Oncogenic KRAS and BRAF Differentially Regulate Hypoxia-Inducible Factor-1α and -2α in Colon Cancer

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Abstract
KRAS and BRAF mutations are frequently observed in human colon cancers. These mutations occur in a mutually exclusive manner, and each is associated with distinctive biological features. We showed previously that K-ras can interact with hypoxia to activate multiple signaling pathways. Many hypoxic responses are mediated by hypoxia-inducible factor (HIF)-1α and HIF-2α, and we sought to define the roles of mutant KRAS and BRAF in the induction of HIF-1α and HIF-2α in colon cancer cells. Ectopic expression of mutant K-ras in Caco2 cells enhanced the hypoxic induction of only HIF-1α, whereas mutant BRAF enhanced both HIF-1α and HIF-2α. Knockout or knockdown of mutant KRAS in DLD-1 and HCT116 cells impaired the hypoxic induction of only HIF-1α. HIF-1α mRNA levels were comparable in cells with and without a KRAS mutation. However, the rate of HIF-1α protein synthesis was higher in cells with a KRAS mutation, and this was suppressed by the phosphoinositide 3-kinase inhibitor LY294002. In contrast, knockdown of mutant BRAF in HT29 cells suppressed both HIF-1α and HIF-2α. Although BRAF regulated mRNA levels of both HIF-1α and HIF-2α, knockdown of BRAF or treatment with the MEK inhibitor PD98059 impaired the translation of only HIF-2α. Our data reveal that oncogenic KRAS and BRAF mutations differentially regulate the hypoxic induction of HIF-1α and HIF-2α in colon cancer, and this may potentially contribute to the phenotypic differences of KRAS and BRAF mutations in colon tumors. [Cancer Res 2009;69(21):8499–8506]

Introduction
Activating mutations of KRAS are found in ∼45% of colorectal cancers (1, 2). Oncogenic activation of KRAS can influence several cellular processes that regulate morphology, proliferation, and motility, and KRAS mutations are clinically associated with a poor prognosis (3, 4). K-ras interacts with several effector proteins, including Raf kinases and phosphoinositide 3-kinases (PI3K). BRAF, one of the Raf kinases, is also mutated in ∼15% of colorectal cancers (5–7). Interestingly, KRAS and BRAF mutations are mutually exclusive (5–7), suggesting that they may have similar functions. However, the biological properties of tumors with KRAS or BRAF mutations are quite distinctive. For example, BRAF mutant tumors are more likely to exhibit microsatellite instability, poor histologic grade, mucinous histology, and a better prognosis (7, 8). However, it remains unclear how these oncogenes that signal through the same pathways can give rise to such distinct tumor phenotypes.

As tumor cells proliferate, their oxygen and nutrient demands increase, and cells adapt to this hypoxic environment through a switch to anaerobic glycolysis and induction of survival factors and angiogenic growth factors such as vascular endothelial growth factor (9). Hypoxia-inducible factors (HIF) are thought to play a major role in controlling the transcriptional responses to hypoxia (10, 11). HIF-1 was the first HIF isoform to be recognized, and it is composed of two subunits, HIF-1α and HIF-1β. HIF-2α and HIF-3α were identified by homology searches or screens for interaction partners with HIF-1α (12). Whereas HIF-3α is the most distantly related isoform, HIF-1α and HIF-2α are closely related and both activate hypoxia-responsive element–dependent gene expression. Although the two isoforms are frequently expressed in human cancers, their functions vary. In colon cancer, we have reported previously that HIF-1α may promote the growth of cancer cells both in vitro and in vivo. However, HIF-2α appears to restrain tumor growth, and loss of expression of HIF-2α but not HIF-1α in human colon cancer tissues is strongly correlated with advanced tumor stage (13). The mechanisms that regulate the expression of HIF-1α versus HIF-2α in colon cancer are unknown.

