RAPID COMMUNICATION

Induction of Vascular Endothelial Growth Factor by Hypoxia Is Modulated by a Phosphatidylinositol 3-Kinase/Akt Signaling Pathway in Ha-ras-Transformed Cells Through a Hypoxia Inducible Factor-1 Transcriptional Element

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Tumor angiogenesis, the development of new blood vessels, is a highly regulated process that is controlled genetically by alterations in oncogene and tumor suppressor gene expression and physiologically by the tumor microenvironment. Previous studies indicate that the angiogenic switch in Ras-transformed cells may be physiologically promoted by the tumor microenvironment through the induction of the angiogenic mitogen, vascular endothelial growth factor (VEGF). In this report, we show Ras-transformed cells do not use the downstream effectors c-Raf-1 or mitogen activated protein kinases (MAPK) in signaling VEGF induction by hypoxia as overexpression of kinase-defective alleles of these genes does not inhibit VEGF induction under low oxygen conditions. In contrast to the c-Raf-1/MAP kinase pathway, hypoxia increases phosphatidylinositol 3-kinase (PI 3-kinase) activity in a Ras-dependent manner, and inhibition of PI 3-kinase activity genetically and pharmacologically results in inhibition of VEGF induction. We propose that hypoxia modulates VEGF induction in Ras-transformed cells through the activation of a stress inducible PI 3-kinase/Akt pathway and the hypoxia inducible factor-1 (HIF-1) transcriptional response element.

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stream of PI 3-kinase are still under investigation, recent studies have shown that the proto-oncogene Akt is activated by lipid products of PI 3-kinase.25

We investigated whether a PI 3-kinase/Akt pathway signaled VEGF induction through the HIF-1 transcription factor under hypoxic conditions in cells expressing oncogenic Ha-ras (NIH3T3R). Because at least two potential pathways involving Ras have been implicated in the response of VEGF in cells to low oxygen conditions, one involving Src/Raf-1/MAPK and a second involving Ras and PI 3-kinase, we examined what contribution each potential pathway had on transcriptional activation of VEGF.

MATERIALS AND METHODS

Cell culture and hypoxia treatment. NIH3T3 cells were maintained in culture in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum. NIH3T3R and Rat-1 (Ha-ras transformed). RasN17-, Raf301-cells were maintained in culture with DMEM media that was additionally supplemented with 400 µg/mL G418. NIH3T3R cells that were stably transfected with a mutant inhibitory allele of Ras [Ras(N17)] or Raf-1 [Raf301] were tested for functionality by assaying for inhibition of NF-eB activation by hypoxia and Ha-ras-induced anchorage independent cell growth in soft agar.26 ThOSE stably transfected cell lines which showed the strongest suppression of both phenotypic responses by Ras and Raf-1 mutant inhibitory alleles were used in transient transfection experiments to determine their effect on VEGF. Cells were cultured in a well-humidiﬁed 95% air, 5% CO2 incubator of activation of known downstream effectors. Except for hypoxia, cell treatment.

Cell treatment. NIH3T3R cells were left untreated or treated with hypoxia (HYP), tumor necrosis factor-a (TNF-a; 200 U/mL), epidermal growth factor (EGF; 30 ng/mL), anisomycin (Anis.; 30 ng/mL), sodium chloride (NaCl; 0.1 mol/L), and sorbitol (0.2 mol/L) for 7 hours before luciferase measurement. UV light was applied at 40 J/m2 and assayed for protein concentration using the bicinchoninic acid technique (Pierce Biochemicals, Rockford, IL). Equal amounts (300 µg) of a plasmid containing a defective VEGF reporter plasmid and assayed for luciferase activity. One or 5 µg of VEGF reporter plasmid was cotransfected with 0.1 and 1 µg of the mutant ERK expression vectors. A mutant form of the p85 (Δp85) subunit of PI 3-kinase that lacks amino acids from 479 to 513 and contains two extra amino acids (Ser-Arg), was generously provided by Dr M. Kasuga (University of Kobe School of Medicine, Kobe, Japan),27 was cotransfected with 5 µg of 385-bp, HIF-1 wt polymer- and HIF-1 mut. polymer-reporter plasmids. One-tenth, 1, or 10 µg of a plasmid containing the wt p110 coding sequence, kindly provided by Dr T. Frank (Harvard Medical School, Boston, MA), was cotransfected with 2.5 µg of 1,511-bp, 385-bp-, and HIF-1 wt polymer-reporter plasmids. Overexpression of wt or dominant negative constructs were verified for activity by assaying the appropriate downstream effector.

