells, we investigated if such a correlation is recapitulated biochemically using isolated RAF proteins. A panel of RAF compounds was screened for their activity against purified $BRAF^{V600E}$, $BRAF^{WT}$, and $CRAF^{WT}$ kinase domains. All compounds blocked $BRAF^{V600E}$ phosphorylation of MEK (Figure 1C; Figure S1B). Each compound also inhibited $BRAF^{WT}$ and CRAF enzymes but only at a subphysiological ATP concentration (1 μM; Figures 1D, 1E, and S1B). It is surprising that adding compound to $BRAF^{WT}$ and CRAF at physiological ATP concentrations promoted MEK phosphorylation in a biphasic manner, reminiscent of the cellular phenomenon. Although previous studies demonstrate that CRAF undergoes rapid inhibition following activation (Stokoe and McCormick, 1997), the mechanism is unknown. As shown in Figure 2A, at conditions approaching cellular ATP concentrations of 1–5 mM (Traut, 1994) the basal activity of both RAF^{WT} isoforms was greatly diminished, in contrast to $BRAF^{V600E}$, which increased activity at 1 mM ATP. These data suggest that RAF inhibition is ATP dependent. Furthermore, compound potency for $BRAF^{WT}$ and CRAF stimulation correlated with compound half-maximal inhibitory concentration (IC_{50}) for $BRAF^{V600E}$ inhibition, suggesting again that compound activation of RAF^{WT} may be linked directly to inhibition of RAF kinase.

$BRAF^{WT}$ and CRAF Are Autoinhibited at Cellular ATP Concentrations

The activating V600E substitution in BRAF appears to alter the apparent $Km_{(ATP)}$ [$Km_{(ATP)app}$] (Hatzivassiliou et al., 2010). Our observation that basal kinase activities of $BRAF^{WT}$ and CRAF were diminished at physiological ATP concentrations suggests additional kinetic differences between $BRAF^{V600E}$ and $BRAF^{WT}$. Whereas $BRAF^{V600E}$ reached a maximum velocity with a calculated $Km_{(ATP)app}$ of 44 μM, initial rates of catalysis for $BRAF^{WT}$ and CRAF were dramatically reduced at ATP concentrations above 40 μM (Figure 2A). The catalytic rates of both WT enzymes did not fit a standard hyperbolic model; thus, we were unable to

