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Negative Regulation of the Serine/Threonine Kinase B-Raf by Akt*

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B-Raf contains multiple Akt consensus sites located within its amino-terminal regulatory domain. One site, Ser^{364}, is conserved with c-Raf but two additional sites, Ser^{428} and Thr^{439}, are unique to B-Raf. We have investigated the role of both the conserved and unique phosphorylation sites in the regulation of B-Raf activity *in vitro* and *in vivo*. We show that phosphorylation of B-Raf by Akt occurs at multiple residues within its amino-terminal regulatory domain, at both the conserved and unique phosphorylation sites. The alteration of the serine residues within the Akt consensus sites to alanines results in a progressive increase in enzymatic activity *in vitro* and *in vivo*. Furthermore, expression of Akt inhibits epidermal growth factor-induced B-Raf activity and inhibition of Akt with LY294002 up-regulates B-Raf activity, suggesting that Akt negatively regulates B-Raf *in vivo*. Our results demonstrate that B-Raf activity can be negatively regulated by Akt through phosphorylation in the amino-terminal regulatory domain of B-Raf. This cross-talk between the B-Raf and Akt serine/threonine kinases is likely to play an important role in modulating the signaling specificity of the Ras/Raf pathway and in promoting biological outcome.

Diverse extracellular stimuli, including growth factors, cytokines, and hormones, promote the formation of active, GTP-Ras. GTP-Ras directly interacts with the Raf family of serine threonine kinases and type I phosphatidylinositol 3-kinases (PI3K)[1] (1–3). Upon activation, Raf phosphorylates mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, which in turn phosphorylates and activates ERK1/2. The ERKs phosphorylate cytoplasmic targets, including the kinases Rsk and Mnk, and translocate to the nucleus where they stimulate the activity of various transcription factors, such as Elk-1, Fos, Jun, and Myc (4). Activation of phosphatidylinositol 3-kinase by both Ras-dependent and Ras-independent mechanisms leads to the increased production of phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate. These lipids regulate the activity and/or localization of a number of target proteins, including those that contain pleckstrin homology domains. One such pleckstrin homology domain-containing protein regulated by lipids is the serine/threonine kinase Akt (also called protein kinase B). Akt was identified as the viral transforming agent of a T-cell lymphoma, and subsequent studies revealed a central role for Akt in promoting cell survival (5). Targets for Akt include kinases, glycogen-synthase kinase 3 and p70S6 kinase, transcription factors, FKHR and cAMP-response element-binding protein, as well as proteins associated with apoptosis, caspase 9 and BAD (6).

Ras promotes cell growth, in part by activation of the Raf/ERK pathway, and unrestrained activation of the Ras pathway is a common occurrence in many human tumors (7). In addition to its role in cell growth, Ras promotes cell survival through activation of the Raf/ERK and PI3K/Akt cascades (8). Ras also regulates differentiation; in the committed neuronal PC12 cell line, Ras/Raf promotes differentiation, whereas in C2C12 myoblasts, the Ras/Raf/ERK pathway blocks skeletal muscle differentiation (9, 10). Ras also promotes cell senescence and cell death, through activation of the Raf kinases (8). Thus, many contrary effects of Ras, promoting differentiation *versus* blocking differentiation and promoting cell proliferation, cell death, or cell survival, require the action of the Raf/ERK pathway. Cross-talk between signaling pathways is likely to be one mechanism by which such divergent biological outcomes are achieved through the use of the Ras signaling pathway. The presence of three Akt consensus sites in the amino-terminal regulatory domain of B-Raf led us to investigate cross-talk between the Ras/Raf pathway and Akt.

Mammalian cells contain three Raf proteins: c-Raf (or Raf-1), A-Raf, and B-Raf. B-Raf exists in multiple spliced forms, which exhibit tissue-specific expression patterns (11). Although all three Rafs are activated by receptor tyrosine kinases through their ability to associate with Ras, the three isoforms display differences in their regulation. Maximal activation of B-Raf requires only signals that activate Ras, whereas maximal activation of c-Raf and A-Raf require signals that activate Ras and signals that lead to their phosphorylation at tyrosine residue 341 (12, 13). Moreover, in PC12 cells, the sustained activation of ERKs in response to nerve growth factor is mediated by Rap1 acting not on c-Raf but on B-Raf (9). Thus, the three Raf proteins are differentially regulated.

Much is known about the role of phosphorylation in c-Raf regulation. Both stimulatory and inhibitory sites have been identified, and sites for serine/threonine and tyrosine phosphorylations have been mapped in c-Raf. The regulation of B-Raf by phosphorylation has diverged considerably from that of c-Raf. c-Raf contains tyrosine residues at 340 and 341, and Tyr^{341} is the major site of tyrosine phosphorylation when c-Raf is coexpressed with activated Ras and Src in mammalian cells

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[1] The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; HEK293 cells, human embryonic kidney 293 cells; HA, hemagglutinin; MOPS, 4-morpholinepropanesulfonic acid; K/D, kinase dead.

