Insulin-like Growth Factor 1 Induces Hypoxia-inducible Factor 1-mediated Vascular Endothelial Growth Factor Expression, Which is Dependent on MAP Kinase and Phosphatidylinositol 3-Kinase Signaling in Colon Cancer Cells*

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Stimulation of human colon cancer cells with insulin-like growth factor 1 (IGF-1) induces expression of the VEGF gene, encoding vascular endothelial growth factor. In this article we demonstrate that exposure of HCT116 human colon carcinoma cells to IGF-1 induces the expression of HIF-1α, the regulated subunit of hypoxia-inducible factor 1, a known transactivator of the VEGF gene. In contrast to hypoxia, which induces HIF-1α expression by inhibiting its ubiquitination and degradation, IGF-1 did not inhibit these processes, indicating an effect on HIF-1α protein synthesis. IGF-1 stimulation of HIF-1α protein and VEGF mRNA expression was inhibited by treating cells with inhibitors of phosphatidylinositol 3-kinase and MAP kinase signaling pathways. These inhibitors also blocked the IGF-1-induced phosphorylation of the translational regulatory proteins 4E-BP1, p70 S6 kinase, and eIF-4E, thus providing a mechanism for the modulation of HIF-1α protein synthesis. Forced expression of a constitutively active form of the MAP kinase kinase, MEK2, was sufficient to induce HIF-1α protein and VEGF mRNA expression. Involvement of the MAP kinase pathway represents a novel mechanism for the induction of HIF-1α protein expression in human cancer cells.

The insulin-like growth factor-1 (IGF-1) receptor tyrosine kinase (IGF-1R) is activated by binding either of its ligands, IGF-1 or IGF-2. IGF-1R signaling through the mitogen-activated protein (MAP) kinase and phosphatidylinositol 3-kinase (PI3-kinase) pathways plays a critical role in transformation and tumorigenesis (1). IGF2 gene expression is up-regulated to the greatest extent of any gene in colon cancer cells relative to normal colonic epithelium (2), resulting in autocrine stimulation of cells which express both receptor and ligand. In addition to the effects of IGF-1R on cell transformation and proliferation, treatment of colon cancer cells with IGF-1 also induces transcription of the VEGF gene encoding vascular endothelial growth factor, which is essential for tumor angiogenesis (3, 4). Treatment of mice with IGF-1 increases colon cancer growth and metastasis as well as tumor VEGF expression and vascularization (5). A variety of growth factor-receptor tyrosine kinase signaling pathways induce VEGF expression in cancer cells. In the case of oncoenetic RAS signaling, VEGF expression is dependent upon the activity of the MAP kinase/extracellular signal-regulated kinase (ERK) kinase 1 (MEK-1) in fibroblasts but is dependent upon PI3-kinase activity in epithelial cells (6).

Cellular signaling pathways modulate gene expression by altering the activity or expression of specific transcription factors. The major physiological stimulus for VEGF expression is cellular hypoxia; hypoxia-induced transcription of the VEGF gene is mediated by hypoxia-inducible factor 1 (HIF-1) (7–10). Recently, the expression of VEGF in response to heregulin-induced activation of the HER2mamt receptor tyrosine kinase in breast cancer cells was shown to be mediated by HIF-1 via the PI3-kinase pathway (11), demonstrating that HIF-1 regulates both hypoxia- and growth factor-induced VEGF expression in tumor cells. HIF-1 is a heterodimer composed of a constitutively expressed HIF-1β subunit and an inducibly expressed HIF-1α subunit (12). Under nonhypoxic conditions, HIF-1α is subject to O2-dependent prolyl hydroxylation (13, 14), which is required for binding of the von Hippel-Lindau tumor suppressor protein (VHL), the recognition component of an E3 ubiquitin-protein ligase, which targets HIF-1α for proteosomal degradation (15). Under hypoxic conditions, O2 becomes limiting for prolyl hydroxylase activity (16) and ubiquitination of HIF-1α is inhibited (17). As a result, HIF-1α accumulates, dimerizes with HIF-1β, and activates transcription of target genes.

