Non-small cell lung cancer (NSCLC) expresses a particularly aggressive metastatic phenotype, and patients with this disease have a poor prognosis. CXC chemokine receptor 4 (CXCR4) is a cell surface receptor that has been shown to mediate the metastasis of many solid tumors including lung, breast, kidney, and prostate. In addition, overexpression of the epidermal growth factor receptor (EGFR) is associated with the majority of NSCLC and has been implicated in the process of malignant transformation by promoting cell proliferation, cell survival, and motility. Here we show for the first time that activation of the EGFR by EGF increases CXCR4 expression and the migratory capacity of NSCLC cells. Furthermore, many solid tumors are associated with low oxygen tension, and when NSCLC cells were cultured with EGF under hypoxic conditions, CXCR4 expression was dramatically enhanced. A molecular analysis of these events indicated that augmented CXCR4 expression was regulated by the phosphatidylinositol 3-kinase/PTEN/AKT/mammalian target of rapamycin signal transduction pathway, activation of hypoxia inducible factor (HIF) 1α, and ultimately HIF-1-dependent transcription of the CXCR4 gene. Thus, a combination of low oxygen tension and overexpression of EGFR within the primary tumor of NSCLC may provide the microenvironmental signals necessary to up-regulate CXCR4 expression and promote metastasis.

Non-small cell lung cancer (NSCLC) is one of the leading causes of malignancy-related mortality in the United States; indeed fewer than 15% patients survive beyond 5 years after diagnosis. The virulence of this cancer is mediated in part by the specific and aggressive metastatic pattern of primary neoplastic cells to regional lymph nodes, liver, adrenal glands, contralateral lung, brain, and the bone marrow (1–4).

In this respect, we and others have now demonstrated that the metastatic propensity of tumors from several different types of cancer including lung, breast, ovarian, renal, and prostate is related to the expression of the chemokine receptor CXCR4 (5–12). In fact, in human NSCLC-SCID mouse chimera we have observed that the neoplastic cells present at the sites of the secondary metastases express dramatically up-regulated levels of this chemokine receptor in comparison with the cancerous cells present in the primary tumor (11). Furthermore, in both NSCLC and breast cancer it has been shown that the ligand for CXCR4, CXCL12, exhibited peak levels of expression in organs that were the preferred destination for their respective metastases (5, 11). Moreover, when the CXCR4/CXCL12 biological axis was perturbed in these systems using either neutralizing anti-CXCR4 or neutralizing anti-CXCL12 antibodies, the host metastatic burden was significantly reduced, whereas the size of the primary tumor was unaffected (5, 11). Thus, it appears that the normal physiology of CXCR4 and CXCL12 has been usurped by several different types of cancer to promote the specific metastasis of neoplastic cells to distant organs.

Tumors such as NSCLC typically require neovascularization to mediate growth and promote metastasis, yet paradoxically the most malignant tumors have been found to prosper under conditions of low oxygen tension or hypoxia (13–18). This paradox occurs because the tumor vasculature is structurally and functionally abnormal, resulting in perfusion that is characterized by marked spatial and temporal heterogeneity. Thus tumor progression requires an increased adaptation to hypoxia, and the master switch that appears to regulate this phenomenon is the transcription factor, hypoxia inducible factor-1 (HIF-1) (14, 15, 19, 20). Indeed, an extensive body of work has already shown that HIF-1 regulates the transcription of several gene clusters that are crucial to tumor progression including angiogenesis, cell survival, glucose metabolism, and invasion/metastasis (14, 18, 21).

HIF-1 is a heterodimer comprising a constitutively expressed HIF-1α subunit and a highly regulated HIF-1α subunit (14–16). Classically, ambient oxygen tension regulates the rate at which HIF-1α protein is degraded; under normoxic conditions...
specific proline residues in the HIF-1α protein are hydroxylated, facilitating the binding of the von Hippel-Lindau (VHL) tumor suppressor protein (22, 23). VHL is the recognition component of the E3 ubiquitin-protein ligase, and ubiquination of HIF-1α targets the protein for rapid degradation by the 26 S proteasome. By contrast, under hypoxic conditions the rates of proline hydroxylation decreases, thus preventing the binding of VHL to HIF-1α and promoting HIF-1-mediated transcription of target genes (13, 14, 24).