(13). In contrast, B-Raf does not have tyrosines at the positions equivalent to 340 and 341, but rather aspartic acid occupies these positions (Asp^{447} and Asp^{448}). These aspartic acid residues in B-Raf contribute to the elevated basal kinase activity observed with B-Raf (13). The elevated kinase activity of B-Raf is normally 15–20 times higher than an equivalent amount of c-Raf. Thus, in striking contrast to c-Raf, tyrosine kinases are not involved in the activation of B-Raf. Moreover, Ser^{445} in B-Raf (the equivalent residue in c-Raf is Ser^{338}) is constitutively phosphorylated (13). In c-Raf, there is a low level of phosphorylation at Ser^{338} in serum-starved cells, which is then greatly increased following stimulation with growth factors, including epidermal growth factor (14). Alteration of Ser^{445} in B-Raf reduces its basal kinase activity; thus, constitutive phosphorylation of B-Raf at Ser^{445}, together with the aspartic acid residues at 447 and 448, is responsible for the elevated basal kinase activity of B-Raf.

Recently, while the work that is presented here was in progress, Rommel *et al.* (15) demonstrated that in one biological cell context, that of muscle cell hypertrophy, the Ras/Raf/ERK pathway and Akt have opposing effects. In addition, Zimmerman and Moelling (16) showed that Akt could phosphorylate c-Raf at Ser^{259}. The alteration of Ser^{259} to A resulted in a relatively modest 2-fold increase in enzymatic activity. Phosphorylation of Ser^{259} in the amino-terminal domain of c-Raf has been previously shown to decrease the enzymatic activity of c-Raf by promoting its association with 14−3−3 proteins (12). The serine residue at 259 in c-Raf is conserved in B-Raf, Ser^{364}, and as in c-Raf, this residue is part of a consensus site for Akt phosphorylation. Intriguingly, B-Raf has two additional Akt consensus sites, Ser^{428} and Thr^{439}. Moreover, the two Akt consensus sites unique to B-Raf do not meet the consensus for 14-3-3 binding.

Here we demonstrate that B-Raf is phosphorylated by Akt. In contrast to c-Raf, phosphorylation of B-Raf occurs at multiple residues within its amino-terminal regulatory domain. The alteration of the serine residues within the Akt consensus sites to alanines results in a progressive increase in enzymatic activity both *in vitro* and *in vivo*. Our results demonstrate that B-Raf activity can be negatively regulated by Akt through phosphorylation in the amino-terminal regulatory domain of B-Raf.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis—B-Raf was expressed as a fusion protein in HEK293 cells to either glutathione *S*-transferase (GST) in the expression vector pEBG-4X or to HA in the expression vector pcDNA3 (Invitrogen). Mutations in B-Raf at the ATP binding site (K482M) and at the Akt consensus phosphorylation sites were generated by site-directed mutagenesis and confirmed by restriction enzyme and sequence analysis.

Kinase Assays—For HA-B-Raf activity assays, 100 ng of pcDNA3-HA-B-Raf was transfected into HEK293 cells in 6-well plates using LipofectAMINE (Life Technologies, Inc.). 24 h after transfection, cells were starved in 0.1% fetal bovine serum medium for 12 h. Cells were lysed in radioimmune precipitation buffer. HA-B-Raf was immunoprecipitated with 2 μg of anti-HA antibody (BabCo). The immunoprecipitated HA-B-Raf was assayed by a coupled *in vitro* kinase assay (17). Briefly, 0.1 μg of GST- mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 was incubated with the precipitated HA-B-Raf in 20 μl of kinase buffer for 20 min at 30 °C. The reaction was briefly centrifuged to pellet the HA-B-Raf, which was bound to protein G-agarose. 15 μl of the supernatant was mixed with 0.1 μg of GST-ERK1 in 20 μl of kinase reaction buffer and incubated for 10 min at 30 °C. Then 10 μl of a mixture containing 3 μg of GST-Elk-1 and [γ-^{32}P]ATP was added to the reaction and incubated for 20 min at 30 °C. Phosphorylation of GST-Elk was determined by SDS-polyacrylamide gel electrophoresis and phosphorimage analysis.

Myc-ERK plasmid (300 ng) was transfected into HEK293 cells in the presence or absence of B-Raf or Ras. 24 h after transfection, cells were starved in 0.1% fetal bovine serum medium for 12 h. Myc-ERK were immunoprecipitated, and kinase activity was determined using GST-Elk as a substrate (18). The amount of Myc-ERK used for kinase assays was analyzed by anti-ERK immunoblot.