Cell treatment. NIH3T3R cells were left untreated or treated with hypoxia (HYP), tumor necrosis factor-a (TNF-a; 200 U/mL), epidermal growth factor (EGF; 30 ng/mL), anisomycin (Anis.; 30 ng/mL), sodium chloride (NaCl; 0.1 mol/L), and sorbitol (0.2 mol/L) for 7 hours before luciferase measurement. UV light was applied at 40 J/m2 and assayed for protein concentration using the bicinchoninic acid technique (Pierce Biochemicals, Rockford, IL). Equal amounts (300 µg) of a plasmid containing a defective VEGF reporter plasmid and assayed for luciferase activity. One or 5 µg of VEGF reporter plasmid was cotransfected with 0.1 and 1 µg of the mutant ERK expression vectors. A mutant form of the p85 (Δp85) subunit of PI 3-kinase that lacks amino acids from 479 to 513 and contains two extra amino acids (Ser-Arg), was generously provided by Dr M. Kasuga (University of Kobe School of Medicine, Kobe, Japan),27 was cotransfected with 5 µg of 385-bp, HIF-1 wt polymer- and HIF-1 mut. polymer-reporter plasmids. One-tenth, 1, or 10 µg of a plasmid containing the wt p110 coding sequence, kindly provided by Dr T. Frank (Harvard Medical School, Boston, MA), was cotransfected with 2.5 µg of 1,511-bp, 385-bp-, and HIF-1 wt polymer-reporter plasmids. Overexpression of wt or dominant negative constructs were verified for activity by assaying the appropriate downstream effector.

RESULTS

Hypoxia-responsive element of the human VEGF promoter in Ha-ras-transformed cells. We have previously
used VEGF reporter constructs to demonstrate that a hypoxia inducible factor-1–like (HIF-1-like) sequence promotes VEGF activity under low oxygen conditions in Ha-ras–transformed cells.1 In this study, we used the 1,511-bp VEGF promoter-reporter gene that includes 1,175 bp from the start site of transcription and 336 bp of the 5′ untranslated region of VEGF promoter (Fig 1A). To further investigate VEGF promoter activity under hypoxic conditions in cells expressing an oncogenic form of Ha-ras (NIH3T3R cells), we also used the 385-bp deletion fragment of the 1,511-bp promoter, located at position −1175 (Sac I) to −790 (Pst I), as well as the HIF-1 polymer ligated to the luciferase reporter gene.

They both contain at least one HIF-1 element (385-bp, one copy; HIF-1 polymer, four copies). This series of promoter-reporter genes allows us to investigate the effect of hypoxia on the full-length VEGF promoter as well as on specific regulatory elements. As with many regulatory elements, the addition of multiple copies increases the magnitude of the readout. The HIF-1 polymer construct was induced to comparable levels by either cotransfection with HIF-1α and HIF-1β-expression vectors or by hypoxia alone (Fig 1B). A mutant HIF-1 element was unresponsive to either hypoxia or cotransfection with HIF-1α and HIF-1β.

A Raf/MAPK or SAPK pathway is not used in signaling VEGF induction under hypoxia. Ras represents a critical junction in the downstream transmission of signals from growth factor receptors and from exposure of cells to stress-inducing agents.30 In many different cell types, activated Ras triggers a kinase cascade involving the serine-threonine kinase Raf-1 which phosphorylates and activates the dual-function specificity kinase MEK (MAP Kinase Kinase) which then phosphorylates and activates MAP Kinase via tyrosine and threonine phosphorylation.37,38 To determine if Ha-ras–transformed cells use an Raf-1/MAP Kinase pathway to signal VEGF induction under low-oxygen conditions, we transfected NIH3T3R cells either stably or transiently with HIF-1 ± expression vectors or by hypoxia alone (Fig 1B). A dominant inhibitory mutant of c-Raf-1, Raf301,39 was found to neither stable overexpression of Raf301 (Fig 2A), nor transient overexpression of an Raf301 plasmid (Fig 2B), which have both been shown to be active in inhibiting NF-κB signaling,15 had a significant effect on VEGF reporter gene induction in response to hypoxia. In comparison, the dominant inhibitory allele of Ras, N17,40 was able to completely inhibit VEGF induction by hypoxia (Fig 2A).

