V-SRC Induces Expression of Hypoxia-inducible Factor 1 (HIF-1) and Transcription of Genes Encoding Vascular Endothelial Growth Factor and Enolase 1: Involvement of HIF-1 in Tumor Progression

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ABSTRACT

Adaptation to hypoxia represents an important aspect of tumor progression. Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that mediates essential homeostatic responses to cellular and systemic hypoxia by activating transcription of multiple genes including those encoding glycolytic enzymes and vascular endothelial growth factor (VEGF). In this report, we demonstrate that whereas C-SRC expression is not required for expression of HIF-1 or transcriptional activation of genes encoding VEGF and enolase 1 (ENO1), cells expressing the v-Src oncogene have increased expression of HIF-1, VEGF, and ENO1 under both hypoxic and nonhypoxic conditions. Expression of V-SRC was associated with increased transcription of reporter genes containing cis-acting hypoxia-response elements from the VEGF and ENO1 genes, and this transcriptional activation required an intact HIF-1 binding site. When three rat hepatoma subclones that differed with respect to the level of HIF-1 expression were injected into nude mice, tumor growth correlated with HIF-1 expression, suggesting that HIF-1 may be generally involved in tumor progression. These studies link an oncogene to the induction of HIF-1 expression, thus providing a mechanism for hypoxic adaptation by tumor cells.

INTRODUCTION

Recent studies support the hypothesis that tissue hypoxia acts at several different physiological levels as a selective agent during tumorogenesis. Tumors must establish an adequate blood supply to grow beyond a volume of several mm³ (reviewed in Ref. 1). The degree of tumor angiogenesis is inversely related to patient survival (reviewed in Ref. 2). VEGF is a protein that is essential for angiogenesis (3, 4). Inhibition of VEGF expression in tumor xenografts resulted in vascular regression and decreased growth (5–10). A major stimulus for VEGF expression, under normal physiological conditions and in tumors, is tissue hypoxia (11–14). In tumor xenografts, mean P<sub>O2</sub> declined from 14 mm Hg (2% O<sub>2</sub>) in cells adjacent to blood vessels to 0–1 mm Hg in cells located 200–400 μm from the nearest capillary (15). However, even some cells closest to blood vessels were found to be hypoxic, indicative of the compromised microcirculation that is characteristic of tumors (16).

Cells subjected to mean P<sub>O2</sub> values of <10 mm Hg undergo metabolic adaptation to survive, such as the transition from oxidative phosphorylation to glycolysis, which is associated with increased levels of glycolytic enzyme mRNA and protein (17, 18). Glucose uptake and expression of GLUT1 is also increased in cancer cells (19). Whereas tumor cells are classically considered to be highly glycolytic even under aerobic conditions [the Warburg effect (20)], these cells can further increase the expression of glycolytic genes under hypoxic conditions (21, 22). Adaptation to low P<sub>O2</sub> is an important step in tumor progression and metastasis. Cervical cancers with P<sub>O2</sub> <10 mm Hg exhibited larger tumor extensions and more frequent parametral, lymphatic, or vascular involvement relative to tumors with P<sub>O2</sub> >10 mm Hg, and patients with well-oxygenated tumors had higher disease-free and overall survival rates (23). The probability of metastasis was 2-fold greater for soft tissue sarcomas with P<sub>O2</sub> <10 mm Hg compared to tumors with P<sub>O2</sub> >10 mm Hg (24). Genetic changes resulting in an increased rate of glycolysis in tumor cells may represent an essential adaptation to hypoxia. Lactate concentrations >20 μmol/g were 6.5-fold more common in cervical cancers with metastatic spread compared to nonmetastatic tumors (25).