For the Akt kinase assays, HEK293 cells in 60 mm dishes were transfected with 6 μg of pCS2+-N-FLAG-Akt using a calcium phosphate transfection method. 48 h after transfection, cells were starved for 16 h in 0.1% fetal bovine serum medium. Following treatment with 100 mM LY294002 (or Me_2SO vehicle) for 1 h, cells were stimulated with insulin (20 μg/ml medium; 3.5 μM) for 10 min and immediately lysed in buffer A (19). Clarified extracts were incubated for 2 h with anti-FLAG M2-agarose resin to purify FLAG-Akt. The immunoprecipitated Akt was washed three times in buffer A containing 0.5 M NaCl, twice with buffer B, and once with assay dilution buffer before being evenly aliquotted to tubes for kinase assay. GST-tagged, kinase-inactive B-Raf and B-Raf mutant cDNAs (15 μg) were transfected into 100-mm dishes of HEK293 cells. 36 h after transfection, actively growing cells were lysed in radioimmune precipitation buffer, and GST-B-Raf proteins were collected with glutathione-Sepharose beads. The beads were washed three times in buffer A containing 0.5 M NaCl, twice with buffer B, and GST-B-Raf substrates were then eluted using 10 mM glutathione in 50 mM Tris (pH 8.0) and quantitated using a Bradford assay (Bio-Rad). Approximately 1.0 μg of each GST-B-Raf mutant was incubated with immunoprecipitated Akt at 30 °C for 30 min in a kinase reaction containing 6.7 mM MOPS (pH 7.2), 8.3 mM β-glycerol phosphate (pH 7.0), 0.33 mM Na_3VO_4, 0.33 mM dithiothreitol, 25 mM $MgCl_2$, 167 mM ATP, and 10 μCi of [γ-^{32}P]ATP. One-third of the reaction product was subjected to SDS-polyacrylamide gel electrophoresis followed by transfer to Immobilon filter, phosphorimager analysis, and Western blotting to verify equivalent GST-B-Raf substrate levels.

Coimmunoprecipitation of B-Raf and Akt—GST-Akt and HA-B-Raf were cotransfected into HEK293 cells using a calcium phosphate transfection protocol. Approximately 36 h after transfection, cells were lysed in 10 mM HEPES, pH 7.4, 50 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.1% β-mercaptoethanol, 1% aprotinin, 50 mM NaF. Glutathione-agarose beads were added to the cell lysates to purify GST-Akt and associated protein(s). GST-Akt and associated protein(s) were eluted in 10 mM glutathione in 50 mM Tris, pH 8.0. Similar experiments were performed with cells transfected with GST-B-Raf and HA-Akt, except that the cells were lysed in 10 mM sodium phosphate, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mg/ml leupeptin, 5 mg/ml aprotinin, 50 mM NaF, 1 mM sodium vanadate. A GST expression vector was included as a negative control. The glutathione-eluted samples were analyzed by Western blot with anti-GST and anti-HA antibodies.

Reporter Assays—In general, HEK293 cells in 35-mm dishes were transfected using a standard calcium phosphate transfection protocol with 36 ng of Gal4-ElkC chimera, 290 ng of a 5xGal4-luciferase reporter, and 145 ng of a B-Raf expression vector or 15 ng of a K-RasV12 expression vector. Total DNA was kept constant by the addition of the appropriate amount of pcDNA3 for all transfections. Luciferase assays were performed using the dual-light luciferase and β-galactosidase reporter gene assay system (Tropix) and were normalized for transfection efficiency using a cotransfected β-galactosidase expression vector (15 ng).

RESULTS

The presence of multiple Akt consensus sites in the amino-terminal regulatory domain of B-Raf led us to investigate whether cross-talk between the Ras signaling pathway and Akt occurs *in vivo*. To determine whether Akt can modulate B-Raf function *in vivo*, we examined the effect of Akt activation and inhibition on B-Raf activity (Fig. 1). Expression of a membrane localized, constitutively active Akt (myrAkt) inhibits B-Raf activity in both actively growing HEK293 cells (Fig. 1*A*, compare *lane 2* with *lane 1*) and in cells stimulated with epidermal growth factor (Fig. 1*A*, compare *lane 3* with *lane 4*). The inhibition of B-Raf activity by Akt is dose-dependent (Fig. 1*B*). A kinase dead version of Akt has no effect on B-Raf activity, suggesting that the catalytic activity of Akt is required to inhibit B-Raf (Fig. 1*C*). To further test the involvement of endogenous Akt in B-Raf regulation, the PI3K inhibitor LY294002 was used to block the PI3K/Akt pathway. As shown in Fig. 1*D*, the addition of LY294002 elevates B-Raf activity, suggesting that Akt negatively regulates B-Raf enzymatic activity.