We also investigated the role of MAPK in VEGF induction by hypoxia in Ha-ras–transformed cells. Kinase dead alleles of MAP Kinases (also referred to as extracellular signal-regulated kinases, ERK-1 and ERK-2)41 are functionally able to inhibit c-fos promoter reporter (Fos-Luc plasmid derived from human c-fos promoter) activity induced by Ha-ras42 (Fig 2C), but do not block hypoxic inducibility of VEGF (Fig 2D). These observations argue against the involvement of ERK-1 and ERK-2 in VEGF signaling in Ha-ras–transformed cells. The mammalian MAP Kinase family (see Fig 7) also includes the members of the stress response pathway, p3829,43 and c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK).29,30,32,43,44 They all require phosphorylation on threonine and tyrosine residues for activation by different upstream effector kinases. The dual phosphorylation of each of MAP Kinase is promoted by a family of dual-function specificity kinases, MAP Kinase Kinase. In some cell types, p38 (MKK) is phosphorylated by MKK3/ MKK629 and JNK/SAPK by MKK4/SEK1.29,30,32,44 The stress response pathways can be activated by cytokines such as TNF-α and interleukin-1 (IL-1).29,32,45 growth factors, and
Fig 2. Raf-1 is not a downstream effector in the signal transduction pathway used to signal VEGF induction in Ha-ras-transformed cells (NIH3T3R). (A) NIH3T3R cells were stably transfected with a mutant inhibitory allele of Ras [Ras(N17)] or Raf-1 [Raf301] before transient transfection with 5 μg of the 385-bp VEGF reporter plasmid and exposure to hypoxia. Values represent at least five independent transfections. Error bars represent ± SD of the mean. (B) Transient transfections with Raf301 (0, 1, 10 μg) and 385-bp reporter construct (2.5 μg) and exposure to hypoxia. (C and D) The effect of MAPKs on signaling VEGF induction by hypoxia. Kinase defective alleles of ERK1 and ERK2 were cotransfected with VEGF reporter constructs to examine their effect on inhibiting VEGF induction by low-oxygen conditions. 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. Error bars represent ± SD of the mean.

UV light.31,32 The mechanism of JNK/SAPK activation by mitogenic stimuli is complex. Because the activation of JNK/SAPKs by growth factors is blocked by a dominant negative allele of Ha-ras (N17),46 growth factor signaling of JNK/SAPK activation might also be mediated through Ras. Treatment of our NIH3T3 cells with stimuli known to activate the JNK/SAPK pathway,29-33 the p38 kinase pathway,34,35 or the NF-κB pathway,15 indicated that these signaling pathways are not used to induce VEGF promoter activity through HIF-1 transcrip tional regulatory sequences (Fig 3A). Furthermore, neither short-term hypoxia nor the combination of hypoxia and reoxygenation stimulates c-Jun phosphorylation when compared to a treatment with the protein synthesis inhibitor anisomycin, a known inducer of the JNK/SAPK pathway (Fig 3B). Thus, JNK/SAPK and p38 kinases do not modulate the Ras-dependent VEGF signaling pathway under low-oxygen conditions.

PI 3-kinase plays a pivotal role in VEGF induction under hypoxic stress. Studies have shown that Ras, in the active GTP-bound conformation, interacts with PI 3-kinase.24 To determine whether PI 3-kinase was involved in the VEGF signaling cascade, we first used wortmannin, a specific inhibitor of PI 3-kinase activity. Wortmannin binds to the 110-kD subunit and irreversibly inhibits PI 3-kinase activity when added at nanomolar concentration to mammalian cells.23 NIH3T3R cells were pretreated 2 hours before hypoxia with increasing concentrations of wortmannin (1 to 100 nM). As shown in Fig 4A, wortmannin inhibited VEGF induction by hypoxia in a concentration-dependent manner. At 1 nM/L, VEGF response to hypoxia was reduced to 77%. Results found with the 385-bp VEGF reporter construct (Fig 4A) were similar to those obtained with a reporter construct containing a polymer of HIF-1 in NIH3T3R or Rat-1R cells that were treated with increasing concentrations of wortmannin (Fig 4B). To determine if PI 3-kinase stimulated VEGF induction through the p70 ribosomal protein S6 kinase (p70^{S6k}), one of the putative targets of PI 3-kinase activation, we examined the effects of rapamycin, a specific inhibitor of the p70^{S6k} pathway, on VEGF induction (Fig 4A).
Fig 3. Inducers of JNK/SAPK and p38 kinase do not activate the same hypoxia-responsive elements that stimulate VEGF promoter activity in Ha-ras transformed cells (NIH3T3R). (A) Effect of JNK/SAPK and p38 kinase activators on the induction of a 385-bp VEGF reporter construct in NIH3T3R cells. NIH3T3R cells were left untreated or treated with 0.02% oxygen (HYP), TNF-α (200 U/mL), epidermal growth factor (EGF, 30 ng/mL), anisomycin (Anis, 30 ng/mL), sodium chloride (NaCl, 0.1 mol/L), or sorbitol (0.2 mol/L) for 7 hours before luciferase measurement. UV light was applied at 40 J/m² 24 hours before luciferase measurement. 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 experimental results. Error bars represent ±1 SD of the mean. (B) Comparison of JNK/SAPK activity induced by anisomycin with JNK/SAPK activity induced by 0.02% oxygen (HYP 0, 30, and 60 minutes) and 3.9% oxygen or 0.02% oxygen with or without reoxygenation after 0 minutes or 30 minutes. Values represent at least three independent experiments.

