Hypoxic Regulation of Vascular Endothelial Growth Factor through the Induction of Phosphatidylinositol 3-Kinase/Rho/ROCK and c-Myc

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The induction of vascular endothelial growth factor (VEGF) is an essential feature of tumor angiogenesis. Hypoxia is a potent stimulator of VEGF expression, and hypoxia-inducible factor-1 (HIF-1) is considered to be critical for this induction. However, we have previously demonstrated that induction of VEGF by hypoxia was preserved when HIF-1α was silenced. We sought to better define the molecular basis of this HIF-1-independent regulation. In colon cancer cells, hypoxia stimulated multiple K-ras effector pathways including phosphatidylinositol 3-kinase. VEGF promoter deletion studies identified a novel promoter region between −418 and −223 bp that was responsive to hypoxia in a PI3K/Rho/ROCK-dependent manner. Electrophoretic mobility shift assays identified a fragment between −300 and −251 bp that demonstrated a unique shift only in hypoxic conditions. Inhibition of PI3K or ROCK blocked the formation of this complex. A binding site for c-Myc, a target of ROCK, was identified at −271 bp. A role for c-Myc in the hypoxic induction of VEGF was demonstrated by site-directed mutagenesis of the VEGF promoter and silencing of c-Myc by small interfering RNA. Collectively, these findings suggest an alternative mechanism for the hypoxic induction of VEGF in colon cancer that does not depend upon HIF-1α but instead requires the activation of PI3K/Rho/ROCK and c-Myc.

Rapidly growing tumors routinely outstrip their supply of oxygen and nutrients, and the induction of new blood vessels is critical to sustain neoplastic proliferation (1). New blood vessels can be stimulated to grow when factors that promote angiogenesis are up-regulated or those that inhibit angiogenesis are down-regulated (2). Vascular endothelial growth factor (VEGF) is a key pro-angiogenic factor, and therapeutic approaches that inhibit VEGF in human malignancies have been approved for clinical use (3–5).

The Wnt and K-ras signaling pathways are frequently activated in early stages of colon carcinogenesis, and we previously demonstrated their role in the regulation of VEGF expression (6). An additional environmental factor that can enhance VEGF expression in advanced tumors is hypoxia. Most solid tumors develop regions of low oxygen tension caused by an imbalance in oxygen supply and consumption, and hypoxia is a potent stimulator of VEGF. This induction is considered to be primarily mediated through hypoxia-inducible factor-1 (HIF-1α) (7). HIF-1 is a heterodimeric basic helix-loop-helix transcription factor composed of two subunits, HIF-1α and HIF-1β. HIF-1α is the key regulatory component, because it is rapidly degraded in normoxic conditions but stabilized and activated in hypoxia (8, 9). The HIF-1 complex recognizes a consensus hypoxia response element in the promoter of a broad range of target genes (10), and VEGF is a key transcriptional target. However, we and others have shown that HIF-1α is not the only regulator of the hypoxic induction of VEGF (11, 12). Cells derived from HIF-1α “knock-out” embryos still demonstrated a significant, albeit reduced, induction of VEGF in response to hypoxia (13, 14). Also, colon cancer cell lines stably expressing an siRNA construct against HIF-1α exhibited significant levels of VEGF in hypoxia (11). Furthermore, the VEGF promoter can be induced by hypoxia when canonical hypoxia response elements are mutated or deleted in human cancer cell lines (7, 15, 16). These findings imply the existence of alternative transcriptional mechanisms that do not depend upon HIF-1α.

