B-Raf and Raf-1 Are Regulated by Distinct Autoregulatory Mechanisms*

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B-Raf is a key regulator of the ERK pathway and is mutationally activated in two-thirds of human melanomas. In this work, we have investigated the activation mechanism of B-Raf and characterized the roles of Ras and of B-Raf phosphorylation in this regulation. Raf-1 is regulated by an N-terminal autoinhibitory domain whose actions are blocked by interaction with Ras and subsequent phosphorylation of Ser\(^{259}\). We observed that B-Raf also contains an N-terminal autoinhibitory domain and that the interaction of this domain with the catalytic domain was inhibited by binding to active H-Ras. However, unlike Raf-1, the phosphorylation of B-Raf at Ser\(^{445}\) was constitutive and was only moderately increased by expression of constitutively active H-Ras or constitutively active PAK1. Ser\(^{445}\) phosphorylation is important to the B-Raf activation mechanism, however, because mutation of this site to alanine increased the affinity of the regulatory domain for the catalytic domain and increased autoinhibition. Similarly, expression of constitutively active PAK1 also decreased autoinhibition. B-Raf autoinhibition was negatively regulated by acidic substitutions at phosphorylation sites within the activation loop of B-Raf and by the oncogenic substitution V599E. However, these substitutions did not affect the ability of the regulatory domain to co-immunoprecipitate with the catalytic domain. These data demonstrate that B-Raf activity is autoregulated, that constitutive phosphorylation of Ser\(^{445}\) primes B-Raf for activation, and that a key feature of phosphorylation within the activation loop or of oncogenic mutations within this region is to block autoinhibition.

Raf proteins are Ras-regulated serine/threonine protein kinases that control the activation of the ERK\(^{1}\)/MAPK cascade and, as such, control a basic mechanism by which cells respond to extracellular ligands (1–4). They regulate ERK activation through the direct phosphorylation of MEK1 and MEK2, which then phosphorylate and activate ERK1 and ERK2. Once activated, ERKs phosphorylate a large number of cytoplasmic and nuclear substrates that enable Ras to control such diverse processes as cellular proliferation, transformation, and differentiation (5).

The Raf family consists of three isoforms, A-Raf, B-Raf, and Raf-1 (C-Raf), which exhibit a high degree of homology within three conserved regions (CR) known as CR1, CR2, and CR3. CR1 contains a Ras-binding domain (RBD) as well as an adjacent cysteine-rich domain (CRD). CR2 comprises a small region with a conserved Akt phosphorylation site, and CR3 encompasses the catalytic domain. Outside of these regions, Raf proteins exhibit little identity to each other. Raf family members also display different abilities to phosphorylate and activate MEK1 and MEK2, with B-Raf being the most active, followed by Raf-1 and then A-Raf (6, 7). In fact, in many cell types, B-Raf appears to constitute the majority of detectable MEK kinase activity (8–11). These findings, coupled with the normal ERK activation kinetics displayed in A-Raf or Raf-1 knockout mice, suggest that B-Raf may be the most important Raf family member in the ERK activation cascade (12–14).

It was recently shown that B-Raf is mutationally activated in two-thirds of all human melanomas (15) as well as in a smaller percentage of other human cancers (16–18). Interestingly, >90% of the oncogenic mutations found in B-Raf are accounted for by a single amino acid change of glutamate for valine at residue 599, which renders the kinase constitutively active (17). In the case of melanoma, such mutations appear to occur early in the development of cancer because the majority of benign nevi also contain activating mutations in the B-Raf gene (19). These observations, coupled with the finding that mutationally activated ras and B-Raf genes generally occur in the same types of cancer and are almost never identified in the same tumors concurrently, underscore the importance of ERK activation to the process of tumorigenesis in select tissues (16, 19–21).

The discovery that a single point mutation can impart full catalytic activity to B-Raf suggests that the activation mechanism of B-Raf is relatively simple and distinct from that of Raf-1 and A-Raf, which cannot be activated in this manner. Wild-type B-Raf activation clearly requires binding to an active small G protein such as Ras or Rap1 (8, 10, 22, 23) as well as phosphorylation of Thr\(^{389}\) and Ser\(^{607}\) (24, 25). These phosphorylation sites are contained within the activation loop of the catalytic domain and are believed to contribute directly to an increase in B-Raf catalytic activity (26). In this respect, the activation requirements of B-Raf are similar to those of Raf-1 because Raf-1 must also bind to Ras and requires phosphorylation of sites within the activation loop of its catalytic domain (Thr\(^{403}\) and Ser\(^{401}\)) (27). However, full activation of Raf-1 also requires phosphorylation of Ser\(^{259}\) and Tyr\(^{251}\), which lie outside the catalytic domain and contribute to the Raf-1 activation mechanism by blocking the actions of the autoinhibitory do-

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* This work was supported by American Cancer Society Research Grant RSG TBE-104121. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; CR, conserved region; RBD, Ras-binding domain; CRD, cysteine-rich domain; PAK1, p21-activated kinase-1; HEK, human embryonic kidney; HA, hemagglutinin; MBP, myelin basic protein; EGF, epidermal growth factor.
main (28, 29). Interestingly, the residues in B-Raf corresponding to Tyr337 and Tyr338 in Raf-1 are substituted with aspartic acids, and Ser445, which corresponds to Ser338 in Raf-1, appears to be constitutively phosphorylated in COS and PC12 cells (30). These data suggest either that B-Raf does not contain a functional N-terminal autoinhibitory domain or that its interaction with the catalytic domain is regulated in a manner that is distinct from Raf-1.

In this study, we have investigated the mechanism of B-Raf regulation by its N terminus and have characterized the roles of Ras and of phosphorylation of Ser445 in this regulation. We show that the phosphorylation of B-Raf at Ser445 is constitutive, but that expression of constitutively active H-Ras or constitutively active PAK1 can modestly increase the phosphorylation of this site. We have also found that B-Raf contains an N-terminal autoinhibitory domain and that the interaction of this domain with the B-Raf catalytic domain is negatively regulated by binding to active Ras. Autoinhibition is sensitive to the phosphorylation state of Ser445 because mutation of this residue to alanine increased the affinity of the autoinhibitory domain for the catalytic domain and increased the level of autoinhibition observed. On the other hand, the ability of the B-Raf autoinhibitory domain to block ERK2 activation was negatively regulated by acidic amino acid substitutions at the activating phosphorylation sites Thr598 and Ser601 as well as by the oncogenic substitution V599E. These data demonstrate that B-Raf does not contain a functional autoinhibitory domain.