Contrast to cells treated with wortmannin, cells pretreated with concentrations of rapamycin up to 20 ng/mL still exhibited VEGF induction under hypoxic conditions.

To genetically demonstrate a role for a PI 3-kinase signaling pathway in VEGF induction by hypoxia, we cotransfected either wt p110Δp85 or mutant p85 (Δp85) expression vectors with VEGF reporter constructs. Previous reports showed that overexpression of p110, the catalytic subunit of PI 3-kinase, results in increased PI 3-kinase activity. Using a similar approach, we examined the involvement of PI 3-kinase in VEGF induction by coexpression of wt p110 and a HIF-1 reporter construct in NIH3T3R cells (Fig 5A). Ectopic overexpression of the p110 subunit resulted in the enhancement of the HIF-1 reporter expression, suggesting a link between PI 3-kinase activity induced by the p110 subunit and the hypoxic response element in the VEGF promoter. Coexpression of the p85 regulatory subunit of PI 3-kinase did not increase the activity of VEGF reporter constructs (data not shown). This result is consistent with several studies showing that overexpression of p85 might uncouple the signal-dependent receptor/PI 3-kinase interaction resulting in an inhibitory effect. However, cotransfection of a dominant negative mutant Δp85 suppressed the activity of the 1,511-bp-, 385-bp-, and HIF-1-VEGF reporter constructs under hypoxic conditions (Fig 5B), but had little effect on Fos-luciferase reporter activity induced by Ha-ras. To more directly investigate the relationship between hypoxia, Ha-ras, and PI 3-kinase, we first compared the levels of PI 3-kinase activity in NIH3T3- and NIH3T3R-cells under oxic conditions. PI 3-kinase activity, as measured by an in vitro immune complex kinase assay that uses antiphosphotyrosine to immunoprecipitate activated PI 3-kinase through the SH2 domains of p85, in control untransformed NIH3T3 cells was low (Fig 5C). In contrast, PI 3-kinase activity in Ha-ras-transformed cells (NIH3T3R) under control conditions was already elevated because it is a downstream effector of Ha-ras. However, when NIH3T3R cells were exposed to hypoxia or to a known agonist of PI 3-kinase, platelet-derived growth factor BB (PDGF BB), PI 3-kinase activity was increased twofold (Fig 5C). These data indicate that a small increase of PI 3-kinase activity is sufficient to induce VEGF/HIF-1 promoter activity under hypoxia and is consistent with studies by Klippel et al. showing that low amounts of PI 3-kinase activity were sufficient to activate p70 S6 kinase. Taken together these results suggest that PI 3-kinase is a critical downstream effector of Ras in the induction of VEGF under hypoxic conditions.

Although many of the downstream targets of PI 3-kinase
are still unknown, Akt (protein kinase B) has recently been identified as being a target of PI 3-kinase when cells are stimulated with PDGF BB. We examined the role of Akt in VEGF induction by examining the overexpression of catalytically inactive mutant Akt [HA-Akt(K179M)] on VEGF promoter activity. Overexpression of HA-Akt(K179M) inhibited the hypoxic induction of all three VEGF reporter genes transfected in NIH3T3R cells (Fig 6). These results strongly suggest that Akt is used in the hypoxia signaling pathway for VEGF induction in cells possessing an activated Ha-ras oncogene and supports our hypothesis that PI 3-kinase and Akt are downstream effectors of Ras in modulating the transcriptional regulators of VEGF by low-oxygen conditions.

