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MAPK and Akt Act Cooperatively but Independently on Hypoxia Inducible Factor-1α in *rasV12* Upregulation of VEGF

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Oncogenic *ras* upregulates the expression of VEGF through the activation of the transcriptional enhancer hypoxia inducible factor-1α (HIF-1α) by a still poorly understood mechanism. Here, we demonstrate that both the Raf/MEK/MAPK and the PI3 kinase/Akt signaling pathways potently and additively stimulate the expression from a hypoxia response element (HRE) within the 5′flanking region of the VEGF promoter. Interestingly, while MAPK appears to specifically upregulate the transactivation activity of HIF-1α through direct phosphorylation of its regulatory/inhibitory domain, GSK-3, a downstream target of Akt, directly phosphorylates the HIF-1α oxygen-dependent degradation domain. These results suggest a novel mechanism whereby two divergent signaling pathways emerging from Ras may cooperatively but independently regulate the activity of a HIF-1α, thereby promoting the expression of a potent angiogenic mediator.

Key Words: **hypoxia inducible factor-1; vascular endothelial growth factor; ras; MAPK; Akt kinase; neovascularization.**

Neovascularization and increased glycolysis in solid tumors are necessary adaptations to a hypoxic environment and are correlated with tumor invasion, metastasis, and lethality (1). While malignancy in tumors represents the phenotypic endpoint of successive genetic lesions that affect the function and regulation of many different oncogenes and tumor-suppressor genes, the prevalence of mutations in certain key oncogenes argues in favor of a multipotent role for these genes in tumorigenesis and tumor progression. Of note, mutations in the *ras* oncogene are found in 30% of all human malignancies, and are reportedly found in a disproportionately higher number of more aggressive cancers, including lung cancer (up to 48%), colorectal carcinoma (up to 80%), and pancreatic adenocarcinoma (up to 87%) (2). Oncogenic *ras* has been shown to be important in both the genesis and maintenance of solid tumors (3). Previous studies indicate that the angiogenic switch in Ras-transformed cells may be physiologically promoted by the tumor microenvironment through the induction of vascular endothelial growth factor (VEGF) by both tumor and the surrounding stromal cells (4, 5). VEGF, a potent angiogenic stimulator, is known to induce angiogenesis and permeabilization of blood vessels, and plays a central role in the regulation of vasculogenesis (1). Recent data suggest that hypoxic induction of VEGF expression is potentiated by activated oncogenes, including Ras, and by a variety of cytokines (6, 7). Deregulated VEGF expression may therefore contribute to the development and progression of Ras-induced solid tumors by promoting neovascularization.

Recent evidence has implicated the transcriptional enhancer, hypoxia-inducible factor-1 (HIF-1) in the upregulation of VEGEF transcription by activated Ras (8). HIF-1 consists of a heterodimer of two basic helix-loop-helix PAS proteins, HIF-1α and HIF-1β (or ARNT) (9). Under low oxygen tension, the activated HIF-1α/HIF-1β complex functions as a transcription

Abbreviations used: HIF, hypoxia inducible factor; HRE, hypoxia response element; HID, HIF-1α regulatory/inhibitory domain; ODD, oxygen-dependent degradation domain; VEGF, vascular endothelial growth factor; MAPK, mitogen-activated protein kinase; GSK-3, glycogen synthase kinase 3; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline.

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factor to control the expression of genes encoding products essential for cell metabolism and tissue growth including several glycolytic enzymes and angiogenic growth factors. Whereas HIF-1β is constitutively expressed, HIF-1α expression is induced in hypoxic cells (10). Furthermore, HIF-1α is extremely unstable ($t_{1/2} <$ 5 min) and is quickly degraded by the ubiquitin-proteasome system (11, 12). Regulation of HIF-1α expression and protein stability appears to involve direct interaction with the tumor suppressor protein, p53, and with the von Hippel-Lindau (VHL) protein (13–16). Regulation of HIF-1α additionally involves the hypoxic induction of HIF-1α nuclear translocation transactivation (17, 18).

Emerging evidence has also suggested a role for phosphorylation of HIF-1α in the regulation of its activity (19, 20). Nonetheless, the mechanism by which this regulation occurs is still under debate, and the contribution of the implicated signaling pathways to VEGF transcription remains poorly understood. Here, we provide evidence that Ras-induced VEGF expression is mediated by the concomitant activation of the Raf/MEK/MAPK and the PI3 kinase/Akt signaling pathways acting on a hypoxia response element (HRE) within the 5′flanking region of the VEGF promoter. These pathways stimulate enhanced activity of HIF-1 by complementary mechanisms. Whereas the MAPK pathway appears to specifically upregulate HIF-1α transactivation activity, the PI3 kinase/Akt pathway appears to regulate HIF-1α protein stability. These results suggest that oncogenic *ras*, through both the MAPK and Akt signaling pathways, may regulate the activity of a single transcription factor by two independent mechanisms acting on distinct regulatory domains, thereby promoting the expression of potent angiogenic mediators.

MATERIALS AND METHODS

Expression plasmids. The pGL-VEGF/K, pGL-VEGF/P, pGL-HRE, and pGL-HREmut reporter plasmids, and the pcDNAIII GAL4/HIF and pGEX GST-HID expression plasmids, were prepared as previously described (19). The HIF-1α oxygen-dependent degradation domain (ODD) (12) was subcloned into the pGEX 4T3 vector, as a *Bam*HI–*Not*I fragment, thus generating the pGEX GST-ODD expression plasmid. The pGL3.TATA GAL-driven luciferase reporter plasmid and expression vectors for activated forms of Ras(V12), Raf(CAAX), MEK(EE), PI3K(CAAX), and (myr)Akt have been previously described (21, 22). Ras partial loss of function mutants S35 and C40 have been previously described (23).