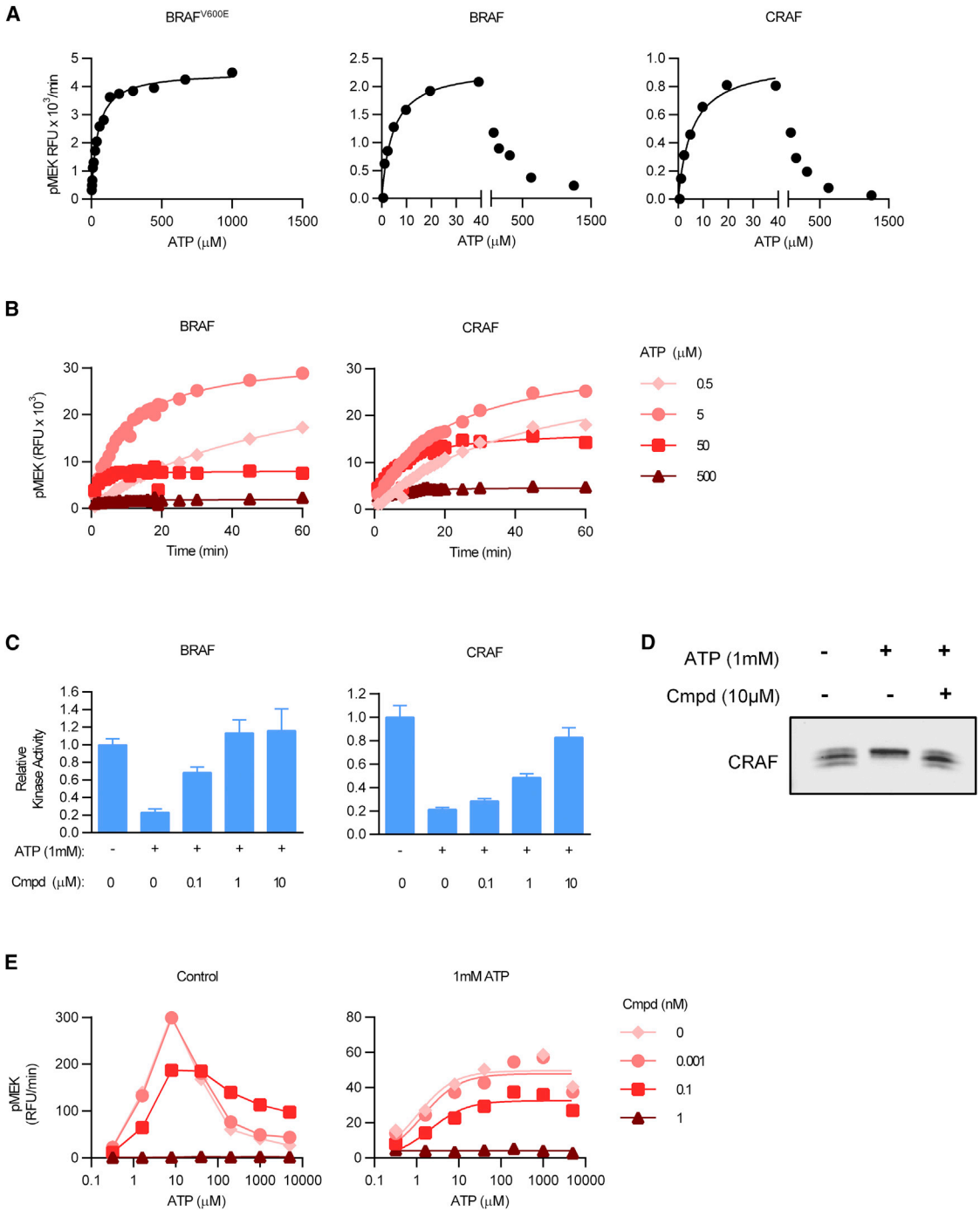

Figure 2. RAF^{WT} Autoinhibition Is Rescued by Catalytic Inhibitors

(A) Enzyme velocity (rate of MEK phosphorylation versus ATP concentration) for BRAF^{V600E}, BRAF^{WT}, and CRAF^{WT} kinase domains. $Km_{(ATP)app}$ was calculated for BRAF^{V600E} using a standard hyperbolic curve fit [$Km_{(ATP)app}$ = 44 μM]. Fitted curves are shown for BRAF^{WT} and CRAF, excluding values above 40 μM ATP.

(B) Reaction progress curves for BRAF and CRAF kinase domain.

(C) 50 pM BRAF^{WT} or 4 pM CRAF kinase domains were incubated with 1 mM ATP and Cmpd E for 1 hr. Excess ATP was removed over buffer exchange column. Initial rates of MEK phosphorylation measured at 1 μM ATP, relative to untreated controls. Error bars denote 95% CI.

(D) CRAF kinase domain (20 nM) was incubated with 1 mM ATP and Cmpd E and run on a 10% SDS-Tris-glycine gel and stained with Coomassie blue.

(E) CRAF kinase domain was treated with 1 mM ATP or no ATP buffer control before removing excess ATP by buffer exchange column. Initial enzyme rates were then measured in the presence of Cmpd C and ATP. Preincubation in 1 mM ATP reduced apparent K_{cat} by more than 85% compared to control. $Km_{(ATP)app}$ values after ATP treatment were consistent with published values for CRAF (Hatzivassiliou et al., 2010).

See also Figure S2.

determine a $Km_{(ATP)app}$ for $BRAF^{WT}$ and CRAF. Although other kinases such as KSR have been shown to modulate RAF activity and affect paradoxical activation (Brennan et al., 2011; McKay et al., 2011), KSR was not observed with the purified RAF preparations. All wild-type RAF constructs tested were inactivated at high concentrations of ATP, including BRAF kinase domain isolated to a single band (>95% purity; Figure S2A), suggesting a RAF-autonomous mechanism.

Although the initial enzymatic rate only appeared diminished at concentrations approaching 100 μM ATP, evidence of the catalytic rate change was observed for both $BRAF^{WT}$ and CRAF even at 0.5 μM ATP (Figure 2B), suggesting that RAF autophosphorylation occurs, albeit slowly, at concentrations below the observed initial rate change around 40 μM ATP. The rate change occurred faster as ATP increased and was nearly instantaneous at the highest concentrations of ATP, causing the observed initial enzymatic rate to appear diminished. These data strongly suggest that $BRAF^{WT}$ and CRAF are negatively regulated by autophosphorylation.

To determine if RAF catalytic activity is required, $BRAF^{WT}$ and CRAF were incubated at 1 mM ATP to promote inhibition. Excess ATP was then removed before kinase activity was measured by adding MEK substrate and a lower ATP concentration allowing for maximal RAF^{WT} activity. Preincubation with 1 mM ATP resulted in >80% inhibition of either $BRAF^{WT}$ or CRAF, indicating that inhibition is a stable modification and is not reversed after removing ATP (Figure 2C). Therefore, inhibition of RAF^{WT} is not transient but is consistent with a covalent modification such as phosphorylation. An electrophoretic mobility shift of CRAF was also observed only after ATP treatment and without compound (Figure 2D), again consistent with a phosphorylation event. Finally, to determine whether RAF kinase activity is required for ATP inhibition, a selective inhibitor (Cmpd E) was added to the preincubation step and then removed along with excess ATP (due to the uniquely low compound dissociation half-life of <10 min). Preincubation with compound blocked RAF inhibition (Figure 2C) and prevented CRAF mobility shift (Figure 2D). Therefore, ATP and RAF catalytic activity are required for inhibition and putative phosphorylation of WT enzymes. Together, these data strongly support an inhibitory autokinase mechanism regulating RAF^{WT} activity.

