Different Effects of Point Mutations within the B-Raf Glycine-Rich Loop in Colorectal Tumors on Mitogen-Activated Protein/Extracellular Signal-Regulated Kinase Kinase/Extracellular Signal-Regulated Kinase and Nuclear Factor κB Pathway and Cellular Transformation

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ABSTRACT

Recently, mutations in the B-Raf gene have been identified in a variety of human cancers, such as melanoma and colorectal carcinoma, and more than 80% of the B-Raf mutations have been V599E. Although other mutations have been reported, their functional consequences are poorly understood. In our earlier study, we demonstrated that colon tumor-associated B-Raf mutations within the kinase activation segment are not necessarily associated with an increase in mitogen-activated protein/ extracellular signal-regulated kinase extracellular signal-regulated kinase (MEK/Erk) or nuclear factor κB (NFκB) signaling activity or in NIH3T3-transforming ability [T. Ikenoue et al., Cancer Res., 63: 8132–8137, 2003]. In this study, we examined the effect of colon tumor-associated mutations within the B-Raf glycine-rich loop (G loop) on MEK/Erk and NFκB signaling and on the transformation of NIH3T3 fibroblasts or IEC-6 intestinal epithelial cells. Of the six G loop mutations examined, only the B-Raf G468A significantly increased MEK/Erk and NFκB signaling and NIH3T3 transformation. Only this mutation induced transformed phenotypes of IEC-6 cells. In contrast, the B-Raf G468E mutation significantly decreased MEK/Erk signaling and NIH3T3 transformation and had no effect on NFκB signaling. The B-Raf F467C mutation moderately elevated MEK/Erk signaling and NIH3T3 transformation. The other three B-Raf mutations, R461I, I462S, and G463E, did not increase MEK/Erk or NFκB signaling or NIH3T3 transformation. Except for F467C, none of the tumors with B-Raf mutations examined in this study had K-Ras mutations. These results suggest that some of the B-Raf G loop mutations reported in colorectal tumors do not increase kinase or transforming activities but might contribute to carcinogenesis via other mechanisms or be irrelevant to carcinogenesis.

INTRODUCTION

The Raf serine/threonine kinases are key signal transducers in the Ras/Raf/mitogen-activated protein/extracellular signal-regulated kinase (Erk) kinase (MEK/Erk cascade, which regulates a variety of physiological processes, including cell growth, differentiation, and apoptosis (1–4). The Raf kinases are also involved in nuclear factor κB (NFκB) signaling, which plays important roles in distinct cellular functions, such as immune responses, inflammation, cell proliferation, and apoptosis (5–11). Activation of the NFκB signaling pathway is reported to be critical for cellular transformation by Raf (9, 10).

Activation of the Ras/Raf/MEK/Erk pathway by Ras commonly observed in human cancers. B-Raf, which is a human Raf isoform, is reportedly mutated in a high proportion of melanomas and in a small proportion of other cancers (12–26). B-Raf mutations are restricted to two regions of the B-Raf kinase domain: the glycine-rich loop (G loop) and the kinase activation segment. More than 80% of the reported B-Raf mutations were T to A transversions at nucleotide 1796, which results in a substitution of glutamic acid for valine at codon 599 (V599E) in the kinase activation segment. Approximately 10% of the colon cancer-associated B-Raf mutations have been found in the G loop (13, 14).

The G loop, with the consensus sequence GXGXGXG, is located in the first of the 12 subdomains of the catalytic domain and is a highly conserved protein kinase motif (27). The three glycine residues of the motif are conserved in most of the known human kinases. The G loop is a part of a β-strand-loop-β-strand nucleotide-positioning motif with a critical role in ATP binding. This motif covers and anchors the ATP, shielding it from solvents (27). The previously described mutations in any of the conserved glycines generally lead to a reduction in kinase activity and are responsible for enzymatic defects that lead to human diseases. For example, a form of diabetes, in which the third glycine in the G loop of the insulin receptor kinase is substituted by valine, leads to insulin resistance (28). In contrast, the Raf-1 mutant G361S, in which the third glycine of the GXGXGXG motif is replaced by serine, has recently been shown to possess higher catalytic activity than the wild-type Raf-1 (29).