In the present study, we sought to characterize the molecular mechanisms independent of HIF-1 that may regulate hypoxic expression of VEGF in colon cancer. In particular, we sought to define the cis-regulatory elements and transcription factors that are critical for the hypoxic response as well as the upstream signaling pathways that regulate them. Hypoxia activated multiple K-ras effector pathways including ERK, PI3K/Akt, and Rho. Inhibition of PI3K, Rho, and ROCK, but not ERK or Akt, attenuated the hypoxic induction of VEGF. c-Myc is a target of the PI3K/ROCK signaling pathway, and it can regulate the VEGF promoter through a binding element at −271 bp.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—Human VEGF promoter luciferase constructs were prepared as previously described (6). The reporter constructs, 0.75–, 0.56–, and 0.43-kb VEGF-luc, contained VEGF promoter sequences from −418 bp to +350 bp, −223 bp to +350 bp, and −90 bp to +350 bp relative to the transcription initiation site, respectively. Site-directed mutagenesis was performed using the 0.75-kb VEGF-luc construct. A Myc-Max-binding site at −271 bp was selectively mutated (5′-GCGGCGCGTGTTCTC→5′-GCGGCGAAATGCTTC) and is designated mut-271-luc. The previously described K-rasV12 (17), dominant negative RhoA-T19N (dnRho; Guthrie Research Institute, Sayre, PA), kinase mutant ERK-1/2 (dnERK), and dominant negative p85 component of the PI3K complex (dnPI3K) (19), dominant negative Akt-
K179A (dnAkt) (20), and c-Myc (pCEP.c-Myc) (21) expression plasmids were also used for transient transfections.

Cell Culture—The human colon cancer cell lines Caco2 and DLD-1 (American Type Culture Collection) were maintained in recommended growth medium. Two independent HIF-1α knock-down clones of each cell line (HIF-kd1470 and HIF-kd2192) as well as a control cell that was transfected with empty pSuper.retro (HIF-wt) were also utilized (11). Hypoxic conditions were achieved by culturing cell lines in a sealed hypoxia chamber (Billups-Rothenberg) after flushing with a mixture of 1% O₂, 5% CO₂, and 94% N₂ (11). To minimize the effect of serum growth factors, the cell culture medium was switched to serum-free UltraCulture (Chambrex) before the cells were subjected to hypoxia. The specific inhibitors PD98059, LY294002, and Y27632 (Calbiochem) were added 1–2 h prior to exposure to normoxia or hypoxia at the concentrations indicated. In selected experiments, the cells were treated with 2.5 μg/ml Clostridium botulinum exoenzyme C3 (Calbiochem) for 12 h prior to incubation in hypoxic conditions.

Northern Blot Analysis—Total RNA was prepared using TRIzol reagent (Invitrogen). Fifteen μg of total RNA was analyzed using a random primer-labeled 400-bp human VEGF cDNA (6), and 18 S ribosomal RNA was used as a loading control. VEGF mRNA decay rates were analyzed utilizing cells that were cultured under normoxic or hypoxic conditions for 10 h prior to the addition of 10 μg/ml actinomycin D (Sigma). Total RNA was isolated at 0, 2, 4, and 6 h, and the level of VEGF mRNA was normalized to the amount of 18 S rRNA after densitometry of Northern blots. All of the time points were performed in triplicate.

Transfections and Reporter Assays—Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s specifications. All of the experiments were performed in 24-well tissue culture plates with cells plated to reach 50–60% confluence on the day of transfection. The cells were allowed to recover in regular culture medium overnight after transfection, switched to UltraCulture medium, and then exposed to normoxia or hypoxia for 24 h.

0.4–0.6 μg of VEGF-luciferase reporter constructs were co-transfected with 2 ng of pRL-CMV (Promega) as a transfection control. pRL-null, a promoter-less Renilla construct, was used when cells were co-transfected with a K-ras expression vector, because Ras has been shown to induce the pRL-CMV plasmid (22). As indicated, 0.2 μg of expression vector was co-transfected, and the total amount of transfected DNA was kept constant by adding corresponding empty plasmid. Luciferase activity was measured with a dual luciferase reporter assay system (Promega). The experiments were performed in duplicate wells a minimum of three times.