RAF Compounds Relieve Autoinhibition of RAF^{WT}

Since compound-mediated activation and autoinhibition of RAF are readily observed at cellular concentrations of ATP, we hypothesized that RAF compounds activate RAF^{WT} by relieving RAF autoinhibition. If correct, RAF inhibitors should reduce rates of MEK phosphorylation at subphysiological ATP concentrations but rescue activity at higher ATP concentrations. Indeed, small amounts of a potent RAF inhibitor decreased the catalytic rate of CRAF at low ATP concentrations (Figure 2E). However, at 200 μM and 1 mM ATP, the RAF inhibitor partly rescued CRAF autoinhibition. These data demonstrate that RAF compounds block MEK phosphorylation under conditions in which CRAF autoinhibition is not readily observed and suggests that the apparent activation of CRAF by compound treatment takes place through partial rescue of CRAF autoinhibition.

If compound activation of RAF^{WT} can be explained through preventing autophosphorylation, RAF compounds should neither reverse autoinhibition nor activate the autoinhibited form of enzyme. Rather, compound should only reduce the remaining catalytic activity of the autoinhibited form of RAF. To test this, rates of MEK phosphorylation were measured under several ATP and inhibitor concentrations and compound using both autoinhibited and control CRAF enzymes. Consistent with our hypothesis, autoinhibited CRAF exhibited reduced catalytic activity with standard Michaelis Menten kinetics. Autoinhibited CRAF was not further inhibited by high ATP concentrations but reached an apparent V_{max}, thereby making calculations for kinetic rate constants possible (Figure 2E). Furthermore, although pretreatment with ATP did not affect compound potency (Figure S2B), the compound did not stimulate CRAF kinase activity at any tested concentration of compound or ATP but only decreased the enzymatic rate in a manner consistent with ATP-competitive inhibitor kinetics (Figure 2E), demonstrating that a catalytic RAF inhibitor cannot activate the autoinhibited form of CRAF.

P-Loop Autophosphorylation

Preliminary mass spectrometry experiments of BRAF and CRAF proteins incubated in 1 mM ATP revealed two phosphorylated peptides that reside in the DFG activation loop and the P loop (Figures S3A–S3F). To confirm specific inhibitory phosphorylation site(s), alanine substitutions were made for each serine in each domain, as well as phosphomimetic (aspartic acid or glutamic acid) substitutions and relative kinase activities were measured. Phosphomimetic substitutions in the activation loop of BRAF and CRAF showed no inhibition but were slightly activating with the exception of $CRAF^{S499D}$ (Figure 3A). However, BRAF P-loop mutations S465D and S467E had reduced kinase activity, suggesting that phosphorylation of these serines may be inhibitory. Curiously, $BRAF^{S465D}$ and $BRAF^{S467E}$ caused only partial inhibition, raising the question of whether phosphorylation at both sites may be required for complete inactivation. This hypothesis is further supported by the tandem mass spectrometry spectra, which showed phosphorylation of both serines in the P loop (Figures S3A–S3B, S3E, and S3F). Alternatively, $CRAF^{S359D}$ blocked nearly all activity, yet $CRAF^{S357D}$ had almost no effect when compared to the alanine mutation at the same site (Figure 3A), indicating that S359 may be the primary site of inhibitory phosphorylation. Each alanine-substituted enzyme was assayed under low and high ATP concentrations as well (1 μM ATP and 1 mM ATP, respectively). Although both WT enzymes and each of the activation-loop mutants were inhibited at 1 mM ATP, $BRAF^{S465A}$, $BRAF^{S467A}$, and $CRAF^{S359A}$ showed higher catalytic activity at 1 mM ATP (Figure 3B). Further profiling showed that both BRAF P-loop mutations largely rescued ATP-dependent autoinhibition, and the $CRAF^{S359A}$ mutation completely reversed the effect while $CRAF^{S357A}$ had no effect (Figure S3G), demonstrating that serine residues in the P loop of both enzymes are required for RAF autoinhibition.