Of the G loop mutants that have been detected in human cancer, only two, G463V and G468A, have been examined with respect to their functional consequences; the replacement of these conserved glycine residues was found to increase B-Raf kinase activity (12). In three other colon tumor-related G loop mutations, i.e., R461I, I462S, and F467C, the residues adjacent to the conserved glycines, not the glycines themselves, are mutated (13, 14).

In a previous study, we revealed that some of the B-Raf mutations within the kinase activation segment do not activate MEK/Erk or NFκB signaling or NIH3T3 transformation (30). In the present study, we analyzed the functional consequences of colon cancer-associated mutations in the G loop of B-Raf and we demonstrate that they do not necessarily activate MEK/Erk or NFκB signaling pathways or increase transforming ability.

MATERIALS AND METHODS

Plasmids. The expression vectors for FLAG-tagged wild-type B-Raf and the B-Raf V599E mutant, constitutively active MEK kinase, dominant-negative inhibitor of nuclear factor-κB α (IκBα), the wild-type and dominant-negative IκB kinase (IKK) α or β, the NFκB-inducible reporter, and the Renilla luciferase control vector have been described previously (30). Hemagglutinin-tagged wild-type B-Raf expression vector, pcDNA3-HA-B-Raf, was kindly provided by Dr. Anne Vojtek (University of Michigan, Ann
Arbor, MI). The B-Raf G loop mutant constructs found in colorectal tumors (R461L, I462S, G468E, and G468A;Refs. 12–16) and B-Raf K482M, which is considered a kinase-dead mutant, were created by oligonucleotide-directed mutagenesis as described previously (30). To create dominant-negative B-Raf, the cDNA fragment encoding amino acids 1–442 of B-Raf was amplified by PCR and subcloned into the pcDNA3-HA vector. To create bacterially expressed glutathione S-transferase (GST) fusion IxBo (2–54) expression vector pGEX-IxBo (2–54), the cDNA fragment encoding amino acids 2–54 of IxBo was amplified by PCR and subcloned into the pGEX-2T vector (Amersham Biosciences, Buckinghamshire, United Kingdom). The constitutively active H-Ras expression vector pCMV-Ras V12 was purchased from Clontech (Palo Alto, CA). The pEA-EIk1 encoding the DNA-binding domain of yeast Gal4 fused to the activation domain of Elk1 and the pFR-luc, in which a promoter containing Gal4-binding sites controls the luciferase expression, were purchased from Stratagene (La Jolla, CA).

Cell Culture and Transfection. COS7 and NIH3T3 cells were cultured and transfected as described previously (30). IEC-6 cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan) and cultured in DMEM containing 5% fetal bovine serum and 4 μg/ml insulin.

To generate NIH3T3 and IEC-6 cells stably expressing the FLAG-B-Raf wild type or mutants, cells were maintained in growth medium containing 400 μg/ml Geneticin (Invitrogen, Carlsbad, CA). Multiple Geneticin-resistant colonies were then pooled together (>50 colonies) for use for proliferation and soft agar assays as described previously (31).

In Vitro Kinase Assay. The B-Raf activity assays were performed as described previously (30). In brief, COS7 cells were transfected with the FLAG-B-Raf constructs in the presence or absence of H-Ras V12. Three h after transfection, the cells were transferred to medium containing 0.1% serum and cultured for an additional 24 h. The cells were lysed in radioimmunoprecipitation assay buffer. FLAG-B-Raf was immunoprecipitated using anti-FLAG M2 affinity gel (Sigma, St. Louis, MO) and assayed with the B-Raf kinase cascade assay (Upstate Biotechnology, Lake Placid, NY), using GST-MEK1, GST-Erk2, and myelin basic protein as sequential substrates, according to the manufacturer’s protocol.