Western Blotting—Protein lysates were harvested from cells subjected to normoxia or hypoxia for the indicated periods. The cells were lysed in chilled lysis buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM Na₂ EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 mM leupeptin) supplemented with the Pefabloc SC (Roche Applied Science). 20–30 μg of protein extracts were resolved on a 4–12% NuPAGE Bis-Tris polyacrylamide gel (Invitrogen) and transferred onto a polyvinylidene difluoride membrane (Millipore). The blots were probed with a HIF-1α (Transduction Laboratories; 1:250), HIF-2α (Novus; 1:250), phospho- and total ERK1/2, phospho- and total Akt, phospho- and total p38, or phospho- and total JNK antibody (all from Cell Signalling; 1:1000). Immunoreactive proteins were visualized using the Western Lighting Chemiluminescence Reagent Plus (PerkinElmer Life Sciences).

siRNA Analysis—To silence c-Myc, siRNA against c-Myc was utilized (SignaSilence c-Myc siRNA kit, Cell Signaling; sequences originally described and validated in Ref. 23). A control siRNA that does not correspond to any known human gene (5′-CGUAAGGGAAUACUCGUCA-3′) was also utilized. Caco2 cells were transfected with 200 nM siRNA duplexes using Lipofectamine 2000 (Invitrogen), and the silencing effect was confirmed by Western blotting 48 h after transfection. VEGF promoter reporter constructs were co-transfected to examine the effect of c-Myc on VEGF promoter activity, and dual luciferase assays were performed 48 h after transfection.

Real Time PCR Assay—RNA was extracted using RNeasy kit (Qiagen) and quantitative reverse transcription PCR was performed using Super Script III platinum Two-Step qRT-PCR Kit (Invitrogen). The 18 S rRNA served as endogenous control. Primer sequences for VEGF and 18 S rRNA were available upon request. PCR cycles were: 2 min at 95°C, followed by 40 cycles with annealing temperature, 55°C. A fluorogenic SYBR Green and MJ research detection system were used for real-time quantification.

The results were presented as parameter threshold cycle (Cₚ) values. ΔCₚ was the difference in the Cₚ values derived from the specific gene being assayed and 18 S rRNA, whereas ΔΔCₚ represented the difference between the paired samples, as calculated by the formula ΔΔCₚ = ΔCₚ of a sample − ΔCₚ of a reference. The amount of target, normalized to an 18 S and relative to a reference, was expressed as 2−ΔΔCₚ.

GST-Rhotekin Pulldown Assay—The level of activated, GTP-bound Rho was assessed utilizing a Rho activation assay kit (Upstate). Briefly, 1 mg of whole cell lysate was incubated with GST-tagged recombinant Rho-binding domain of Rhotekin. Precipitated GTP-bound Rho was detected by Western blotting using a RhoA antibody. Twenty μg of lysates were used for Western blotting to determine expression levels of RhoA protein for each sample.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared from cells cultured in either normoxic or hypoxic conditions for 10 h utilizing NE-PER nuclear extraction reagent (Pierce). Sequences of the VEGF promoter between −418 and −223 bp were divided into five fragments and utilized as oligonucleotide probes (Table 1). The 3′-ends of the oligonucleotides were labeled with biotin during synthesis, and complementary oligonucleotides were annealed to generate double-stranded fragments. EMSA was performed using LightShift chemiluminescent kit (Pierce) according to the manufacturer’s protocol. Briefly, 5 μg of nuclear extracts were incubated with 20 fmol of biotinylated oligonucleotides in binding buffer including 50 ng/μl poly(dI-dC), 0.05% Nonidet P-40, 2.5% glycerol, and 5 mM MgCl₂, and the reaction mix was loaded onto 6% DNA retardation gels (Invitrogen). DNA-protein complexes were transferred onto nylon membranes (Roche Applied Science), and the mobility shift was detected using a streptavidin-horseradish peroxidase conjugate and a chemiluminescent substrate. Specificity of shifts was confirmed by utilizing 200-fold molar

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**TABLE 1**

Sequences of oligonucleotide probes used for EMSA

| Oligonucleotides | Sequences |
|------------------|-----------|
| 1                | 5′-CCAAATGATCTGTCCTCCCTCCACCCGTCCTCGGCCCTCC |
| 2                | 5′-CTCTCCGGCCTTCCTCCCCCTCCCTCAGTTCTAACCCGCTCCT |
| 3                | 5′-AAGAGGGAGAGGTCCTGCCCTGGCCTCCCGAATCCACTCACCTC |
| 4                | 5′-CTTCGCTTCCTCCCTCCCTCCCTCCCTCCCTCCCTCCCTCCCT |
| 5                | 5′-GGACAGAGTTTCGCCGGGCTGGAATTTTCTAGCT |
| 4mut-271         | 5′-CTCCTCGCTCCCTCCCTCCCTCCCTCCCTCCCTCCCTCCCTCCCT |