Signaling via receptor tyrosine kinases can induce HIF-1α expression by an independent mechanism. HER2mamt activation in breast cancer cells stimulates increased rates of HIF-1α protein synthesis via PI3-kinase and the downstream serine-threonine kinases, AKT (protein kinase B) and FRAP (FKBP/rapamycin-associated protein), which is also known as mTOR (mammalian target of rapamycin) (11). FRAP/mTOR phosphorylates and activates the translational regulatory proteins eIF-
4E-binding protein 1 (4E-BP1) and p70 S6 kinase (p70 S6K) (18–20). Phosphorylation of 4E-BP1 disrupts its inhibitory interaction with eukaryotic initiation factor 4E (eIF-4E), whereas activated p70S6K phosphorylates the 40 S ribosomal protein S6. The effect of HER neu signaling on the translation of HIF-1α/HIF-1 protein is dependent upon the presence of the 5′-untranslated region of HIF-1α mRNA (11). These pathways thus provide a

**FIG. 1.** Effect of IGF-1 treatment on HIF-1α and VEGF expression in HCT116 cells. A, analysis of HIF-1α expression as a function of IGF-1 concentration. Duplicate plates of HCT116 cells were cultured in the absence of serum for 24 h, exposed to vehicle (lane 1), 1–1000 ng/ml IGF-1 (lanes 2–5), or 100 μM CoCl2 (lane 6) for 6 h. Then either whole cell lysates were subject to immunoblot assay for expression of HIF-1α protein (top) or total cellular RNA was isolated and analyzed by blot hybridization using a HIF-1α cDNA probe (middle) following RNA transfer from an ethidium bromide (EtBr)-stained gel (bottom; migration of 28 S and 18 S rRNA indicated).

**B**, kinetics of HIF-1α induction. Serum-starved cells were exposed to vehicle (lane 1) or 100 ng/ml IGF-1 for 2–24 h (lanes 2–8) prior to immunoblot analysis of whole cell lysates using monoclonal antibodies specific for HIF-1α (top panel) or HIF-1β (bottom panel). C, analysis of VEGF mRNA expression. Serum-starved cells were exposed to vehicle (lane 1), 10–100 ng/ml IGF-1 (lanes 2–3), or 1% O2 (lane 4) for 24 h; total cellular RNA was isolated and analyzed by blot hybridization using a VEGF cDNA probe (top) following transfer of RNA from an EtBr-stained gel (bottom).

**D**, effect of IGF-1R inhibitor. Cells were pretreated with vehicle (lanes 1 and 2) or 1–100 μM H-1356 (lanes 3–6), exposed to IGF-1 (lanes 2–5) or 1% O2 (lane 6), and harvested after 6 h for analysis of HIF-1α protein expression by immunoblot assay (top) or at 24 h for analysis of VEGF mRNA expression by blot hybridization (middle) following transfer of RNA from an EtBr-stained gel (bottom).

**FIG. 2.** Effect of IGF-1, CoCl2, and 1% O2, on HIF-1α expression and stability. A, analysis of HIF-1α stability. HCT116 cells were exposed to 100 μM CoCl2 (top panel) or 100 ng/ml IGF-1 (bottom panel) for 4 h, cycloheximide (CHX) was added to a final concentration of 100 μM, the cells were incubated for 0–60 min, and whole cell lysates were subject to immunoblot assay using an anti-HIF-1α monoclonal antibody. The proportion of HIF-1α remaining at each time point relative to time 0 is indicated. B, induction of HIF-1α protein and VEGF mRNA expression by CoCl2 or 1% O2 in the presence or absence of IGF-1. Serum-starved HCT116 cells were exposed to 100 μM CoCl2 (lanes 3 and 4), 1% O2 (lanes 5 and 6), or neither (lanes 1 and 2) in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of 100 ng/ml IGF-1 for 4 or 24 h prior to analysis of HIF-1α protein or VEGF mRNA expression, respectively.