**EXPERIMENTAL PROCEDURES**

**Cells, Plasmids, and Recombinant Proteins**—Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were transfected either by CaPO4 coprecipitation (31) or using Lipofectamine Plus (Invitrogen) according to the manufacturer’s instructions. FLAG-tagged wild-type B-Raf was contained in pLNX. FLAG-tagged wild-type A-Raf, B-Raf (1–435), B-Raf (1–374), B-Raf (1–345), B-Raf (1–281), B-Raf (100–435), B-Raf (100–345), and B-Raf (148–435) were amplified with Pfu polymerase (Stratagene) and subcloned into pCMV-FLAG/mCherry (Sigma). B-Raf (1–435) R187L was produced by PCR using B-Raf (1–435) as a template. The Myc-tagged B-Raf catalytic domain (B-RafCAT) was amplified with Pfu polymerase and subcloned into pCMV5M. All cDNAs amplified by PCR were sequenced to confirm correct amplification. B-RafCAT S445A, B-RafCAT S445D, and subcloned into pCMV5M. All cDNAs amplified by PCR were sequenced to confirm correct amplification. Hamagatain (HA) epitope-tagged wild-type ERK2 was contained in pCEP4l (31). Constitutively active HA-Ras (H-RasV12) was contained in pRCMV (31). Constitutively active B-Raf (B-RafCAT1) was contained in pCMV5M (32). glutathione S-transferase-MEK1 and hexahistidine-tagged ERK2 K2H were expressed in Escherichia coli BL21(DE3) cells and purified using glutathione-agarose or nickel-nitrilotriacetic acid affinity chromatography as described previously.

**Immunoprecipitations and in Vitro Kinase Assays**—HEK 293 cells were transfected with HA-ERK2 (2 μg) with or without the appropriate B-RafCAT (20 ng) or N-terminal expression constructs (varying amounts) by CaPO4 coprecipitation. The next day, the cell medium was changed to Dulbecco’s modified Eagle’s medium plus penicillin/streptomycin without fetal bovine serum, and the cells were incubated for another 24 h. Cells were then lysed in Triton lysis buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.5% Triton X-100, 80 μM β-glycerolphosphate, 0.5 mM sodium orthovanadate, 20 mM NaF, 1 μg/ml insulin, 1 μg/ml leupeptin, 2 μg/ml apropin, and 1 mM phenylmethylsulfonyl fluoride), and insoluble material was precipitated by centrifugation at 16,000 × g for 10 min at 4 °C. Supernatants were stored at ~80 °C for later analysis. To measure HA-ERK2 activity, HA-ERK2 was immunoprecipitated from cell lysates using 2 μg of mouse anti-HA antibody (Santa Cruz Biotechnology, Inc.) and 40 μl of a 50% slurry of protein A-Sepharose (Amersham Biosciences). Precipitates were washed three times with 1 ml of high salt buffer (20 mM Tris-HCl (pH 8.0) and 500 mM NaCl) and twice with 1 ml of low salt buffer (20 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 1 mM dithiothreitol, 100 μM ATP, and 2 μg/ml of [γ-32P]ATP (PerkinElmer Life Sciences)). Proteins were then resolved by 15% SDS-PAGE and visualized by Coomasie Blue staining. SDS-polycrylamide gels were dried, and phosphorylated proteins were visualized by autoradiography. Radioactive phosphate incorporated into MBP was determined by scintillation counting of the excised protein bands. B-Raf autoinhibitory domain to block ERK2 activation was negatively regulated by binding to active Ras. Autoinhibition is sensitive to the phosphorylation state of Ser445 because mutation of this residue to alanine increased the affinity of the autoinhibitory domain for the catalytic domain and increased the level of autoinhibition observed. On the other hand, the ability of the B-Raf autoinhibitory domain to block ERK2 activation was negatively regulated by acidic amino acid substitutions at the activating phosphorylation sites Thr598 and Ser601 as well as by the oncogenic substitution V599E. These data demonstrate that B-Raf does not contain a functional autoinhibitory domain.

**RESULTS**

**Regulation of Phosphorylation of A-Raf, B-Raf, and Raf-1 at Ser299, Ser445, and Ser338**—Raf-1 activation is regulated in part by phosphorylation of Ser338, and subtitution of this site with alanine precludes Raf-1 activation by physiologic stimuli (34–39). Phosphorylation of this site contributes to Raf-1 activation by blocking the ability of the N-terminal autoinhibitory domain to negatively regulate Raf-1 catalytic activity (28, 29). Because this residue is conserved...
B-Raf is constitutively phosphorylated at Ser445 in COS cells in a ligand-dependent manner. Previously, it was shown that transfected B-Raf proteins were immunoprecipitated with an antibody previously shown to recognize both B-Raf and Raf-1. We first examined whether expression of the B-Raf catalytic domain (B-RafCatalytic Domain, amino acids 436–765) would stimulate phosphorylation of Raf-1 at Ser445 and Ser338, respectively (data not shown). We then tested whether the phosphorylation of Ser445 could be increased by expression of constitutively active Ras or constitutively active Pak1 because Ras stimulates B-Raf activity and because Pak1 phosphorylates Raf-1 at the analogous site (Ser445) (28, 36, 38, 42). HEK 293 cells were transfected with FLAG epitope-tagged wild-type B-Raf and increasing amounts of constitutively active Ras (V12H-Ras) or constitutively active Pak1. After serum starvation, B-Raf was immunoprecipitated and tested for phosphorylation of Ser445 by Western blotting. As shown in Fig. 1C (lane 2), transfected B-Raf exhibited a high degree of Ser445 phosphorylation, similar to endogenous B-Raf (Fig. 1B). However, expression of active Ras did stimulate a modest increase in this phosphorylation (Fig. 1C, compare lanes 2–5 with lane 2). On the other hand, constitutively active Pak1 was more effective at stimulating Ser445 phosphorylation (Fig. 1D, compare lanes 2 and 5). This indicates that the phosphorylation of B-Raf at Ser445 in serum-starved cells is not maximal, and indicates that Ras stimulates the phosphorylation of this site. It also suggests that Pak1 is a potential B-Raf kinase.