Oxygen-independent regulation of HIF-1α has also been shown to occur, although this is thought to be cell type-specific. Here, growth factors stimulate HIF-1α synthesis via activation of the phosphatidylinositol 3-kinase (PI 3-kinase) and mitogen-activated protein kinase pathways (14, 25, 26). PI 3-kinase activates the downstream serine/threonine kinase AKT and the mammalian target of rapamycin, mTOR (27–30). PI 3-kinase itself is regulated by the phosphatase activity of the tumor suppressor gene PTEN (27–30). Both the PI 3-kinase and mitogen-activated protein kinase pathways converge on the p70 S6 kinase, which initiates a cascade of events that ultimately leads to an increase in the rate at which HIF-1α mRNA is translated into protein (14). Other studies have also suggested the potential involvement of NF-κB (32, 33).

In an effort to address the mechanisms governing the up-regulation of CXCR4 in NSCLC we have used an in vitro model system to study this pivotal chemokine receptor. Our data indicate that exposure of NSCLC cells to hypoxia or EGFR results in a significant up-regulation of CXCR4 expression and chemotactic behavior. In addition, both hypoxia and EGFR activate HIF-1α, and this in turn increases transcription at the CXCR4 promoter. The PI 3-kinase inhibitors wortmannin and LY294002 and the mTOR inhibitor, rapamycin, inhibit activation of HIF-1α and, hence, up-regulation of CXCR4 expression. Moreover, introduction of wild type PTEN into NSCLC cells also inhibits hypoxia-induced up-regulation of CXCR4 expression. Taken together, these data suggest that dysregulated signal transduction through the PI 3-kinase pathway in NSCLC leads to activation of HIF-1α, up-regulation of CXCR4, and increased metastatic potential.

**EXPERIMENTAL PROCEDURES**

**Human NSCLC Cell Lines**—The H157 and A549 non-small cell lung cancer cell lines were obtained from the ATCC. These cell lines were cultured in RPMI 1640 media (Whitaker Biomedical Products, Whittaker, CA) together with 1 mM l-glutamine, 25 mM HEPES buffer, 100 units/ml penicillin, 100 ng/ml streptomycin, and 10% FCS (RPMI complete media). Before assay the cells were transferred to RPMI starvation media, which comprises 1 mM l-glutamine, 25 mM HEPES buffer, 100 units/ml penicillin, 100 ng/ml streptomycin, and 1% FCS or 0.25% human serum albumin. Where appropriate, the PI 3-kinase inhibitors, LY294002 (20–50 μM), wortmannin (100–250 μM), and the mTOR inhibitor rapamycin (10 ng/ml) were preincubated with cells for 2 h before exposure to hypoxia and stimulation with EGF (20 ng/ml).

**RNA Isolation and Real-time PCR**—Total RNA was isolated from both A549 and H157 cells using TRIzol (Invitrogen) and by following the manufacturer’s instructions. Briefly, cells were lysed in TRIzol and then mixed with chloroform. The lysate was then centrifuged to separate RNA, DNA, and protein. Total RNA was recovered, precipitated with isopropanol, washed in 75% ethanol to remove impurities and finally dissolved in water. Next, 1.5 μg of RNA was taken and DNase-treated to remove contaminating DNA before reverse transcription to cDNA using a ProSTAR first strand reverse transcription-PCR kit (Stratagene) and by following the manufacturer’s instructions. Subsequently, the cDNA was assayed for changes in CXCR4 expression by real-time PCR using the ABI Prism 7700 sequence detector and SDS analysis software (Applied Biosystems, Foster City, CA) as previously described (34).