Cell lines and transfection. For transient transfections for reporter gene assays, NIH 3T3 cells and MDCK cells were transfected by using the calcium-phosphate precipitation technique. COS-7 cells were transfected by the DEAE-dextran method. In each experiment, total amount of DNA was adjusted to 3–5 μg/plate using pCEFL GFP.

ELISA. Conditioned media from either NIH 3T3 cells transiently transfected with DNA encoding wild-type Ras, activated Ras, Ras partial loss of function mutants, or Ras signaling pathway molecules was collected after 24 h. VEGF secretion was detected in the media using a VEGF immunoassay kit (R&D Laboratories) as indicated in the standard protocol provided by the manufacturer.

Reporter gene assays. NIH 3T3, COS-7, and MDCK cells were transfected with the indicated expression and reporter plasmids together with 0.5 μg of pcDNAIII-βgal, a plasmid expressing the enzyme β-galactosidase. After overnight incubation, cells were washed once with PBS, and kept for approximately 36 h in DMEM. Cells were then lysed using reporter lysis buffer (Promega). Luciferase activity and β-galactosidase activity present in cellular lysates were assayed as previously described (19). The data for luciferase activity, normalized by the β-galactosidase activity, is expressed as fold induction with respect to control cells, and are the mean ± SEM of triplicate samples from a typical experiment.

Kinase assays. The phosphorylating activity of epitope-tagged MAPK and Akt was assayed as previously described (21, 22), using as substrates either 5 μg of myelin basic protein (MBP) (Sigma) for MAPK, 5 μg of histone 2B (H2B) for Akt or 5 μg of purified, bacterially expressed GST-HID. Samples were analyzed by SDS–gel electrophoresis on 12% acrylamide gels, and autoradiography was performed with the aid of an intensifying screen. GST-fusion proteins were expressed in bacteria and purified by affinity chromatography using standard procedures.

Western blot. HA-Immunoprecipitates from transiently transfected COS-7 cells carrying HA-MAPK or HA-Akt DNAs were analyzed by Western blotting after SDS–polyacrylamide gels using an anti-HA monoclonal antibody as previously described (21).

Kinase inhibitors. The MEK inhibitor (PD98059) (Calbiochem, Inc.) and wortmannin were dissolved in DMSO as 1000-fold concentrated stock solutions, and used at the indicated concentrations. Cells were then treated with a single dose of kinase inhibitor for 8 h prior to lysis. In each case, the final concentration of DMSO was $<$0.1%.

RESULTS AND DISCUSSION

Effect of Oncogenic ras Signaling Pathways on VEGF Expression

Oncogenic *ras* upregulates VEGF secretion in expressing cells (4). To explore whether enhanced steady-state level of VEGF secretion results from an increase in VEGF transcription, we transfected MDCK cells with oncogenic RasV12 or its inactive mutant RasN17 along with pGL-VEGF/K (19), a reporter plasmid expressing luciferase under the control of the full-length VEGF promoter. As shown in Fig. 1A, the VEGF promoter was strongly induced by activated Ras. Furthermore, when plasmids for HIF-1 α and β were cotransfected into these cells, we observed a synergistic increase in the expression from the VEGF promoter (data not shown). This prompted us to further explore the role of this transcription factor in oncogenic *ras*-induced VEGF transcription. The stimulation by oncogenic *ras* was markedly reduced when we used a truncated VEGF reporter construct, pGL-VEGF/P (19), lacking the hypoxia responsive element (HRE), thus suggesting that the ability for oncogenic *ras* to stimulate expression from the VEGF promoter requires an intact HRE. We next asked whether Ras could activate transcription from an isolated HRE, using a reporter plasmid under the control of a single VEGF HRE con-