**The B-Raf N Terminal Contains an Autoinhibitory Domain—**

Raf-1 contains an autoinhibitory domain that minimally encompasses residues 1–147 and that includes the entire RBD and part of the CRD. We have shown previously that coexpression of this domain with the catalytic domain of Raf-1 blocks its ability to stimulate ERK2 activation (28). Therefore, we tested whether B-Raf also contains an autoinhibitory domain in its N terminus.

We first examined whether expression of the B-Raf catalytic domain (B-RafCAT, amino acids 436–765) would stimulate ERK2 activity. HEK 293 cells were transfected with HA epitope-tagged ERK2 plus increasing amounts of B-RafCAT. After serum starvation, the cells were lysed, and HA-ERK2 was immunoprecipitated and tested for kinase activity using MBP as a substrate. B-RafCAT expressed very well in these cells, and transfection of as little as 10 ng of the B-RafCAT plasmid stimulated a robust activation of ERK2 as measured by MBP phosphorylation of Raf-1 at Ser338 (30), we first examined whether EGF stimulates the phosphorylation of endogenous A-Raf or B-Raf at the corresponding residues in HEK 293 cells. Cells were treated with EGF for different periods of time and lysed, and the endogenous A-Raf and B-Raf proteins were immunoprecipitated. After immunoprecipitation, the phosphorylation of A-Raf and B-Raf at Ser299 and Ser445 was examined by Western blotting. Phosphorylation of endogenous Raf-1 at Ser338 was monitored by Western blotting without prior immunoprecipitation. As shown in Fig. 1B, addition of EGF stimulated a robust phosphorylation of Raf-1 at Ser338 as early as 1 min after stimulation and lasted past 15 min. This time course corresponds well with the reported activation kinetics of both endogenous and transfected Raf-1 following EGF stimulation and also with the phosphorylation kinetics of transfected Raf-1 at Ser338 in COS cells (40, 41). Interestingly, we found that the phosphorylation of A-Raf at Ser299 was also stimulated by EGF, although the duration of phosphorylation of this site was much shorter than for Raf-1. Thus, by analogy with Raf-1, phosphorylation of this site may play an important role in the A-Raf activation mechanism. On the other hand, endogenous B-Raf was phosphorylated at Ser445 even in the absence of EGF, and the level of Ser445 phosphorylation was not stimulated addition of ligand. Similar results were obtained using a rat monoclonal antibody previously shown to recognize both B-Raf and Raf-1 phosphorylated at Ser445 and Ser338, respectively (data not shown) (30). Thus, these data correlate well with previous findings demonstrating that transfected B-Raf is constitutively phosphorylated in COS cells (30).

**Autoregulation of B-Raf**

![Image](105x388 to 260x737)

**FIG. 1. Phosphorylation of A-Raf, B-Raf, and Raf-1 at homologous sites is differentially regulated.** A, alignment of the amino acid sequences surrounding Ser299, Ser445, and Ser338 in human A-Raf, B-Raf, and Raf-1, respectively. Amino acids in boldface correspond to the Raf-1 phosphorylation sites Ser338 and Tyr341. B, phosphorylation of endogenous A-Raf, B-Raf, and Raf-1 at Ser299, Ser445, and Ser338 in HEK 293 cells. Cells were serum-starved and then stimulated with EGF (50 ng/ml) for the time periods shown. Endogenous A-Raf and B-Raf proteins were immunoprecipitated (IP) prior to immunoblotting (IB) for phosphorylation of Ser299 and Ser445. Phosphorylation of Raf-1 at Ser338 was analyzed by immunoblotting without prior immunoprecipitation. For each protein, the upper panel shows an immunoblot for phosphorylation of the indicated residue, and the lower panel shows an immunoblot for the indicated non-phosphorylated Raf protein. The results from a representative experiment are shown. C and D, expression of constitutively active H-Ras or constitutively active Pak1, respectively, stimulates phosphorylation of B-Raf at Ser445. HEK 293 cells were transfected with FLAG epitope-tagged wild-type B-Raf and either a control vector or increasing amounts of constitutively active H-Ras (V12-H-Ras) or constitutively active Pak1 (PAK1***)*. After serum starvation, the cells were lysed, and B-Raf was immunoprecipitated and tested for phosphorylation of Ser445 by Western blotting. In C and D, the upper panels are immunoblots for B-Raf phosphorylated at Ser445, and the middle panels are immunoblots for immunoprecipitated B-Raf. The results from a representative experiment are shown.

among Raf family members (Fig. 1A), we examined whether the corresponding residues in A-Raf and B-Raf (Ser299 and Ser445, respectively) are also phosphorylated in a ligand-dependent manner. Previously, it was shown that transfected B-Raf is constitutively phosphorylated at Ser445 in COS cells and that endogenous B-Raf is highly phosphorylated in cycling PC12 cells (30). However, the phosphorylation status of endogenous B-Raf at Ser445 in other cell types has not been reported, nor has the phosphorylation of endogenous or transfected A-Raf at Ser299 been examined.

Because treatment of COS cells with EGF stimulates the phosphorylation of Raf-1 at Ser338 (30), we first examined whether EGF stimulates the phosphorylation of endogenous A-Raf or B-Raf at the corresponding residues in HEK 293 cells. Cells were treated with EGF for different periods of time and lysed, and the endogenous A-Raf and B-Raf proteins were immunoprecipitated. After immunoprecipitation, the phosphorylation of A-Raf and B-Raf at Ser299 and Ser445 was examined by Western blotting. Phosphorylation of endogenous Raf-1 at Ser338 was monitored by Western blotting without prior immunoprecipitation. As shown in Fig. 1B, addition of EGF stimulated a robust phosphorylation of Raf-1 at Ser338 as early as 1 min after stimulation and lasted past 15 min. This time course corresponds well with the reported activation kinetics of both endogenous and transfected Raf-1 following EGF stimulation and also with the phosphorylation kinetics of transfected Raf-1 at Ser338 in COS cells (40, 41). Interestingly, we found that the phosphorylation of A-Raf at Ser299 was also stimulated by EGF, although the duration of phosphorylation of this site was much shorter than for Raf-1. Thus, by analogy with Raf-1, phosphorylation of this site may play an important role in the A-Raf activation mechanism. On the other hand, endogenous B-Raf was phosphorylated at Ser445 even in the absence of EGF, and the level of Ser445 phosphorylation was not stimulated addition of ligand. Similar results were obtained using a rat monoclonal antibody previously shown to recognize both B-Raf and Raf-1 phosphorylated at Ser445 and Ser338, respectively (data not shown) (30). Thus, these data correlate well with previous findings demonstrating that transfected B-Raf is constitutively phosphorylated in COS cells (30).