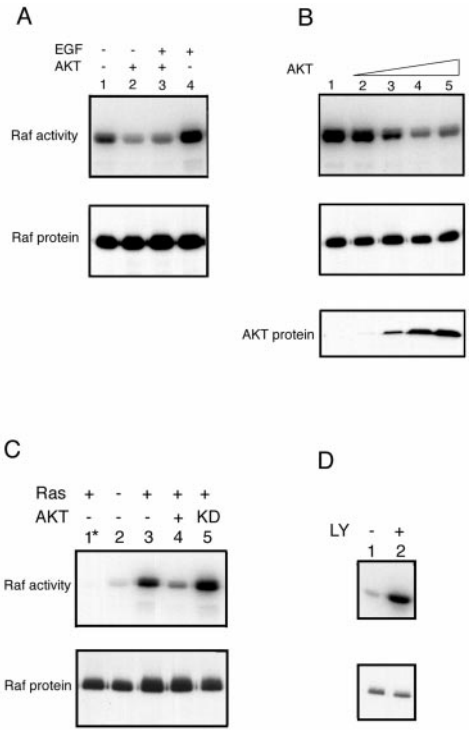

FIG. 1. **Akt inhibits B-Raf activity *in vivo*.** *A,* HEK293 cells were transfected with expression vectors for HA-B-Raf and myrAkt, as indicated. B-Raf was immunoprecipitated from lysates and Raf activity assessed by an *in vitro*-coupled kinase reaction in which bacterially expressed GST-Elk-1 was used as a substrate (*top panel*). *Lanes 1* and *2,* B-Raf was immunoprecipitated from transfected cells. *Lanes 3* and *4,* B-Raf was immunoprecipitated from cells after serum starvation and stimulation with epidermal growth factor for 3 min. Western blot of HA-B-Raf proteins used for the *in vitro* kinase is shown in the *bottom panel*. *B,* Akt inhibits Raf enzymatic activity in a dose-dependent manner. HEK293 cells were transfected with the expression vector for HA-B-Raf and increasing concentrations of myrAkt, as indicated. B-Raf activity was determined by a coupled *in vitro* kinase reaction (*top panel*). Western blot of HA-B-Raf proteins used in the *in vitro* kinase reactions (*middle panel*). Western blot of HA-Akt in cell lysates (*bottom panel*). *C,* Akt kinase activity is required to inhibit B-Raf activity. HA-B-Raf was transfected with expression vectors for K-RasV12 or Akt as indicated. Activated Ras stimulates the co-transfected Raf activity (compare *lanes 2* and *3*). GST-mitogen-activated protein kinase/extracellular signal-regulated kinase kinase was omitted in *lane 1* as a control for the specificity of the *in vitro* Raf kinase assays. The Ras-induced Raf activation is inhibited by wild type (*lane 4*) but not the kinase inactive (*lane 5*) Akt. *D,* B-Raf activity is enhanced upon inhibition of PI3K. Ha-B-Raf-transfected cells were treated with LY294002 for 1 h. HA-B-Raf was isolated, and kinase activity was determined.

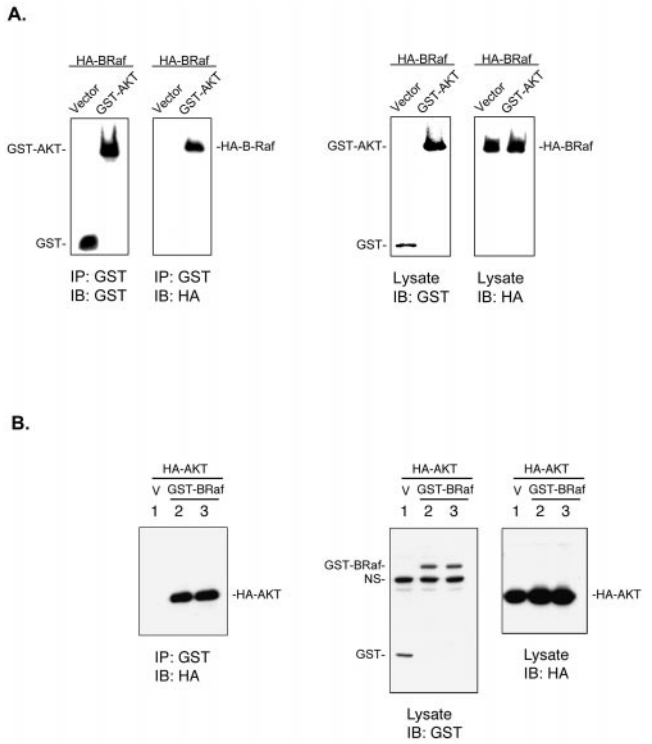

FIG. 2. **B-Raf and Akt co-associate *in vivo*.** GST-Akt (*A*) or GST-B-Raf (*B*) was purified from lysates prepared from HEK293 cells transfected with the indicated constructs using glutathione-Sepharose. The pull-downs were subject to SDS-polyacrylamide gel electrophoresis followed by Western blotting with antibodies directed against the epitope tags on B-Raf and Akt. *IP*, immunoprecipitate; *IB*, immunoblot.