**Antibody Staining and Fluorescence-activated Cell Sorter Analysis**—Cells from each cell line were taken and resuspended in ice-cold staining buffer (phosphate-buffered saline plus 2% FCS plus 0.1% sodium azide) and incubated with Fc block for 5 min at 4 °C. Subsequently, the cells were stained with fluorescein isothiocyanate-conjugated anti-CXCR4 antibodies or the appropriate isotype control at 4 °C for 20 min, after which they were washed twice with staining buffer. Samples were finally analyzed on a FACScan flow cytometer (BD Biosciences) using CellQuest 3.2.1H software.

**Hypoxia Treatment and Extract Preparation**—Cells were cultured to a density of ~80% in complete media and then transferred to starvation media. Next, A549 and H157 cells were exposed to either normoxia (ambient oxygen tension) or hypoxia (94% nitrogen, 5% carbon dioxide, and 1% oxygen) in Modular Incubator Chambers (Bilupee-Rothenberg, Inc., Del Mar, CA) for the times indicated. Subsequently, whole cell extracts or nuclear and cytoplasmic extracts of A549 and H157 cells were prepared. Briefly, whole cell extract lysis buffer was composed of 20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, and 0.2 mM EDTA plus a panel of protease and phosphatase inhibitors (phenylmethylsulfonyl fluoride, dithiothreitol, and NaF at 1 mM; aprotonin, leupeptin, pepstatin, and β-glycerophosphate at 10 μg/ml). A buffer A for extraction of cytoplasmic fractions was composed of 10 mM HEPES, pH 7.9, 10 mM KCl, and 0.1 mM EDTA (plus the above panel of protease and phosphatase inhibitors), and buffer C for extraction of the nuclear fraction was composed of 20 mM HEPES, pH 7.9, 400 mM NaCl, and 1 mM EDTA (plus the protease and phosphatase inhibitors described above).

**Western Blotting**—Immuno blotting was performed on 40 μg of total protein from either whole cell or nuclear and cytoplasmic extracts. After SDS-PAGE the proteins were electrotransferred to a polyvinylidene difluoride membrane at 100 V for 1 h at room temperature and then blocked in BLOTTO for 30 min. Subsequently, the membranes were incubated overnight at 4 °C with either a mouse anti-human HIF-1α (1:500; BD Biosciences), rabbit anti-human CXCR4 (1:500; Oncogene Research Products, Cambridge, MA), or rabbit anti-human phospho-AKT (1:1000; Cell Signaling Technology, Beverly, MA). Subsequently, the blots were washed in TWEEN-20 buffered saline and then incubated with either donkey anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibodies for 45 min at room temperature. After washing in TWEEN-20 buffered saline (~3, 15 min each wash), the immunoreactive proteins were finally visualized using enhanced chemiluminescence (SuperSignal; Pierce Biotechnology) and by following the manufacturer’s instructions. To demonstrate equal loading of each lane, the membranes were then reprobed with a GAPDH antibody (1:500; Abcon) or total AKT antibody (1:1000; Cell Signaling Technology, Beverly, MA).

**Chemotaxis**—A549 cells and H157 cells previously exposed to either hypoxia or normoxia were harvested by trypsinization, counted, and resuspended in RPMI 1640 media containing 10% FCS at a concentration of 10⁶/ml. Neuroprobe filters (5-μm diameter) pretreated with 5 μg/ml fibronectin and 12-well chemotaxis chambers were used for these assays. CXCL12 (30 ng/ml; Peprotech, Rocky Hill, NJ) was added to the lower wells, and 10⁵ cells were added to each of the upper wells. The chemotaxis chambers were then incubated for 6 h at 37 °C. After fixing in methanol and staining in 2% toluidine blue, the number of cells that had migrated through the filters was calculated by counting the total number of cells in 5 separate fields of view under 400× magnification. In similar experiments A549 cells were either left untreated or pretreated with the PI 3-kinase inhibitors wortmannin (100 nM; Upstate Biotechnology) and LY294002 (20 μM; Cell Signal Technology) for 2 h before chemotactic analysis.