To test if P-loop phosphorylation is required for compound activation, alanine mutants and WT controls were tested at 1 mM ATP. As predicted, $BRAF^{S465A}$ and $BRAF^{S467A}$ had higher catalytic activity than $BRAF^{WT}$ and were inhibited and not activated by RAF inhibitor (Figure 3C), demonstrating that both S465 and S467 are required for compound activation of BRAF. Similar results were observed for $CRAF^{S359A}$. To confirm

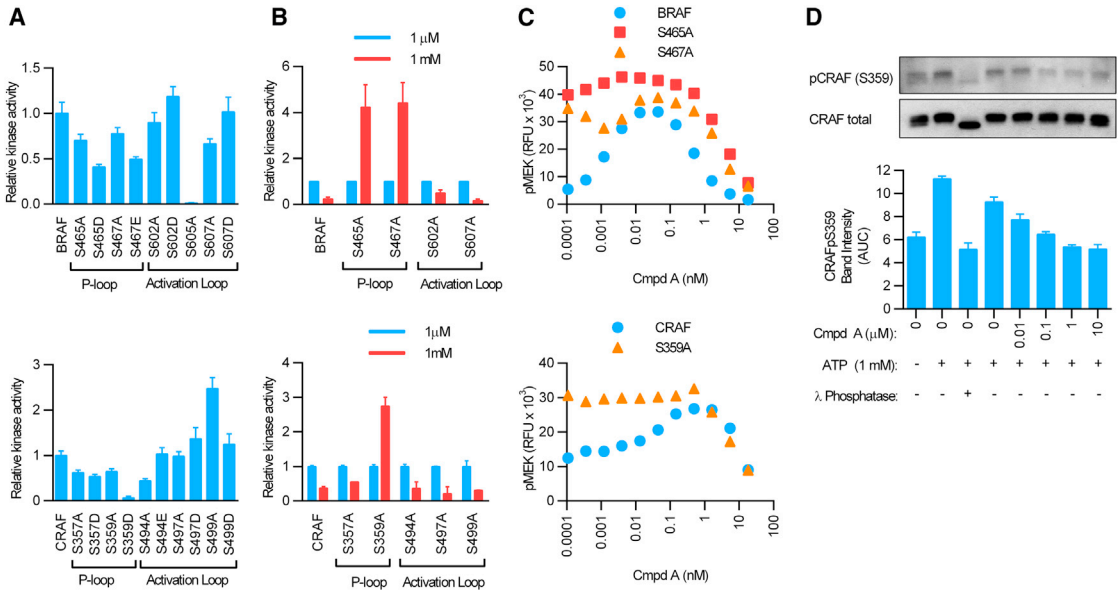

Figure 3. RAF^{WT} Is Autoinhibited through P-Loop Phosphorylation

(A) Serine-to-alanine, aspartic acid, or glutamic acid point mutations were introduced into the P loop and activation loop of BRAF and CRAF. Initial rates of MEK phosphorylation was measured at 10 μM ATP and normalized to WT control. Error bars denote 95% confidence interval (CI).

(B) MEK phosphorylation measured at 1 μM and 1 mM ATP for alanine substitution mutants. Initial enzymatic rates normalized to enzyme activity at 1 μM ATP. Error bars denote 95% CI.

(C) Purified enzymes were treated with Cmpd A and 1 mM ATP.

(D) Kinase domain CRAF (50 nM) was incubated under the indicated conditions for 1 hr. The resulting protein was run on an SDS gel, and immunoblots for pCRAF S359 and for total CRAF (anti-$(His)_6$-tag) are shown. Bands were semiquantified by image intensity area under the curve. Error bars denote SEM.

See also Figure S3.

P-loop phosphorylation, a polyclonal antibody was raised against the CRAF phospho-S359 peptide and affinity purified. Purified CRAF kinase domain was treated with ATP and immunoblotted using the phospho-specific antibody. CRAF S359 phosphorylation increased after treatment with ATP (Figure 3D) and diminished after λ phosphatase treatment. Phosphorylation was also prevented by cotreatment with a RAF inhibitor, again confirming that CRAF S359 is indeed an autophosphorylation site.

CRAF S359 Is Required for Compound Activation in Cells

To confirm P-loop phosphorylation of endogenous CRAF, SW620 cells (*KRAS*^{G12V}) were treated with a RAF inhibitor and CRAF was immunoprecipitated from cell lysates. Compound treatment inhibited CRAF S359 phosphorylation (Figure 4A), consistent with the hypothesis that CRAF S359 is an endogenous autophosphorylation site. To test the functional role of P-loop phosphorylation in cells, HEK293 cells expressing cMyc-epitope-tagged CRAF or CRAF^{S359A} point mutant were treated with RAF inhibitor. Overexpression of CRAF^{WT} increased the ability of compound to stimulate pERK above levels in endogenous, control cells expressing green fluorescent protein (GFP) (Figure 4B). However, compound activation was not observed in CRAF^{S359A}-expressing cells, demonstrating that CRAF S359 phosphorylation is required. Furthermore, basal pERK was elevated in CRAF^{S359A}-expressing cells compared to overexpressed CRAF^{WT}, consistent with the hypothesis that S359 is an inhibitory phosphorylation site.