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excess of unbiotinylated oligonucleotides as a specific competitor. Mutagenesis was performed to further define the elements responsible for the specific shifts obtained, as described in Table 1. Oligonucleotide 4mut-271 includes mutations that disrupt a Myc-Max-binding site.

Statistical Analysis—Statistical differences were analyzed by Student’s t test, and p values <0.05 were considered statistically significant.

RESULTS

Effect of Hypoxia on Ras Effector Pathways—We previously demonstrated that hypoxia and signaling through oncogenic K-ras can synergistically up-regulate VEGF (11). We thus sought to identify which K-ras effector pathways may be activated during hypoxia. In Caco2 cells, ERK and Akt, but not p38 and JNK, were activated by exposure to hypoxic conditions (Fig. 1A). The role of the PI3K pathway in hypoxia was examined utilizing the specific inhibitor LY294002. As shown in Fig. 1B, hypoxia up-regulated the levels of GTP-bound Rho, and this activation of Rho/ROCK is essential for the hypoxic induction of the VEGF promoter activity by hypoxia, normalized to 1. The mean values from three independent transfections are shown. DMSO, dimethyl sulfoxide.

hypoxia can induce VEGF through PI3K in human colon cancer cells. A, protein lysates from Caco2 cells grown in normoxic (21% O2) or hypoxic (1% O2) conditions were utilized for immunoblotting (IB) with anti-HIF-1α, anti-phospho-ERK1/2, phospho-p38, phospho-JNK, or phospho-Akt antibodies. B, Northern blotting for VEGF mRNA and Western blotting for HIF-1α were performed. Caco2 cells were incubated 12 h under normoxic (N) or hypoxic (H) conditions in serum-free medium with UltraCulture. 15 μg of total RNA was probed with a 400-bp human VEGF cDNA fragment, and 20 μg of protein lysates from cells incubated in normoxia or hypoxia for 8 h were utilized for Western blotting. C, the 2.3-kb VEGF promoter reporter construct was co-transfected with dominant negative mutant ERK1/2 (dnERK), dominant negative PI3K (dnPI3K), or dominant negative Akt (dnAkt), or treated with 20 μM PD98059 or 50 μM LY294002. The first bar (basal) represents the induction of the VEGF promoter by hypoxia, normalized to 1. The mean values from three independent transfections are shown. DMSO, dimethyl sulfoxide.

hypoxia (data not shown). These results imply that the PI3K pathway can regulate VEGF expression in hypoxia and that this occurs primarily through transcriptional but not post-transcriptional mechanisms.