**DISCUSSION**

Ras mutations are the most common oncogenic mutation found in over 40% of all human solid tumors. Previous studies have indicated that oncogenic forms of Ras increase VEGF expression and that it is further increased under a microenvironment low in oxygen. The purpose of this study was to identify the immediate downstream effectors of Ras that are used in inducing VEGF under hypoxic conditions and to determine whether they act through the hypoxic-responsive element (HIF-1 binding site) in the VEGF promoter. Although a recent study has implicated a role for PI 3-kinase in VEGF mRNA accumulation based on an inhibitor of PI 3-kinase, it is unknown whether this modulation occurs at the level of VEGF mRNA stability or induction, both of which can be modified by hypoxia. Using a genetic approach to modulate VEGF promoter activity, we conclude that PI 3-kinase is a downstream activator of Ras which mediates the rapid induction of VEGF through the HIF-1 regulatory region (Fig 7). The evidence supporting these conclusions is based on three observations. Concentrations of wortmannin as low as 1 nmol/L, which specifically inhibits PI 3-kinase activity, significantly block VEGF induction under hypoxic conditions. Overexpression of a dominant negative mutant of the p85 subunit of PI 3-kinase inhibits VEGF/HIF-1 induction under low-oxygen conditions and
Fig 5. Modulation of VEGF activity by the p110 and p85 subunits of PI 3-kinase in Ha-ras±transformed cells (NIH3T3R). (A) Stimulation of VEGF expression by cotransfection of increasing concentrations of the wt p110 subunit (0.1, 1, and 10 μg) of PI 3-kinase and 5 μg of wt HIF-1 expression vector. (B) Inhibition of VEGF expression under low-oxygen conditions by a mutant p85 subunit of PI 3-kinase (Δp85). NIH3T3R cells were cotransfected with the mutant p85 (2.5 μg) subunit of PI 3-kinase and a constant amount of reporter plasmid (5 μg; 385 bp; HIF-1 wt [×4]; HIF-1 mut [×5]; Fos-luc) and exposed to hypoxia (except Fos-luciferase) before assaying 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. Error bars represent 1 SD of the mean. (C) Thin-layer chromatogram of PI 3-kinase activity from lysates of NIH3T3 and NIH3T3R cells treated with hypoxia and PDGF BB.

overexpression of a wt p110 subunit induces VEGF/HIF-1 induction. Third, Ha-ras–transformed cells exposed to low oxygen exhibit a rapid increase in PI 3-kinase activity.

Based on our results, we propose that the p110 catalytic subunit of PI 3-kinase plays a pivotal role in transmitting the signal to activate VEGF by the combination of oncogenic Ha-ras and hypoxic stress. We have previously shown that Ha-ras–transformed cells possess increased basal levels of VEGF mRNA and a significant increase in VEGF promoter activity under hypoxic conditions. The induction of HIF-1 activity after coexpression of the wt of p110 and HIF-1 reporter construct (Fig 1B) is similar to that obtained under hypoxic conditions, suggesting that the activated p110 subunit is sufficient to mimic hypoxia induced transcriptional activity. According to previous studies, p110 acts as the catalytic subunit of PI 3-kinase. We propose that, in NIH3T3R cells, Ha-ras will directly interact with the p110 catalytic subunit of PI 3-kinase to stimulate a partial PI 3-
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kinase activity and increase the basal expression of VEGF. Hypoxia increases PI 3-kinase activity further most probably by altering the interaction of the p85 subunit with other phosphotyrosine-containing proteins. Studies to test this hypothesis are presently under way.

If PI 3-kinase lies downstream of Ras in signaling VEGF induction, what are its downstream effectors? At present, we can exclude p70S6k as one such effector because the macroline rapamycin that inhibits p70S6k by inducing its dephosphorylation, had little effect on VEGF induction. A second potential downstream effector of PI 3-kinase is the serine/threonine protein kinase Akt that is indirectly activated by PI 3-kinase through its lipid products. Activated Akt, and its effector PI 3-kinase, have been shown to block apoptosis by transducing a survival signal.26 Our studies show that Akt activity is important in the induction of VEGF by hypoxia as overexpression of catalytically inactive mutant Akt inhibits VEGF reporter gene activity in a concentration-dependent manner (Fig 6).

The involvement of PI 3-kinase in the angiogenic and apoptotic programs of Ha-ras±transformed cells has important clinical implications. If PI 3-kinase activity is able to act as transducer of a survival signal to inhibit apoptosis induced by serum withdrawal,57 then increasing the expression of angiogenic mitogens such as VEGF could also act as an additional survival signal to tumor cells exposed to a low-oxygen environment. Thus, PI 3-kinase may not only protect cells from adverse environmental conditions, it may also act to promote tumor evolution by stimulating the angiogenic switch in Ha-ras±transformed cells and allowing the expansion of apoptotically resistant tumor cells. Therefore, PI 3-kinase may represent a selective target for anticancer therapy in cells possessing oncogenic Ras mutations.

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