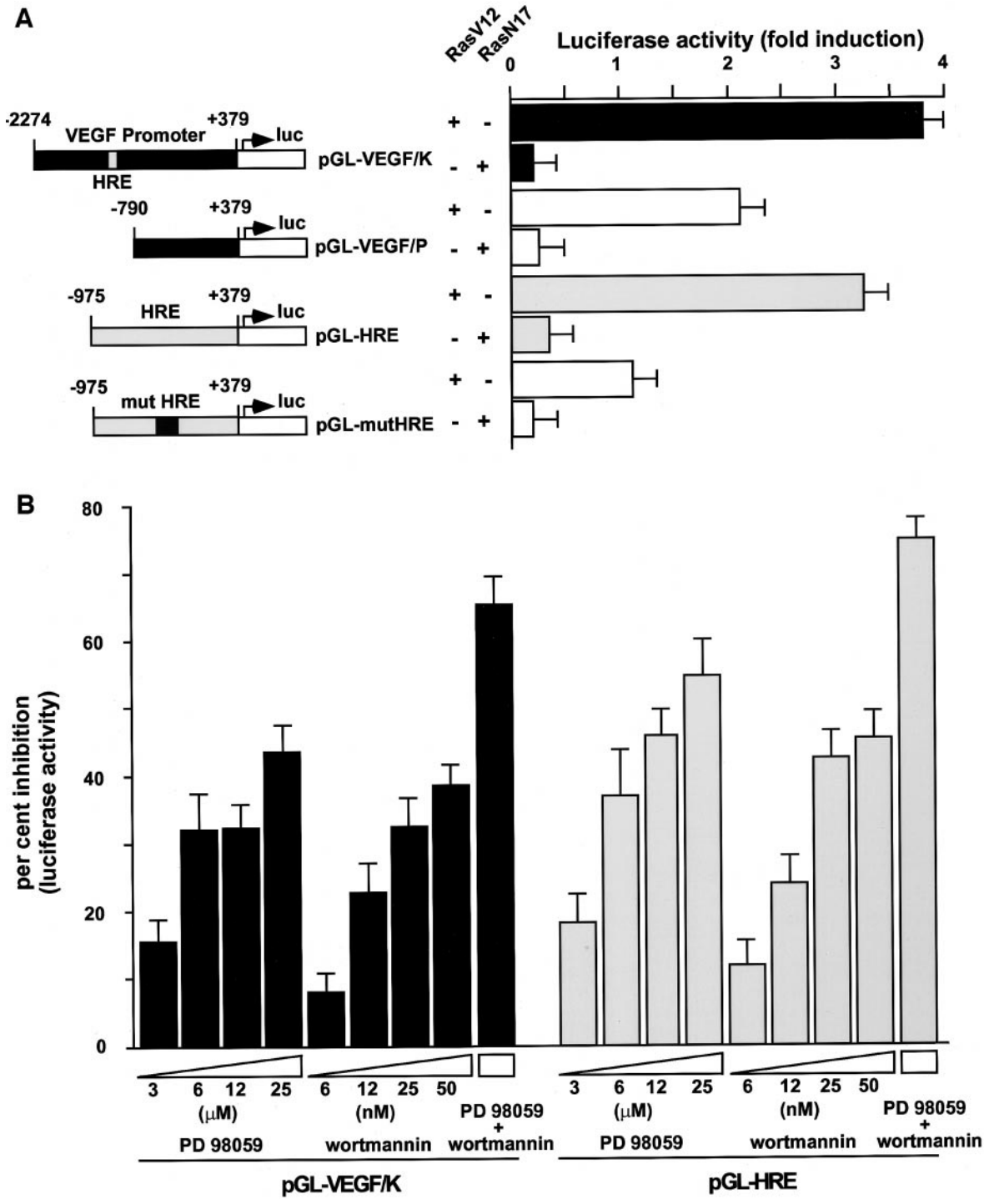


FIG. 1. Upregulation of VEGF transcription by both the Raf/MEK and PI3 kinase/Akt signaling pathways is dependent on an intact HRE. (A) The four reporter constructs, the full-length VEGF promoter (pGL-VEGF/K), a truncated VEGF promoter lacking the HRE (pGL-VEGF/P), a hypoxia responsive element (HRE) (pGL-HRE), and a mutated HRE, lacking the HIF-1-binding site (pGL-HREmut) are depicted. A histogram compares the induction of transcription from these reporter constructs by oncogenic RasV12 and its inactive mutant RasN17. Data represent the mean ± SEM of triplicate samples from a typical experiment, expressed as fold increase with respect to pCEFL GFP-transfected cells. (B) A histogram compares per cent inhibition of transcription from the pGL-VEGF/K and pGL-HRE reporter constructs by oncogenic *ras* in response to increasing concentrations of the MEK inhibitor (PD 98059) or the PI3 kinase inhibitor (wortmannin) with respect to cells treated with vehicle alone. Data represent the mean ± SEM of triplicate samples from a typical experiment.