**Electrophoretic Mobility Shift Assay**—A549 cells were either exposed to hypoxia or normoxia for 6 h, and then nuclear extracts were prepared. Oligonucleotide probes for HIF-1α were generated by 5’ end labeling of the sense strand with [γ₃₂P]ATP (Amersham Biosciences) and T4 polynucleotide kinase. Subsequently, labeled wild type (WT; 5’-attgCCCTCAATGCTGCTTCAG-3’) and mutant (M; 5’-attgCCCTCAATGCTGCTTCAG-3’) probes (600 pmol) were purified using the MERmaid kit (Bio 101 Systems, Irvine, CA) and by following the manufacturer’s instructions. Binding reactions were performed in a total volume of 20 μl containing 10 μg of nuclear extract and 0.5 μg of poly(dI-dC)·(dI-dC) in 10 mM Tris-HCl, pH 7.5, 50 mM KCl, pH 7.5, 50 mM NaCl, 1 mM MgCl₂, 1 mM EDTA, 5 mM dithiothreitol, and 5% glycerol. Probe (5 x 10⁸ cpms) was then added to the reaction mixture and incubated for 10 min on ice. From CXCR4 expression by real-time PCR using the ABI Prism 7700 sequence detector and SDS analysis software (Applied Biosystems, Foster City, CA) as previously described (34).

**Antibody Staining and Fluorescence-activated Cell Sorter Analysis**—Cells from each cell line were taken and resuspended in ice-cold staining buffer (phosphate-buffered saline plus 2% FCS plus 0.1% sodium azide) and incubated with Fc block for 5 min at 4 °C. Subsequently, the
Hypoxia induces CXCR4 expression and enhances CXCR4 function. A. A549 cells and H157 cells were serum-starved and exposed to either normoxia or hypoxia (94% N2, 5% CO2, and 1% O2) for the times indicated. Changes in gene expression were then determined by real-time quantitative PCR. Fold induction represents increases in CXCR4 expression under hypoxic conditions compared with the normoxic controls. Results are representative of five separate experiments. B, A549 cells and H157 cells were treated as in A and then subjected to Western analysis to examine changes in CXCR4 protein levels. Results are representative of five separate experiments. C, A549 cells and H157 cells were serum-starved and exposed to either normoxia or hypoxia for 24 h and stained for cell surface expression of CXCR4. Surface expression was determined by flow cytometry using a rabbit anti-human CXCR4 monoclonal antibody. Results are representative of three separate experiments. D, A549 cells and H157 cells were treated as in C and then subjected to chemotaxis in response to the indicated concentrations of CXCL12 for 6 h. Data represent the mean ± S.E. from five high power fields. *, p < 0.05.

**RESULTS**

**Hypoxia Promotes Up-regulation of CXCR4 Expression in Non-small Cell Lung Cancer Cells**—We have previously shown that up-regulation of CXCR4 expression is a key component in the metastasis of NSCLC cells *in vivo* (11), but the mechanisms that regulate expression of this chemokine receptor are unclear. Initial evidence in other cancerous cells has indicated that hypoxia-induced HIF-1 activation may be involved (10, 36). To address this phenomenon in non-small cell lung cancer, therefore, we exposed tumor cells (cultured in RPMI starvation media) to a hypoxic environment (94% N2, 5% CO2, 1% O2) and performed a kinetic analysis to examine changes in CXCR4 expression and function (Fig. 1). Using real-time PCR, our data revealed that the expression of CXCR4 mRNA was strongly elevated in hypoxia-exposed A549 and H157 NSCLC cells by 6 h when compared with the normoxic control (Fig. 1A). This expression remained elevated until at least 24 h (Fig. 1A).

Next, we wanted to determine whether the increase in CXCR4 mRNA correlated with an increase in protein levels of CXCR4. We examined this both at the level of intracellular expression (Fig. 1B) and cell surface expression (Fig. 1C). Our results indicated that NSCLC cells exposed to hypoxia showed a significant increase in intracellular CXCR4 protein levels when compared with the normoxic control by 6 h, and these levels remained elevated until at least 24 h (Fig. 1B). Indeed, by 24 h both A549 cells and H157 cells showed significantly greater expression of CXCR4 at the cell surface (Fig. 1C).
To determine whether this increased expression of CXCR4 was functional, we performed chemotaxis assays (Fig. 1D). Here, NSCLC cells were exposed to hypoxia or normoxia for 24 h and then treated with CXCL12 for 6 h. Although both A549 and H157 cells demonstrated chemotactic behavior in response to CXCL12 under normoxic conditions, the magnitude of these responses was dramatically enhanced in those cells exposed to hypoxia for 24 h (Fig. 1D). Thus, we have demonstrated that hypoxia not only increases expression of CXCR4 on NSCLC cells but also enhances the migratory ability of these cells in response to CXCL12.

