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Oncogenic Transformation and Hypoxia Synergistically Act to Modulate Vascular Endothelial Growth Factor Expression[1]

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Abstract

Hypoxia can select for cells that have lost their apoptotic potential, thereby making them resistant to adverse conditions. However, long-term survival of transformed cells which have diminished apoptotic sensitivity when exposed to low oxygen conditions would require the activation of their angiogenic program to compensate for an insufficient oxygen supply. In this report, we show that the activity of oncogenic Ha-*ras*, either constitutively or transiently, enhances the induction of the angiogenic mitogen, vascular endothelial growth factor (VEGF), by hypoxia. Analysis of the 5′ flanking region of the VEGF promoter indicates that a HIF-1-like sequence is to promote a 15-fold increase in reporter gene activity in Ha-*ras*-transformed cells when exposed to hypoxia, whereas mutations in the same site totally inhibited VEGF induction. Under low oxygen conditions, VEGF induction is inhibited in cells expressing a mutant inhibitory allele of Ha-*ras* (*RasN17*), indicating a direct role for Ras in modulating VEGF activity. We propose that the angiogenic switch in Ras-transformed cells may be physiologically promoted by the tumor microenvironment through VEGF induction.

Introduction

Recent studies have indicated that oncogene activation and the tumor microenvironment play critical and possibly synergistic roles in regulating cell viability (1–4). During tumor development, transformed cells not only accumulate genetic alterations in proto-oncogenes and tumor suppressor genes, they are also exposed to a changing tumor microenvironment that exerts its own influence on malignant progression. For example, low oxygen conditions (hypoxia) induce apoptosis in oncogenically transformed cells that possess a functional *p53* tumor suppressor gene (3). Transformed cells that have lost functional p53 or have accrued mutations in antiapoptotic genes are significantly more resistant to hypoxic stress in both cell culture and transplantable tumors, strongly indicating that the physiological stress of hypoxia can select for tumor cells that have lost their apoptotic program. However, for apoptotically resistant transformed cells to clonally expand from this hypoxic environment and become the predominant genotype in the tumor, they must either migrate to an oxic region or increase their expression of angiogenic growth factors to promote the development of new oxygen-supplying blood vessels.

This switch to the angiogenic phenotype is highly associated with the net increase in expression of angiogenic promoting cytokines such as tumor necrosis factor α (1) and growth factor mitogens such as basic fibroblast growth factor (2), platelet-derived growth factor (5), and VEGF[3] (2). Previous studies have demonstrated that oncogenic transformation with activated forms of the *Src* (6), *Ras* (4, 7), and *Raf-1* (6, 7) oncogenes could increase the expression of VEGF, suggesting a link between tumorigenesis and angiogenesis. Although an increase in angiogenic growth factor expression by oncogenes can in part explain the development of a small multicellular mass of tumor cells, many studies have shown that hypoxia is also a potent activator of VEGF (8, 9) and many proangiogenic mitogens. Thus, if oncogenic transformation primes cells for increased VEGF expression, tumor hypoxia would provide the necessary signal to increase or maintain this state of angiogenic growth factor production.

In this article, we examine how oncogenic transformation influenced the induction of VEGF by hypoxia. We focused our studies on cells transformed by the Ha-*ras* oncogene since it is the most common oncogenic mutation found in over 40% of all solid tumors (10). Using genetically matched rodent fibroblasts that were untransformed or transformed with the Ha-*ras* oncogene, we found that transformation of cells with Ha-*ras* substantially increases VEGF induction under hypoxic conditions. Using VEGF promoter-reporter constructs, we investigated whether this increase in VEGF transcription under hypoxic conditions was due in part to VEGF promoter activity and what were the elements in the promoter that responded to hypoxia. This analysis revealed that a region of the promoter containing a HIF-1-like binding site exhibited the strongest induction by hypoxia in Ha-*ras*-transformed cells. Furthermore, overexpression of a dominant interfering allele of Ha-*ras* (*RasN17*) inhibited the induction of VEGF under hypoxia conditions. Taken together, these results suggest that oncogenic Ha-*ras* and hypoxia can act synergistically to induce VEGF through a HIF-1-like sequence.