For the IKKβ kinase assay, NIH3T3 cells in 6-well plates were transfected with the indicated amount of the FLAG-B-Raf G468A mutant, constitutively active MEK kinase, or empty vector in the presence of wild-type IKKβ expression vector (0.5 μg). IKKβ was immunoprecipitated using the protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-IKKβ polyclonal antibody (H-470; Santa Cruz Biotechnology). The immunoprecipitated IKKβ complex was added to a mixture that contained 2 μg of GST-IκBα (2–54) and 10 μCi of [γ-32P]ATP in kinase assay buffer (20 mM 4-morpholinepropanesulfonic acid (pH 7.2), 25 mM β-glycerophosphate, 15 mM MgCl₂, 5 mM EGTA, 1 mM sodium vanadate, 1 mM DTT, and 100 μM ATnP), and the mixture was incubated for 20 min at 30°C. The samples were then subjected to SDS-PAGE and then detected with the GS-525 Molecular Imager using Screen-CH (Bio-Rad, Hercules, CA).

Erk Activation Assay. The Erk activation assay was performed as described previously (30). In brief, COS7 cells in 6-well plates were transfected with FLAG-B-Raf constructs, oncogenic H-Ras, or empty vector. The cells were further cultured for 24 h in medium containing 0.1% fetal bovine serum. The cell lysates were then analyzed using SDS-PAGE and immunoblotted using anti-phospho-p44/42 mitogen-activated protein kinase and anti-p44/42 mitogen-activated protein kinase antibodies (Cell Signaling Technology, Beverly, MA).

Luciferase Reporter Assay. For the Elk1-dependent luciferase reporter assay, COS7 or IEC-6 cells in 12-well plates were cotransfected with 0.03 μg of pEA-EIk1, 0.3 μg of pFR-luc, 0.05 μg of pRL-TK, and 0.5 μg of the wild-type or mutant FLAG-B-Raf constructs or 0.05 μg of H-Ras V12. The NFkB-dependent luciferase reporter assay was performed as described previously (30). NIH3T3 cells were cotransfected with pNFκB-luc, pRL-TK, and the wild-type or mutant FLAG-B-Raf constructs. To examine the effects of the dominant-negative mutants IxBo, IKKα, or IKKβ in B-Raf-induced NFκB-dependent transcription, NIH3T3 cells were cotransfected with pNFκB-Luc, pRL-TK, B-Raf mutants, and dominant-negative vectors.

Focus Formation Assay. The focus-formation assay was performed as described previously (30). In brief, the cells in 10-cm dishes were transfected with 0.5 μg (NIH3T3) or 5.0 μg (IEC-6) of empty vector, FLAG-B-Raf constructs, or H-Ras V12 expression vector, cultured in DMEM containing 5% serum, and stained with crystal violet (NIH3T3) or 21 (IEC-6) days later, and the number of transformed foci was counted.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide (MTT) Assay. NIH3T3 cells stably expressing FLAG-B-Raf constructs were seeded in 96-well plates at a density of 1 × 10³ cells/well and grown in medium containing 1% calf serum. One, 4, and 7 days after seeding, MTT solution (Sigma) was added at 0.5 mg/ml, and after an additional 4-h incubation, 100 μl of isopropanol containing 0.04 n HCl was added to extract the reduced formazan product. The reduction of MTT was quantified by measuring the absorbance at 570 nm.

Soft Agar Assay. NIH3T3 cells stably expressing FLAG-B-Raf constructs were trypsinized, suspended in medium containing 0.3% agar and 10% calf serum, and plated onto a bottom layer containing 0.6% agar. The cells were plated at a density of 3 × 10³ cells/6-cm dish, and the number of colonies >0.5 mm in diameter was counted 14 days later.