BRAF P-Loop Mutations Disrupt Compound Activation

Although CRAF overexpression is sufficient to mediate compound activation in HEK293 cells, similar experiments with BRAF did not promote compound activation of pERK (Figure S4). However, *BRAF* mutations have previously been identified in patient tumor samples that cover nearly every residue of the P loop, including serine 467 (Akslen et al., 2005), and mutations of the neighboring glycines (464, 466, and 469) are among the most frequently occurring non-V600E *BRAF* mutations (Davies et al., 2002). This suggested to us not only that the P loop is an important regulatory domain but also that mutations in this region may activate BRAF by relieving or preventing P-loop autophosphorylation. To test if glycine mutations disrupt P-loop autophosphorylation, we cloned and purified BRAF kinase domains with a G464V or G464E mutation. Both enzymes were highly active and displayed no signs of ATP-dependent autoinhibition (Figure 5A). $Km_{(ATP)app}$ values were calculated and were equivalent to the $Km_{(ATP)app}$ for BRAF^{V600E}. Furthermore, treatment with compound did not activate either BRAF^{G464V} or BRAF^{G464E} in the presence of low (1 μM) or high (1 mM) ATP concentration, suggesting again that autoinhibition through P-loop phosphorylation is required for compound activation in vitro (Figure 5B).

Having shown that BRAF^{G464V} and BRAF^{G464E} disrupt P-loop autoinhibition and compound activation in vitro, we wanted to test if this is also true in a cellular system. Because compound activation also appears to be exaggerated in context of activated RAS (Carnahan et al., 2010; Hatzivassiliou et al., 2010; Poulikakos et al., 2010), two cell lines were identified with co-occurring *BRAF*

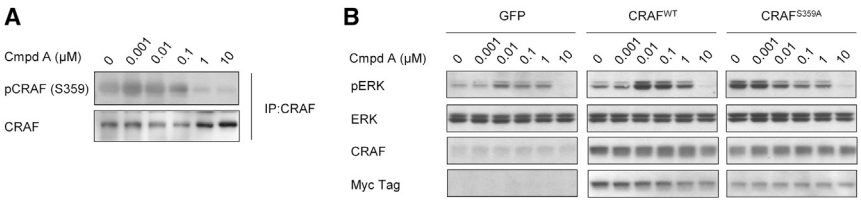

Figure 4. CRAF S359 Phosphorylation Is Required for Compound Activation in Cells

(A) SW620 cells were treated with Cmpd A. Total endogenous CRAF was immunoprecipitated and then immunoblotted for total and pCRAF S359.

(B) HEK293 cells expressing full-length $CRAF^{WT}$ or $CRAF^{S359A}$ point mutant harboring a Myc epitope tag or expressing GFP as a control were treated with Cmpd A for 1 hr. Cell lysates were immunoblotted for pERK, total ERK, total CRAF, and Myc tag.

See also Figure S4.

G464 and *KRAS* mutations. As predicted, treatment with RAF inhibitor reduced pERK levels, and no activation was observed in HEY-A8 ($KRAS^{G12D}$/$BRAF^{G464E}$) and MDA-MB-231 ($KRAS^{G13D}$/$BRAF^{G464V}$) cells (Figure 5C), suggesting that P-loop phosphorylation of BRAF is critical for compound activation in cells.

Inhibition of P-Loop Phosphorylation Rescues Catalytic Activity of RAF Monomers

Previous studies have shown that RAF inhibitors promote BRAF/CRAF coimmunoprecipitation and promote association with RAS (Hatzivassiliou et al., 2010; Heidorn et al., 2010), and it is therefore thought that induced dimerization and/or association with RAS may contribute to the paradoxical activation. However, although treatment with Cmpd A promoted coimmunoprecipitation of the BRAF/CRAF complex, no paradoxical activation was observed in HEY-A8 or MDA-MB-231 cells (Figure 5C), suggesting that any compound-induced dimerization was insufficient to activate endogenous RAF. Similar experiments were performed with SW620 cells treated with RAF inhibitor, which increased pMEK/pERK levels and promoted BRAF/CRAF and RAF/RAS coimmunoprecipitation as expected (Figure 6A). However, these phenomena were frequently not correlated. Figure 6A shows that .01–.1 μM of inhibitor was sufficient to strongly elevate pMEK and pERK, yet little or no BRAF/CRAF coimmunoprecipitation was observed until relatively high inhibitor concentrations were used (between 0.3 and 3 μM). Similar observations were made for RAS/RAF coimmunoprecipitation, suggesting that most cellular RAF is cytoplasmic and monomeric at inhibitor concentrations that cause very strong pathway activation. This observation suggests either that very low amounts of induced dimerization and RAS association is sufficient for pathway activation or that another mechanism contributes to paradoxical activation in cells.