4E-binding protein 1 (4E-BP1) and p70 S6 kinase (p70 S6K) (18–20). Phosphorylation of 4E-BP1 disrupts its inhibitory interaction with eukaryotic initiation factor 4E (eIF-4E), whereas activated p70 S6K phosphorylates the 40 S ribosomal protein S6. The effect of HER neu signaling on the translation of HIF-1α protein is dependent upon the presence of the 5′-untranslated region of HIF-1α mRNA (11). These pathways thus provide a
molecular basis for stimulation of HIF-1α protein synthesis in response to HER2\textsuperscript{neo} activation. Treatment of cultured cells with IGF-1 or IGF-2 also induces HIF-1α protein expression, HIF-1 DNA binding activity, and transactivation of target genes (21, 22). The demonstration that IGF2 is a HIF-1 target gene (22), that HIF-1α is overexpressed in human colon cancers (23), and that forced overexpression of HIF-1α in HCT116 colon carcinoma cells increases tumor growth and vascularization in vivo (24) suggest that HIF-1 may play an important role in autocrine IGF-1R signaling and angiogenesis in colon cancer. We therefore investigated the mechanisms by which IGF-1 stimulation increases the expression of HIF-1 and VEGF.

**EXPERIMENTAL PROCEDURES**

**Tissue Culture and Reagents—**HCT116 cells were cultured in McCoy’s 5A medium with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Unless otherwise stated, cells were main-
tained at 37 °C in a humidified 5% CO\textsubscript{2}, 95% air incubator. IGF-1, PD98059, wortmannin, rapamycin, cycloheximide (CHX), and cobalt chloride (CoCl\textsubscript{2}) were purchased from Sigma. H-1356 (JB1) was pur-
chased from Bachem Biochemica GmbH. CHX was dissolved in ethanol to a concentration of 10 mg/ml, 1 mm dithiothreitol), and lysed in a Dounce homogenizer. The cell extract was centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant was stored in aliquots at −70 °C. Ubiquitination assays were performed as described previously (26) at 30 °C in a total volume of 40 μl containing 27 μl (50 μg) of cell extract, 4 μl of 10 × ATP-regenerating system (20 μM Tris (pH 7.5), 10 μM ATP, 10 mM magnesium acetate, 300 μM creatine phosphate, 0.5 mg/ml creatine phosphokinase), 4 μl of 5 mg/ml ubiquitin (Sigma), 0.83 μl of 150 μM ubiquitin aldehyde (Sigma), and 2 μl of HA-HIF-1α that was in vitro translated (TNT Quick Coupled Transcription/Translation System, Promega) in the presence of [\textsuperscript{35}S]methionine. HA-HIF-1α was recovered using anti-HA-agarose beads, which were then mixed with SDS sample buffer and boiled for 5 min; the eluates were then analyzed by SDS-
PAGE and autoradiography.

**In Vitro Ubiquitination Assay—**HCT116 cells were serum-starved, treated with IGF-1 for 0, 30 or 150 min, washed twice with cold hypotonic extraction buffer (20 mM Tris (pH 7.5), 5 mM KCl, 1.5 mM MgCl\textsubscript{2}, 1 mM dithiothreitol), and lysed in a Dounce homogenizer. The cell extract was centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant was stored in aliquots at −70 °C. Ubiquitination assays were performed as described previously (26) at 30 °C in a total volume of 40 μl containing 27 μl (50 μg) of cell extract, 4 μl of 10 × ATP-regenerating system (20 μM Tris (pH 7.5), 10 μM ATP, 10 mM magnesium acetate, 300 μM creatine phosphate, 0.5 mg/ml creatine phosphokinase), 4 μl of 5 mg/ml ubiquitin (Sigma), 0.83 μl of 150 μM ubiquitin aldehyde (Sigma), and 2 μl of HA-HIF-1α that was in vitro translated (TNT Quick Coupled Transcription/Translation System, Promega) in the presence of [\textsuperscript{35}S]methionine. HA-HIF-1α was recovered using anti-HA-agarose beads, which were then mixed with SDS sample buffer and boiled for 5 min; the eluates were then analyzed by SDS-
PAGE and autoradiography.