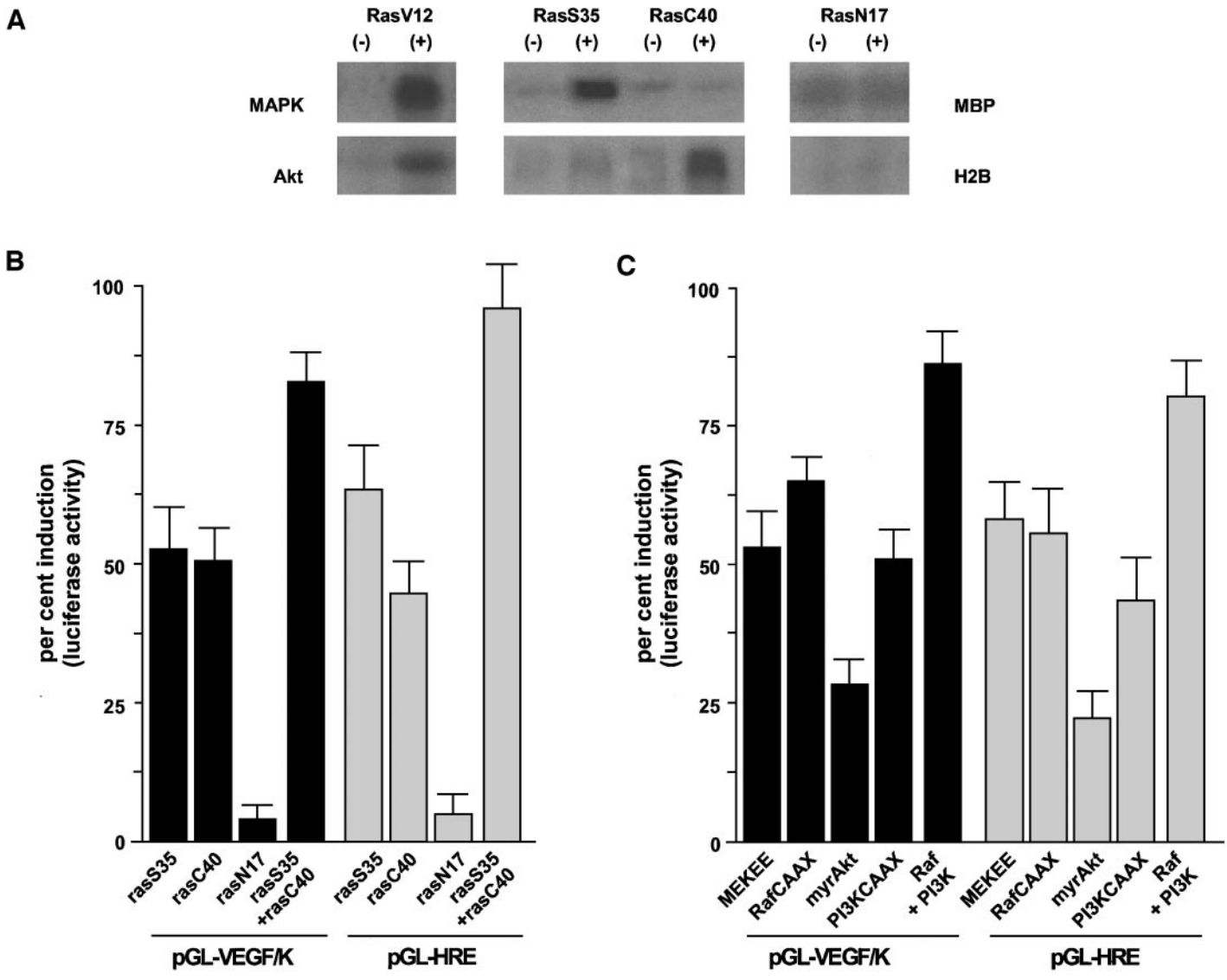


FIG. 2. Oncogenic *ras* stimulates VEGF transcription by the Raf/MEK and PI3 kinase/Akt signaling pathways independently. (A) Activation of MAPK or Akt by Ras mutants. COS-7 cells were transfected with HA-tagged MAPK or Akt for MAP kinase assays, together with pCEFL GFP vector (−), pCEFL oncogenic *ras* (V12), Ras partial loss of function mutants (S35 and C40), or inactive Ras (N17). Kinase reactions were performed in anti-HA immunoprecipitates from the corresponding cellular lysates using either MBP (MAPK) or H2B (Akt) as a substrate. Autoradiograms correspond to representative experiments. (B, C) A histogram compares per cent induction of transcription from the pGL-VEGF/K and pGL-HRE reporter constructs by (B) constitutively activated forms of Ras partial loss of function mutants (S35 and C40) and wild-type (inactive) Ras, or (C) constitutively activated forms of Raf, MEK, PI3 kinase, and Akt, with respect to oncogenic *ras*. Data represent the mean ± SEM of triplicate samples from a typical experiment.

sensus sequence, which was designated pGL-HRE (19). As shown in Fig. 1A, RasV12 strongly induced pGL-HRE, but failed to stimulate a control reporter plasmid, pGL-HREmut (19), which contains a mutation in the HIF-1-binding site, thus supporting the specificity of the transcriptional response.

To explore the role of the MAP kinase and PI3 kinase pathways in Ras-induced upregulation of VEGF transcription, we employed the use of specific chemical inhibitors. As shown in Fig. 1B, transcription from both the full-length VEGF promoter and from an intact HRE was significantly inhibited by increasing concentrations of either the MEK inhibitor (PD 98059) or the PI3 kinase inhibitor (wortmannin) with respect to cells treated with vehicle alone. Furthermore, when the two drugs are used in combination their effects were additive, nearly completely blocking oncogenic *ras*-induced transcription. To determine the relative contribution of each signaling pathway on oncogenic *ras* activation of VEGF transcription, we took advantage of the availability of Ras partial loss of function mutants. Mutations in the effector region of Ras, residues 32–40 lead to partial loss of function mutants in which interaction with some effectors is maintained but with others is