Under normoxic conditions, HIF-1α and HIF-2α are hydroxylated by O2-dependent prolyl hydroxylases within the oxygen-dependent degradation domain and targeted by the von Hippel-Lindau protein (pVHL) E3 ubiquitin ligase complex, leading to proteasomal degradation. Under hypoxic conditions, prolyl hydroxylase activity is inhibited, thereby allowing stabilization and accumulation of HIF-1α and HIF-2α protein (12). In addition to hypoxia, HIF-1α expression can be regulated by certain oncogenic pathways. For instance, insulin, insulin-like growth factor, epidermal growth factor, and fibroblast growth factor can induce expression of HIF-1α protein in normoxia via mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways (14–16). Transformation by H-ras or mutant K-ras has also been shown to enhance the levels of HIF-1α protein (17, 18). In melanoma cells, mutant BRAF (V600E) can enhance HIF-1α expression in normoxia (19). However, the relevance of these mechanisms in hypoxia as well as the effects on the HIF-2α isoform are unclear. We therefore sought to identify the roles of mutant KRAS and BRAF on the hypoxic induction of HIF-1α and HIF-2α in colon cancer.

Materials and Methods
Cell culture. DLD-1, HCT116, HT29, Caco2, DKS-5, DKO-3, HK2-10, and HKe-3 cells were maintained in DMEM supplemented with 10% fetal bovine serum. Hypoxic conditions were achieved by culturing cell lines in a sealed hypoxia chamber (Billups-Rothenberg) after flushing with a mixture of 1% or 5% O2 and 5% CO2 balanced with N2 as described previously (20).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Plasmid construction and establishment of stable cells. Lentivirus vector pH-Neo-CGW GFP-LC3 was kindly provided by Dr. Rammik Xavier and the GFP-LC3 fragment was excised with BamHI and NotI (21). Human K-ras and BRAF cDNA were amplified by reverse transcription-PCR (RT-PCR) using RNA from Caco2 cells for wild-type K-ras, SW480 cells for mutant K-ras V12, DLD-1 cells for wild-type BRAF, and HT29 cells for mutant BRAF V600E. The PCR primers used were 5’-AAGGAAGGATCCAGCCCATGTGAATGGCAGCTG-3’ and 5’-AAACAAGCGGCGACAGCCA- CGCATCATACACACACAG-3’ for K-ras and 5’-AAGGAAGGATCCATTTGCGGG-3’ and 5’-AAACAAGCGGCGACAGCCA- CGCATCATACACACACAG-3’ for BRAF. The PCR-amplified products were digested with BamHI and NotI for K-ras and BglII and NotI for BRAF and subcloned into the pCGW vector.

pCGW K-ras wild-type, K-ras V12, BRAF wild-type, BRAF V600E, or empty vector were introduced along with packaging plasmid pCMVΔr8.91 and envelope plasmid pMD2G into HEK293T cells by transfection with the Fugene 6 (Roche). The culture medium containing lentivirus was harvested and filtered, and polybrene was added at 8 μg/mL. Caco2 cells were infected with the virus for 48 h.

pSicoR shBRAF plasmids were kindly provided by Dr. Kevin M. Haigis. pSicoR shBRAF or empty vector was introduced into HEK293T cells and lentivirus was harvested as described above. HT29, DLD-1, and Caco2 cells were infected with the virus and then selected by puromycin resistance.

RNA interference. Cells were transfected with 20 nmol/L small interfering RNA (siRNA) duplex oligos using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. Nucleotide sequences of siRNAs were as follows: 5’-r(GGUGACUUAGGUUCUAGAU)d(TT)-3’ for K-ras #1, 5’-r(GGAACAGAUAGUAUAGA)d(TT)-3’ for K-ras #2, 5’-r(GGCCCUUAGGAAAUUUU)d(TT)-3’ for BRAF #1, 5’-r(GAGGGUGUGAAUACAAA)d(TT)-3’ for BRAF #2, and 5’-r(GGCGCUUUGUAGAUCG)d(TT)-3’ for control. The resulting cDNA was amplified by real-time PCR using the iQ5 Real-time PCR Detection System (Bio-Rad) and Power SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences were 5’-CGGCGGACGACAGAAGAAAAG-3’ and 5’-CCCTATCAAGATCGGCAACTCA-3’ for HIF-1α, 5’-GGAGGTGTCTTATGAGCTTG-3’ and 5’-GCAAGAGATCATGTGCGCA-3’ for HIF-2α, 5’-AGGCCAGCAGAAGAGAAGAG-3’ and 5’-TTTCTTGGGCTTTCTTTTTT-3’ for 18S rRNA.