Increased expression of VEGF and glycolytic enzymes in hypoxic cells is mediated in part by increased transcription (26–28). Transcriptional activation of genes encoding VEGF, GLUT1, and the glycolytic enzymes aldolase A, ENO1, lactate dehydrogenase A, phosphofructokinase L, phosphoglycerate kinase 1, and pyruvate kinase M is mediated by binding of HIF-1 to cis-acting HREs located primarily within 5'-flanking regions of these genes (21, 22, 29–35). HIF-1 is a heterodimeric basic-helix-loop-helix transcription factor (36) consisting of HIF-1α, which is unique to HIF-1, and HIF-1β, which is identical to the aryl hydrocarbon receptor nuclear translocator and can heterodimerize with the aryl hydrocarbon receptor in cells exposed to xenobiotic agents (37). HIF-1α protein levels, which determine the level of HIF-1 DNA-binding and transcriptional activity, increase exponentially as cellular O<sub>2</sub> concentration is reduced (21, 38). HIF-1 mRNAs are ubiquitously expressed in mammalian tissues, and HIF-1 DNA-binding activity is induced by hypoxia in all transformed and primary cell lines tested (39–41). Under hypoxic conditions, both HIF-1α protein levels and activity of the HIF-1α transcriptional domains increase (33, 38, 42–44). The therapeutic utility of this transcriptional regulatory system for targeting gene expression to hypoxic tumor cells has been demonstrated (45).

The essential role of HIF-1 in O<sub>2</sub> homeostasis suggests that genetic changes in tumor cells leading to increased HIF-1 activity may be selected for during the process of adaptation to hypoxia. The report that VEGF expression was correlated with expression of the mammalian proto-oncogene C-SRC or V-SRC, the transforming oncogene of RSV (46), suggested that SRC proteins may affect VEGF expression via HIF-1. Expression of V-SRC was associated with increased transcription of a reporter gene containing a 2.6-kb human VEGF promoter fragment (47). We, therefore, examined the effect of C-SRC and V-SRC expression on HIF-1 activity and on transcription of the HIF-1-regulated VEGF and ENO1 genes. The results of these studies have implications for models of hypoxia signal-transduction in normal...
cells and the potential role of HIF-1 in hypoxic adaptation by tumor cells.

MATERIALS AND METHODS

Nuclear Extract Preparation and EMSA. Mouse embryo fibroblast lines 8sp74src(+/+) and 1sp74src(−/−) (provided by P. Soriano, Fred Hutchinson Cancer Research Center, Seattle, WA) and rat fibroblast lines Ratl and v-Src/Ratl (provided by S. Meloche, Hotel-Dieu de Montreal, Montreal, Quebec, Canada) were cultured in DMEM supplemented with 10% FCS (Life Technologies, Inc.). Confluent cells were exposed to 20 or 1% O2 for 4 h, nuclear extracts were prepared, and EMSA was performed using oligonucleotide probe W18 (48, 49).

Immunoblot Analysis. Confluent mouse embryo fibroblasts were exposed to 20 or 1% O2 for 4 h and lysed in RIPA buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, and 30 mM sodium phosphate] supplemented with 2 mM DTT, 0.4 mM phenylmethylsulfonyl fluoride, and 1 mM Na3VO4. Lysates were fractionated by electrophoresis through a 7% acrylamide-0.23% bisacrylamide gel and transferred to nitrocellulose membranes. Proteins were detected with 1:500 (v/v) dilution of monoclonal antibody to C-SRC protein (provided by S. Meloche) followed by incubation with a 1:2000 dilution of peroxidase-labeled goat anti-mouse IgG (Amersham Corp.). Nuclear extracts prepared from Ratl and v-Src/Ratl cells were detected with anti-HIF-1α and anti-HIF-1β antibodies (38).

Northern Blot Analysis. Cultured cells were exposed to 20 or 1% O2 for 16 h, and total RNA was isolated (50). Fifteen-μg aliquots of RNA were fractionated, transferred to nylon membranes, and hybridized with 32P-labeled VEGF, ENO1, or HIF-1α cDNAs (32). Blots werestripped and re-probed with a 32P end-labeled oligonucleotide complementary to 18S rRNA. Autoradiographic signals were quantitated by scanning laser densitometry (Molecular Dynamics, Sunnyvale, CA). The densitometric values of VEGF, ENO1, and HIF-1α mRNA were normalized to the value of 18S rRNA to control for variation in sample loading and transfer.