RESULTS

In Vitro Kinase Activities of B-Raf G Loop Mutants. In this study, we analyzed the biological effects of B-Raf mutations within the G loop that have been previously reported in colorectal cancer cases (Fig. 1). As listed in Table 1, the wild-type K-Ras gene was present in all of the tumors with B-Raf mutations except F467C. Both the B-Raf G468A mutation, which was reported in a colorectal polyp as well as in lung and oropharynx cancers (12, 15, 26), and the B-Raf V599E mutation, which is the mutation that is most commonly found in human cancers (12–26), have been reported to constitutively activate the MEK/Erk signaling pathway (12).

First, we examined whether individual B-Raf mutants could activate the MEK/Erk signaling cascade using the in vitro-coupled B-Raf kinase assay (Fig. 2). Consistent with a previous report (12), both the B-Raf V599E and G468A mutants showed high basal kinase activities (6.3- and 7.2-fold increases, respectively, compared with the wild type). B-Raf F467C had moderately high basal kinase activity (2.5 times that of the wild type); the B-Raf R461L, I462S, and G463E mutants had basal kinase activities that were not higher than the wild-type B-Raf activity (0.89-, 0.82-, and 0.77-fold increases, respectively, versus the wild type). In contrast to the B-Raf G468A mutant, the B-Raf G468E mutant showed reduced basal kinase activity (0.24...
time that of the wild type), although the activity was higher than that of kinase-dead B-Raf, B-Raf K482M.

Moreover, as shown in Fig. 2, oncogenic H-Ras increased the kinase activity of the wild-type B-Raf 6.2-fold. The B-Raf mutants were stimulated to different degrees by oncogenic H-Ras, but the fold-activation levels of the individual G loop mutants examined were not higher than that of the wild type (1.5–6.1-fold; Fig. 2), which was not the case for B-Raf K482M.

**Effects of B-Raf G Loop Mutants on Erk Activation.** Next, we examined whether the B-Raf G loop mutants activate the MEK/Erk cascade in vivo. COS7 cells were transfected with wild-type or B-Raf mutant constructs, and the Erk activities were determined by immunoblotting using phosphospecific Erk antibody. As expected, the Erk activity was elevated in cells expressing B-Raf G468A and F467C and was comparable with that in H-Ras V12-expressing cells, whereas the Erk activity was not increased significantly in the other G loop mutants compared with wild-type B-Raf (Fig. 3A).

Then we assessed whether the B-Raf G468E mutant acts as dominant negative and inhibits Ras-induced Erk activation by the Elk1-dependent reporter assay. When the cells were cotransfected with the H-Ras V12 and wild-type B-Raf, the Elk1-dependent reporter activity was increased further (Fig. 3B). Cotransfection of dominant-negative B-Raf strongly inhibited H-Ras V12-induced reporter activity, whereas cotransfection of the B-Raf G468E did not significantly decrease the H-Ras V12-induced reporter activity, indicating that this mutant does not have a dominant-negative effect (Fig. 3B).

We also assessed whether the B-Raf G loop mutants also activate the MEK/Erk cascade in intestinal cells by measuring the Elk1-dependent transcriptional activity. As shown in Fig. 3C, only B-Raf G468A showed significantly elevated Elk1-dependent reporter activity in IEC-6 cells, whereas the other B-Raf G loop mutants did not.

These results indicate that the B-Raf G loop mutants reported in colorectal tumors do not necessarily elevate MEK/Erk signaling activity, as compared with wild-type B-Raf.