**In Vitro HIF-1α-VHL Interaction Assay—**[\textsuperscript{35}S]Methionine-labeled VHL protein was synthesized in vitro and glutathione S-transferase (GST)-HIF-1α (429–608) fusion protein was expressed in E. coli as described previously (27). HCT116 cells were serum-starved and treated with IGF-1 or CoCl\textsubscript{2} for 4 h prior to lysate preparation. GST-HIF-1α (429–608) was preincubated with 10 μl of the HCT116 lysate for 30 min at 30 °C. Five-μl aliquots of the GST-HIF-1α (429–608) preincubation and VHL in vitro translation reactions were mixed in 150 μl of NETN buffer (150 mM NaCl, 0.5 mM EDTA, 20 mM Tris-HCl (pH 8.0), 0.5% (v/v) Nonidet P-40). After 90 min at 4 °C, 20 μl of glutathione-Sepharose-4B (Amersham Biosciences) was added. After 30 min of mixing on a rotator, beads were washed three times with NETN buffer. Proteins were eluted in 2× SDS sample buffer, fractionated by SDS-
PAGE, and detected by autoradiography.

**Transient Transfection—**8.6 × 10\textsuperscript{5} HCT116 cells were plated in 6-cm dish, cultured overnight, and transfected with 1.25 μg of pCMV-HA-
MEK2-DD (kind gift of S. Moleo, Institut de Recherches Cliniques de
IGF-1 Induces HIF-1 and VEGF via MAP Kinase and PI3-kinase Signaling

**RESULTS**

Exposure of serum-starved HCT116 human colon carcinoma cells to IGF-1 for 6 h resulted in a concentration-dependent induction of HIF-1α protein expression with a maximal effect observed in the presence of 100 ng/ml of IGF-1 (Fig. 1A). Similar results were obtained with IGF-2 (data not shown). HIF-1α expression was also induced by exposure of cells to CoCl2 (Fig. 1A, lane 6), which blocks HIF-1α degradation. In contrast, neither IGF-1 nor CoCl2 induced HIF-1α mRNA expression (Fig. 1A, middle), demonstrating the specific effects of these agents on HIF-1α protein expression. In the presence of IGF-1, HIF-1α protein levels peaked at 8 h and declined thereafter (Fig. 1B, top). HIF-1α levels were unaffected by IGF-1 treatment (Fig. 1B, bottom). IGF-1 treatment also induced VEGF mRNA expression in a concentration-dependent manner (Fig. 1C). H-1356, a selective inhibitor of IGF-1R tyrosine kinase activity, inhibited the induction of HIF-1α protein and VEGF mRNA expression in IGF-1-treated cells in a dose-dependent manner (Fig. 1D, lanes 3–5), thus demonstrating a requirement for signal transduction via the IGF-1R. In contrast, hypoxic cells expressed HIF-1α protein and VEGF mRNA at high levels even in the presence of H-1356 (Fig. 1D, lane 6). Under all conditions, there was a strong correlation between the levels of HIF-1α protein and VEGF mRNA (Fig. 1D, compare top and middle panels).

To determine whether IGF-1 treatment affected HIF-1α protein half-life, HCT116 cells were treated with CoCl2 or IGF-1 for 4 h to induce HIF-1α expression, and then CHX was added to block ongoing protein synthesis. In the presence of CHX, the half-life of HIF-1α was >60 min in CoCl2-treated cells but <30 min in IGF-1-treated cells (Fig. 2A). These results indicate that HIF-1α expression in IGF-1-treated cells is dependent upon ongoing protein synthesis. If IGF-1 induces HIF-1α expression by stimulating synthesis of the protein, then it would be expected to have an additive effect with that of CoCl2 or hypoxia, which act by increasing the stability of the protein. Exposure of HCT116 cells to the combination of IGF-1 and either CoCl2 or hypoxia resulted in a greater induction of HIF-1α protein (Fig. 2B, top) and VEGF mRNA (Fig. 2B, middle) expression than exposure of cells to IGF-1, CoCl2, or hypoxia alone.