To confirm the role of PI3K in the regulation of gene transcription, a VEGF promoter reporter construct was utilized. Hypoxia up-regulated the 2.3-kb VEGF-luc construct almost 3-fold (Fig. 1C). Consistent with the effects on endogenous mRNA levels, LY294002 blocked the hypoxic induction of VEGF promoter activity by 89%, whereas PD98059 had no inhibitory activity (Fig. 1C). Consistent results were also obtained when dominant negative PI3K or ERK, respectively, were expressed. We then sought to identify the downstream effectors of PI3K. Akt is an important target of PI3K and is activated by hypoxia (24). However, dominant negative-Akt, which was previously shown to inhibit VEGF promoter activity in normoxia (6), did not block the hypoxic induction of VEGF (Fig. 1C). Rho is another downstream target of PI3K that is also upstream of ROCK (25, 26). A Rho activation assay was performed to determine whether it may be a target of PI3K in hypoxia. As shown in Fig. 2A, hypoxia up-regulated the levels of GTP-bound Rho, and this was inhibited by LY294002, indicating that hypoxia can activate Rho in a PI3K-dependent manner. There was also a synergistic activation of Rho by hypoxia and K-rasV12 (Fig. 2B), suggesting that oncogenic K-ras can further enhance PI3K-Rho signaling induced by hypoxia. When Rho was inhibited by C3 exoenzyme or a dominant negative construct or when ROCK was inhibited by the specific inhibitor Y27632 (27), the hypoxic induction of the VEGF promoter was strongly down-regulated (Fig. 2C). Thus, activation of Rho/ROCK is essential for the hypoxic induction of the VEGF promoter by PI3K.
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Identification of a Novel Regulatory Region of the VEGF Promoter Responsive to Hypoxia—To identify regions of the VEGF promoter that mediate its induction by hypoxia, we previously performed serial 5′ deletions (11). Although deletion of the consensus hypoxia response element at −418 bp failed to block the hypoxic induction, a dramatic reduction in the hypoxic induction was observed when the promoter region between −418 and −90 bp was deleted (11). To further define the element responsible for this regulation, additional deletions were performed. 0.75-kb VEGF-luc, which contains the promoter region between −418 and +350 bp, responded with a 2.2-fold induction by hypoxia in Caco2 colon cancer cells (Fig. 3A). However, the hypoxic induction fell to 1.3-fold when sequences from −418 to −223 bp were deleted. The VEGF promoter fragment between −418 and −223 bp does not contain a consensus HIF-1-binding element (11) and may thus contain a novel element that is activated by hypoxia. When Caco2 and DLD-1 cells deficient in HIF-1α were utilized (Fig. 3B and data not shown), similar results were obtained, confirming that transcription factors other than HIF-1 can also regulate VEGF in hypoxia. To verify

that this hypoxic induction of VEGF in the absence of HIF-1 was not mediated by a compensatory up-regulation of HIF-2, immunoblots were performed. In Caco2 cells deficient in HIF-1α, the levels of HIF-2α were nearly undetectable, and no up-regulation of HIF-2α was observed in hypoxic conditions (supplemental Fig. S1).

Next we determined the effect of oncogenic K-rasV12 on this 195-bp promoter fragment. Hypoxia up-regulated the 0.75-kb VEGF promoter construct 2.3-fold, but there was a stronger synergistic activation when K-rasV12 was also expressed (7-fold up-regulation) (Fig. 3C). Inhibition of either PI3K or ROCK attenuated this synergistic response. No such induction was observed with the 0.56-kb VEGF-luc construct. K-ras is thus a potent regulator of this promoter region during hypoxia, and the effect is mediated through the PI3K/ROCK pathway.

Identification of a Critical Regulatory Element Responsive to Hypoxia at −271 bp—To further characterize the regulatory element in the VEGF promoter between −418 and −223 bp that is responsive to hypoxia, EMSAs were performed. The 195-bp region was divided into five fragments that were utilized as probes for EMSA (Table 1). Nuclear extracts were isolated from Caco2 cells growing in either normoxic or hypoxic conditions. A unique band shift was obtained only when nuclear extracts from cells grown in hypoxic conditions were incubated with oligonucleotide (Fig. 4A). The shift was also observed utilizing extracts from Caco2 cells deficient in HIF-1α (Caco2-HIF-kd1470 cells) (Fig. 4B). There are several consensus transcription factor-binding sites for AP2, Egr-1, Ets-1, and c-Myc in this promoter fragment. We were particularly curious about c-Myc, because c-Myc is a known target of the Rho/ROCK pathway (27). As shown in Table 1, there is a putative Myc-Max-binding site at −271 bp, 5′-GCGGGCGCGTGTCCT-3′. When probes with specific mutations in these binding sites were utilized (5′-GGGCGCGAGTCTC-3′), the novel bands obtained in hypoxic conditions were lost, suggesting that this element may play an important role in regulating the hypoxic response (Fig. 4C).