TABLE I
Akt consensus sites RXRXXS/Tϕ

Protein	Site	Position
Glycogen-synthase kinase 3β	RPRTT**S**F	9
BAD	RGRSR**S**A	136
FKHR	RRRAA**S**M	253
c-Raf	RQRST**S**T	259
B-Raf	RDRSS**S**A	364
	RERKS**S**S	428
	RNRMK**T**L	439

We also examined the association of B-Raf with Akt *in vivo*. As shown in Fig. 2, B-Raf and Akt associate in HEK293 cells overexpressing these proteins. The association between Akt and B-Raf is observed in actively growing HEK293 cells and in HEK293 cells that have been serum-starved or serum-starved and then stimulated with insulin. However, in one orientation, when GST-B-Raf is isolated from HEK293 cells expressing Flag epitope-tagged Akt, the association between B-Raf and Akt is enhanced upon insulin stimulation (data not shown). This may suggest that the association between B-Raf and Akt is sensitive to, but not absolutely dependent on, whether one or both of the proteins is in an activated state.

B-Raf contains three Akt consensus sites, Table I. One site, Ser^{364} is conserved with c-Raf; however, two sites, Ser^{428} and Thr^{439}, are unique to B-Raf. To begin to examine the physiological significance of phosphorylation at these sites, the conserved and unique sites were altered to alanine alone and in combination. The effect of the mutations on enzymatic activity *in vitro* and *in vivo* was assessed.

Mutation of Ser^{364} to Ala (A), of both 428 and 439 to Ala (AA), or of all three Akt consensus sites to Ala (AAA) resulted in a progressive increase in enzymatic activity: B-Raf < B-Raf A < B-Raf AA < B-Raf AAA (Fig. 3). Alteration of Ser^{364} to Ala (conserved with c-Raf) leads to a modest 2X elevation in enzymatic activity, whereas alteration of both Ser^{428} and Thr^{439} to Ala (unique to B-Raf) leads to at least a 19 times elevation of enzymatic activity. Thus, phosphorylation of B-Raf at both the unique and conserved phosphorylation sites is likely to negatively regulate B-Raf enzymatic activity.

Activation of the Ras/Raf/ERK pathway culminates in the phosphorylation of transcription factors, including the ternary complex factor Elk-1 (4). Thus, the degree of activation of the Ras/Raf/ERK pathway can be assessed by examining the transcriptional activity of Elk-1. To address whether the increase in enzymatic activity of the B-Raf alanine mutants *in vitro* correlates with deregulation of enzymatic activity *in vivo*, the effect of the B-Raf mutants on Elk-1-mediated transcriptional activity was assessed by examining the activity of a Gal4-ElkC reporter. Gal4-ElkC contains the carboxyl-terminal domain of

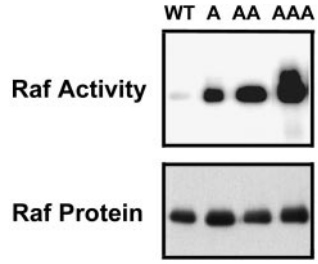

FIG. 3. **B-Raf kinase activity is progressively increased upon mutation of the Akt consensus sites.** HA epitope-tagged B-Raf, wild type, or mutant at the Akt consensus sites was immunoprecipitated from HEK293 cells, and its enzymatic activity assessed by a coupled *in vitro* kinase reaction in which bacterially expressed GST-Elk-1 was used as substrate. *WT,* wild type HA-B-Raf; *A,* HA-B-Raf S364A; *AA,* HA-B-Raf S428A/T439A; *AAA,* S364A/S428A/T439A. Raf kinase activity and protein are shown in the *top* and *bottom panels*, respectively.

Elk-1, which includes the transactivation domain of Elk and the ERK phosphorylation sites, fused to the DNA binding domain of the yeast Gal4 protein. The transcriptional activity of Gal4-ElkC is dependent on the phosphorylation of the Elk transactivation domain by ERK (20). As shown in Fig. 4, the B-Raf mutants activate the Elk reporter to a greater extent than wild type B-Raf. Furthermore, B-Raf mutant at all three of the Akt consensus sites activates the Elk-1 reporter to a greater extent than B-Raf mutant at only 364 or B-Raf mutant at both S428A and T439A. The observed increase in reporter activity with the mutants correlates with the progressive increase in enzymatic activity observed in the *in vitro* kinase reactions shown in Fig. 3. Thus, B-Raf proteins altered so as to prevent phosphorylation at the Akt consensus sites activate downstream signaling events.

We also examined the effect of the phosphatidylinositol 3-kinase inhibitor LY294002 on activation of the Elk-1 reporter by wild type B-Raf. We consistently observe a modest increase in the ability of B-Raf to activate the Elk-1 reporter in the presence of LY294002 (Fig. 4). This result is consistent with the data in Fig. 1 showing that Akt can negatively regulate B-Raf activity *in vivo*.