**Hypoxia Activates HIF-1α Expression in NSCLC Cells and Promotes HIF-1-mediated Transcription at the CXCR4 Promoter**—Having established that a physiological event such as hypoxia is capable of up-regulating CXCR4 expression in NSCLC cells, we next wanted to examine the underlying biochemistry that mediates this phenomenon. It has been well established that hypoxia regulates the expression of HIF-1α, which is a key component of the transcription factor HIF-1 (14–16). And HIF-1 itself is thought to regulate the transcription of several gene clusters crucial to tumor progression including angiogenesis, cell survival, glucose metabolism, and invasion/metastasis (13, 14, 18, 21). Thus, we exposed A549 cells and H157 cells to normoxia or hypoxia for the times indicated and then examined intranuclear HIF-1α expression by Western analysis (Fig. 2A). Under normoxic conditions little or no intranuclear expression of HIF-1α was observed. This is in keeping with known data, which has suggested that under normal ambient conditions the tumor suppressor gene, VHL, binds to HIF-1α and targets it for degradation (22, 23). However, under hypoxic conditions, strong intranuclear expression of HIF-1α was observed within 2 h, and this expression remained elevated for a total of 6 h; by 24 h, HIF-1α expression had returned to background levels (Fig. 2A).

Next, we wanted to determine whether hypoxia promoted an increase in the binding of the HIF-1 transcription factor (which comprises inducibly expressed HIF-1α and constitutively expressed HIF-1β) to its cognate DNA binding motif (Fig. 2B). Therefore, we exposed A549 cells to normoxia or hypoxia for 4 h and then prepared nuclear extracts for analysis by electrophoretic mobility shift assay. Our results indicate that under normoxic conditions there is little or no inducible binding of HIF-1 to its cognate binding motif (Fig. 2B, lane 1), whereas a 4-h exposure to hypoxia mediated a strong signal (Fig. 2B, lane 2). To demonstrate the specificity of this binding activity, we added nuclear extract from hypoxia-treated cells to a labeled probe containing a mutated form of the core binding motif (5′-AAAAA-3′ instead of 5′-ACGTG-3′; Fig. 2B, lane 3) or included an excess of cold WT probe with the 32P-labeled WT probe (Fig. 2B, lane 4). Under both conditions specific binding of the HIF-1 transcription factor was abrogated.

To further verify that HIF-1 contributed to the transcription and up-regulation of CXCR4 gene expression, we transfected a luciferase reporter construct containing a 2.6-kb fragment of the wild type CXCR4 promoter (WT-CXCR4) into A549 cells and H157 cells (Fig. 2C). In addition, we co-transfected either a random control cDNA (GFP; Fig. 2C, lanes 1 and 5) or HIF-1α cDNA (Fig. 2C, lanes 2 and 6). Under these conditions significant transactivation of the CXCR4 promoter was only observed in those cells receiving the HIF-1α cDNA but not the GFP
Regulation of CXCR4 Expression in NSCLC Cells

The PI 3-Kinase Pathway Contributes to the Regulation of CXCR4 Expression Mediated by HIF-1. To more fully elucidate the signaling pathways involved in the regulation of CXCR4 expression, we treated A549 cells and H157 cells with the PI 3-kinase inhibitors wortmannin and LY294002. Previous studies in prostate cancer cells and glioblastoma cells have implicated a role for PI 3-kinase hypoxia-induced HIF-1α activation (26, 38). To that end we pretreated NSCLC cells with either wortmannin (250 nM) or LY294002 (50 μM) for 2 h and then subjected these cell lines to either normoxic or hypoxic conditions for a further 6 h (Fig. 3, Ai and Bi). Under these conditions the up-regulation of CXCR4 expression mediated by hypoxia was indeed abrogated by the PI 3-kinase inhibitors, although some basal expression of CXCR4 remained (compare Fig. 3, Ai, lanes 4–6, and Bi, lanes 4–6). This data were further verified at the level of CXCR4 mRNA expression. Here, LY294002 strongly inhibited hypoxia-induced CXCR4 expression (Fig. 3, C and D).