To test the relationship between dimerization and catalytic activity, point mutations of the putative RAF dimer interface (Poulikakos et al., 2010; Rajakulendran et al., 2009), which disrupt dimerization, were introduced into the kinase domains of BRAF and CRAF ($CRAF^{R401A}$ and $BRAF^{R509A}$). Consistent with results published by others (Röring et al., 2012; Baljuls et al., 2011), arginine-to-alanine substitution ablated the kinase activity of the purified enzyme (Figure 6B), suggesting that monomeric BRAF and CRAF are inactive. However, $BRAF^{V600E/R509A}$ had similar activity to $BRAF^{V600E}$, demonstrating that monomeric $BRAF^{V600E}$ is active. Since $BRAF^{V600E}$ also does not respond to inhibitory P-loop autophosphorylation, we hypothesized that disrupting P-loop phosphorylation, even of RAF monomers, may be sufficient to strongly activate the pathway. Although $CRAF^{R401A}$ was inactive at all concentrations of ATP tested, mutating the P-loop autophosphorylation site rendered CRAF unresponsive to ATP-dependent autoinhibition and strongly rescued kinase

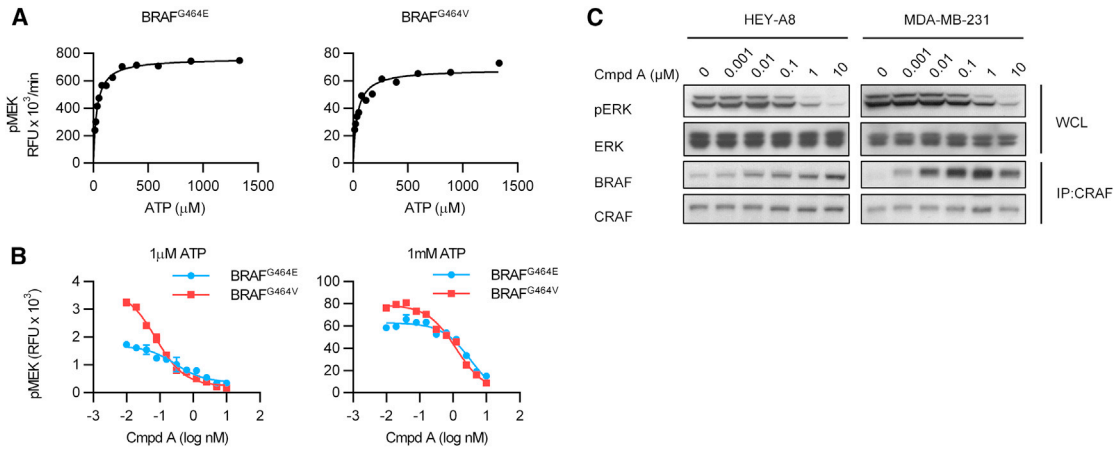

Figure 5. P-Loop Glycine Mutations Prevent Autoinhibition and Constitutively Activate BRAF

(A) $Km_{(ATP)app}$ was calculated for purified $BRAF^{G464V}$ [$Km_{(ATP)app}$ = 38 μM] and $BRAF^{G464E}$ [$Km_{(ATP)app}$ = 33 μM] kinase domains.

(B) Cmpd A IC_{50} values were calculated for $BRAF^{G464V}$ and $BRAF^{G464E}$ at 1 μM and 1 mM ATP (Cmpd A IC_{50} calculated at 1 μM ATP, $BRAF^{G464V}$ = .07 nM, and $BRAF^{G464E}$ = 0.3 nM).

(C) *KRAS* mutant cells with co-occurring *BRAF* P-loop mutations (HEY-A8 and MDA-MB-231) were treated with Cmpd A. Increased coimmunoprecipitation of BRAF with CRAF is shown (IP:CRAF) together with pERK immunoblots from whole cell lysate (WCL).