**Effects of B-Raf G Loop Mutants on NFκB-Dependent Transcription.** Because activation of NFκB signaling pathway is reported to be critical for Raf-induced transformation (9, 10), we examined whether colon cancer-associated B-Raf G loop mutants activate the NFκB pathway using the luciferase reporter assay. The NFκB-inducible reporter activity was elevated slightly by transfection with the wild-type B-Raf (1.2-fold compared with the empty vector; Fig. 4A). The positive control B-Raf V599E increased NFκB reporter activity more than the wild type did (4.8-fold compared with wild-type B-Raf). Of the G loop mutants examined in this study, only B-Raf G468A strongly induced NFκB reporter activity (4.1-fold compared with the wild type). The other G loop mutants did not signifi-

### Table 1  
**B-Raf G loop mutations, their basal kinase activities, and K-Ras status of the colon tumors examined in this study**

<table>
<thead>
<tr>
<th>B-Raf</th>
<th>B-Raf kinase activity&lt;sup&gt;a&lt;/sup&gt; (fold increase over wild type)</th>
<th>K-Ras&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>R461I</td>
<td>0.89</td>
<td>Wild type</td>
</tr>
<tr>
<td>I462S</td>
<td>0.82</td>
<td>Wild type</td>
</tr>
<tr>
<td>G463E</td>
<td>0.77</td>
<td>Wild type</td>
</tr>
<tr>
<td>F467C</td>
<td>5.77</td>
<td>G12D</td>
</tr>
<tr>
<td>G468E</td>
<td>0.24</td>
<td>Wild type</td>
</tr>
<tr>
<td>G468A</td>
<td>7.2</td>
<td>Wild type</td>
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</table>

<sup>a</sup> Data from the results of the in vitro-coupled B-Raf kinase assay (Fig. 2).

<sup>b</sup> Data from Refs. 12–16.
Examined in our previous study (30). As shown in Fig. 5, as did the B-Raf V599E and K600E activation segment mutants, dominant-negative IκBα/H9260A activity (Fig. 5). Transfection of the B-Raf G468A mutant with the dominant-negative G468A-induced NFκB activity (Fig. 5). SS32/36AA. After 24 h, dual luciferase assay was performed. The results are mean ± SD from three independent experiments.

NFκB is regulated by inhibitor proteins, IκBs, which reside in the cytoplasm. To examine the specificity of the reporter construct, cotransfection of the B-Raf G468A mutant with the dominant-negative form of IκBα, IκBα SS32/36AA, was performed. The B-Raf G468A-induced NFκB reporter activity was completely blocked by the dominant-negative IκBα (Fig. 4B). These results indicate that some B-Raf G loop mutants can activate NFκB signaling.

Activation of IKKβ by B-Raf G Loop Mutants. The signal-induced phosphorylation of the IκBs by IKKα and IKKβ and their subsequent degradation by ubiquitination-dependent processes are required for NFκB activation (5–7). Next, we examined whether the G loop mutant G468A could activate NFκB signaling through IKKβ, as did the B-Raf V599E and K600E activation segment mutants, examined in our previous study (30). As shown in Fig. 5A, the B-Raf G468A-induced NFκB reporter activity was completely blocked by the dominant-negative form of IKKβ, but only partially blocked by that of IKKα. Furthermore, coexpression of wild-type IKKβ, but not IKKα, with the B-Raf G468A mutant further induced NFκB reporter activity (Fig. 5A).

The ability of the B-Raf G468A mutant to induce IKKβ activation was then determined using in vitro kinase assays, with GST-IκBα (2–54) as the substrate. As shown in Fig. 5B, the B-Raf G468A mutant increased IKKβ kinase activity in a dose-dependent manner; B-Raf V599E also increased the kinase activity of IKKβ (data not shown). These results suggest that the B-Raf G468A mutant activates NFκB signaling pathway primarily through IKKβ.

Morphologically Transforming Activities of B-Raf G Loop Mutants. We then examined the transforming ability of each B-Raf G loop mutant. The morphology of NIH3T3 fibroblasts stably expressing wild-type or mutant FLAG-B-Raf is shown in Fig. 6A. The cells expressing B-Raf G468A and F467C were more refractile and less flattened, constituting typical morphological changes of transformed cells. In contrast, the NIH3T3 cells expressing the other G loop mutants or wild-type B-Raf were unlikely to be morphologically transformed. Oncogenic H-Ras displayed morphological changes not only in NIH3T3 fibroblasts but also in IEC-6 intestinal epithelial cells.