In addition, we assessed the deregulation of the enzymatic activity of the B-Raf mutants *in vivo* by assaying ERK activity in the presence and absence of the B-Raf mutants. As expected, ERK activity is elevated in HEK293 cells expressing the B-Raf mutants: B-Raf AAA > B-Raf AA > B-Raf A > B-Raf (Fig. 5).

To determine whether B-Raf is a substrate of Akt, *in vitro* kinase reactions were performed. A kinase dead version of B-Raf fused to glutathione *S*-transferase (K/D GST-B-Raf) was used in the *in vitro* kinase reactions. Akt phosphorylates K/D GST-B-Raf but not the control protein GST (Fig. 6). Phosphorylation of K/D GST-B-Raf requires active Akt because phosphorylation was not observed when Akt was prepared from cells in the presence of the phosphatidylinositol 3-kinase inhibitor LY294002.

To determine which of the Akt consensus sites in B-Raf were utilized by Akt, each of the sites was altered to alanine alone and in combination in the context of a K/D GST-B-Raf (see Table I). Each of the mutant proteins was purified from HEK293 cells and used as substrate in *in vitro* kinase reactions with active Akt (harvested from cells after insulin stimulation) or inactive Akt (harvested from cells after treatment with LY294002 and insulin) (Fig. 6*A*). Quantitation of the *in vitro* kinase reactions by phosphorimage analysis indicates that each of the single mutants B-Raf S364A and B-Raf S428A is phosphorylated to approximately the same extent and that the extent of phosphorylation of each of these mutants is approximately one half that observed for the B-Raf control (Fig. 6*B*). Taken together, these observations suggest that Akt phosphorylates both Ser^{364} and Ser^{428}. In contrast, the single mutant B-Raf T439A is not significantly decreased in its phosphorylation compared with the B-Raf control, suggesting that Thr^{439} is not subject to phosphorylation by Akt. Consistent with the idea that Thr^{439} is not phosphorylated by Akt, the double B-Raf mutant S428A/T439A is phosphorylated to the same extent as the single B-Raf mutant S428A. Moreover, the extent of phosphorylation of the triple B-Raf mutant S364A/S428A/T439A is approximately one half that observed for each of the single mutants at residues 364 or 428, confirming that Akt phospho-

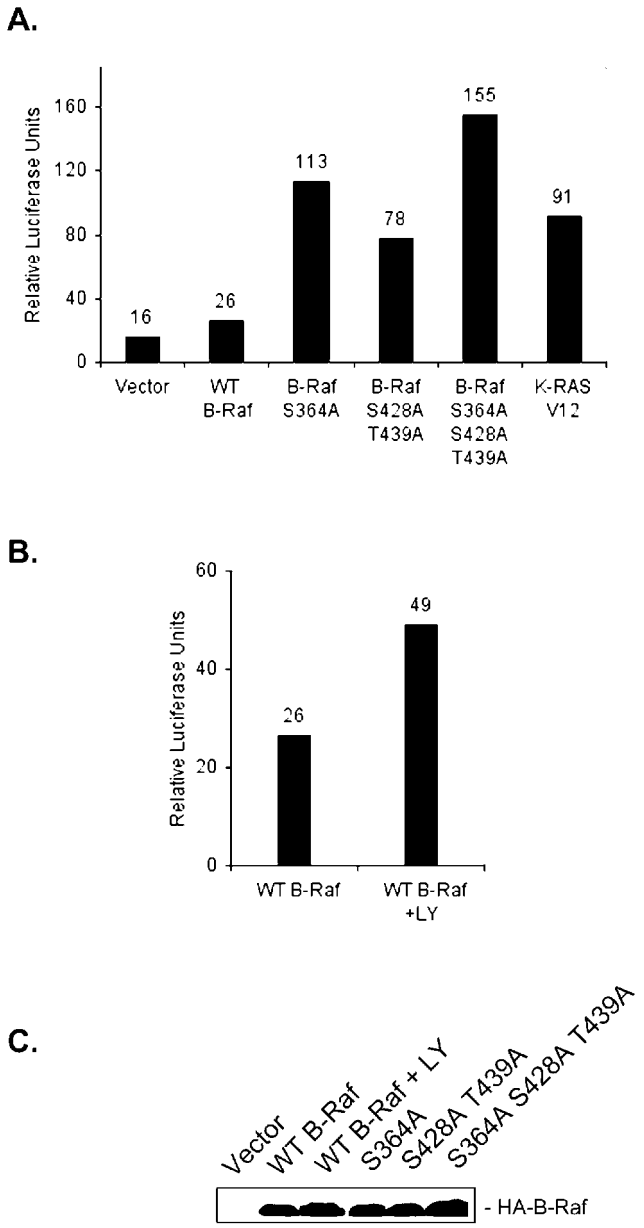

FIG. 4. **Activation of Elk-mediated transcription by B-Raf mutants.** *A,* HEK293 cells were transfected with expression vectors for Gal4-ElkC, Gal4-luciferase, and either pcDNA3 (vector control), K-Ras V12 (positive control), wild type (WT)-B-Raf, B-Raf S364A, B-Raf S428A/T439A, or B-Raf S364A/S428A/T439A. *B,* LY294002 (LY) was added for 24 h prior to harvest, WT B-Raf + LY. Luciferase activity was assayed 36 h after transfection. Luciferase activity was normalized to a co-transfected β-galactosidase expression vector. Shown is the average of two experiments performed in duplicate. *C,* Western blot of extracts showing expression of HA-B-Raf wild type and mutants.