**FIG. 4.** Effect of kinase inhibitors on the induction of HIF-1α and VEGF. A, serum-starved HCT116 cells were exposed to vehicle (lane 1) or 100 ng/ml IGF-1 in the presence of no kinase inhibitor (lane 2) or a 1-h pretreatment with 50 μM PD98059 (lane 3), 200 nM wortmannin (lane 4), or 100 nM rapamycin (lane 5). Cells were harvested after 6 h for analysis of HIF-1α protein and mRNA or after 24 h for analysis of VEGF mRNA. B, HCT116 cells were exposed to vehicle (lane 1) or 100 ng/ml IGF-1 in the presence of 0–50 μM PD98059 (lanes 2–5) for 6 h, and HIF-1α protein expression was determined by immunoblot assay. C, cells were exposed to vehicle (lane 1) or 100 ng/ml IGF-1 in the presence of 0–200 nM wortmannin (lanes 2–6). D, cells were exposed to IGF-1 after pretreatment with the indicated concentrations of PD98059 and wortmannin. E, cells were exposed to 100 μM CoCl2 (lanes 1–3) or 100 ng/ml IGF-1 (lanes 4–6) in the presence of no kinase inhibitor (lanes 1 and 4), 50 μM PD98059 (lanes 2 and 5), or 200 nM wortmannin (lanes 3 and 6).

**FIG. 5.** MAP kinase and PI3-kinase pathway signaling in IGF-1-treated cells. HCT116 cells were pretreated for 1 h with 50 μM PD98059, 200 nM wortmannin, or 100 nM rapamycin and then exposed to 100 ng/ml IGF-1 as indicated. Whole cell extracts were prepared after 15 min (left) or 6 h (right) of IGF-1 stimulation and subject to immunoblot assays using antibodies specific for phosphorylated (Thr-202/Tyr-204) or total p42/p44 MAP kinase and phosphorylated (Ser-473) or total AKT.

Montreal) or empty pCMV (Stratagene) in the presence of Fugene-6 (Roche Molecular Biochemicals). After 24 h, cells were cultured in 0.1% FBS for an additional 24 h. Whole cell extracts and total RNA were prepared for immunoblot and slot blot hybridization assays, respectively. For transfected cells exposed to PD98059, the drug was added at the time of serum starvation.
IGF-1 Induces HIF-1 and VEGF via MAP Kinase and PI3-kinase Signaling

Ubiquitination of HIF-1α is inhibited under hypoxic conditions (13–17). To determine whether IGF-1 treatment affects ubiquitination, an in vitro assay was performed using lysates prepared from control and IGF-1-treated cells. The lysates were incubated with 35S-labeled in vitro translated HIF-1α in the presence of ubiquitin and ATP for 0, 30, or 150 min followed by SDSPAGE to resolve non-ubiquitinated and ubiquitinated forms of HIF-1α. Prior to incubation (time 0), no ubiquitinated HIF-1α was detected, whereas the ratio of ubiquitinated to non-ubiquitinated forms of HIF-1α increased over time with no difference observed between IGF-1-treated and untreated lysates (Fig. 3A). Incubation of a GST-HIF-1α fusion protein with control lysate from untreated cells resulted in prolyl hydroxylation of HIF-1α, which is required for its interaction with VHL (Fig. 3B, lane 2). Lysate from CoCl2-treated cells did not promote the interaction of GST-HIF-1α with VHL (Fig. 3B, lane 4). In contrast, lysates from IGF-1-treated cells (Fig. 3B, lane 3) promoted the interaction of GST-HIF-1α with VHL as efficiently as control lysates, providing further evidence that IGF-1 treatment does not induce HIF-1α expression by inhibiting VHL-mediated ubiquitination.