DNA was isolated from cultured cells using the Isogen kit (Wako) and reverse transcription with random hexanucleotide primers and SuperScript reverse transcriptase III (Invitrogen) according to the manufacturer’s instructions. Nucleotide sequences of primers were: 5’-GGCAACAGGATGAGAAAACG-3’ for K-ras and 5’-AACCATGCAACGACCTCTTT-GAACCCTTCACC-3’ for BRAF. The PCR-amplified products were digested with BamHI and NotI (21). Human K-ras and BRAF gene (Fig. 1A) were cloned into the pCMVΔr8.91 and envelope plasmid pMD2G into HEK293T cells by transfection with the Fugene 6 reagent (Roche). The culture medium containing lentivirus was harvested and filtered, and polybrene was added at 8 μg/mL. Caco2 cells were infected with the virus for 48 h.

Lentivirus was harvested as described above. HT29, DLD-1, and Caco2 cells were infected with the virus for 48 h.

Statistical analysis. Statistical differences were analyzed by Student’s t test, and P values < 0.05 were considered statistically significant.

Results

Mutant KRAS enhances the hypoxic induction of HIF-1α, but mutant BRAF induces both HIF-1α and HIF-2α. To determine whether activating mutations of KRAS or BRAF regulate the hypoxic induction of HIF-1α and HIF-2α, lentiviral vectors expressing wild-type K-ras, mutant K-ras (V12), wild-type BRAF, or mutant BRAF (V600E) were transduced into Caco2 cells that do not carry mutations in either KRAS or BRAF gene (Fig. 1A). Stably transected
cells were cultured in either normoxia or hypoxia for 12 h, and hypoxic induction of HIF proteins was analyzed by Western blotting. The induction of HIF-1α was enhanced by ectopic expression of K-ras (V12) or BRAF (V600E; 2.11- and 1.71-fold, respectively; both P < 0.05) in hypoxic conditions (Fig. 1B). However, overexpression of wild-type K-ras or wild-type BRAF did not increase levels of HIF-1α mRNA expression in normoxic induction (1.81-fold; P < 0.05) but also induced expression in normoxic conditions. K-ras (V12) did not upregulate HIF-2α in either condition (Fig. 1B).

We then evaluated whether the hypoxic induction of HIF-1α and HIF-2α was regulated by endogenous KRAS mutations in colon cancer cells. DKO-3 and HKe-3 cells are derived from DLD-1 and HCT116 cells, respectively, in which the endogenous KRAS mutations in colon cancer cells. DKO-3 and HK2-10 cells have a mutant KRAS allele (22). These cells were incubated in either normoxia or hypoxia. HIF-1α induction was impaired by genetic disruption of mutant KRAS in both DKO-3 and HKe-3 cells (62% and 74% reduction, respectively; both P < 0.05), whereas the induction of HIF-2α was unaffected (Fig. 2A).

To verify this observation, DLD-1 and HCT116 cells were treated with siRNA oligos targeting K-ras (K-ras #1 and #2) or control siRNA. Thirty-six hours after transfection, cells were subjected to either normoxia or hypoxia for 12 h. The weak K-ras bands seen in the DKO-3 and HKe-3 cells correspond to the remaining wild-type KRAS allele. DLD-1 and HCT116 cells were treated with siRNA targeting K-ras (K-ras #1 and #2) or control siRNA. Thirty-six hours after transfection, cells were subjected to either normoxia or hypoxia for 12 h. However, overexpression of wild-type K-ras or wild-type BRAF did not increase levels of HIF-1α mRNA levels were comparable between DKs-5 and HK2-10 cells (62% and 74% reduction, respectively; both P < 0.05), whereas the induction of HIF-2α was unaffected (Fig. 2A).