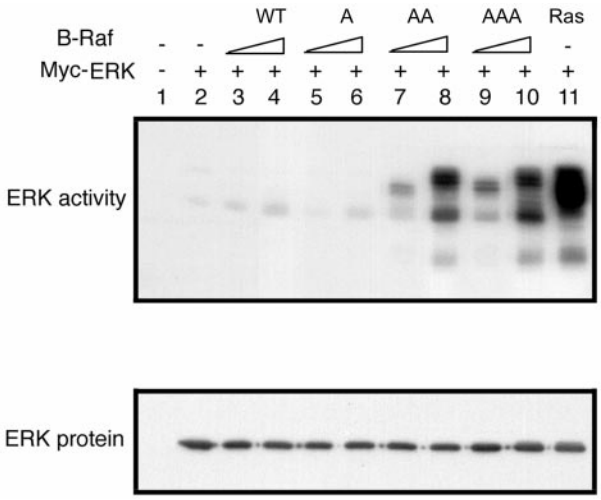

FIG. 5. **Mutant Raf has enhanced ability to stimulate ERK activity *in vivo*.** HEK293 cells were transfected with expression vectors for wild type or mutant B-Raf proteins or RasV12 and Myc-ERK, as indicated. Myc-ERK was immunoprecipitated, and kinase activity was determined. Western blotting with anti-ERK antibody shows the level of Myc-ERK used in the kinase assay (*bottom panel*).

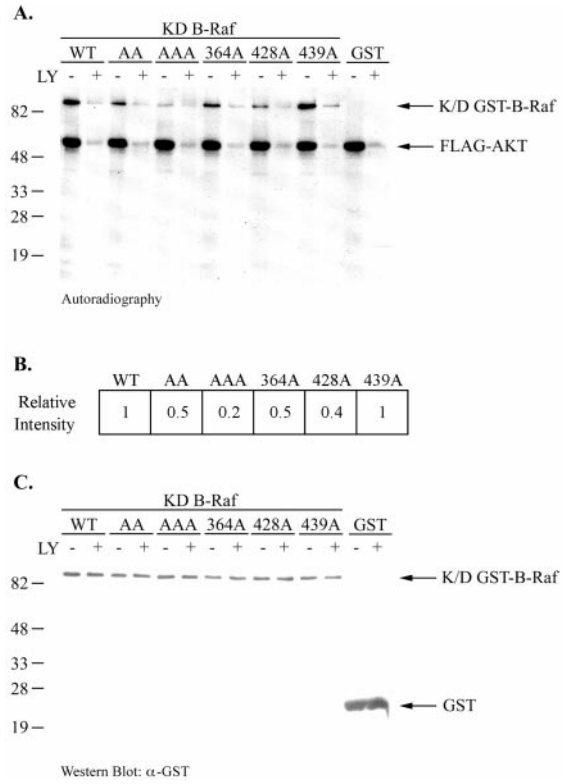

FIG. 6. **B-Raf is phosphorylated *in vitro* by Akt at residues 364 and 428.** Kinase inactive (K/D) GST-B-Raf fusion proteins were subjected to an *in vitro* kinase reaction with active Akt (−LY) or inactive Akt (+LY). In addition to the K482M mutation to create a kinase-inactive B-Raf, the B-Raf proteins either do not contain additional mutations and are designated WT, for wild type at the Akt consensus sites, or contain mutations at the Akt consensus sites alone (S364A, S428A, or T439A) or in combination (*AA,* S428A/T439A; *AAA,* S364A/S428A/T439A). *A,* autoradiography of the *in vitro* kinase reactions after SDS-polyacrylamide gel electrophoresis and transfer to an Immobilon filter. *Arrows* show the position of the phosphorylated K/D GST-B-Raf and the autophosphorylated Flag-Akt. *B,* relative intensity of the phosphorimage signal (−LY lanes) of wild type and mutant K/D GST-B-Raf from the filter in *A*. The intensity of the signal for each of the mutant proteins was determined relative to K/D GST-B-Raf, which was set to 1.0. *C,* Western blot analysis of the filter in *A*, using an anti-GST antibody showing equal loading of the GST-B-Raf substrates.

rylates both Ser^{364} and Ser^{428}. Thus, Akt phosphorylates B-Raf *in vitro* at residues Ser^{364} and Ser^{428}.