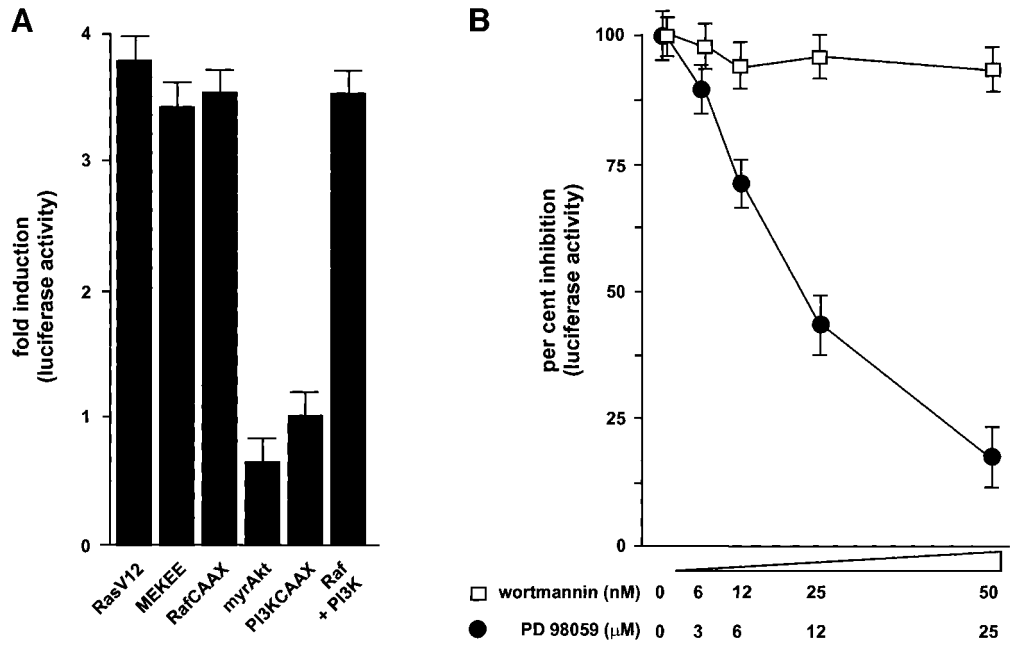


FIG. 3. Raf and MEK but not PI3 kinase nor Akt activate the transactivation domain of HIF-1α. (A) A histogram compares the fold induction of transcription from the pGL3.TATA GAL reporter plasmid by GAL4/HIF in response to constitutively active forms of Raf, MEK, PI3 kinase, or Akt with respect to oncogenic *ras*. Data represent the mean ± SEM of triplicate samples from a typical experiment, expressed as fold increase with respect to pCEFL GFP-transfected cells. (B) A graph shows per cent inhibition of transcription from the pGL3.TATA GAL reporter plasmid by GAL4/HIF in response to oncogenic *ras* by increasing concentrations of the MEK inhibitor (PD 98059) or the PI3 kinase inhibitor (wortmannin) with respect to cells treated with vehicle alone. Data represent the mean ± SEM of triplicate samples from a typical experiment.

lost (23). As shown in Fig. 2A, the Ras partial loss of function mutants S35 and C40 signal only through either the MAPK or PI3 kinase signaling pathways, respectively (23). Figure 2B demonstrates significant induction of both the full-length VEGF promoter and an intact HRE by both Ras partial loss of function mutants. Similarly, cells expressing activated forms of Raf, MEK, PI3 kinase, and Akt (21, 22) potently induced transcription from both promoters (Fig. 2C). Coexpression of either Ras S35 with C40 or active Raf with PI3 kinase-induced transcription from both promoters at levels similar to that of oncogenic *ras*. Taken together, these data indicate that oncogenic *ras* can activate transcription from an HRE within the 5′ flanking region of the VEGF promoter through both the Raf/MEK/MAPK and the PI3 kinase/Akt signaling pathways.

Effect of Oncogenic ras Signaling Pathways on the Transactivation Domain of HIF-1α

To begin exploring whether Ras-induced HIF-1-dependent transcription results in the activation of the HIF-1α transactivation domain, we constructed a chimeric GAL4-core HIF-1α fusion molecule, GAL4/HIF (19), containing both the N-terminal and C-terminal transactivation domains and the regulatory/inhibitory domain (amino acids 575–786), which inhibits the transcriptional activity of the adjacent transactivating regions under normoxic conditions (17, 18). This the fusion protein was stable under normoxic conditions and demonstrated minimal enhancement of nuclear localization upon addition of cobalt chloride or activation by oncogenic *ras* (results not shown), suggesting that activation of GAL4/HIF occurs solely by enhancing HIF-1α transcriptional activity. We expressed activated Ras together with GAL4/HIF and the pGL3-TATA GAL4 reporter plasmid. As shown in Fig. 3A, oncogenic *ras* potently induced the transcriptional activity of GAL4/HIF. We then expressed the activated mutants of Raf, MEK, PI3 kinase, or Akt together with GAL4/HIF and the pGL3-TATA GAL4 reporter plasmid. As shown in Fig. 3A, activated Raf and MEK potently induced the transcriptional activity of GAL4/HIF. In contrast, the activated mutants of PI3 kinase and Akt had little effect on GAL4/HIF. In line with these observations, Fig. 3B shows a clear dose-dependent inhibition of the activation of GAL4/HIF by Ras when treated with PD 98059, although wortmannin had no affect. Furthermore, when used in combination, the addition of wortmannin did not affect the inhibition seen with PD 98059 alone. Similarly, dominant negative mutants of both Raf and MEK demonstrated potent inhibition of Ras activa-