We then tested whether the phosphorylation of Ser445 could be increased by expression of constitutively active Ras or constitutively active Pak1 because Ras stimulates B-Raf activity and because Pak1 phosphorylates Raf-1 at the analogous site (Ser338) (28, 36, 38, 42). HEK 293 cells were transfected with FLAG epitope-tagged wild-type B-Raf and increasing amounts of constitutively active Ras (V12-H-Ras) or constitutively active Pak1. After serum starvation, B-Raf was immunoprecipitated and tested for phosphorylation of Ser445 by Western blotting. As shown in Fig. 1C (lane 2), transfected B-Raf exhibited a high degree of Ser445 phosphorylation, similar to endogenous B-Raf (Fig. 1B). However, expression of active Ras did stimulate a modest increase in this phosphorylation (Fig. 1C, compare lanes 2–5 with lane 2). On the other hand, constitutively active Pak1 was more effective at stimulating Ser445 phosphorylation (Fig. 1D, compare lanes 2 and 5). This indicates that the phosphorylation of B-Raf at Ser445 in serum-starved cells is not maximal, and indicates that Ras stimulates the phosphorylation of this site. It also suggests that Pak1 is a potential B-Raf kinase.
kinase activity and by Western blotting for active ERK2 (Fig. 2A, upper and middle panels, respectively). The average results from three independent experiments are shown in Fig. 2B.

We then examined whether coexpression of the B-Raf N terminus (amino acids 1–435) would inhibit ERK2 activation stimulated by B-RafCAT. For these assays, 20 ng of B-RafCAT were transfected, which stimulated half-maximal activation of ERK2. As shown in Fig. 2C, the ability of B-RafCAT to stimulate ERK2 activity was progressively inhibited by coexpression of increasing amounts of the B-Raf N terminus. The average results from five separate experiments are shown in Fig. 2D. Because ERK2 activation by B-RafCAT is independent of upstream stimuli, these data indicate that the B-Raf N terminus contains an autoinhibitory domain that blocks the activity of the catalytic domain, similar to Raf-1. In the course of these experiments, we noted that the degree of autoinhibition observed following transfection of higher amounts of B-RafCAT (100 ng of plasmid and above) was reduced (data not shown). This was presumably because B-RafCAT expressed so well, it was extraordinarily efficient at stimulating ERK2 activity, and because B-Raf-(1–435) did not express as efficiently as B-RafCAT. Therefore, for all experiments monitoring the autoinhibition of ERK2 activation by B-RafCAT, we continued to transfect only 20 ng of plasmid.

Using this assay, we then determined the minimal region constituting the autoinhibitory domain of B-Raf. In Raf-1, the autoinhibitory domain encompasses both the RBD and a portion of the CRD, but not CR2 (28). Therefore, we constructed a series of deletion mutants of the B-Raf N terminus to define the region responsible for autoinhibition (Fig. 3A). As shown in Fig. 3A, deletion of the B-Raf regulatory domain up to and including CR2 (B-Raf-(1–374) and B-Raf-(1–345), respectively) did not affect autoinhibition. However, further deletion

![FIG. 2. Activation of ERK2 by the B-Raf catalytic domain and autoinhibition by the B-Raf N terminus.](image)

**A.** Titration of B-RafCAT expression for ERK2 activation. HEK 293 cells were transfected with HA epitope-tagged ERK2 (lanes 2–7) without (lanes 1 and 2) or with (lanes 3–7) increasing amounts of the B-Raf catalytic domain (B-RafCAT). After serum starvation, the cells were lysed, and HA-ERK2 was immunoprecipitated (IP) with anti-HA antibody and tested for kinase activity using MBP as a substrate. An autoradiograph of phosphorylated MBP (pMBP) is shown in the first panel. The immunoprecipitates were also tested for the amounts of ERK2 precipitated (second panel) and the phosphorylation of ERK2 at the activating phosphorylation sites Thr183 and Tyr185 (third panel). Lysates were immunoblotted (IB) for Myc epitope-tagged B-RafCAT expression (fourth panel). Fold Activation refers to the fold increase in MBP kinase activity compared with HA-ERK2 alone. The results from a representative experiment are shown. B, quantification of HA-ERK2 activation by B-RafCAT. ERK2 activity is represented as the fold activation over ERK2 in the absence of transfected B-RafCAT. The results are means ± S.E. of three independent experiments. C, ERK2 activation by B-RafCAT is blocked by coexpression of the B-Raf regulatory domain. HEK 293 cells were transfected with HA-ERK2 (lanes 2–6) without (lanes 1 and 2) or with B-RafCAT (lanes 3–6) and increasing amounts of the B-Raf N terminus (amino acids 1–435) (lanes 4–6). HA-ERK2 activity was tested as described for A. D, quantification of autoinhibition. The results are means ± S.E. of five independent experiments.
B-Raf catalytic domain. B-Raf N-terminal constructs shown. After serum starvation, the cells were lysed, and B-RafCAT was immunoprecipitated (IP) with anti-Myc antibody. The immunoprecipitates were then probed by immunoblotting (IB) for the presence of the N-terminal constructs (upper panel) or immunoprecipitated B-RafCAT (middle panel). An immunoblot for FLAG epitope-tagged B-Raf N-terminal proteins present in the cell lysate prior to immunoprecipitation is shown (lower panel).

The B-Raf N terminus begins with a unique 100-amino acid segment, followed by the RBD, which begins at residue 148. We observed that deletion of the first 100 residues of the N terminus did not affect the ability of the N terminus to block ERK2 activation because B-Raf-(100–435) and B-Raf-(100–345) were as effective as their full-length counterparts at blocking ERK2 activation (Fig. 3B). However, further deletion of residues between amino acid 100 and the RBD (amino acid 148) significantly impaired autoinhibition (data not shown). This was not unexpected, given that the Raf-1 autoinhibitory domain also requires residues N-terminal to its RBD (28). Thus, these data demonstrate that residues C-terminal to the CRD, but not including CR2, are required for autoregulation.