Materials and Methods

Cell Culture and Hypoxic Treatment. NIH3T3 and Rat-1 cells were maintained in culture in DMEM supplemented with 10% (v/v) FCS. NIH3T3R, RasN17, and NIH3T3 cells harboring an IPTG-inducible form of Ha-*ras* have been described previously (11) and were grown in DMEM supplemented with 400 μg/ml G418. Rat-1 cells were stably transfected with oncogenic Ha-*ras* as described previously (3). All cells were cultured in a well-humidified 95% air/5% CO_2 incubator at 37°C and were subconfluent at the beginning of hypoxia incubation. To determine the effect of hypoxia on VEGF induction, cells plated on glass dishes were placed into specially designed aluminum hypoxia chambers that were prewarmed at 37°C overnight. These chambers were flushed with a gas mixture containing 5% CO_2 and 95% N_2 until the final level of oxygen was 0.02%, as determined by an oxygen electrode, then sealed and incubated for 5 h at 37°C followed by 2 h of reoxygenation (11). Some experiments were also performed in an anaerobic tissue culture hood (Anaerobie Systems, Portland, OR). All experiments were performed at least three times in duplicate.

Plasmid Constructs and Transfections. To generate VEGF promoter-reporter constructs, a *Sac*I-*Sac*I fragment, which included 1175 bp of the promoter from the start site of transcription and 336 bp of untranslated mRNA,

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[3] The abbreviation used is: VEGF, vascular endothelial growth factor.