Interestingly, BRAF (V600E) enhanced HIF-2α expression in hypoxia (1.81-fold; P < 0.05) but also induced expression in normoxic conditions. K-ras (V12) did not upregulate HIF-2α in either condition (Fig. 1B).

We next addressed the mechanisms by which mutant KRAS enhances the hypoxic induction of HIF-1α. DKO-3, HK2-10, and HKe-3 cells were cultured in normoxia and relative levels of HIF-1α mRNA were measured by quantitative PCR. HIF-1α mRNA levels were comparable between DKs-5 and DKO-3 cells as well as between HK2-10 and HKe-3 cells (Fig. 3A). DLD-1 and HCT116 cells were treated with siRNA oligos against K-ras, and HIF-1α mRNA levels were measured. HIF-1α mRNA expression was not affected by knockdown of K-ras in either cell line.
was extracted from DKs-5 and DKO-3 cells (Fig. 3A). This resulted in higher induction of vascular endothelial growth factor mRNA (Supplementary Fig. S2B). These data suggest that mutant KRAS may not play a key role in the hypoxic induction of HIF-1α mRNA but may regulate the accumulation of HIF-1α protein at the translational level. To further address this hypothesis, DKs-5 and DKO-3 cells were treated with MG132 for 0, 10, 20, and 30 min to block proteasomal degradation of HIF-1α protein. The rate of translation of HIF-1α in normoxia was measured by its accumulation during MG132 treatment. The rate of HIF-1α protein synthesis was significantly lower in DKO-3 cells (45% and 39% reductions at 10 and 20 min, respectively; both P < 0.05) compared with DKs-5 cells (Fig. 4A). DLD-1 cells were then treated with the MEK inhibitor PD98059 or the PI3K inhibitor LY294002 for 9 h followed by MG132 treatment. HIF-1α protein synthesis was inhibited by LY294002 (63% and 55% reductions at 20 and 30 min, respectively; both P < 0.05) but not PD98059 (Fig. 4B). DLD-1 cells were treated with PD98059 or LY294002 and then incubated in hypoxia for 6 h. Hypoxic induction of HIF-1α protein was reduced by 61% (P < 0.05) with LY294002 but not PD98059 despite the activation of extracellular signal-regulated kinase (ERK) in hypoxia (Fig. 4C). These data suggest that mutant KRAS may regulate the translation of HIF-1α through the PI3K pathway.

HIF-1α protein is unstable in normoxic conditions, and the half-life of HIF-1α protein is estimated at 2 to 5 min. To examine whether mutant KRAS affects the stability of HIF-1α protein, DKs-5 and DKO-3 cells were incubated in hypoxia for 6 h and then cultured in the presence of the translational inhibitor cycloheximide. The half-life of HIF-1α protein was comparable between DKs-5 and DKO-3 cells (Supplementary Fig. S3A). Furthermore, pVHL protein levels were not affected by either knockout of mutant KRAS or knockdown of K-ras in DLD-1 and HCT116 cells (Supplementary Fig. S3B). These data indicate that mutant KRAS does not regulate HIF-1α protein stability.

Mutant BRAF enhances mRNA expression of HIF-1α and HIF-2α in normoxia. We next addressed the mechanisms by which mutant BRAF enhances the hypoxic induction of both HIF-1α and HIF-2α. HT29 pSicoR cells were cultured in normoxia and HIF mRNA levels were measured. The mRNA levels of both HIF-1α and HIF-2α were significantly reduced in BRAF knockdown cells (57-72% reduction in HIF-1α (P < 0.05) and 80-89% reduction in HIF-2α (P < 0.005) compared with control cells (Fig. 5A). BRAF signals through MAPK, and we tested the significance of this pathway with the MEK inhibitor PD98059. The mRNA levels of both HIF-1α and HIF-2α in HT29 cells were suppressed by PD98059 [33% reduction in HIF-1α (P < 0.05) and 43% reduction in HIF-2α (P < 0.005); Fig. 5B]. To investigate whether the early induction of HIF mRNA is enhanced by mutant BRAF, HT29 pSicoR cells were cultured in hypoxia for 1 to 12 h. HIF-1α and HIF-2α mRNA were induced 2- and 2.5-fold, respectively, at 3 h after exposure to hypoxia, but these changes were not affected by knockdown of mutant BRAF (Fig. 5C). These data suggest that although mutant BRAF can enhance HIF-1α and HIF-2α mRNA expression in basal normoxic conditions, it does not enhance the induction of HIF mRNA in hypoxia.