Transient Transfection Assays. Ratl cells, v-Src/Ratl1 cells, and NIH3T3 cells were maintained in DMEM supplemented with 10% FCS. Ratl and v-Src/Ratl1 cells were transfected by calcium phosphate coprecipitation (51) with 15 μg of pSVβgal and 15 μg of pl1w, pl1m, p2.1, or p2.4 (21, 32). NIH 3T3 cells (4.5 × 106) were transfected by electroporation in 300 μl of DMEM supplemented with 7.5 μg/ml DEAE-dextran and 10 μg of pSVβgal, 10 μg of pl1w, pl1m, p2.1 or p2.4, and 15 μg of v-Src expression plasmid, tsUP1 (52), or parental plasmid, pLCN1 (provided by S. Meloche). Twenty-four hour after transfection, cells were exposed to 20 or 1% O2 for 24 h. Cell extracts were analyzed for βgal and luciferase activity as described (32). A GAL4 fusion-protein expression plasmid, pG.HIF1A, was constructed by PCR amplification of cDNA sequences encoding HIF-1α amino acids 531 to 826 and cloning into pGALO (provided by C. V. Dang, Johns Hopkins University) to allow synthesis of an in-frame fusion protein with the GAL4 (1-147) DNA-binding domain (53). The reporter plasmid, GALAE1βLuc, containing five GAL4 binding sites upstream of a minimal adenovirus E1b gene TATA sequence and the luciferase gene (54), was provided by R. A. Maurer (Oregon Health Sciences University). Ratl and v-Src/Ratl1 cells were transfected with 3 μg of pSVβgal, 5 μg of pG.HIF1A, and 6 μg of GALAE1βLuc by calcium phosphate coprecipitation (51) and exposed to 20 and 1% O2.

Immunocytochemistry. Ratl cells grown overnight on coverslips were cultured for 6 h at 20 or 1% O2, washed in PBS, fixed in 3% formaldehyde in PBS for 20 min at room temperature, washed, and incubated with 1% Triton X-100 in PBS for 5 min at room temperature. Cells were washed in PBS and incubated overnight in 3% BSA and 5% goat serum (Sigma) in PBS at 4°C, followed by incubation with a 1:30 (v/v) dilution of anti-HIF-1α antibodies for 1 h at room temperature, and washed in PBS. For immunofluorescence, the cells were then incubated for 2 h in a 1:100 (v/v) dilution of FITC-labeled goat anti-rabbit IgG (Sigma) in 3% BSA and 5% goat serum in PBS. 3,3′-Diaminobenzidine staining was performed using the Vectastain Elite kit (Vector Laboratories).

Tumor Xenografts. Mouse hepatoma Hepa1, C4, and RB13 cells (provided by O. Hankinson, University of California at Los Angeles, Los Angeles, California) were harvested with trypsin-EDTA. Viability of cells as determined by trypan blue exclusion was >95%. Cells were washed in serum-free DMEM and resuspended at a density of 2 × 107 cell/ml in serum-free DMEM. Athymic nude mice (NIH strain from Frederick Cancer Research Center, Frederick, MD) were housed in micro-isolator cages in a pathogen-free facility under established institutional guidelines. Mice (6–8 weeks old) were injected s.c. in the right dorsal region with 0.25 or 0.50 ml of cells. For each experiment, five mice per cell line were injected. Tumor volume was determined by caliper measurements of tumors in three dimensions as soon as tumors became visible. Mice that developed necrotic tumors were euthanized. For statistical comparisons, the unpaired t test was used to calculate significance value (P) from the mean and SDs of each data set.