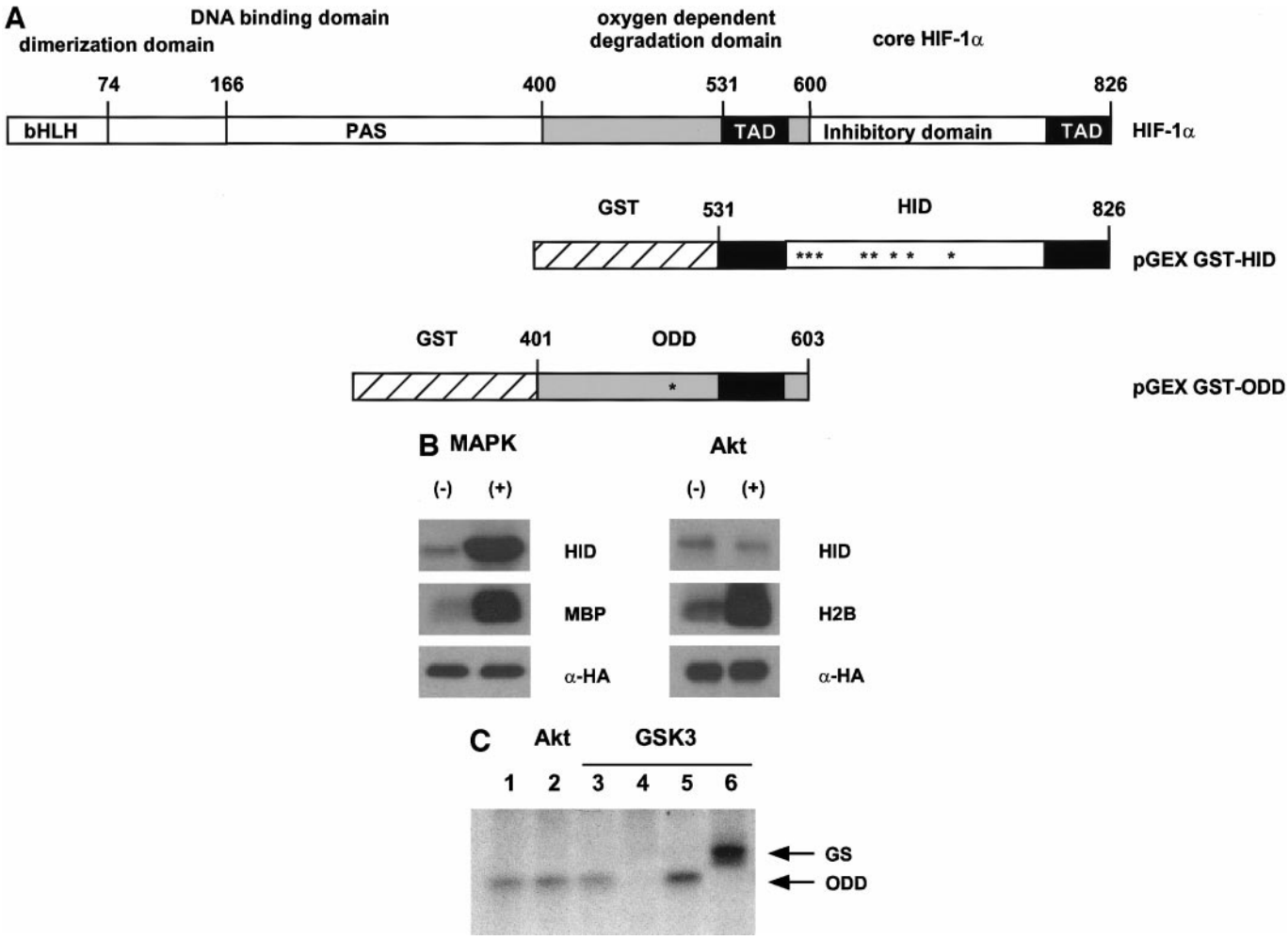


FIG. 4. MAPK and GSK3 phosphorylate HIF-1α at different functional domains. (A) Above, the structure of full-length HIF-1α, demonstrating the DNA-binding domain, the oxygen-dependent degradation domain, and the inhibitory/regulatory domain. Below, the pGEX-GST-HID construct highlighting the eight putative proline-directed serine phosphorylation sites (*) in the HIF-1α inhibitory/regulatory domain (HID), and the pGEX GST-ODD construct highlighting the single putative GSK3 phosphorylation site (*) in the oxygen-dependent degradation domain (ODD). (B) Phosphorylation of HIF-1α inhibitory/regulatory domain (HID) by MAPK or Akt. COS-7 cells were transfected with HA-tagged MAPK or Akt for MAP kinase assays, together with the pCEFL GFP vector (−) or pCEFL oncogenic *ras* (+). Kinase reactions were performed in anti-HA immunoprecipitates using the GST-HID fusion protein as a substrate. Phosphorylation of either MBP (MAPK) or H2B (Akt) were used as controls. Autoradiograms correspond to representative experiments. Anti-HA Western blots (WB) were performed in the corresponding cellular lysates. (C) Phosphorylation of HIF-1α oxygen-dependent degradation domain (ODD) by GSK-3. COS-7 cells were transfected with pCEFL GFP vector (lane 1), HA-tagged Akt together with pCEFL oncogenic *ras* (lane 2), or HA-tagged GSK-3 (lanes 2–6) for MAP kinase assays. Kinase reactions were performed in anti-HA (lanes 1, 2, and 4–6) or anti-AU5 (lane 3) immunoprecipitates from the corresponding cellular lysates using the GST-ODD fusion protein (lanes 1–3 and 5) or no substrate (lane 4). Phosphorylation of glycogen synthase (GS) by GSK-3 (lane 6) was used as a control. Autoradiograms correspond to representative experiments.

tion of GAL4/HIF (data not shown). Taken together, these data strongly suggest that Ras induces the expression of VEGF by stimulating the activity of the VEGF promoter through both the Raf/MEK/MAPK and the PI3 kinase/Akt signaling pathways. However, only the MAPK signaling pathway leads to activation of the transactivating domain of HIF-1α.

Phosphorylation of HIF-1α at Distinct Domains by MAPK and GSK-3

Kinase activation of transcriptional enhancers can occur through direct phosphorylation of the transcription factor. To examine whether HIF-1α can be a direct substrate for either MAPK or Akt, we constructed a fusion protein containing the N-terminal domain of glutathione *S* transferase (GST) and the HIF-1α regulatory/inhibitory domain (HID) (19), containing the eight proline-directed serine residues which may represent potential targets for members of the MAP kinase superfamily (Fig. 4A), and tested its ability to be phosphorylated *in vitro* by MAPK or Akt. As shown in Fig. 4B, activated MAPK was able to phosphorylate the GST/HID fusion protein but not the GST protein alone (results not shown). Activated Akt, however, was

unable to use the GST/HID as a substrate, although it did phosphorylate H2B when used as a control. These data strongly suggest that HIF-1α can be a direct target for phosphorylation by MAPK, thus providing a likely mechanism for the induction of HIF-1α-dependent transcription by the Raf/MEK/MAPK signaling pathway.