To determine the signal transduction pathways mediating the effects of IGF-1 on HIF-1α protein and VEGF mRNA expression, HCT116 cells were pretreated with PD98059, wortmannin, or rapamycin, which are selective pharmacologic inhibitors of MEK, PI3-kinase, and FRAP/mTOR kinase activity, respectively. All three agents inhibited the induction of HIF-1α protein expression in IGF-1-treated cells (Fig. 4A). At the concentrations used, the rank inhibitory effect of these agents was PD98059 > wortmannin > rapamycin. None of the inhibitors had any effect on the expression of HIF-1α mRNA. However, the induction of VEGF mRNA expression was inhibited by these agents with the same rank potency as seen for the inhibition of HIF-1α protein expression. The induction of HIF-1α by IGF-1 was inhibited in a dose-dependent manner by PD98059 (Fig. 4B) or wortmannin (Fig. 4C). The effects of these inhibitors were synergistic: 10 μM PD98059 or 25 nM wortmannin had little effect alone but in combination markedly inhibited IGF-1-induced HIF-1α expression (Fig. 4D). In contrast to their effects on the expression of HIF-1α induced by IGF-1 treatment, PD98059 or wortmannin had little inhibitory effect on the expression of HIF-1α in CoCl2-treated HCT116 cells (Fig. 4E), providing further evidence that IGF-1 and CoCl2 act by distinct molecular mechanisms.

To determine whether the MAP kinase and PI3-kinase pathways were activated serially or independently in IGF-1-treated cells, the phosphorylation of p42ERK/p44ERK and AKT were analyzed. The increased phosphorylation of p42ERK/p44ERK that was induced by IGF-1 treatment was blocked by PD98059 but not by wortmannin or rapamycin (Fig. 5). The increased phosphorylation of AKT that was induced by IGF-1 treatment was blocked by wortmannin, but neither PD98059 nor rapamycin affected the ratio of phosphorylated to total AKT. Thus, whereas both MAP kinase and PI3-kinase activities are required for induction of HIF-1α protein expression, IGF-1 induces the activity of each pathway independently.

The signal transduction pathway involving PI3-kinase, AKT, and FRAP has been shown to regulate protein translation via phosphorylation of 4E-BP1 and p70s6k (18–20). In HCT116 cells, the phosphorylation of both 4E-BP1 and p70s6k, which was induced by IGF-1 stimulation, could be blocked by wortmannin or rapamycin (Fig. 6) as expected. PD98059 also blocked the phosphorylation of 4E-BP1 and p70s6k, an effect consistent with its inhibition of IGF-1-induced HIF-1α protein and VEGF mRNA expression. The mRNA cap-binding protein, eIF4E, was also transiently phosphorylated by IGF-1 treatment of HCT116 cells, and this process was inhibited by PD98059. This result is consistent with studies indicating that ERK activates the MAP kinase signal integrating kinases, MNK1 and MNK2, which in turn phosphorylate eIF4E (28, 29).

Involvement of MEK and ERK in the induction of HIF-1α expression in IGF-1-treated colon cancer cells represents a novel signaling pathway. We investigated whether activation of this pathway was sufficient to induce HIF-1α and VEGF expression. Transient transfection of HCT116 cells with a plasmid encoding a constitutively active form of MEK-2 (MEK-2DD) resulted in increased levels of phosphorylated p42ERK/p44ERK MAP kinases and increased expression of HIF-1α protein and VEGF mRNA (Fig. 7A). PD98059 has previously been shown to block the phosphorylation of ERK1 and ERK2 by constitutively active forms of MEK (30, 31). The activation of p42ERK/p44ERK and the induction of HIF-1α protein expression in MEK-2DD-transfected cells were inhibited by PD98059 in a dose-dependent manner (Fig. 7B). These results indicate that constitutive MAP kinase kinase ac-
IGF-1 Induces HIF-1 and VEGF via MAP Kinase and PI3-kinase Signaling

Recent studies have demonstrated that in addition to mediating proliferative and anti-apoptotic signals, receptor tyrosine kinases also promote tumor angiogenesis and that the therapeutic efficacy of receptor tyrosine kinase inhibitors may derive in part from their anti-angiogenic effects (32, 33). A principal mediator of tumor angiogenesis is VEGF, and a major transcriptional activator of the VEGF gene is HIF-1 (34). We previously demonstrated that whereas hypoxia decreases HIF-1α protein degradation, heregulin stimulation of breast cancer cells increases HIF-1α synthesis, an effect that is dependent on HER2/neu, PI3-kinase, AKT, and FRAP/mTOR (but not MEK-1) activity and the 5’-untranslated region of HIF-1α mRNA (11).