RESULTS

Effect of C-SRC on HIF-1 Activity and Expression of Downstream Genes. We first determined whether induction of HIF-1 in hypoxic cells was affected by the presence or absence of C-SRC expression. Wild-type C-SRC-expressing mouse embryo fibroblasts and C-SRC-deficient cells obtained from mice homozygous for a null allele at the c-src locus (55) were exposed to 20 or 1% O2 for 4 h prior to isolation of total cellular or nuclear protein for analysis of C-SRC and HIF-1, respectively (Fig. 1). Immunoblot assays revealed the absence of C-SRC in mutant cells and the presence of C-SRC in wild-type cells. In contrast, EMSA revealed no significant difference in the induction of HIF-1 DNA-binding activity by hypoxia in the two cell lines, which both demonstrated induction of HIF-1 DNA-binding activity under hypoxic conditions. It was, therefore, important to confirm the previously reported observation that C-SRC-deficient cells manifested decreased induction of VEGF mRNA under hypoxic conditions (46). However, in four independent experiments, C-Src genotype had no significant effect on VEGF mRNA expression under nonhypoxic or hypoxic conditions (Fig. 2). ENO1 mRNA expression under nonhypoxic and hypoxic conditions was also unaffected by loss of C-SRC expression. While this work was in progress, another group reported that C-SRC deficiency had no effect on VEGF mRNA expression or HIF-1 DNA-binding activity and demonstrated that
V-SRC induces transcriptional activation by HIF-1

C-SRC:  
Hypoxia:  

VEGF  

18S rRNA  

ENO1  

overexpression of wild-type or dominant-negative forms of C-SRC had no effect on VEGF mRNA expression (56).

Effect of V-SRC on HIF-1 Expression. We next analyzed HIF-1 activity levels in Rat1 fibroblasts and V-SRC-transformed Rat1 cells (57). Cells were exposed to 20 or 1% O₂ for 4 h, and nuclear extracts were prepared for analysis of HIF-1 DNA-binding activity and immunoblot assays of HIF-1α and HIF-1β protein levels. Little HIF-1 DNA-binding activity or HIF-1α protein was detected in nonhypoxic Rat1 cells, whereas both were markedly induced by hypoxia (Fig. 3A, Lanes 1 and 2). Immunocytochemistry demonstrated nuclear localization of HIF-1α protein in hypoxic cells (Fig. 4). Compared to Rat1 cells, levels of HIF-1α protein and HIF-1 DNA-binding activity were markedly increased in nuclear extracts of V-SRC-expressing cells under both nonhypoxic and hypoxic conditions (Fig. 3A, Lanes 3 and 4). HIF-1α mRNA was transiently induced by hypoxia in Rat1 cells (Fig. 3B, Lanes 1–3), as described previously for Hep3B cells (36). In v-Src/Rat1 cells, HIF-1α mRNA levels were increased under both nonhypoxic and hypoxic conditions (Fig. 3B, Lanes 4–6). Thus, in contrast to C-SRC, expression of the V-SRC oncoprotein had a dramatic stimulatory effect on the expression of HIF-1α mRNA, HIF-1α protein, and HIF-1 DNA-binding activity that was independent of cellular pO₂.

Effect of V-SRC on VEGF and ENO1 mRNA Expression. To determine whether V-SRC affected expression of HIF-1-regulated genes, we exposed Rat1 and v-Src/Rat1 cells to 1% O₂ for 0, 2, or 16 h prior to isolation of total RNA. Consistent with the induction of HIF-1 activity at both 20 and 1% O₂, V-SRC expression was associated with increased VEGF and ENO1 mRNA levels at all three time points (Fig. 5). Quantitation of mRNA levels normalized to 18S rRNA levels revealed that the stimulatory effect of V-SRC (under nonhypoxic or hypoxic conditions) was 1.7- to 2.5-fold for ENO1 and 4.6- to 4.8-fold for VEGF mRNA.

Effect of V-SRC on Transcriptional Activation Mediated by HIF-1. VEGF mRNA expression is regulated at both the transcriptional and posttranscriptional levels (26, 27, 58, 59). It was thus important to determine whether the effect of V-SRC on VEGF mRNA expression involved transcriptional activation and, if so, whether this effect was mediated by HIF-1. We performed transient transfection assays using the reporter plasmids p11w, in which a 47-bp HRE from the human VEGF gene 5'-flanking region was cloned 5' to an SV40 promoter-luciferase transcription unit, and p11m, which was identical to p11w except for a 3-bp mutation within the HIF-1 binding site of the HRE (32). Rat1 cells were cotransfected with p11w or p11m and pSVβgal, exposed to 1 or 20% O₂ for 24 h, and then harvested. The
V-SRC INDUCES TRANSCRIPTIONAL ACTIVATION BY HIF-1