The B-Raf N terminus begins with a unique 100-amino acid segment, followed by the RBD, which begins at residue 148. We observed that deletion of the first 100 residues of the N terminus did not affect the ability of the N terminus to block ERK2 activation because B-Raf-(100–435) and B-Raf-(100–345) were as effective as their full-length counterparts at blocking ERK2 activation (Fig. 3B). However, further deletion of residues between amino acid 100 and the RBD (amino acid 148) significantly impaired autoinhibition (data not shown). This was not unexpected, given that the Raf-1 autoinhibitory domain also requires residues N-terminal to its RBD (28). Thus, these data demonstrate that the B-Raf autoinhibitory domain is minimally contained between residues 100 and 345. This region encompasses the RBD and CRD as well as additional residues C-terminal to the CRD. This primary structure distinguishes the autoinhibitory domain of B-Raf from that of Raf-1 because the corresponding domain in Raf-1 did not include the complete CRD or residues C-terminal to the CRD.

The B-Raf Autoinhibitory Domain Forms a Complex with the Catalytic Domain in Cells—When coexpressed in cells, the Raf-1 regulatory domain forms a tight complex with the catalytic domain such that it can be co-immunoprecipitated with the B-Raf catalytic domain and whether this binding correlates with the ability of this domain to inhibit B-Raf-stimulated ERK2 activity. HEK 293 cells were transfected with Myc epitope-tagged B-RafCAT and various FLAG epitope-tagged B-Raf N-terminal constructs. B-RafCAT was then immunoprecipitated and tested for coprecipitation of the N-terminal constructs by Western blotting. As shown in Fig. 4 (upper panel), the B-Raf-(1–435), B-Raf-(1–374), and B-Raf-(100–345) N-terminal constructs coprecipitated with the B-RafCAT protein. This correlated well with their ability to block ERK2 activation by B-RafCAT (Fig. 3). On the other hand, the B-Raf-(1–281) N-terminal construct, which did not block ERK2 activation by B-RafCAT, also did not coprecipitate efficiently with B-RafCAT. These data demonstrate that the B-Raf autoinhibitory domain interacts tightly with the catalytic domain in cells and suggest that this interaction must occur for efficient autoinhibition.

The Raf-1 Autoinhibitory Domain Interacts with the B-Raf Catalytic Domain—B-Raf and Raf-1 have been reported to form a complex in the cell that may afford an opportunity for cross-regulation between isoforms (26, 43). Because the B-Raf and Raf-1 autoinhibitory domains share a high degree of homology, we examined whether the Raf-1 autoinhibitory domain could interact with the B-Raf catalytic domain and block its activity. Therefore, HEK 293 cells were cotransfected with HA-ERK2 without (lanes 1 and 2) or with Myc epitope-tagged B-Raf or Raf-1 regulatory domains from B-Raf (B-Raf-(1–435)) (lanes 3–9) and increasing amounts of FLAG epitope-tagged regulatory domains from Raf-1 (Raf-1-(1–330)) (lanes 7–9). After serum starvation, the cells were lysed, and HA-ERK2 was immunoprecipitated (IP) and tested for activity as described. pMBP, phosphorylated MBP, B, the Raf-1 regulatory domain binds to B-RafCAT in cells. HEK 293 cells were cotransfected with Myc epitope-tagged B-RafCAT with or without FLAG epitope-tagged B-Raf-(1–435) or FLAG epitope-tagged Raf-1-(1–330). The cells were starved and lysed, and B-RafCAT was immunoprecipitated with anti-Myc antibody (IB: -Myc). The immunoprecipitates were then tested for the presence of B-RafCAT (IB: -Myc) and the B-Raf or Raf-1 regulatory domain (IB: -Flag), which did not block ERK2 activation by B-RafCAT, also did not coprecipitate efficiently with B-RafCAT. These data demonstrate that the B-Raf autoinhibitory domain interacts tightly with the catalytic domain in cells and suggest that this interaction must occur for efficient autoinhibition.
We then examined whether the Raf-1 regulatory domain forms a complex with B-RafCAT. Therefore, HEK 293 cells were cotransfected with B-RafCAT with or without the B-Raf or Raf-1 regulatory domain. B-RafCAT was then immunoprecipitated and tested for the presence of either regulatory domain. As shown in Fig. 5B, the Raf-1 regulatory domain also coprecipitated with the B-Raf catalytic domain, although again with lower affinity compared with the B-Raf regulatory domain. Thus, these data demonstrate that the Raf-1 regulatory domain can interact with the B-Raf catalytic domain, albeit with lower affinity, and suggest that B-Raf and Raf-1 may regulate the activity of one another through mutual autoinhibitory mechanisms.

Binding of the B-Raf Autoinhibitory Domain to the Catalytic Domain Is Disrupted by Expression of Active Ras—We then examined whether the interaction between the B-Raf autoinhibitory and catalytic domains is regulated by Ras. In the case of Raf-1, this interaction is blocked by coexpression of constitutively active Ras (29). Therefore, HEK 293 cells were transfected with B-RafCAT and B-Raf-(1–435) with or without constitutively active H-Ras (V12H-Ras). B-RafCAT was then immunoprecipitated and tested for coprecipitation of the B-Raf N terminus. B-Raf-(1–435) efficiently coprecipitated with the B-Raf catalytic domain, and this coprecipitation was inhibited by the expression of V12H-Ras (Fig. 6A, first panel, compare lanes 4 and 5). This effect depended on the ability of Ras to interact with B-Raf-(1–435) because a B-Raf-(1–435) mutant incapable of binding to Ras (B-Raf-(1–435) R187L) coprecipitated with B-RafCAT even in the presence of active H-Ras (compare lanes 6 and 7). Interestingly, the B-RafCAT protein was highly phosphorylated at Ser445, and expression of active Ras did not further significantly alter this phosphorylation. This suggests that a change in the phosphorylation of this site is not required for Ras to block the interaction between the regulatory and catalytic domains. To test this, we examined whether B-RafCAT containing an alanine substitution at Ser445 is still subject to regulation by Ras. Therefore, HEK 293 cells were transfected with wild-type B-RafCAT or B-RafCAT S445A with or without B-Raf-(1–435) and V12H-Ras. The B-RafCAT proteins were then immunoprecipitated and tested for coprecipitation of the B-Raf N terminus. As expected, wild-type B-RafCAT was highly phosphorylated at Ser445, whereas B-RafCAT S445A was not (Fig. 6B, second panel). Importantly, expression of active Ras disrupted the association between the B-Raf regulatory domain and the wild-type and mutant B-RafCAT proteins. Thus, these data demonstrate that Ras blocks the interaction between the regulatory and catalytic domains by binding to the B-Raf N terminus and does so irrespective of the presence of Ser445. Interestingly, more B-Raf-(1–435) appeared to coprecipitate with B-RafCAT S445A than with wild-type B-RafCAT (compare lanes 4 and 6), suggesting that phosphorylation of Ser445 may decrease the binding of the regulatory domain to the catalytic domain.