The tumor suppressor gene PTEN is also known to be a key regulatory component in the PI3-signaling cascade (26, 27, 30). We, therefore, transfected A549 cells with either WT-PTEN or a catalytically inactive form of PTEN containing a C124S missense mutation (C124S-PTEN) (35). Next, we exposed these transfected cells to either normoxia or hypoxia for 24 h and then extracted RNA to examine changes in CXCR4 mRNA expression by real-time PCR (Fig. 4). Our results indicate that overexpression of WT-PTEN, but not the catalytically inactive form, C124S-PTEN, significantly curtailed hypoxia-induced activation of CXCR4. Taken together, these data suggest that hypoxia-induced HIF-1α activation and CXCR4 expression are regulated at least in part by PI 3-kinase and the PTEN tumor suppressor gene.

Epidermal Growth Factor-activated PI 3-kinase Signaling Synergizes with Hypoxia Treatment to Dramatically Up-regulate CXCR4 Expression. Gain of function mutations in receptor-tyrosine kinases such as the epidermal growth factor receptor are known to be a feature of the majority of non-small cell lung cancers (39–41). Furthermore, these receptor-tyrosine kinases are also known to signal through the PI 3-kinase pathway (39–42). Therefore, we decided to treat A549 cells and H157 cells with EGF in the presence or absence of LY294002 and under normoxic and hypoxic conditions to assess changes in CXCR4 mRNA expression by real-time PCR (Fig. 5A). Under normoxic conditions, EGF alone induced a 5–10-fold induction in CXCR4 mRNA expression (Compare Fig. 5A, panels i and ii) alone. In both cell lines the observed induction of CXCR4 mRNA expression was strongly inhibited by LY294002. Hypoxia-induced CXCR4 mRNA expression was of a similar magnitude to that observed by EGF treatment under normoxic conditions, and although LY294002 abrogated hypoxia-induced CXCR4 mRNA expression, the inhibition was not complete (compare Fig. 5A, panels i and ii and also Fig. 3, C and D). The combined treatments of EGF plus hypoxia produced a dramatic increase in CXCR4 mRNA expression in both NSCLC cell lines, and this synergistic increase was also susceptible to inhibition by LY294002 (compare Fig. 5A, panels i and ii). In addition, treatment with either EGF or hypoxia or EGF plus hypoxia resulted in a significant up-regulation of CXCR4 protein levels in both A549 cells and H157 cells (Fig. 5B and data not shown); moreover, pretreatment with LY294002 modulated the EGF-in-
duced increase in CXCR4 protein expression (Fig. 5).

The substrate for PI 3-kinase is phosphatidylinositol 4,5-bisphosphate to generate the second messenger phosphatidylinositol3,4,5-triphosphate, which activates phosphatidylinositol-dependent kinase, which in turn phosphorylates and activates the serine/threonine kinase, AKT (protein kinase B) (28, 29). To further characterize the signaling pathway that mediates EGF-induced up-regulation of CXCR4, we therefore examined changes in the activation state of AKT (Fig. 6). Cells were cultured in RPMI starvation media with 0.25% human serum albumin for 24 h pretreated with LY294002 (50 μM; lanes 2, 5, 8, 11, 14, and 17) or wortmannin (250 nM; lanes 3, 6, 9, 12, 15, and 18) for 2 h and then either left unstimulated (lanes 1–3 and 10–12) or stimulated with 10% FCS (lanes 4–6 and 13–15) or 20 ng/ml EGF (lanes 7–9 and 16–18) for 10 min. We then examined AKT activity by Western blot for CXCR4 and GAPDH protein levels. Results are representative of three separate experiments.