Fig. 4. Nuclear localization of HIF-1α protein. Rat1 cells were exposed to 20% (A and C) or 1% (B and D) O₂ for 6 h and analyzed by immunocytochemistry using affinity-purified antibodies against HIF-1α, which were visualized by 3,3'-diaminobenzidine staining (A and B) or immunofluorescence (C and D).

luciferase:βgal ratio was determined and normalized to the value obtained for cells transfected with p11m and exposed to 20% O₂. Whereas expression of p11w was induced 3-fold in hypoxic cells, there was no significant increase in the expression of p11m in hypoxic compared to nonhypoxic Rat1 cells (Fig. 6A). Compared to Rat1 cells, expression of p11w in v-Src/Rat1 cells was increased 3- to 4-fold at 20 and 1% O₂, such that expression of p11w in hypoxic v-Src/Rat1 cells was 9-fold higher than in nonhypoxic Rat1 cells. In contrast, V-SRC had no effect on the expression of p11m. Thus, V-SRC stimulation of reporter gene transcription is dependent upon an intact HIF-1 binding site.

To further explore the effect of V-SRC on HIF-1-mediated transcription, we cotransfected Rat1 cells with pSVβgal and the reporter plasmid p2.1, which contained a 68-bp HRE from the ENO1 gene 5’-flanking region cloned 5’ to the SV40 promoter-luciferase transcription unit, or p2.4, which was identical to p2.1 except for 3-bp mutations in two HIF-1 binding sites within the HRE (21). Expression of p2.1, but not p2.4, was induced 2-fold by exposure of Rat1 cells to 1% O₂ (Fig. 6B). Compared to Rat1 cells, expression of p2.1 in v-Src/Rat1 cells was increased approximately 2-fold at 20 and 1% O₂, such that expression of p2.1 in hypoxic v-Src/Rat1 cells was over 4-fold higher than in nonhypoxic Rat1 cells. Because the sequences of the VEGF and ENO1 HREs share only HIF-1 binding sites in common, this result and other evidence show that V-SRC increases the expression of VEGF and ENO1 mRNA at the transcriptional level by increasing HIF-1 activity in both hypoxic and nonhypoxic cells.

To demonstrate that the differences in reporter gene expression in v-Src/Rat1 and Rat1 cells were in fact due to expression of V-SRC rather than some other genetic difference between these two lines, we transiently cotransfected NIH 3T3 cells with pSVβgal, reporter p11w or p2.1, and an expression vector containing either no insert or V-SRC coding sequences. Compared to reporter transcription in cells transfected with empty vector, in cells expressing V-SRC, transcription of p11w and p2.1 was increased 2- and 3-fold, respectively, both at 20 and 1% O₂ (Fig. 6C), whereas expression of V-SRC had no effect on transcription of p11m or p2.4 (data not shown). Fig. 6, A–C, demonstrate that in two different transformed cell lines, V-SRC expression results in transcriptional activation that is dependent upon the presence of HIF-1 binding sites.