Some Geneticin-resistant clones of IEC-6 cells expressing the G468A mutant induced morphological changes that were easily distinguishable from the cells expressing empty vector and wild-type B-Raf (Fig. 6B). However, there were far fewer colonies of G468A-expressing IEC-6 cells that were morphologically transformed than there were those of oncogenic-Ras-expressing cells. The other G loop mutants expressing IEC-6 cells did not show any morphological changes (data not shown).

Loss of Contact Inhibition Induced by B-Raf G Loop Mutants. Next, we quantified the transforming ability of the B-Raf mutants. First, we performed a focus-formation assay to test for loss of contact inhibition. Wild-type B-Raf induced a small number of foci in the subline of NIH3T3 cells used for the focus formation assay as described previously (30). The B-Raf G468A mutant induced approximately 28 times more foci than did the wild-type B-Raf (Fig. 7A); this was comparable with the results with the B-Raf V599E mutant. The
B-Raf F467C mutant induced 5.8-fold more foci than did wild-type B-Raf. The B-Raf G468E mutant induced no foci in this study. The other three G loop mutants, R461I, I462S, and G463E, induced only a small number of foci (Fig. 7A), which was similar to the number of foci produced by the wild type.

Of the G loop mutants, only the G468A mutant induced transformed foci in IEC-6 epithelial cells; however, the number of foci was much less than that induced by oncogenic H-Ras (Fig. 7, B and C).

Proliferation Abilities Induced by B-Raf G Loop Mutants under Low-Serum Conditions. Next, we examined the proliferation ability of the cells stably expressing wild type or each mutant B-Raf under low-serum conditions. Cells expressing each B-Raf construct were seeded and grown in 1% serum, and cell proliferation was analyzed using MTT assays 1, 4, and 7 days later. Of the NIH3T3 cells expressing the B-Raf G loop mutants, the cells expressing B-Raf G468A and F467C mutants showed significantly increased proliferation rates, as compared with the cells expressing wild-type B-Raf (Fig. 8). By contrast, the cells expressing other B-Raf G loop mutants showed no increase in proliferation rates.

We also examined the proliferation ability of the IEC-6 cells expressing each G loop mutant in low-serum medium. However, the cells expressing each B-Raf mutant did not have a significantly increased proliferation rate, as compared with the cells expressing wild-type B-Raf (data not shown).

Anchorage-Independent Growth Induced by B-Raf G Loop Mutants. Finally, we used a soft agar assay to examine the effects of the B-Raf G loop mutants on anchorage-independent growth. The B-Raf G468A mutant-expressing NIH3T3 cells had the highest colony-forming activity (14.6 times that of wild-type B-Raf); this was comparable with that of the V599E-expressing cells (Fig. 9, A and B). The F467C mutant showed moderately increased colony formation ability (3.2 times that of wild-type B-Raf). The other B-Raf mutants did not show significantly elevated colony-forming activity in NIH3T3 cells, as compared with the wild type (Fig. 9B).

As shown in Fig. 9C, the IEC-6 cells expressing oncogenic H-Ras induced a large number of colonies in soft agar. Of the G loop mutants expressing IEC-6 cells, only the G468A-expressing cells induced a considerable number of colonies in soft agar (Fig. 9C); however, there were fewer colonies than induced by oncogenic-Ras-expressing cells. By contrast, the IEC cells expressing the other G loop mutants induced no colonies in soft agar (data not shown).