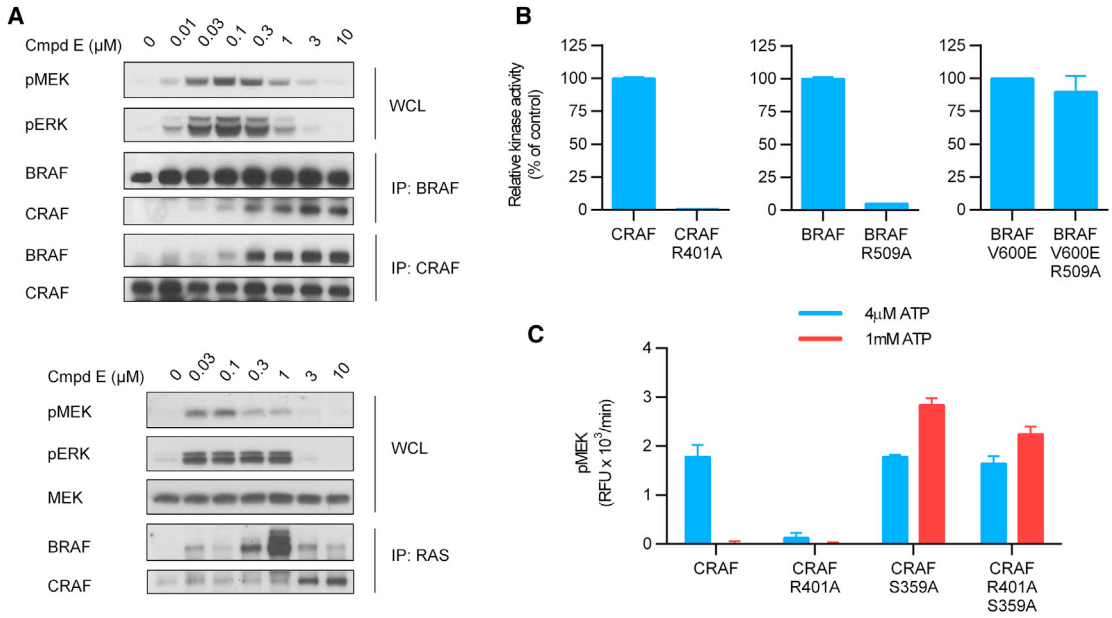

Figure 6. Loss of P-Loop Phosphorylation Rescues Catalytic Activity of RAF Monomers

(A) SW620 cells were treated with Cmpd E for 1 hr. Immunoblots of pMEK, pERK from whole cell lysates (WCL), and immunoblots of BRAF/CRAF from immunoprecipitatated (IP) proteins are shown.

(B) Relative kinase activity of dimer-impaired CRAF (R401A), BRAF (R509A), and $BRAF^{V600E}$ (R509A) (pMEK relative fluorescent units % of control). Error bars denote SEM.

(C) Catalytic rates of MEK phosphorylation were measured for CRAF constructs at 4 μM and 1 mM ATP. CRAF S359A restores catalytic activity of dimer-impaired CRAF ($CRAF^{R401A/S359A}$). Error bars denote SEM.

activity of the R401A mutant (Figure 6C), suggesting that preventing autoinhibition through blocking P-loop autophosphorylation is sufficient to strongly activate CRAF monomers.

DISCUSSION

These findings demonstrate that P-loop autophosphorylation is a critical mechanism regulating RAF kinase activity. Although P-loop autophosphorylation has not been described previously, P-loop phosphorylation is known to inhibit several other kinases. Tyr 15 phosphorylation of CDK1 (Gould and Nurse, 1989; Morla et al., 1989) and CDK2 (Welburn et al., 2007) inhibits kinase activity through steric blockade of ATP and/or peptide substrate binding. A similar phenomenon has been observed for BCR-ABL P-loop phosphorylation, though the structural mechanism of inhibition is unknown but likely differs from CDK regulation (Skaggs et al., 2006). In the case of BRAF and CRAF, P-loop phosphorylation did not alter the $Km_{(ATP)app}$ (Figures 2E and S3G), suggesting that ATP binding is unaffected though blocked peptide substrate binding, as in the case of CDK2, is a possibility.

P-loop phosphorylation may also have consequences for activation-loop conformation. The BRAF crystal structure shows an interaction between the P loop and the DFG activation loop through F468 and V600 (Wan et al., 2004). Phosphorylation of T599 and S602 (or the V600E mutation) is thought to destabilize this interaction, allowing a shift to the "DFG-in" conformation, which is required for kinase activity (Chong et al., 2001; Kolch et al., 1993). Phosphorylation of the P loop at BRAF S465/S467 (CRAF S359) may then affect the activation-loop/P-loop interaction, thereby stabilizing the inactive, DFG-out confirmation. Similarly, since $BRAF^{V600E}$, $BRAF^{G464E}$, and $BRAF^{G464V}$ were neither activated by RAF inhibitor nor sensitive to autoinhibition, this suggests that the mechanism of oncogene activation may be not only to mimic T599/S601 phosphorylation but also to render the enzyme unresponsive to P-loop autophosphorylation.

We propose that RAF^{WT} exists in an autoinhibited state at physiological conditions through autophosphorylation of the P loop and a competing reaction such as a phosphatase must be required to frequently renew the pool of active RAF within the cell. Therefore, catalytic inhibition of RAF would shift the equilibrium to stabilize the active form by relieving RAF autoinhibition. The inhibitor potency shift observed in RAF^{WT} cells could therefore be explained by the very high concentrations of RAF compound required to occupy both RAF molecules and inhibit MAPK signaling in RAF^{WT} cells.