Hypoxia increases both HIF-1α protein levels and the specific transcriptional activity of HIF-1α (43, 44). To determine whether V-SRC expression affects transactivation domain function as well as HIF-1α protein expression, Rat1 and v-Src/Rat1 cells were cotransfected with: pSVβgal; reporter pGAL4E1bLUC, which contains five GAL4 binding sites upstream of the adenovirus E1b minimal promoter and luciferase coding sequences; and expression vectors encoding either the GAL4 DNA-binding domain (pG.0) or the GAL4 DNA-binding domain fused to the HIF-1α transactivation domain (pG.HIF1A) (44). Transfected cells were exposed to 20 or 1% O₂ for 24 h, and the luciferase:βgal ratios were normalized to the value obtained for pG.0 in cells at 20% O₂. Compared to Rat1 cells transfected with pG.0, in cells transfected with pG.HIF1A, reporter expression was 21- and 31-fold higher at 20 and 1% O₂, respectively (Fig. 6D). The effect of pG.HIF1A on reporter expression in Rat1 and v-Src/Rat1 cells was not significantly different. These results suggest...
Mechanisms of Hypoxia Signal Transduction and HIF-1α Induction. We demonstrated previously that the protein tyrosine kinase inhibitor genistein completely blocked the induction of HIF-1α protein and HIF-1 DNA-binding activity in hypoxic Hep3B cells (60). Genistein also inhibited hypoxic induction of VEGF mRNA expression in U87 cells (46). C-SRC does not appear to be the target of genistein based on the results presented here (Figs. 1 and 2) and elsewhere (56). The basis for decreased induction of VEGF mRNA in C-SRC-deficient cells reported previously (46) is unclear but may have been an artifact of the culture conditions used. In contrast, V-SRC expression resulted in a marked increase in HIF-1, ENO1, and VEGF expression, indicating that V-SRC activates a signal transduction pathway leading to induction of HIF-1α expression, which determines HIF-1 DNA-binding activity (21, 42, 43). These results suggest that V-SRC may activate the downstream target(s) of a tyrosine kinase that is normally active only under hypoxic conditions. Furthermore, the positive effect of V-SRC on HIF-1 expression suggests that hypoxia signal transduction begins at the cell membrane.

Previous studies have demonstrated that hypoxia stimulates both HIF-1α protein expression and HIF-1α transactivation domain function (33, 42–44). All known chemical inducers of HIF-1α protein expression (1% O₂, cobalt chloride, and desferrioxamine) also induced HIF-1α transactivation domain function, suggesting that these two processes occur via the same signal transduction pathway (44). The present data indicate that V-SRC activates a pathway that leads to induction of HIF-1α mRNA and protein expression but not HIF-1α transactivation domain function. V-SRC expression will thus provide a useful tool for examining the effects of increased HIF-1α protein expression under nonhypoxic conditions.

V-SRC, HIF-1, and Hypoxic Adaptation by Tumor Cells. The present study provides the first evidence that expression of an oncogene is associated with induction of HIF-1α protein and HIF-1 DNA-binding activity under nonhypoxic conditions and superinduction under hypoxic conditions. This increased HIF-1 activity is reflected in the augmented expression of downstream target genes affecting angiogenesis and cellular energy metabolism, two processes that are subject to significant alteration during tumor progression. Experiments performed over 20 years ago demonstrated that RSV infection of normal chick or rat cells resulted in an increased rate of aerobic glycolysis, which was temporally and genetically correlated with V-SRC-mediated transformation (61–63). Our present results suggest a molecular basis for these observations. In contrast to the parental Rat1 line, v-Src/Rat1 cells are capable of anchorage-independent growth in soft agar (57). Cells in the center of an expanding soft agar colony are likely to encounter hypoxic conditions that require increased glycolytic activity for continued cellular proliferation and survival (64). Taken together, the results of previous studies of RSV-infected cells and the data presented in this report suggest that in addition to affecting cellular proliferation, V-SRC activates signal transduction pathways that lead to the expression of HIF-1 and downstream genes, which provide a molecular mechanism for adaptation of tumor cells to a hypoxic microenvironment.

V-SRC may not be unique among oncogene products with respect to its effects on HIF-1. Expression of activated H-RAS has also been studied in various cell types, and its effects on HIF-1 expression and hypoxic adaptation have been reported. For example, the transfection of H-RAS into C4 cells resulted in the induction of HIF-1α expression (46). These findings suggest that V-SRC may not be unique among oncogene products with respect to its effects on HIF-1.

V-SRC: -  
Hypoxia (h): 0 2 16 0 2 16

VEGF →

18S rRNA →

ENO1 →

Fig. 5. Expression of VEGF and ENO1 mRNA in V-SRC expressing and nonexpressing cells. Rat1 (Lanes 1–3) and v-Src/Rat1 (Lanes 4–6) cells were exposed to 1% O₂ for 0, 2, or 16 h prior to isolation of total cellular RNA. Fifteen-μg aliquots were analyzed by blot hybridization using probes for VEGF mRNA (top), 18S rRNA (middle), or ENO1 mRNA (bottom).

that V-SRC selectively increases HIF-1α protein expression without affecting transactivation domain function.