Ultimately, several transformation assays showed that the G468A and F467C mutations rendered B-Raf transforming, as compared with the wild type in NIH3T3 fibroblasts, whereas only the former had that effect in IEC-6 intestinal epithelial cells. Moreover, the IEC-6 transforming activity of the B-Raf G468A mutant was modest compared with that of oncogenic H-Ras. Conversely, the other B-Raf G loop mutations examined in this study did not increase the transforming activity in either NIH3T3 or IEC-6 cells.
DISCUSSION

In our earlier study, we analyzed the functional consequences of B-Raf mutations within the kinase activation segment (30). Although several types of non-V599E mutations have been reported, they do not necessarily increase kinase activity or NIH3T3 transformation (30). In this study, we analyzed the association between mutations within the G loop of B-Raf that have been identified in colorectal tumors and the up-regulation of B-Raf kinase activity and transforming ability. Two of the B-Raf G loop mutations found in colorectal tumors, G463V and G468A, were analyzed in the previous study and were found to have high basal kinase activities, as compared with the wild-type B-Raf, according to the in vitro B-Raf kinase cascade assay. In addition, both mutants had much higher NIH3T3 focus-forming activities than the wild-type B-Raf (12).

In this study, the basal kinase activity of the B-Raf V599E mutant, which is the most common mutation in human cancers, was 6.2-fold higher than that of wild-type B-Raf. Three of the G loop mutants examined in this study, B-Raf G463E, G468E, and G468A, had mutations in conserved glycine residues. Intriguingly, the B-Raf G468E mutant had reduced basal kinase activity in the MEK/Erk pathway; it did not up-regulate NFκB-dependent transcription, and it did not exhibit increased NIH3T3 focus-forming activity. This mutant contrasts with the B-Raf G468A mutant, which strongly activated MEK/Erk and NFκB signaling and increased NIH3T3 transformation.

The G loop forms a β-hairpin, which is thought to act as a highly flexible molecular lid that anchors ATP and shields it from water. The three glycines in the G loop are among the most highly conserved residues in the protein kinase family. The first, second, and third G are reportedly conserved in 94.4, 99.8, and 84.7% of known protein kinases, respectively (32). Although the third glycine is the least conserved, greater than 99% of all protein kinases have a small amino acid (G, A, or S) at this position. A truncated form of phosphorylase kinase, which naturally has a serine at the third position, maintained the same activity when the S was replaced with G (Fig. 1; Ref. 33); the atypical protein kinase C-1 naturally contains an A residue instead of the third G (Fig. 1; Ref. 34). Furthermore, the Raf-1 mutant G361S, in which the third glycine of the G loop is replaced by serine, has recently been shown to possess a level of catalytic activity that is higher than that of the wild-type Raf-1 (29). In contrast, a mutant form of the insulin receptor kinase, in which V replaces the third G, showed a 90% decrease in kinase activity (28). These facts indicate that residues larger than S cannot maintain the β-hairpin conformation of the phosphate-binding loop that is necessary for the avoidance of steric interference with ATP binding. This is consistent with our
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finding that replacement of the third G by E in the B-Raf G loop decreases enzymatic activity, whereas the replacement of the third G by A increases enzymatic activity.

In the B-Raf G463E mutant, the first G of the G loop is affected. Based on studies of the PKA catalytic subunit, the first G is thought to be in direct contact with the ribose ring of ATP. Although the first G in the G loop is reportedly more highly conserved than the third G, it is naturally replaced by a larger residue than A or S in approximately 3% of human protein kinases (33). The substitution of the first G with A or S has been reported to decrease PKA kinase activity (32). In this study, the B-Raf G463E mutant showed slightly decreased kinase activity. In contrast, the B-Raf G463V mutant has been reported to have increased enzymatic activity (12). Therefore, the mutation of the first G residue does not seem to have equivalent effects on the catalytic activity of protein kinases.