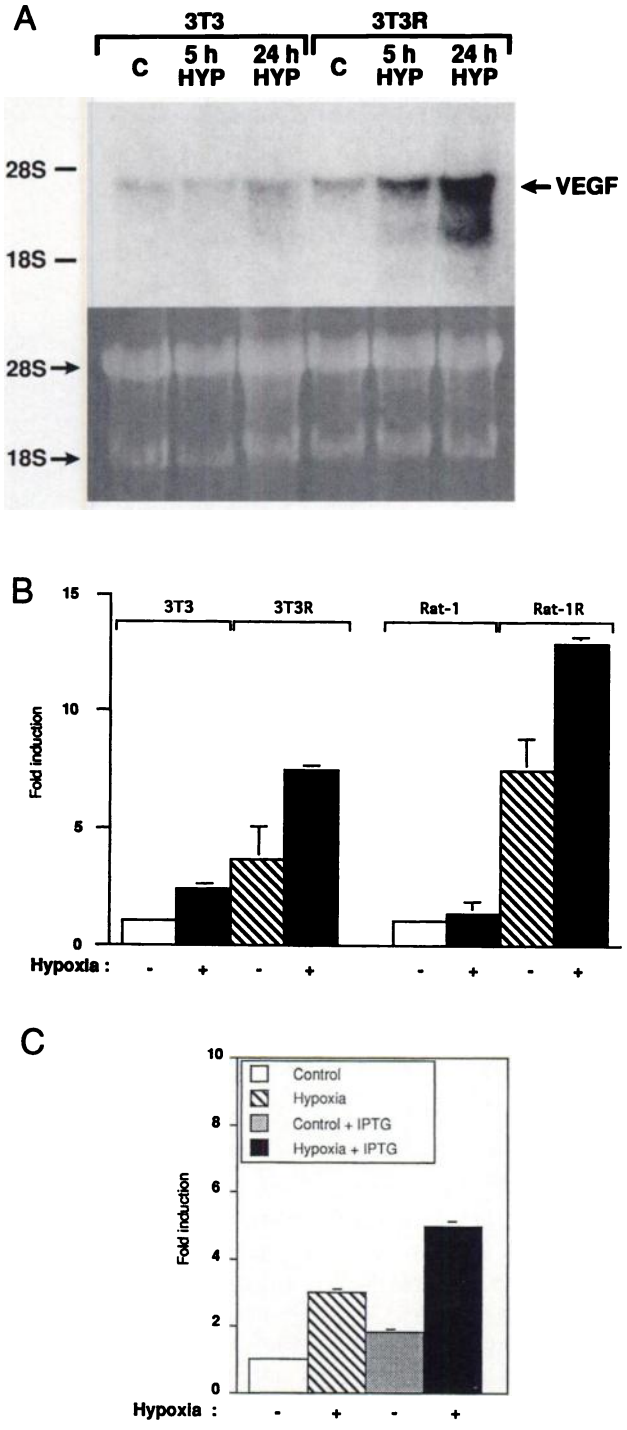

Fig. 1. Effect of Ha-*ras* oncogene. *A*, endogenous VEGF mRNA expression under low oxygen conditions. *Top panel*, endogenous VEGF mRNA expression after 0, 5, and 24 h of hypoxia (0.02% O_2) in NIH3T3 cells and Ha-*ras*-transformed NIH3T3 cells (NIH3T3R) hybridized with a cDNA to the $VEGF_{165}$ probe. *Arrows*, position of the ribosomal genes 28*S* and 18*S*. *Bottom panel*, ethidium bromide-stained photograph of the same gel before transfer to demonstrate equal loading of RNA. *B*, VEGF promoter activity under hypoxic conditions. NIH3T3, NIH3T3R, Rat-1, and Rat-1R cells were transfected with 5 μg of the 1511-bp reporter gene, allowed to recover for 17 h after transfection in air, exposed to hypoxia (0.02% 0_2) for 5 h, and assayed for luciferase activity. The relative fold induction refers to the ratio of luciferase activity measured in hypoxia-treated cells (+) relative to the activity observed in the untreated controls (−). Values represent the means of at least three independent transfections. *Bars*, SD. *C*, inducible Ha-*ras* activity on VEGF promoter activity. Conditionally, Ha-*ras*-inducible NIH3T3 cells were transfected with 5 μg of VEGF reporter plasmid (1511 bp), incubated in 20 nM IPTG for 24 h to induce Ha-*ras* activity, exposed to hypoxia (0.02% 0_2) for 5 h, and then assayed for luciferase activity. The relative fold induction refers to the ratio of luciferase activity measured in hypoxia-treated cells compared to aerobic cells either in the presence or absence of inducer (+IPTG). Values represent the means of at least three independent transfections. *Bars*, SD.

was inserted into pGl_2 basic (Luciferase Expression System; Promega, Madison, WI) at the *Sac*I site. The correct orientation of the promoter insert was verified by restriction mapping. A series of deletions was derived from this 1511-bp fragment by restriction digestion: the 1275-, 764-, 671, and 385-bp fragments are located at position −1175 to +100, −411, −504, and −790, respectively; the 601-bp fragment is located at position −1012 to −411, the 379-bp fragment is located at position −790 to −411, the 93-bp fragment is located at position −504 to −411, and, finally, the 286-bp fragment is located at position −790 to −504. A HIF-1 polymer was generated by PCR amplification from the *Sac*I-*Sac*I fragment by using the forward primers, HIF-1 wild-type (5′-ccacagtgcatacgtgggctcc-3′) and HIF-1 mutant (5′-ccacagtgcataAAAAggctcc-3′), and a reverse primer at position −782 (*Pst*I; 5′-ctggcctgcagacatc-3′). In all transfection experiments, 3×10^6 exponentially growing cells were electroporated with the indicated plasmid DNA and incubated for 17 h in DMEM (10% serum) before treatment. Before harvesting, cells were washed with PBS and lysed in 100 μl luciferase lysis buffer (Luciferase Expression System; Promega).

RNA Extraction and Northern Blot Analysis. Induction of endogenous VEGF mRNA expression during hypoxia in NIH3T3 and Ha-*ras*-transformed NIH3T3 cells (NIH3T3R) was performed as described by Laderoute *et al.* (12). Total RNA from NIH3T3 and NIH3T3R cells that were exposed to 0.02% oxygen for 0, 5 (5-h HYP), and 24 h (24-h HYP) was hybridized with a human $VEGF_{165}$ cDNA probe (13). The ethidium bromide-stained gel is included to indicate equal loading and transfer of RNA from each sample. Blots were also probed with β-actin to demonstrate equal transfer (data not shown).