Mutant BRAF enhances the translation of HIF-2α through the MAPK pathway. Although there were no changes in the hypoxic induction of HIF mRNA, mutant BRAF did regulate the hypoxic induction of HIF-1α and HIF-2α protein (Supplementary Fig. S2C), which was accompanied by an increase in the levels of vascular endothelial growth factor mRNA (Supplementary Fig. S2D). This induction was significantly lower in DKO-3 cells (45% and 39% reductions at 10 and 20 min, respectively; both P < 0.05) compared with DKs-5 cells (Fig. 4A). HIF-1α protein synthesis was inhibited by LY294002 (63% and 55% reductions at 20 and 30 min, respectively; both P < 0.05) but not PD98059 (Fig. 4B). HIF-1α protein was induced by hypoxia to a greater extent in
Fig. S2D). To examine whether mutant BRAF controlled the translation of HIF protein, HT29 pSicoR cells were treated with MG132 for 0, 10, 20, and 30 min. Interestingly, the rate of HIF-1α protein synthesis was not affected, but the rate of HIF-2α protein synthesis was reduced by the knockdown of BRAF at 20 min ($P < 0.05$; Fig. 6A). The rate of HIF-2α protein synthesis was also suppressed at 10 and 20 min when HT29 cells were treated with PD98059 (both $P < 0.05$; Fig. 6B). Interestingly, PD98059 treatment suppressed the hypoxic induction of HIF-1α protein by 68% and HIF-2α by 58% in HT29 cells (both $P < 0.05$; Fig. 6C). These data suggest that mutant BRAF regulates the translation of HIF-2α through MAPK pathways.

To examine whether mutant BRAF affects the stability of HIF proteins, HT29 pSicoR cells were incubated in hypoxia for 6 h and then cultured in the presence of cycloheximide. The half-life of HIF-1α or HIF-2α protein was not altered by stable knockdown of BRAF (Supplementary Fig. S3C). Furthermore, pVHL expression was not affected by either stable or transient knockdown of BRAF in HT29 cells (Supplementary Fig. S3D). These data indicate that mutant BRAF does not affect the stability of either HIF-1α or HIF-2α protein.

**Discussion**

Although HIF-1α and HIF-2α are both induced by hypoxia in colon cancer cells, they have distinctive biological functions (13). We sought to determine whether the induction of these factors may be influenced by the underlying tumor genotype. In particular, KRAS and BRAF are among the most frequently mutated oncoproteins in colon cancer, and tumors with these mutations are associated with distinct clinical phenotypes (1, 2, 5–8). Our previous studies have shown a unique interaction between K-ras and hypoxia (20, 24). We now show that oncogenic KRAS functions primarily to induce HIF-1α at the level of translation. In contrast, oncogenic BRAF can enhance the mRNA expression of both HIF-1α and HIF-2α, but at the translational level, only HIF2α is induced by BRAF.

Transformation with H-ras or mutant K-ras in fibroblasts enhances protein levels of HIF-1α (17, 18), which is consistent with our results in Caco2 cells. However, ectopic overexpression of mutant K-ras can produce misleading results (25, 26). Thus, it is essential to evaluate the oncogenic regulation of HIF-1α at physiologic levels of mutant K-ras. In the present study, we used
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mutant KRAS knockout cells or transiently knocked down endogenous K-ras in DLD-1 and HT116 cells. The effects of endogenous mutant KRAS on the hypoxic induction of HIFs were consistent. Importantly, these effects on HIF appear to be specific to mutant KRAS, as no such regulation of HIF was seen with the wild-type KRAS gene.