The studies reported above demonstrate that IGF-1 stimulation of human colon cancer cells also increases HIF-1α protein and VEGF mRNA expression via effects on the translational machinery (Fig. 8). In the previous study of MCF-7 breast cancer cells, the effect on protein synthesis was documented by cycloheximide inhibition and by pulse-chase experiments. In the present study of colon cancer cells, we have confirmed that IGF-1 treatment had no effect on HIF-1α protein stability in IGF-1-treated HCT116 cells and also demonstrated that IGF-1 did not inhibit the interaction of HIF-1α with VHL or its subsequent ubiquitination. Thus, as in the case of heregulin-treated cells, the increased expression of HIF-1α protein in IGF-1-treated cells is due to stimulation of its synthesis. However, in contrast to heregulin-stimulated breast cancer cells, this effect is dependent upon activity of both the PI3-kinase and MAP kinase pathways in IGF-1-stimulated colon cancer cells (Fig. 8). Whereas signaling from constitutively active forms of a G protein-coupled receptor, RAF-1, or RAS to MEK and MAP kinases has been shown to stimulate HIF-1α transactivation domain function (35–37), the data reported here represent the first demonstration that the MAP kinase pathway can also stimulate HIF-1α expression.

Dependence on MEK activity for phosphorylation of 4E-BP1 and p70s6K has been demonstrated in other cellular contexts (38–40). In the case of interleukin 6-stimulated myeloma cells, both MEK and PI3-kinase are required for activation of p70s6K, with MEK inhibitors preventing the phosphorylation of Thr-389 and Ser-424 in the autoinhibitory domain, which is required for subsequent phosphorylation at Thr-389 by FRAP/mTOR (38). ERK has been shown to phosphorylate 4E-BP1 in vitro (38). Our data demonstrate a striking correlation between the inhibition of IGF-1-induced HIF-1α protein and VEGF mRNA expression and the inhibition of 4E-BP1 and p70s6K phosphorylation by wortmannin, rapamycin, and PD98059 in HCT116 cells. The IGF-1 → MEK → ERK pathway also stimulated the phosphorylation of eIF-4E, which is required for its mRNA cap binding activity. Thus, IGF-1 signaling both de-represses (via phosphorylation of 4E-BP1) and activates (via phosphorylation of eIF-4E and p70s6K) protein synthesis in HCT116 cells (Fig. 8).

In experimental tumors, increased eIF-4E activity stimulates tumor growth, invasion, and metastasis (41). Although it increases global protein synthesis, elevated eIF-4E activity disproportionately stimulates the translation of specific proteins with important roles in tumor progression, including...
VEGF (41). FRAP/mTOR also has a disproportionate effect on the translation of specific proteins (42). In herelgun-treated MCF-7 cells, increased translation of luciferase mRNA was dependent upon the presence of HIF-1α 5′-untranslated sequences, demonstrating that the stimulation of translation was mRNA-specific (11).

Taken together, these results provide evidence that activation of different receptor tyrosine kinases (HER2/neu, IGF-1R) in different human cancers (breast, colon) have in common the stimulation of HIF-1α protein synthesis and increased expression of the downstream target VEGF. The effects of receptor tyrosine kinase activation on HIF-1α expression are additive to the effects of hypoxia, emphasizing the importance of two parallel pathways for induction of HIF-1 in human cancer, one based on physiologic stimulation and the other on genetic alterations. HIF-1α overexpression is associated with tumor angiogenesis and increased mortality in cancers of the breast, central nervous system, oropharynx, ovary, and uterine cervix (34). HIF-1α overexpression is observed in colon cancer (23), and the results presented in this study suggest that HIF-1α overexpression may contribute significantly to angiogenesis and other important aspects of colon cancer progression.

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