Results

Induction of Endogenous VEGF mRNA Expression by Hypoxia Is Enhanced by the Expression of the Ha-*ras* Oncogene. To investigate how oncogenic transformation of cells by Ha-*ras* changes their angiogenic potential, we compared the expression of VEGF mRNA in NIH3T3 cells to that in NIH3T3 cells which express an activated form of Ras, Ha-*ras* (NIH3T3R). Untransformed cells express low basal levels of VEGF mRNA and exhibit a 1.6-fold increase in their steady-state expression of VEGF after a 24-h hypoxic exposure (Fig. 1*A*). In contrast, Ha-*ras*-transformed cells possess increased basal levels of VEGF mRNA, but also exhibit a 3.2 ± 1.8-fold induction after a 5-h hypoxic exposure that remains elevated at this level during a 24-h hypoxic exposure. Thus, Ha-*ras* transformation not only increases the basal levels of VEGF mRNA, it also acts synergistically with hypoxia to maintain a sustained increase in VEGF expression. Similar results were found for Rat-1 and Rat-1R cells (data not shown) and are in good agreement with the past results of other investigators (4).

Both Ha-Ras and Hypoxia Increase the Activity of the 1511-bp Length VEGF Promoter. As a rapid means to determine whether the increased expression of VEGF mRNA by Ha-*ras* and hypoxia was due to increased promoter activity, we transfected VEGF promoter-luciferase constructs that spanned 1175 bp from the start site of transcription and 336 bp of 5′ untranslated region into Ha-*ras*-expressing cells and their parental counterparts. Ha-*ras* alone increases the activity of the VEGF promoter in both NIH3T3 (2.5-fold) and Rat-1 (7.6-fold) cells (Fig. 1*B*). Hypoxia weakly increased VEGF expression in NIH3T3 and Rat-1 cells that did not express oncogenic Ha-*ras*. However, a significant increase in VEGF activity was found in both cell lines which constitutively express Ha-*ras* (Fig. 1*B*). These studies support the hypothesis that oncogenic Ha-*ras* alone increases VEGF expression in Ha-*ras*-transformed cells that can be substantially increased by hypoxic exposure.

To further investigate the role of Ras in VEGF induction by hypoxia, we examined VEGF reporter activity in NIH3T3 cells that possess a conditionally inducible form of Ha-*ras*. In the absence of inducing agent (IPTG; Ref. 14), VEGF reporter activity increased 3-fold in response to hypoxia, an increase over untransfected NIH3T3 cells due to a low loss of repressor activity. Upon induction of Ha-*ras*