DISCUSSION

Here we have demonstrated that *in vivo* and *in vitro* the Ras/Raf signaling pathway is negatively regulated by Akt. First, epidermal growth factor stimulation of B-Raf activity is inhibited by co-expression of Akt. Second, B-Raf enzymatic activity is elevated after treatment of cells with LY294002, a pharmacological inhibitor of PI3K/Akt. Taken together, these observations demonstrate that Akt down-regulates B-Raf activity *in vivo*. Because B-Raf contains three Akt consensus sites located within its amino-terminal regulatory domain, the most likely effect of Akt is a direct phosphorylation at one or more of these sites and a concomitant down-regulation of B-Raf enzymatic activity. We have demonstrated that Akt will phosphorylate two of the three Akt consensus phosphorylation sites *in vitro*, Ser^{364} and Ser^{428}. Ser^{364} is conserved with c-Raf, but Ser^{428} is unique to B-Raf. Substitution of the phosphorylatable residue in the Akt consensus phosphorylation sites with alanine increased B-Raf enzymatic activity, as assessed by *in vitro* coupled kinase assays, activation of the Elk-1 reporter, and activation of ERK activity. Thus, phosphorylation of B-Raf at multiple residues within the amino-terminal regulatory domain negatively regulates its enzymatic activity *in vitro* and *in vivo*.

B-Raf enzymatic activity is further enhanced by combining the alanine substitution mutations within the Akt consensus phosphorylation sites. The seemingly additive nature of the mutations suggests that phosphorylation of these residues is not likely to be ordered but rather phosphorylation at one residue is likely to occur independently of the status of phosphorylation at the other residues. The multiplicity of phosphorylation sites in B-Raf may enable a more flexible regulation (either duration or timing) of B-Raf activity.

Surprisingly, blocking the activation of Akt with the pharmacological inhibitor of phosphatidylinositol 3-kinase, LY294002, did not up-regulate B-Raf activity to the same extent as altering all three Akt consensus sites. The addition of LY294002 only modestly up-regulated B-Raf enzymatic activity (Fig. 1 and data not shown) and Elk-1 reporter activity (Fig. 4), whereas the B-Raf AAA mutant exhibited a striking 20× elevation in enzymatic activity (Fig. 3) and a 6× elevation in Elk-1 reporter activity (Fig. 4). Possibly the addition of LY294002 does not block Akt activity in its entirety, a result consistent with observations that Akt can be activated by lipid-independent mechanisms (6). Alternatively, other kinases, in addition to Akt, may be able to regulate B-Raf enzymatic activity by phosphorylation of these sites. Because Thr^{439} is not phosphorylated by Akt *in vitro*, these other kinases may negatively regulate B-Raf activity at this site.

While our studies were in progress, c-Raf was shown to be phosphorylated by Akt at Ser^{259} (16), equivalent to Ser^{364} in B-Raf. The alteration of Ser^{259} to alanine in c-Raf resulted in a modest 2-fold increase in enzymatic activity. Phosphorylation of Ser^{259} in c-Raf had been previously shown to decrease the enzymatic activity of c-Raf by promoting its association with 14-3-3 proteins (12). Our results support and extend these observations. First, the regulation of Raf kinases extends to multiple family members; both c-Raf (16) and B-Raf (this report) are subject to phosphorylation and regulation by Akt. Second, of the three Akt consensus sites in B-Raf, only the site conserved with c-Raf (Ser^{364}) lies within a 14-3-3 binding motif (RSXS*XP, where S* represents phosphorylated serine) (12). This suggests that an as yet uncharacterized molecular mechanism, in addition to 14-3-3 binding, is contributing to the

negative regulation of B-Raf at Ser[428]. Phosphorylation of B-Raf by Akt does not appear to disrupt the association between B-Raf and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase or heat shock protein 90.[2] Phosphorylation may decrease the association between B-Raf and Ras or Rap or other adaptors and/or signaling molecules, such as Raf kinase inhibitor protein, that regulate Raf activity. Alternatively, phosphorylation may hinder the association of B-Raf with the plasma membrane. Experiments are in progress to test these possibilities. Third, the discordance between the activity of the B-Raf protein mutant at the Akt consensus sites and the activity of B-Raf in the presence of LY294002 might suggest that kinases, in addition to Akt, can act to negatively regulate B-Raf at one or more of these residues.

The Ras signaling pathway regulates cell growth, differentiation, cell survival, cell senescence, and cell death. The sustained *versus* transient activation of the Ras/Raf/ERK pathway is a critically important mediator of signaling specificity and biological outcome (9, 21). We have demonstrated here that Akt can regulate the activity of the kinase cascade downstream of Ras through phosphorylating and inhibiting B-Raf activity. It seems likely that the integration of signals from multiple kinase cascades within a particular cell, such as described here for B-Raf and Akt, will play an important role in modulating the specificity and biological outcome of signal transduction pathways.

[2] K.-L. Guan, C. Figueroa, and A. B. Vojtek, unpublished observations.

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