In the three other B-Raf mutants (R461I, I462S, and F467C), residues adjacent to the conserved G, but not the G residues themselves, were affected. The B-Raf R461I and I462S mutants did not up-regulate MEK/Erk or NfκB signaling or NIH3T3 transformation as compared with the wild-type B-Raf; the B-Raf F467C mutant showed moderate up-regulation. These results indicate that colon cancer-associated mutations of the residues adjacent to the conserved Gs of B-Raf have different effects on enzymatic activity. Additional studies on the structure of the B-Raf catalytic domain are needed to elucidate the mechanisms of these effects.

Of the B-Raf G loop mutations in this study, only the G468A mutation that showed greatly elevated kinase activity and NIH3T3-transforming ability was able to induce morphological changes and generated some foci in IEC-6 intestinal epithelial cells. Furthermore, the G468A-expressing IEC-6 cells induced a considerable number of colonies in soft agar. These results contrast with a previous report, which indicated that both constitutively active Ras and Raf-1 transform NIH3T3 fibroblasts; however, only the former is able to transform epithelial cells including RIE-1 and IEC-6 (31). Nevertheless, after we had submitted this paper, Boucher et al. (35) reported that constitutively active MEK-expressing IEC-6 cells did form foci at higher densities and induced colonies in soft agar, which are the characteristics of transformation. Combined intense activation of the Raf/MEK/Erk cascade by constitutively active Raf or MEK may be sufficient for the transformation of intestinal epithelial cells, but their transforming abilities are modest compared with that of oncogenic Ras.

In this study, we used luciferase reporter assays to evaluate the activation of the NfκB signaling pathway. It is possible for the NfκB activity to be increased when the luciferase reporter activity is not elevated, because NfκB may translocate to the nucleus and display increased DNA binding and transcriptional activity in the absence of increased reporter activity. It is also possible that Erk activity is increased even when the Elk1-dependent reporter activity is not elevated. Therefore, we cannot exclude the possibility that the B-Raf mutant still activates the signaling pathways to some extent without up-regulation of reporter activity.

In our previous study, we demonstrated that B-Raf mutations in the activation segment that were unable to activate signaling were found predominantly in tumors with activating K-Ras mutations (30). Of the two G loop mutations studied previously, the B-Raf G463V mutation was shown to be coincident with a K-Ras mutation, and the cell line that contained the G463V mutation required additional Ras activity for proliferation (12). Of the G loop mutations examined here (Table 1), only F467C was reportedly coincident with a K-Ras mutation (14). It seems reasonable to assume that tumors with the B-Raf G468A mutation, which has greatly increased kinase activity and transforming ability comparable with those of the V599E mutation, do not require additional Ras activity for proliferation. Although the B-Raf F467C mutation moderately increased kinase and transforming activities compared with those of the wild type, the activities of this mutant were at a much lower level than those of the G468A or V599E mutants. Furthermore, this mutant could not transform intestinal epithelial cells, whereas the G468A mutant could. Therefore, tumors with the F467C mutation or G463V mutations may also require Ras for proliferation. The other four B-Raf G loop mutations examined in this study did not significantly activate MEK/Erk or NfκB signaling nor did they activate NIH3T3 transformation, and they were not coincident with the Ras mutation. These results suggest that mechanisms of MEK/Erk pathway activation other than Ras or B-Raf mutations are involved in the tumorigenesis of tumors with the B-Raf mutations that do not increase the signaling activation.

In conclusion, the functional analysis of the colorectal tumorderated mutations within the B-Raf G loop shows that some of the mutations do not activate the MEK/Erk or NfκB signaling pathways or increase transforming ability. The results of this study suggest that the reported mutations within the G loop in colorectal tumors have different effects on kinase activity and transforming ability and that some of them might contribute to carcinogenesis via other mechanisms or might be irrelevant to carcinogenesis.

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REFERENCES

Different Effects of Point Mutations within the B-Raf Glycine-Rich Loop in Colorectal Tumors on Mitogen-Activated Protein/Extracellular Signal-Regulated Kinase Kinase/Extracellular Signal-Regulated Kinase and Nuclear Factor κB Pathway and Cellular Transformation

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