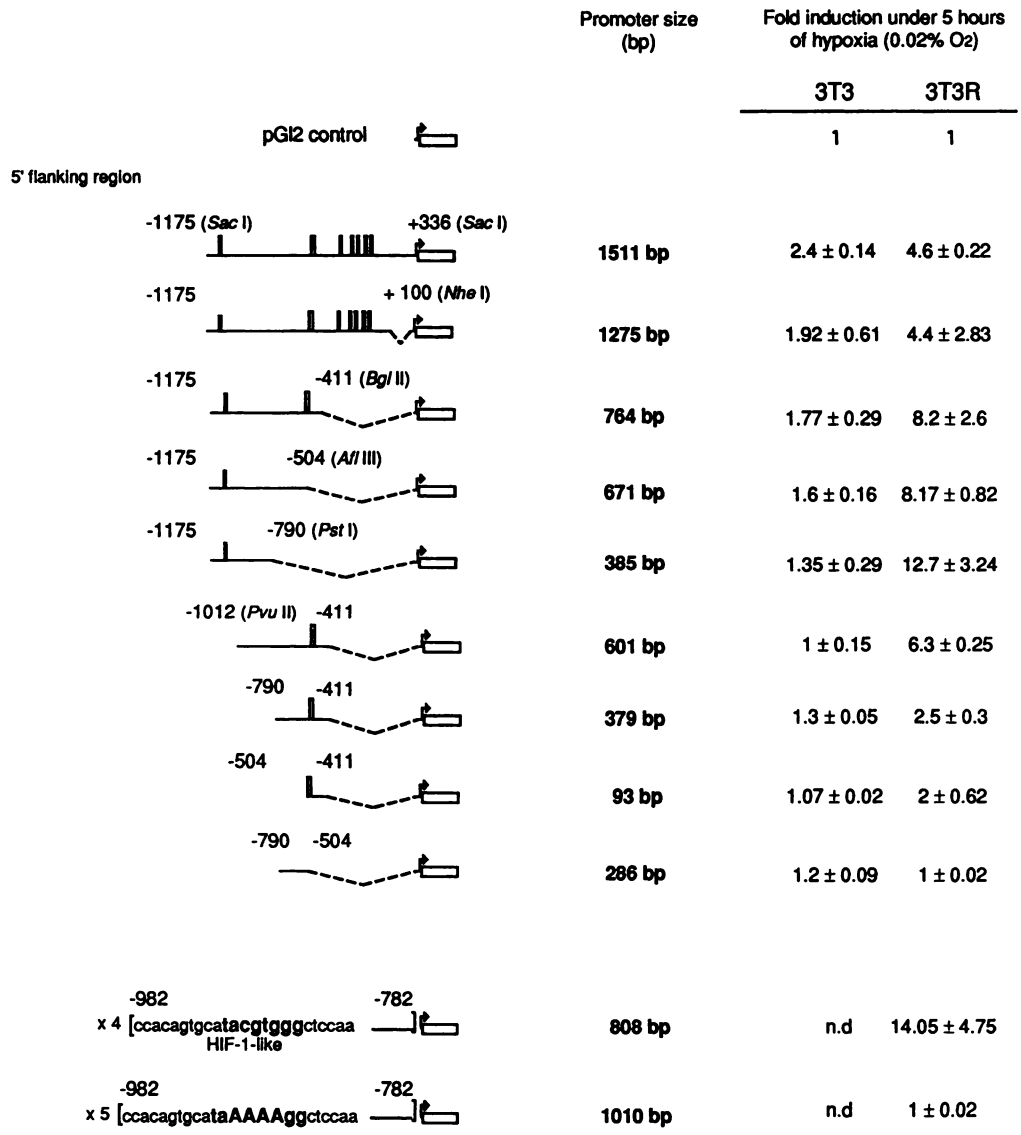

Fig. 2. Characterization of the 5′ flanking region of the human VEGF promoter under hypoxic conditions. A series of promoter deletion constructs were derived from the 1511-bp fragment and tested for hypoxia responsiveness in NIH3T3 cells or NIH3T3R (Ha-*ras*-containing cells). The HIF-1-like wild-type and mutant polymers were generated by PCR amplification from the 1511-bp fragment. *Thin lines*, 5′ sequences used (numbers refer to the distance from the start site of transcription); *plain boxes*, SP-1-binding sites; and *open boxes*, AP-1-binding site. NIH3T3 and NIH3T3R cells were transfected with 5 μg of reporter gene, allowed to recover for 17 h in air, treated in air or hypoxia (0.02% 0_2) for 5 h, and then assayed for luciferase activity. The relative fold induction refers to the ratio of luciferase activity measured in treated cells relative to the activity observed in the untreated controls. Values represent the means of at least three independent transfections. *Bars*, SD.

activity through the addition of IPTG, VEGF reporter activity increased 5-fold under a 5-h exposure to hypoxic conditions (Fig. 1*C*). Therefore, increased expression of Ha-*ras*, even in a transient manner, is sufficient to increase the induction of VEGF to low oxygen conditions.

Analysis of the 5′ Flanking Region of the VEGF Promoter under Hypoxia Stress. To identify and characterize the hypoxia-responsive element in the 5′ flanking region of the *VEGF* gene, a deletion series of the 1511-bp promoter reporter gene was analyzed in transient transfection experiments in both NIH3T3 and NIH3T3R under normoxic and hypoxic conditions (Fig. 2). Several points of this analysis are noteworthy. VEGF promoter activity was weak in NIH3T3 cells with all of the constructs tested, whereas the inducibility of VEGF under hypoxia was 2–10 times higher in Ha-*ras*-transformed NIH3T3 cells. Although the 1511- and 1275-bp VEGF promoters which contain the same 5′ end differ in their 3′ ends, this difference has no effect on their inducibility under hypoxic conditions. Both promoters exhibited a 4.6- and 4.4-fold induction in NIH3T3R cells and a 2.4- and 1.9-fold induction in untransformed NIH3T3 cells, respectively (Fig. 2). The 747- and 840-bp deletions designated the 764- and the 671-bp promoters from the +336 *Sac*I site to the *Bgl*II site at −411 and the *Afl*III site at −504 of the 1511-bp reporter gene showed an 8-fold induction in NIH3T3R cells under hypoxia. These results suggest that the −411 to +100 region, which contains five potential binding sites for the SP-1 transcription factor, has a significant inhibitory effect on VEGF induction under hypoxic conditions. At present, the mechanism for this repressor activity is unknown, but is under investigation. Deletion of a consensus AP-1 site in the 671-bp VEGF promoter had little effect on the activity of VEGF under hypoxic conditions, indicating that AP-1 does not mediate the hypoxia inducibility of VEGF in Ha-*ras*-transformed cells as previously suggested by Finkenzeller *et al.* (15).