Phosphorylation of B-Raf at Ser445 Reduces the Affinity of the Autoinhibitory Domain for the Catalytic Domain—The previous experiment suggested that phosphorylation of B-RafCAT at Ser445 negatively regulates the affinity of the regulatory domain for the catalytic domain. Consistent with the possibility of a higher binding affinity of the autoinhibitory domain for the non-phosphorylated catalytic domain, Mason et al. (30) have shown previously that mutation of Ser445 and Ser446 to alanines reduces the basal and Ras-stimulated activities of B-Raf. Therefore, we first examined whether the presence of Ser445 in B-RafCAT affects the degree of autoinhibition conferred by the N terminus. HEK 293 cells were transfected with HA-ERK2 and either wild-type B-RafCAT or B-RafCAT S445A in the absence or presence of increasing levels of the B-Raf regulatory domain. ERK2 was then immunoprecipitated and tested for MBP kinase activity. In these assays, both wild-type B-RafCAT and B-RafCAT S445A stimulated ERK2 activity, although the S445A mutant was less active in this regard than the wild-type protein (Fig. 7A, compare lanes 3 and 7). Similarly, both the wild-type and mutant proteins were autoinhibited by B-Raf-(1–435). However, B-RafCAT S445A was more sensitive to autoinhibition compared with wild-type B-RafCAT, such that, even at the lowest concentration of regulatory domain transfected, the activity of the S445A mutant was completely blocked. Thus, these data indicate that the presence of Ser445 negatively impacts the ability of the N terminus to regulate the activity of the catalytic domain and suggest that the constitutive phosphorylation of Ser445 primes B-Raf for activation.

We then examined whether elimination of the Ser445 phosphorylation site alters the affinity of the regulatory domain for the catalytic domain. HEK 293 cells were transfected with the absence or presence of increasing levels of the B-Raf regulatory domain. ERK2 was then immunoprecipitated and tested for MBP kinase activity. In these assays, both wild-type B-RafCAT and B-RafCAT S445A stimulated ERK2 activity, although the S445A mutant was less active in this regard than the wild-type protein (Fig. 7A, compare lanes 3 and 7). Similarly, both the wild-type and mutant proteins were autoinhibited by B-Raf-(1–435). However, B-RafCAT S445A was more sensitive to autoinhibition compared with wild-type B-RafCAT, such that, even at the lowest concentration of regulatory domain transfected, the activity of the S445A mutant was completely blocked. Thus, these data indicate that the presence of Ser445 negatively impacts the ability of the N terminus to regulate the activity of the catalytic domain and suggest that the constitutive phosphorylation of Ser445 primes B-Raf for activation.

We then examined whether elimination of the Ser445 phosphorylation site alters the affinity of the regulatory domain for the catalytic domain. HEK 293 cells were transfected with the
wild-type or S445A form of B-RafCAT with or without increasing levels of B-Raf(1–435). B-RafCAT was then immunoprecipitated and tested for the presence of the B-Raf N terminus by Western blotting. The B-Raf N terminus coprecipitated with B-RafCAT S445A to a much higher degree than with wild-type B-RafCAT (Fig. 7B, compare lanes 6–8 with 10–12). As expected, Western blotting with anti-phospho-Ser445 antibody demonstrated that only wild-type B-RafCAT was phosphorylated at Ser445 (second panel). These data indicate that B-Raf(1–435) binds more strongly to B-RafCAT S445A than to wild-type B-RafCAT and suggest that the phosphorylation of Ser445 reduces the affinity of the B-Raf autoinhibitory domain for the catalytic domain.

Because expression of active PAK1 augmented the phosphorylation of wild-type B-Raf at Ser445 (Fig. 1C), we examined whether expression of active PAK1 could further decrease the effectiveness of B-Raf autoinhibition. Expression of a moderate amount of B-Raf(1–435) reduced ERK2 activation by B-RafCAT from 92- to 27-fold (Fig. 7C, compare lanes 3 and 4). However, coexpression of constitutively active PAK1 reduced the degree of autoinhibition such that ERK2 activation was restored to >60% of its uninhibited level (compare lanes 4 and 5). Because the anti-phospho-Ser445 antibody was not of sufficient affinity to detect the phosphorylation of B-RafCAT at this site with the low amounts of B-RafCAT being expressed in this assay, we could not demonstrate that PAK1 expression stimulated the phosphorylation of B-RafCAT in this experiment. However, autoinhibition of B-RafCAT S445A was only slightly relieved by active PAK1 expression (Fig. 7C, lanes 6–8), indicating that PAK1 most likely relieved autoinhibition through the phosphorylation of B-RafCAT at Ser445. Furthermore, PAK1 stimulated the phosphorylation of B-RafCAT at Ser445 when higher amounts of B-RafCAT were transfected (Fig. 7D, compare lanes 2 and 3). Thus, from these experiments, we conclude that the phosphorylation of Ser445 serves to reduce the affinity of the B-Raf regulatory domain for the catalytic domain.

**Phosphomimetic Substitutions in the B-Raf Activation Loop Block Autoinhibition**—Wild-type B-Raf activation requires phosphorylation of Thr208 and Ser603, which are contained within the activation loop of the catalytic domain. Furthermore, acidic substitutions at these sites create a constitutively active version of B-Raf that does not require Ras for activation (24, 25). Therefore, we examined whether acidic or alanine substitutions at these sites affect autoinhibition or association between the regulatory and catalytic domains. HEK 293 cells were transfected with B-RafCAT T598E/S601D (lanes 4 and 5) without (lanes 1, 2, and 4) or with (lanes 3 and 5) constitutively active PAK1. After serum starvation, the cells were lysed, and B-RafCAT was immunoprecipitated and tested for phosphorylation of Ser445 by Western blotting. The results from a representative experiment are shown.
lysed, and the B-RafCAT proteins were immunoprecipitated (first for the expression of B-Raf-(1–435) (third panels through (A) panel most common oncogenic mutation in B-Raf and results in a