To examine the functional interaction between PI3K/ROCK and c-Myc, Western blotting was performed to detect the activated, phosphorylated form of c-Myc (Thr58/Ser62). Incubation of Caco2 cells in hypoxia increased the phosphorylation of c-Myc, and this was blocked when both PI3K and ROCK were specifically inhibited (Fig. 4D). This suggests that c-Myc is a downstream effector of PI3K/ROCK during hypoxia. The effect of these chemical inhibitors on the gel shift patterns was evaluated (Fig. 4E). Both LY294002 and Y27632 blocked the formation of the novel band. To confirm the role of c-Myc in the formation of this unique band identified on gel shift assays, siRNA duplexes against c-Myc were transfected into Caco2 cells prior to harvesting nuclear extracts. Silencing of c-Myc completely blocked the formation of this shifted band (Fig. 4F). A second faster migrating band was also observed in hypoxic conditions, and formation of this band was similarly blocked by both LY294002 and Y27632. However, this band was still identifiable when a mutant probe was used (Fig. 4C). Furthermore, the band was also present in normoxic conditions (Fig. 4A and B), making its possible role in the hypoxic induction of VEGF less clear. Although we cannot rule out an independent factor that may also play a role in the hypoxic induction of VEGF, these results suggest that c-Myc may be the transcription factor that interacts with the element at −271 bp in hypoxia.

Role of c-Myc in the Regulation of VEGF—Site-directed mutagenesis was performed to selectively alter the putative c-Myc-binding site in the 0.75-kb VEGF-luc construct, referred to as mut-271-luc (Fig. 5A). There was a 62% reduction (p < 0.01) in the hypoxic induction of the VEGF promoter in Caco2 cells when the Myc-Max-binding site at −271 bp was mutated. To directly determine its role in the regulation of VEGF transcription during hypoxia, c-Myc was overexpressed in these cells.

![Diagram](image-url)
c-Myc induced the wild-type 0.75-kb VEGF-luc construct 1.6-fold, but there was no induction when the Myc-Max element at −271bp was mutated (Fig. 5A, right panel). To further verify its role, c-Myc was silenced by transient transfection of specific siRNA duplexes. There was a strong silencing effect on c-Myc 48 h after transfection (Fig. 5B). Hypoxia up-regulated the VEGF promoter 2.1-fold when 0.75-kb VEGF-luc was co-transfected with control siRNA, but the induction was attenuated 1.6-fold by c-Myc siRNA (56% reduction, p < 0.01) (Fig. 5B). The reduction in the hypoxic induction by siRNA directed against c-Myc was also demonstrated in HIF-1a-deficient Caco2 cells (HIF-kd1470). Finally, the effect of silencing c-Myc on endogenous levels of VEGF mRNA was determined. Transfection of the c-Myc siRNA resulted in a 43% reduction in VEGF mRNA levels in hypoxia, as measured by real time PCR (Fig. 5C). This level of inhibition was comparable with that achieved by the addition of LY294002 (38% reduction in VEGF mRNA) or Y27632 (29% reduction in VEGF mRNA). These findings indicate that c-Myc can regulate VEGF during hypoxia and can at least partially explain the hypoxic induction of VEGF that is independent of HIF-1.

**Discussion**

In previous studies, we evaluated the role of HIF-1 in the hypoxic induction of VEGF in colon cancer cells and demonstrated that HIF-1 is not necessary (11). Although HIF-1 is generally considered to be a critical mediator of the hypoxic response (28), it appears not to be the only mediator. For example, others have shown that transcription factors including Sp-1 (29), AP-1 (30), NF-kB (31), and Egr-1 (32) may also play a role in the regulation of hypoxia-responsive genes. We sought to characterize the HIF-1-independent signaling pathways that can regulate the hypoxic induction of VEGF in colon cancer. Our studies suggest a role for the activation of c-Myc by PI3K/Rho/ROCK signaling as an alternative, HIF-1-independent mechanism for the induction of VEGF in hypoxia.