To define the hypoxia-responsive element in Ha-*ras*-transformed cells, the 764-bp promoter was divided in three parts: from −1175 to −790 (385 bp), from −790 to −504 (286 bp), and from −504 to −411 (93 bp). The 385-bp fragment derived in this way exhibited a reproducible 13–16-fold increase after hypoxic conditions only in NIH3T3R cells and displayed little detectible activity in untransformed NIH3T3 cells. In comparison to the 385-bp promoter, the 93-bp promoter displayed a 2-fold increase that was also highly reproducible (six independent experiments in duplicate), and the 286-bp region was completely unresponsive to hypoxia in either Ha-*ras* or parental NIH3T3 or Rat-1 cells. Based on sequence analysis, the 385-bp promoter contains a similar binding site (5′-TACGT-

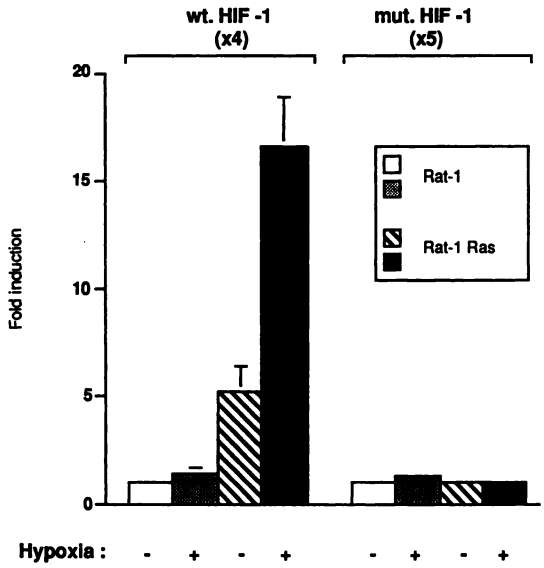

Fig. 3. Responsiveness of HIF-1 polymer in Rat-1 cells under hypoxic conditions. Rat-1 and Rat-1R (Ha-*ras*) cells were transfected with 5 μg of wild-type or mutant HIF-1-like sequences, allowed to recover aerobically for 17 h, treated in air or hypoxia (0.02% 0_2) for 5 h, and assayed for luciferase activity. The relative fold induction refers to the ratio of luciferase activity measured in treated cells relative to the activity observed in the untreated aerobic controls. Values represent the means of five independent transfections. *Bars*, SD. *wt*, wild type; *mut*, mutant.

Ggg-3′) to that of the hypoxia-inducible transcription factor HIF-1 (5′-TACGTGCT-3′; Ref. 16). Interesting, the 93-bp promoter, which exhibits a more diminished but yet reproducible response to low oxygen conditions, contains a truncated HIF-1-like sequence.

To gain more direct evidence that this sequence is an important regulator of VEGF activity under hypoxic conditions, we compared the response of a reporter gene which contains four copies of this HIF-1-like sequence to a mutated version of this sequence. The HIF-1 polymer exhibited a 14-fold increase in activity when exposed to hypoxia in NIH3T3 Ras-transfected cells, whereas the same promoter region containing five copies of a mutation in the putative HIF-1-binding site displayed no increase in activity when exposed to low oxygen conditions (Fig. 2). Similar results were obtained after transfection with both wild-type and mutant HIF-1-like polymers in Rat-1 and Rat-1R cells (Fig. 3). These studies suggest that the hypoxia inducibility of the VEGF promoter in Ha-*ras*-transformed cells is in part mediated by HIF-1 because regions of the promoter which do not possess this HIF-1-like binding sequence are not strongly inducible.

To further support a role for Ras in signaling VEGF under hypoxic conditions, we examined the induction of the 1511-bp VEGF promoter in cells stably or transiently overexpressing a mutant form of Ha-*ras* (*RasN17*, a mutation of Asn-17; Ref. 17) which inhibits Ras activity. Ha-*ras*-transformed cells displayed an 4.6-fold induction of VEGF reporter activity, whereas RasN17-transfected cells showed no induction (Fig. 4*A*). RasN17 also blocked VEGF induction in untransformed NIH3T3 cells (Fig. 4*B*), suggesting that Ras is utilized in signaling VEGF induction by hypoxia in both transformed and untransformed cells alike.