Oncogenic Mutation in B-Raf—

which the autoinhibitory and catalytic domains interact,

mutation blocks autoinhibition by affecting the manner in

tive H-Ras. These findings suggest that the T598E/S601D

were transfected with Myc epitope-tagged wild-type (WT) B-RafCAT (lanes 3–6), Myc epitope-tagged B-RafCAT T598E/S601D (lanes 7–10), or Myc epitope-tagged B-RafCAT T598A/S601A (lanes 11–14) without (lanes 1–3, 7, and 11) or with (lanes 4–6, 8–10, and 12–14) increasing amounts of FLAG epitope-tagged B-Raf(1–435). After serum starvation, the cells were lysed, and HA-ERK2 was immunoprecipitated (IP) and tested for activity as described. The results from a representative experiment are shown. pMBP, phosphorylated MBP; IB, immunoblot. B, acidic substitution of the activation loop phosphorylation sites does not affect the interaction between the B-Raf regulatory and catalytic domains. HEK 293 cells were transfected with Myc epitope-tagged wild-type B-RafCAT (lanes 2, 4, and 5), Myc epitope-tagged B-RafCAT T598A/S601A (lanes 6 and 7), or Myc epitope-tagged B-RafCAT T598E/S601D (lanes 8 and 9) without (lanes 1 and 2) or with FLAG epitope-tagged B-Raf(1–435) (lanes 3–9) and V12H-Ras (lanes 5, 7, and 9). After serum starvation, the cells were lysed, and the B-RafCAT proteins were immunoprecipitated (first through third panels). The immunoprecipitates were tested for the presence of B-Raf(1–435) (first panel), B-RafCAT phosphorylated at Ser445 (second panel), and B-RafCAT (third panel). Lysates were probed for the expression of B-Raf(1–435) (fourth panel) and V12H-Ras (fifth panel). The results from a representative experiment are shown.

length B-Raf containing these mutations (24). However, the modest level of ERK2 activity stimulated by this mutant was still largely inhibited by coexpression of B-Raf(1–435). Thus, these data suggest that phosphorylation of these sites, which is concomitant with B-Raf activation, serves to block autoinhibition by the B-Raf regulatory domain.

We then tested whether acidic substitutions at these sites affects the ability of the B-Raf regulatory and catalytic domains to interact. Surprisingly, B-Raf(1–435) coprecipitated with B-RafCAT T598E/S601D as well as with wild-type B-RafCAT and B-RafCAT T598A/S601A (Fig. 8B). Furthermore, in each case, the coprecipitation B-Raf(1–435) with these catalytic domains was inhibited by coexpression of active H-Ras. These findings suggest that the T598E/S601D mutation blocks autoinhibition by affecting the manner in which the autoinhibitory and catalytic domains interact, rather than precluding their binding altogether.

B-Raf Autoinhibition Is Also Blocked by the Most Common Oncogenic Mutation in B-Raf—The V599E substitution is the most common oncogenic mutation in B-Raf and results in a

constitutively active protein with an intrinsic MEK kinase activity that is greater than that stimulated by active Ras (15, 26). Because the T598E/S601D acidic substitutions blocked autoinhibition, it was possible that the V599E substitution may also have affected autoinhibition. Therefore, we examined whether B-RafCAT containing the V599E mutation is still subject to autoinhibition by the B-Raf N terminus. HEK 293 cells were transfected with HA-ERK2 and either wild-type B-RafCAT or B-RafCAT V599E with or without increasing amounts of B-Raf(1–435). HA-ERK2 was then immunoprecipitated and tested for MBP kinase activity. As shown in Fig. 9A, although ERK2 activation by wild-type B-RafCAT was progressively inhibited by expression of B-Raf(1–435), ERK2 activity stimulated by B-RafCAT V599E was unaffected by expression of the B-Raf N terminus. These data indicate that the V599E mutation blocks autoinhibitory activity. To test whether this mutation also affects the ability of the N terminus to interact with the catalytic domain, we examined the ability of B-Raf(1–435) to coprecipitate with B-RafCAT V599E. As shown in Fig. 9B, the B-Raf N terminus coprecipitated with B-RafCAT V599E as efficiently as with wild-type B-RafCAT. These findings suggest that, similar to the T598E/S601D substitutions, the V599E mutation blocks autoinhibition by affecting the manner in which the autoinhibitory and catalytic domains interact, rather than precluding their binding altogether. Thus, these data
indicate that the V599E mutation must serve two roles in activating full-length B-Raf: blocking autoinhibition as well as directly increasing B-Raf catalytic activity.

**Discussion**

In this work, we have examined the autoregulatory mechanism governing the activity of B-Raf. Previously, it was shown that Raf-1 is regulated by an autoinhibitory mechanism that is relieved by binding to active Ras and subsequent phosphorylation of Ser^{338} (28, 29, 45). Here, we have shown that B-Raf also contains an autoinhibitory domain within its N terminus and that this domain is contained between amino acids 100 and 345. We also found that autoinhibition of B-Raf is relieved by the interaction between active Ras and the regulatory domain. However, the phosphorylation of Ser^{445}, which corresponds to Ser^{338} in Raf-1, is largely constitutive and so does not actively contribute to relief from autoinhibition. Instead, this phosphorylation appears to prime B-Raf for activation because substitution of Ser^{445} with alanine increased the affinity of the regulatory domain for the catalytic domain and augmented autoinhibition. On the other hand, phosphorylation of sites within the activation loop of B-Raf appears to interfere with autoinhibition because acidic substitutions at the activation loop phosphorylation sites as well as the oncogenic mutation V599E blocked the inhibitory effect of the regulatory domain. This mechanism diverges from that regulating activation of Raf-1 because phosphorylation of sites within its activation loop does not affect autoinhibition (28). Thus, these results demonstrate that B-Raf activity is also controlled through the actions of an N-terminal autoinhibitory domain, but that the manner in which its interaction with the catalytic domain is regulated is distinct from Raf-1.