Because a synergistic effect between hypoxia and K-ras on VEGF expression has been observed, we were curious to define the roles that specific Ras effector pathways may play in hypoxic conditions. Hypoxia activated two major Ras effectors, ERK and PI3K/Akt. Although the ERK pathway appeared to regulate HIF-1, the MEK inhibitor PD98059 did not block VEGF mRNA expression or VEGF promoter activity in hypoxia. The effect of PD98059 may not be specific for ERK1/2, because it may potentially also inhibit ERK5, which has recently been shown to suppress the hypoxic induction of VEGF (33, 34). However, studies using dominant negative ERK1/2 also failed to demonstrate a role for ERK1/2 in the hypoxic induction of VEGF. In contrast, inhibition of PI3K strongly attenuated the hypoxic induction of VEGF. This appeared to be a transcriptional effect because PI3K inhibition suppressed VEGF promoter activity but did not alter VEGF mRNA stability. It should be noted that inhibition of PI3K did not suppress the induction of HIF-1α in hypoxia, indicating that the effects of PI3K on the regulation of VEGF are independent of HIF-1.

Akt is a major effector of PI3K that can regulate the VEGF promoter in normoxic conditions (6), but, to our surprise, Akt was not an important regulator of VEGF in hypoxic conditions. Instead, we demonstrated that an alternative target of PI3K, the Rho/ROCK pathway, can mediate the hypoxic induction of VEGF. Hypoxia up-regulated the levels of GTP-bound Rho, and the combination of hypoxia and oncogenic K-ras was synergistic. Inhibition of PI3K by LY294002 attenuated Rho activation in a dose-dependent manner. The precise mechanism of Rho activation remains to be defined, but one possible explanation could involve...
guanine exchange factors for Rho, because guanine exchange factors for Rho family GTPases have been shown to contain a pleckstrin homology domain that can interact with phosphatidylinositol 3,4,5-triphosphate (35). Of note, preliminary microarray studies have indicated that hypoxia can up-regulate Rho-guanine exchange factor expression by 70.4% and down-regulate Rho GDP dissociation inhibitor by 64.8% in Caco2 cells (data not shown).

Watnick et al. (27) recently demonstrated the H-ras-mediated phosphorylation of c-Myc at Ser62 and Ser71 through a signaling cascade also involving PI3K, Rho, and ROCK. In our studies, phosphorylation of c-Myc at residues Thr58 and Ser62 was observed when Caco2 cells were incubated in hypoxia. This depended upon signaling through the PI3K/ROCK pathway, because the phosphorylation was almost completely attenuated by LY294002 and Y27632. Thus, this pathway can also explain the PI3K-mediated induction of VEGF in states of hypoxia. In contrast to previous studies with H-ras, our results demonstrated that the K-ras isoform that is most frequently mutated in human cancers enhanced signaling through this pathway. Thus far, there is no evidence showing a direct interaction between ROCK and c-Myc, and further studies are required to clarify whether ROCK can directly phosphorylate c-Myc or indirectly through another kinase.

c-Myc can positively regulate VEGF expression (36). In addition, c-Myc has been demonstrated to have a role in VEGF mRNA translation (37, 38). However, there are some conflicting results, because inhibitory effects of c-Myc on VEGF expression have also been described (39). Other studies have demonstrated a role for Rho/ROCK signaling in the down-regulation of c-Myc by TGF-β signaling (40). We did not address the molecular events triggered by TGF-β but focused on the regulation by hypoxia. Our results indicated that c-Myc was activated by hypoxia, and we have identified a Myc/Max-binding element in the VEGF promoter that can up-regulate the gene in hypoxia. Its role was verified through the use of siRNAs specifically targeting c-Myc. Of note, c-Myc has been linked to tumor angiogenesis (41), and regulation of thrombospondin-1 expression is one possible mechanism (27). Our findings suggest that VEGF may also be an important target...
of c-Myc, particularly in conditions of hypoxia. This may be particularly relevant in colon cancer, because c-Myc is commonly overexpressed in this tumor type (42).

In summary, we present a novel alternative mechanism for the regulation of VEGF in hypoxic conditions (Fig. 6). This pathway does not depend upon HIF-1α and appears to be mediated through the activation of PI3K, Rho, ROCK, and c-Myc. Oncogenic K-ras can further enhance signaling through this pathway. These results demonstrate that hypoxia, an environmental stimulus frequently observed within solid tumors, can stimulate multiple signaling pathways that regulate tumor angiogenesis, some of which do not depend upon HIF-1.

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