The PI3 kinase/Akt signaling pathway has previously been implicated in the stabilization of HIF-1α, presumably through the regulation of the HIF-1α oxygen-dependent degradation domain (ODD) (24). However, analysis of the HIF-1α ODD reveals that it does not contain an Akt phosphorylation consensus sequence, suggesting that the regulation of HIF-1α protein stability by the PI3 kinase/Akt signaling pathway likely involves a molecule downstream of Akt. Of note, GSK-3, a downstream target of Akt, has been shown to target proteins for degradation and Akt inhibits GSK-3 through direct phosphorylation of the kinase at serine 9 (25). Inspection of the HIF-1α ODD reveals that it contains a single GSK-3 consensus sequence (Fig. 4A). We therefore set out to determine if GSK-3 could use the HIF-1α ODD fused to GST as a substrate. As shown in Fig. 4C, GSK-3, but not Akt, is able to phosphorylate HIF-1α ODD, but neither kinase was able to use core HIF-1α as a substrate (results not shown). These results suggest a mechanism whereby the PI3 kinase/Akt signaling pathway may regulate HIF-1α protein stability through the inhibition of GSK-3 phosphorylation of the HIF-1α ODD.

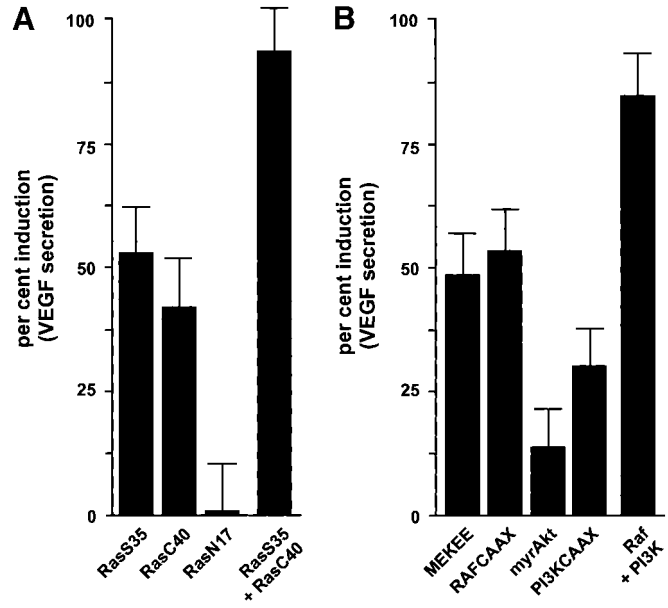


FIG. 5. Oncogenic *ras* stimulates VEGF secretion by the Raf/MEK and PI3 kinase/Akt signaling pathways independently. (A) A histogram compares per cent induction of constitutively activated forms of Ras partial loss of function mutants (S35 and C40)- and inactive Ras (N17)-induced VEGF secretion in NIH 3T3 cells with respect to oncogenic *ras* (V12). Data represent the mean ± SEM of triplicate samples from a typical experiment, expressed as per cent secretion with respect to control. (B) A histogram compares per cent induction of constitutively activated forms of MEK-, Raf-, Akt-, or PI3 kinase-induced VEGF secretion in NIH 3T3 cells with respect to oncogenic *ras*. Data represent the mean ± SEM of triplicate samples from a typical experiment, expressed as per cent secretion with respect to control.

Effect of Oncogenic ras Signaling Pathways on VEGF Secretion in Vivo

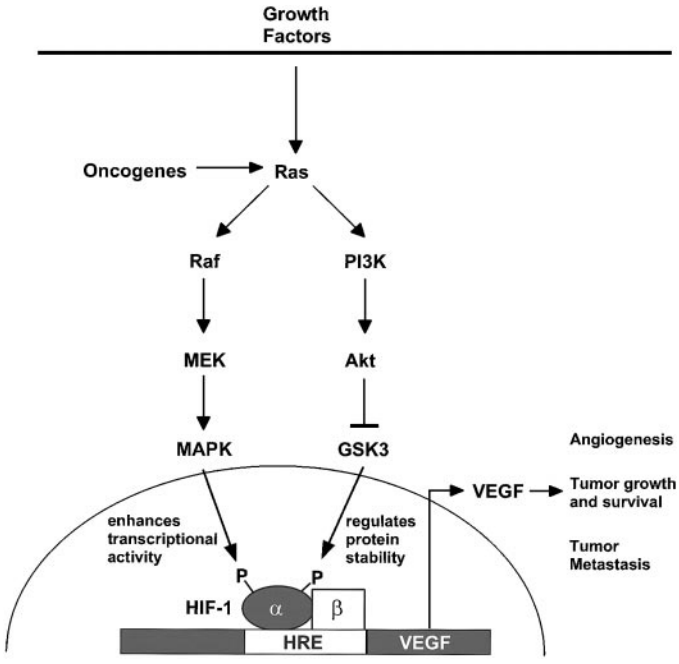
We next set explored whether induction of VEGF transcription through the Ras signaling pathways results in the upregulation of VEGF secretion, using the Ras partial loss of function mutants. Expression of RasV12 in NIH-3T3 cells upregulated VEGF secretion by 4- to 5-fold. As shown in Fig. 5A, although diminished, both the S35 and C40 mutants maintain significant stimulation of VEGF secretion in expressing cells. RasN17, which is inactive, was used as a negative control. To verify the independent role of the two Ras signaling pathways in upregulation of VEGF secretion, we transiently expressed constitutively activated forms of Raf, MEK, PI3 kinase, and Akt in NIH 3T3 cells (Fig. 5B). Cells expressing active Raf, MEK, and to a lesser extent, PI3 kinase and Akt, potently induced VEGF secretion. Coexpression of the S35 with C40 mutants or constitutively active Raf with PI3 kinase induced VEGF secretion at levels similar to that of oncogenic *ras*. Furthermore, as shown in Fig. 6, stimulation of VEGF secretion by NIH 3T3 cells transiently expressing oncogenic *ras* was significantly inhibited by increasing concentrations of both PD 98059 and wortmannin, and when the two drugs are used in combination, their effects were additive, nearly abolishing upregulation of VEGF secretion by oncogenic *ras*. These results verify the cooperative but independent contribution of the Raf/MEK/MAPK and PI3K/Akt signaling pathways in Ras induced upregulation of VEGF secretion.