Discussion

Oncogenic mutations which act to accelerate the growth of transformed cells can also activate a mechanism that promotes survival of these same cells by priming them for a switch to the angiogenic phenotype in response to low oxygen conditions. Because regions of low oxygen tension are a unifying feature of solid tumors, this microenvironmental stress may provide a common signal that induces a prolonged increase in angiogenic gene expression during tumorigenesis. Because Ras-activating mutations are one of the most common oncogenic mutations (10) in solid tumors, especially tumors which contain hypoxic regions and increased vascularity such as breast adenocarcinomas (18, 19), a Ras/HIF-1-like pathway would seem to be important in the regulation of angiogenic mitogens during solid tumor development. The recent identification of a Src/Raf/MAP kinase pathway (6) may also utilize Ras in its signaling or may define a parallel pathway that enhances the switch to the angiogenic phenotype, either by modulating HIF-1, other hypoxic regulatory elements in the VEGF genomic sequence, or the stability of VEGF mRNA. However, VEGF induction through the HIF-1-like transcriptional regulatory element is modulated by a Ras signaling pathway. These results suggest that inhibition of downstream effectors' oncogenic Ras or inhibition of Ras activity through the use of farnesyltransferase inhibitors (4) should have a potent antitumor effect by inhibiting angiogenesis.

Finally, a long but poorly understood feature of solid tumor cells is their increased expression of glycolytic enzymes under aerobic conditions (20). Studies (21) indicate that genes encoding the glycolytic enzymes aldolase A, phosphoglycerate kinase I, and pyruvate kinase M possess HIF-1 regulatory elements and are hypoxia inducible. Thus, oncogenic transformation of cells may not only prime them for the switch to the angiogenic phenotype, it may also prepare them for the adverse and changing conditions of the tumor microenvironment by mediating the transition from oxidative phosphorylation to anaerobic glycolysis.

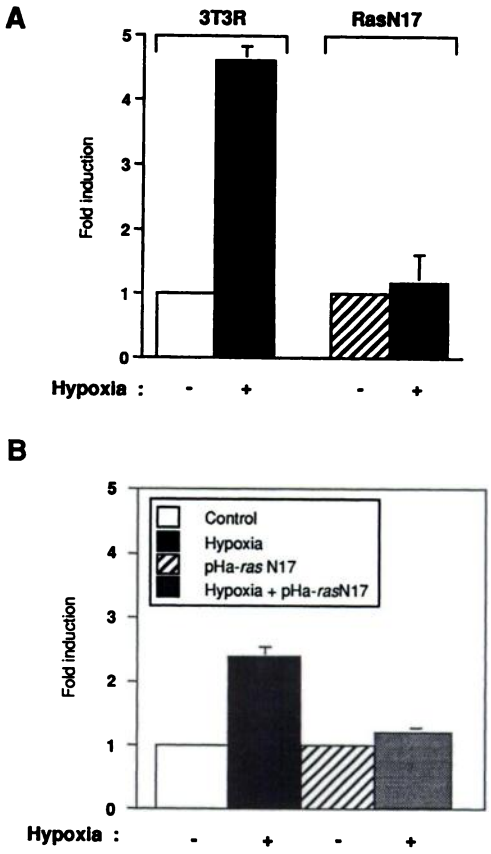

Fig. 4. Effect of a dominant mutant inhibitory allele of Ha-*ras* (*RasN17*) on VEGF induction. *A*, NIH3T3R cells and NIH3T3R cells stably transfected with RasN17 were transfected with 5 μg of VEGF promoter- (1511 bp) reporter construct, allowed to recover aerobically for 17 h, exposed to hypoxic conditions (0.02% 0_2) for 5 h or left untreated, and then assayed for luciferase activity. *B*, the same protocol as described above was performed below, except that the VEGF reporter construct was cotransfected with 1 μg RasN17 plasmid (pHa-*ras* N17) before hypoxic or aerobic exposure. Values represent the means of four independent transfections. *Bars*, SD.

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