This model offers a complete explanation for the activation of RAF kinase by catalytic inhibitors. However, some aspects are consistent with previous models. Heidorn et al. initially reported that expression of catalytically inactive BRAF phenocopies RAF compound treatment and can promote tumor progression in KRAS-mutated cells. They state that only BRAF-isoform-selective inhibitors activate the pathway and invoke a mechanism involving BRAF-mediated regulation of CRAF (Heidorn et al., 2010). Although MAPK activity in KRAS-mutated cells appears to be mediated by CRAF (Hatzivassiliou et al., 2010; Poulikakos et al., 2010), we show that pan RAF inhibitors with high potency for CRAF inhibition strongly promote MEK phosphorylation

in vitro and in cells (Figures 1 and S1B). Poulikakos et al. showed that RAF dimer formation is required for compound activation and hypothesized that binding of compound to one RAF molecule within a RAF dimer transactivates the adjacent RAF protomer through a kinase-independent mechanism. If transactivation in cells is entirely allosteric, it must then persist after inhibitor dissociation, as no inhibitor is present during cell washes, lysis, immunoprecipitation, and subsequent kinase assay. An alternative explanation, which our data support, is that activation involves a covalent modification. While these mechanisms are not mutually exclusive, inhibitor treatment did not activate autoinhibited RAF kinase in vitro (Figure 2E), compound-induced dimerization alone was not sufficient to activate the pathway in cells (Figure 5C), and disrupting P-loop phosphorylation was sufficient to activate RAF monomers (Figure 6C). Whereas RAS activity and dimerization likely play a role, these results suggest that inhibitory P-loop autophosphorylation is dominant to conformational effects of RAF dimers binding compound.

Inhibition of P-loop phosphorylation may also have adverse clinical consequences. Three different clinical RAF inhibitors, Vemurafenib, XL281, and Sorafenib, promoted dermal squamous cell carcinoma (SCC) and keratoacanthomas (Flaherty et al., 2010; Schwartz et al., 2009; Dubauskas et al., 2009), which are likely caused by RAF compound activation of the MAPK pathway in *BRAF*^{WT} skin (Carnahan et al., 2010; Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010). Although SCC is not currently a dose-limiting toxicity for melanoma patients, efficacy is primarily limited due to acquired resistance to RAF drugs. The median progression free survival for melanoma patients taking Vemurafenib is limited to 8 months (Flaherty et al., 2010). In-vitro-acquired resistance modeling shows that *BRAF*^{V600E} mutant melanoma cells become dependent on drug for sustained pERK and cell growth (Tap et al., 2010) through activation of upstream signaling or increased RAF^{WT} expression (Villanueva et al., 2010; Nazarian et al., 2010). Furthermore, a recent report indicates that RAF inhibitors may promote secondary melanomas (Zimmer et al., 2012). RAF inhibitors themselves may, in fact, be promoting tumor growth by relieving RAF autoinhibition.

EXPERIMENTAL PROCEDURES

Biochemical Kinase Assays

RAF enzyme and MEK substrate proteins were isolated from baculoviral expression in Sf9 cells via $(His)_6$ tag and Ni-affinity chromatography. Activity for BRAF and CRAF semipurified protein was determined by AlphaScreen (PerkinElmer) as described elsewhere (Tsai et al., 2008). Enzyme concentrations used were as follows: BRAF (kinase domain), 50 pM; BRAF^{V600E} (kinase domain), 100 pM; CRAF (kinase domain), 4 pM; MEK1 (kinase dead), 20 nM, unless otherwise stated. Compounds were diluted in dimethyl sulfoxide (DMSO) (2.5% final concentration). Zebda Spin Desalting Columns (Pierce) were used to remove excess ATP and compound.

Cell Culture and Compounds

All compounds were provided by Novartis Global Discovery Chemistry or the Genomics Institute of the Novartis Research Foundation and were dissolved in DMSO (0.2% final concentration). Cell proliferation was measured using Cell Titer-Glo (Promega). pERK levels were measured by sandwich immunoassay (Meso Scale Discovery). The following primary antibodies were used for immunoblots: ARAF, BRAF, and KRAS (all from Santa Cruz Biotechnology); CRAF (BD Transduction Laboratories); pCRAF S359 (Covance); MEK1/2, pMEK1/2$^{Ser217/221}$, ERK1/2, pERK1/2$^{Thr202/Tyr204}$, and Myc Tag (all from Cell Signaling Technology); $(His)_6$ (QIAGEN); and for immunoprecipitations, BRAF (BD Transduction Laboratories), CRAF (Abcam), and RAS (Millipore).

Expression Constructs

pBlueBac4.5 (Invitrogen) vectors were used for baculoviral expression. BRAF^{WT} and BRAF^{V600E} catalytic domains (residues 437–765) each contain a C-terminal $(His)_6$ tag, and CRAF catalytic domain (residues 325–648) contains an N-terminal $(His)_6$ tag and two activating point mutations (Y340E and Y341E, which mimic SRC phosphorylation and are required for active enzyme; Fabian et al., 1993). pcDNA3.1/*myc*-His (Invitrogen) was used for expression of full-length BRAF^{WT}, CRAF^{WT}, and CRAF^{S359A} constructs in mammalian cells. Point mutations were introduced using a site-directed mutagenesis kit (Stratagene).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found in this article online at http://dx.doi.org/10.1016/j.ccr.2013.03.033.

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