Effect of HIF-1 Expression on Tumorigenicity of Rat Hepatoma Cells. To obtain evidence for more general involvement of HIF-1 in tumorigenesis, we examined the in vivo growth characteristics of Hepa1 rat hepatoma cells that were either wild-type or completely deficient (clone C4) in their expression of HIF-1 due to a mutation affecting the expression of the HIF-1β (ARNT) subunit (37). We have demonstrated previously that, compared to wild-type Hepa1 cells, HIF-1 DNA-binding activity is not detectable, and VEGF mRNA levels are dramatically reduced in C4 cells incubated in 1% O₂ (32). Athymic mice injected with wild-type Hepa1 cells formed large, rapidly growing tumors that necessitated euthanasia by 4 weeks after injection (Fig. 7A). Mice injected with C4 cells formed tumors that grew at a significantly reduced rate. To demonstrate that this difference in tumor growth was reproducible and was related to the expression of HIF-1, the experiment was repeated with wild-type Hepa1 cells, HIF-1-deficient C4 cells, and RB13 cells that arose as a spontaneous revertant of C4 (37). HIF-1 and VEGF expression was induced by hypoxia in RB13 cells but to a lesser extent than in wild-type cells (32). A significant difference in tumor growth rate was again observed in mice injected with wild-type Hepa1 as compared to C4 cells (Fig. 7B). In mice injected with RB13 cells, the tumor growth rate more closely resembled that of the wild-type Hepa1 cells, providing further evidence that the level of HIF-1 expression had a significant effect on tumorigenesis.

DISCUSSION

Mechanisms of Hypoxia Signal Transduction and HIF-1α Induction. We demonstrated previously that the protein tyrosine kinase inhibitor genistein completely blocked the induction of HIF-1α protein and HIF-1 DNA-binding activity in hypoxic Hep3B cells (60). Genistein also inhibited hypoxic induction of VEGF mRNA expression in U87 cells (46). C-SRC does not appear to be the target of genistein based on the results presented here (Figs. 1 and 2) and elsewhere (56). The basis for decreased induction of VEGF mRNA in C-SRC-deficient cells reported previously (46) is unclear but may have been an artifact of the culture conditions used. In contrast, V-SRC expression resulted in a marked increase in HIF-1, ENO1, and VEGF expression, indicating that V-SRC activated a signal transduction pathway leading to induction of HIF-1α expression, which determines HIF-1 DNA-binding activity (21, 42, 43). These results suggest that V-SRC may activate the downstream target(s) of a tyrosine kinase that is normally active only under hypoxic conditions. Furthermore, the positive effect of V-SRC on HIF-1 expression suggests that hypoxia signal transduction begins at the cell membrane.

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shown to increase VEGF gene transcription, and this effect was also dependent upon the presence of an intact HIF-1 binding site in the VEGF HRE (65). Introduction of an activated H-RAS gene into murine endothelial cells transformed with SV40 T-antigen was associated with increased VEGF expression under both nonhypoxic and hypoxic conditions in tissue culture and the formation of large, well-vascularized tumors when injected into nude mice (66). It is not known whether activated H-RAS induces expression of HIF-1 or another transcription factor that binds (directly or indirectly) to the same DNA sequence.

Oncogenes such as V-SRC and H-RAS activate signal transduction pathways that have profound effects on many aspects of cell biology, and this discussion is not intended to imply that HIP-1 affects processes not directly related to O2 homeostasis. However, tumors adapt to hypoxia to maintain cellular proliferation, avoid apoptosis (67), and undergo metastasis (24). These adaptations represent end points that tumor cells can reach by multiple genetic pathways. Genes involved in these processes can be activated independent of HIF-1, such as amplification of the type II hexokinase gene in AS-30D hepatoma cells (68), transactivation of lactate dehydrogenase A by C-MYC (64), or increased stability of VEGF and GLUT1 mRNA in tumors with inactivated VHL genes (69, 70). Thus, multiple genetic mechanisms may account for the Warburg effect (20).