A previous report suggested that mutant BRAF may regulate HIF-1α expression in melanoma cells, but its role in colon cancer was unknown (19). In our study, stable knockdown of BRAF in HT29 cells suppressed mRNA expression of HIF-1α but did not regulate its translation, which is consistent with the previous report (19). However, HIF-1α protein stability or pVHL protein expression was not affected by knockdown of BRAF in HT29 cells, whereas stable knockdown of BRAF in WM793 melanoma cells increased pVHL protein, resulting in lower stability of HIF-1α protein (19). These findings suggest that the effects of mutant BRAF on HIF-1α protein stability and pVHL protein expression are cell-specific.

In our study, mutant KRAS and BRAF appear to regulate HIF protein primarily at the level of translation. Whereas KRAS primarily regulates the translation of HIF-1α, BRAF may selectively regulate the translation of HIF-2α. Global protein translation is generally suppressed under hypoxic conditions (27), and in our study, p70 S6 kinase, which regulates global translation and can be phosphorylated by mammalian target of rapamycin, was inactivated in hypoxia (data not shown). Recent reports have shown that the translation of HIF-1α may also be regulated by mammalian target of rapamycin–independent pathways (28, 29). In addition, hypoxia increases HIF-2α translation by disrupting the iron-regulatory protein 1-HIF-2α iron-responsive element interaction (30). This may explain how HIF-2α protein can accumulate during hypoxia while global translation is suppressed. However, it is unknown whether oncogenic signaling regulates the binding activity of iron-regulatory protein 1.

Although HIF-1α and HIF-2α are similarly induced by hypoxia and both can bind hypoxia-responsive elements at target gene loci, several reports have shown that they have distinct expression patterns and functions (31–41). Our previous study suggested that HIF-1α may promote the growth of colon cancer cells, whereas HIF-2α may restrain growth. Expression of HIF-2α in human colon cancer tissues was inversely correlated with tumor stage (13). In the present study, we show that mutant BRAF but not KRAS upregulates HIF-2α in hypoxia. These differential effects of oncogenic KRAS and BRAF on the hypoxic induction of HIF-2α may potentially contribute to the more favorable clinical behavior associated with mutant BRAF tumors (7). It is important to recognize that BRAF mutations typically occur in the setting of high levels of MSI, but there are likely to be other genetic alterations associated with MSI as well as additional targets of BRAF that may contribute to the unique clinical phenotype. Of note, our studies of BRAF were done in HT29 and Caco2 cells, neither of which exhibits MSI, suggesting that the observed relationship between BRAF and HIF isoforms does not depend on the presence of MSI.

In conclusion, we have shown that oncogenic KRAS and BRAF mutations differentially regulate the hypoxic induction of HIF-1α and HIF-2α in colon cancer. Mutant KRAS enhances the hypoxic induction of HIF-1α by regulating its translation through PI3K pathways. Mutant BRAF enhances HIF-1α and HIF-2α mRNA expression but, more importantly, regulates HIF-2α translation. These differential effects on HIFs highlight the unique interaction

![Figure 5](https://example.com/f5.png)

**Figure 5.** Knockdown of BRAF and mRNA expression of HIF-1α and HIF-2α. Total RNA was extracted from HT29 pSicoR (vector, shBRAF #1, and shBRAF #2) cells cultured in normoxia (A) or HT29 cells treated with DMSO or PD98059 (B). Relative levels of HIF-1α and HIF-2α mRNA were determined by quantitative reverse transcription-PCR and normalized to the levels of 18S rRNA. All reactions were done in triplicate. Mean ± SD for three different cDNA samples. *, P < 0.05; **, P < 0.005. C, HT29 pSicoR cells were incubated in hypoxia for the indicated times. Relative levels of HIF-1α and HIF-2α mRNA were determined by quantitative reverse transcription-PCR and normalized to the levels of 18S rRNA at each time point. Mean ± SD relative to time 0 in each cell line for two different cDNA samples.
between oncogenes and the tumor microenvironment and may potentially contribute to the some of the phenotypic differences in mutant KRAS and BRAF colon tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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