ras Signaling Pathways Regulating VEGF Expression and Secretion

Ongoing research has implicated the transcriptional enhancer, hypoxia-inducible factor-1α (HIF-1α) in the upregulation of VEGF transcription by oncogenic *ras* (8). Although both the Raf/MEK/MAPK and the PI3 kinase/Akt signaling pathways have been implicated in Ras-induced VEGF secretion, reasonable disagreement exists as to the role(s) of the two pathways in HIF-1α activation and the contribution of each signaling pathway to Ras-induced VEGF transcription (19, 20, 24–27). Herein, we found that the *ras* oncogene can potently induce the expression of VEGF by stimulating transcription from a hypoxia response element (HRE)

within the VEGF promoter through both the Raf/MEK/MAPK and the PI3 kinase/Akt signaling pathways. Ras induction of HIF-1α transactivation specifically requires only the Raf/MEK/MAPK signaling pathway and may involve the direct phosphorylation of the regulatory/inhibitory domain of HIF-1α by MAPK. As shown above, Akt was unable to use this domain as a substrate *in vitro* and was similarly unable to upregulate the transactivation activity of HIF-1α *in vivo.* However, GSK-3 a downstream target of Akt, was able to use the HIF-1α oxygen-dependent degradation domain (ODD) as a substrate, suggesting that while MAPK phosphorylation of HIF-1α may upregulate the transcription factors's transactivation activity, Akt may regulate protein stabilization by inhibiting GSK-3-mediated targeting of HIF-1α for protein degradation. Both MAPK and, more recently, GSK-3, have been shown to be induced by hypoxia, suggesting a role for these two kinases in hypoxic regulation of HIF-1α (28).

These findings may have a broad impact on our understanding of the pathophysiological mechanisms involved in the acquisition of the angiogenic phenotype in human tumors expressing oncogenic *ras.* Our results suggest the *ras* oncogene may regulate the activity of HIF-1α by two distinct mechanisms, through both the MAPK and Akt signaling pathways. Inhibition of either pathway alone was insufficient to block Ras-induced transcription from the VEGF promoter. Similarly, activation of either signaling pathway was able to stimulate VEGF transcription independent of the other pathway. Indeed, as depicted in Scheme 1, this novel divergent mechanism, together with those previously described such as increased half-life of HIF-1α (12), and stabilization of VEGF mRNA (29), may ultimately control VEGF secretion and thus play a critical role in angiogenesis in neoplastic disease.



SCHEME 1. Proposed mechanism whereby the oncogenic *ras* stimulates HIF-1α-mediated VEGF transcription through two independent but cooperative mechanisms. Ras activates downstream kinases through two distinct pathways (Raf/MEK/MAPK and PI3 kinase/Akt) thereby causing the phosphorylation and activation of both MAPK and Akt. MAPK phosphorylates the HIF-1α regulatory/inhibitory domain, thus activating the HIF-1α transactivation domain. Conversely Akt, through the phosphorylation and inhibition of GSK-3, may stabilize the HIF-1α oxygen-dependent degradation domain.

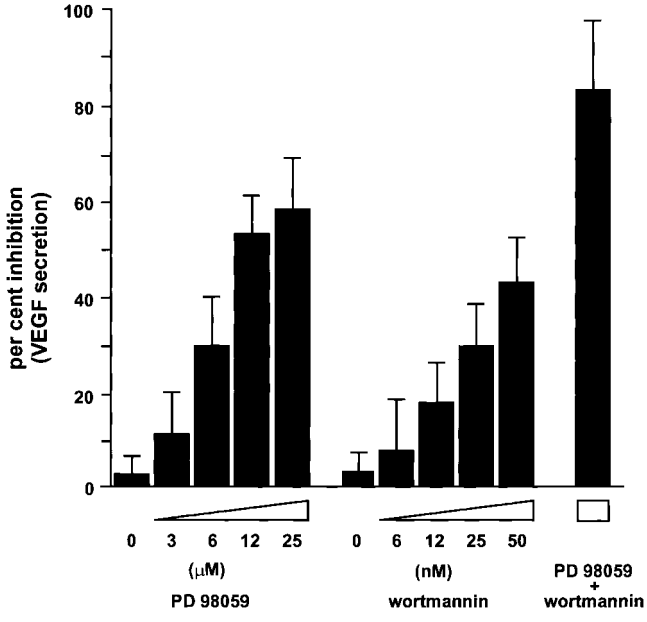


FIG. 6. Oncogenic *ras* upregulation of VEGF secretion is inhibited by the MEK inhibitor, PD 98059 and the PI3 kinase inhibitor, wortmannin. A histogram shows per cent inhibition of oncogenic *ras*-induced VEGF secretion in NIH 3T3 cells in response to increasing concentrations of the MEK inhibitor (PD 98059) or the PI3 kinase inhibitor (wortmannin) with respect to cells treated with vehicle alone. Data represent the mean ± SEM of triplicate samples from a typical experiment.

Interfering with and preventing tumor angiogenesis is an attractive therapeutic approach for treating cancer and thus the application of angiogenesis inhibitors is currently under intense investigation. Our findings suggest that the ability of oncogenic *ras* to induce VEGF secretion may be amenable of pharmacological intervention by inhibiting divergent signaling pathways and their downstream targets. They also imply that inhibition of Ras-induced angiogenesis may require combination therapy to ensure comprehensive suppression of proangiogenic signaling pathways.

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