To provide evidence that HIF-1 plays a general role in tumorigenesis, we examined tumor formation by mouse hepatoma cells expressing different levels of HIF-1. Tumor growth paralleled the previously determined expression of HIF-1 and VEGF under hypoxic culture conditions (32). Additional experiments that are beyond the scope of the present study will be required to determine the basis for the differences in tumor growth, but differences in cellular proliferation were not seen under aerobic tissue culture conditions.4 Because the HIF-1β subunit is common to other transcription factors, it will also be important to repeat these studies in cells that are deficient in HIF-1α (the subunit unique to HIF-1), when such cells become available, to provide more definitive evidence for involvement of

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**Fig. 6.** Effect of V-SRC on transcription of reporter genes containing hypoxia response elements. A, expression of VEGF reporters. Rat1 and v-Src/Rat1 cells were transfected with pSVβgal and p11w or p11m, which contain a wild-type and mutant copy of the 47-bp VEGF HRE, respectively (sequence at bottom with HIF-1 binding site, 5'-TACGTGGG-3', is indicated). Transfected cells were split on to two plates that were exposed to 20 or 1% O2 for 24 h. Luciferase:βgal ratios were determined by analysis of constant protein aliquots of cell lysates and normalized to the value obtained for cells transfected with p11m and exposed to 20% O2 (Relative Luciferase Activity). Bars, SE (n = 3). B, expression of ENO1 reporters. Rat and v-Src/Rat1 cells were transfected with pSVβgal and p2.1 or p2.4, which contain a wild-type or mutant copy of the 68-bp ENO1 HRE (sequence at bottom with HIF-1 binding sites, 5'-GACGTGGG-3' and 5'-TACGTGAC-3', is indicated). C, cotransfection with V-SRC expression vector. NIH 3T3 cells were cotransfected with pSVβgal, p11w or p2.1, and an expression vector containing no insert or V-SRC coding sequences. D, effect of V-SRC on HIF-1α transactivation domain function. Rat1 and v-Src/Rat1 cells were cotransfected with pSVβgal, pGAL4E1bLUC, and expression vector pG.0 or pG.HIF1A, which encoded the GAL4 DNA-binding domain or a GAL4 DNA-binding domain/HIF-1α transactivation domain fusion protein. Luciferase:βgal ratios were normalized to the value obtained for cells transfected with pG.0 and exposed to 20% O2.

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Fig. 7. Tumor formation by HIF-1-expressing and HIF-1-deficient mouse hepatoma cells. A, comparison of wild-type Hepal and HIF-1-deficient C4 cells. Nude mice (n = 5) were injected with $1 \times 10^6$ Hepal (•) or C4 (○) cells on day 0, and tumor volume in cm$^3$ was determined serially for up to 40 days. Mean values are shown; bars, SD. Hepal data points after 19 days are based on three to four mice due to euthanasia of mice with necrotic tumors. Differences between mean Hepal and C4 tumor volumes were statistically significant (P < 0.0005) at all time points. B, comparison of wild-type Hepal, C4, and partially revertant RB13 cells. Nude mice (n = 5) were injected with $1 \times 10^6$ Hepal (•), RB13 (A), or C4 (○) cells on day 0, and tumor volume was determined serially for up to 47 days. Hepal and RB13 data points after 25 days are based on three to four mice due to euthanasia of mice with necrotic tumors. Statistical significance of differences between mean tumor volumes are as follows: Hepal or RB13 versus C4, P < 0.0005 at all time points; Hepal versus RB13, 0.01 < P < 0.025 at all time points.

**HIF-1.** However, these preliminary results are consistent with the hypothesis that because HIF-1 coordinates multiple systemic, local, and cellular responses to hypoxia (71), this transcription factor may be an important target for up-regulation by V-SRC and other activated oncogenes in tumor cells.

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V-SRC Induces Expression of Hypoxia-inducible Factor 1 (HIF-1) and Transcription of Genes Encoding Vascular Endothelial Growth Factor and Enolase 1: Involvement of HIF-1 in Tumor Progression

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