Our data show that the minimal autoinhibitory domain of B-Raf encompasses the RBD and CRD (amino acids 148–281) as well as a C-terminal region of low conservation between Raf isoforms (amino acids 282–345). This makes the composition of the autoinhibitory domain of B-Raf distinct from that of Raf-1, which contains the RBD, but includes only part of the CRD and does not contain residues C-terminal to the CRD (28). Still, the primary structures of these domains are close enough within the RBD and CRD (60% identity overall) that we tested whether a potential for cross-regulation exists. Intriguingly, we observed that the Raf-1 regulatory domain is able to block ERK2 activation by the B-Raf catalytic domain, although with less efficacy compared with the B-Raf regulatory domain (Fig. 5A). Similarly, the Raf-1 regulatory domain coprecipitated with the B-Raf catalytic domain, although again with a lower apparent affinity (Fig. 5B). Thus, these experiments illustrate the potential for cross-regulation between Raf-1 and B-Raf. This may be very important to the activation mechanism of each protein, given the recent observations that Raf-1 and B-Raf form a complex in the cell (43). Additionally, the finding that oncogenic mutations within subdomain I of the kinase domain of B-Raf do not augment its catalytic activity but instead enable B-Raf to activate Raf-1 supports the notion that the activities of these proteins are inextricably linked (15). Future experiments will be required to determine whether the regulatory domain of each protein can control the activity of the other within the context of the wild-type enzymes.

Our data show that the autoregulatory mechanism of B-Raf is similar to that of Raf-1 in that Ras also blocks B-Raf autoinhibition by directly binding to the B-Raf regulatory domain (Fig. 6) (29). However, the regulatory mechanism of each protein diverges in the requirement for phosphorylation of specific residues within the catalytic domain to relieve autoinhibition. In Raf-1, Ser^{338} and Tyr^{341} are phosphorylated in response to Ras activation, and phosphorylation of either of these sites blocks autoinhibition (28, 29). On the other hand, we observed that the corresponding serine in B-Raf (Ser^{445}) was heavily phosphorylated in quiescent cells, and this phosphorylation was only marginally increased by expression of constitutively active H-Ras or constitutively active PAK1 (Fig. 1, C and D, respectively). Thus, phosphorylation of Ser^{445} cannot serve as the signal to relieve autoinhibition. Instead, we found that phosphorylation of this site reduced the apparent affinity of the regulatory domain for the catalytic domain, thus priming B-Raf for activation. This statement is based on three lines of evidence. First, substitution of Ser^{445} with alanine predisposed the B-Raf catalytic domain to autoinhibition such that its ability to stimulate ERK2 activity was inhibited by expression of relatively low amounts of the B-Raf regulatory domain (Fig. 7A). Second, the B-Raf regulatory domain coprecipitated much more efficiently with the B-Raf catalytic domain containing the S445A substitution (Fig. 7B). Third, expression of constitutively active PAK1 partially relieved autoinhibition only when Ser^{445} was intact (Fig. 7C). Thus, these results suggest that the constitutive phosphorylation of Ser^{445} serves to reduce the affinity of the B-Raf autoinhibitory domain for the catalytic domain. These results are consistent with those of Mason et al. (30), who observed that substitution of Ser^{445} and Ser^{446} with alamines reduces the basal and Ras-stimulated activities of B-Raf, and suggest that the phosphorylation of this site may contribute to the higher basal activity observed for B-Raf by reducing autoinhibition.

We then examined whether B-Raf autoinhibition is regulated by phosphorylation of sites within the activation loop of the catalytic domain. Two sites within this subdomain must be phosphorylated for wild-type B-Raf activation (Thr^{598} and Ser^{601}), and substitution of these sites with acidic residues creates a constitutively active version of B-Raf that cannot be further activated by Ras (24, 25). Importantly, we observed that the ability of B-RafCAT T598E/S601D to stimulate ERK2 activity was unaffected by the coexpression of the B-Raf regulatory domain (Fig. 8A). This result suggests either that these sites must be phosphorylated to relieve autoinhibition or that, once these sites are phosphorylated, B-Raf activity cannot be autoinhibited. In either case, this finding sets the autoregulation mechanism of B-Raf farther apart from that of Raf-1, which is unaffected by acidic substitutions at the phosphorylation sites within its activation loop (28). The pivotal role of these activating phosphorylation sites in blocking B-Raf autoinhibition is supported by our observation that the most common oncogenic mutation in B-Raf (V599E) also precluded autoinhibition (Fig. 9A). Interestingly, we observed that the acidic substitutions T598E/S601D and V599E did not affect the coprecipitation of the B-Raf regulatory and catalytic domains (Figs. 8B and 9B). Coupled with the observation that phosphorylation of Raf-1 at Ser^{338} blocks autoinhibition but does not prevent the coprecipitation of the regulatory and catalytic domains (28, 29), these data suggest that relief from autoinhibition can be achieved by changing the way in which these domains interact, rather than precluding their interaction altogether.

Taken together, these data suggest a simplified activation mechanism for B-Raf compared with Raf-1. In this model, GTP-liganded Ras binds to the B-Raf N terminus. This blocks the interaction between the autoinhibitory and catalytic domains and allows for the phosphorylation of B-Raf at Thr^{598} and Ser^{601}. Phosphorylation of these sites directly contributes to an increase in catalytic activity and also precludes rebinding of the autoinhibitory and catalytic domains (26). In this model, B-Raf activity can be down-regulated only when the phosphorylation sites within the activation loop are dephosphorylated,
thus allowing for a renewed interaction between the regulatory and catalytic domains. Furthermore, the phosphorylation of Ser\textsuperscript{445} would prime B-Raf for activation by reducing the affinity of the autoinhibitory domain for the catalytic domain. It should be noted that dephosphorylation of B-Raf at select inhibitory residues may also be necessary for full activation. For example, the activity of Raf-1 is negatively regulated by phosphorylation of Ser\textsuperscript{259}, and Raf-1 activation is accompanied by dephosphorylation of this site (46–48). Similarly, the corresponding residue in B-Raf (Ser\textsuperscript{364}) is phosphorylated by both Akt and serum and glucocorticoid-inducible kinase, and phosphorylation of this site inhibits B-Raf activation by upstream stimuli (49, 50). Thus, although it has not been shown that Ser\textsuperscript{364} is dephosphorylated upon B-Raf activation, by analogy with Raf-1, this might be expected to occur. The simplified activation mechanism of B-Raf may explain why it is subject to autogenetic activation in cancer. A single mutation that mimics the effect of phosphorylation within the activation loop would be sufficient to both block autoinhibition and fully stimulate its kinase activity. On the other hand, Raf-1 requires phosphorylation of multiple residues for activation and thus cannot be activated by a single mutagenic event.

Acknowledgments—We thank Debbie Morrison for the wild-type B-Raf plasmid and Carmen Dessauer for critical reading of the manuscript.

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