Fig. 4. Wild type PTEN, but not mutant PTEN, blocks hypoxia-induced CXCR4 expression. A549 cells were transfected with mammalian expression vectors containing either WT-PTEN or a catalytically inactive form of PTEN (C124S-PTEN). Transfected cells were then exposed to either normoxia or hypoxia (94% N₂, 5% CO₂, and 1% O₂) for 24 h, and RNA was prepared. Changes in CXCR4 mRNA expression were then determined by real-time quantitative PCR. -Fold induction represents increases in CXCR4 expression under hypoxic conditions compared with the normoxic controls (Con). Data are the mean ± S.E. *, p < 0.05.

Fig. 5. EGF induces CXCR4 expression via a pathway that involves PI 3-kinase and HIF-1α. A, A549 cells and H157 cells were serum-starved, left untreated, or pretreated with LY294002 (LY; 50 μM) for 2 h and then exposed to either normoxia or hypoxia (94% N₂, 5% CO₂, and 1% O₂) for 24 h in the presence or absence of EGF (20 ng/ml). RNA was then prepared, and changes in gene expression were determined by real-time quantitative PCR. -Fold induction represents increases in CXCR4 expression under hypoxic conditions compared with the normoxic controls (Con). Results are representative of three separate experiments. B, A549 cells were serum-starved for 24 h and then exposed to either normoxia or hypoxia for the times indicated in the presence or absence of EGF (20 ng/ml). Subsequently, cytoplasmic extracts were prepared and analyzed by immunoblotting for CXCR4 and GAPDH protein levels. Results are representative of three separate experiments. C, A549 cells were serum-starved, left untreated, or pretreated with LY294002 (50 μM) for 2 h and then stimulated with EGF (20 ng/ml) for 24 h under normoxic conditions. Next, cytoplasmic extracts were prepared and analyzed by Western blot for CXCR4 and GAPDH protein levels. Results are representative of three separate experiments.
induced strong phosphorylation and activation of AKT (compare lanes 4 and 7). These phosphorylation events were strongly inhibited by the PI 3-kinase inhibitors (lanes 8 and 9). By contrast, constitutive phosphorylation of AKT was still observed in H157 cells despite prior culture in RPMI starvation media with 0.25% human serum albumin for 24 h (lane 10). However, both the constitutive AKT phosphorylation and the augmented signaling mediated by FCS and EGF were completely modulated by the PI 3-kinase inhibitors (Fig. 6B).

Downstream of the serine/threonine kinase AKT is another key component of the PI 3-kinase signaling cascade, namely mTOR (43). Therefore, to further establish the signaling sequence through which EGF up-regulates expression of CXCR4, we serum-starved A549 cells for 24 h, pretreated with the mTOR-specific inhibitor, rapamycin (10 ng/ml), and then treated with the EGF (20 ng/ml) for an additional 24 h. Subsequently, we prepared RNA and analyzed changes in CXCR4 expression by real-time quantitative PCR (Fig. 7A). EGF-mediated up-regulation of CXCR4 mRNA under both normoxic and hypoxic conditions was strongly inhibited in the presence of rapamycin (Fig. 7A).

Next, we wanted to determine whether EGF itself was capable of activating HIF-1α in NSCLC cells under normoxic conditions. Previous studies have already suggested that growth factors and cytokines are capable of activating HIF-1α in the absence of low oxygen tension (14, 32, 33, 38). Therefore, serum-starved A549 cells were treated with EGF (20 ng/ml) for 6 h under normoxic or hypoxic conditions, and extracts were prepared to examine intranuclear HIF-1α activity by Western analysis (Fig. 7B). Our results revealed that EGF was able of inducing HIF-1α, although the extent of the activation was more modest than that observed by hypoxia. Furthermore, in the presence of rapamycin, EGF-induced HIF-1α activation was inhibited under both normoxic and hypoxic conditions (Fig. 7B).

Finally, we wanted to determine whether the increased expression of CXCR4 observed in the presence of EGF and particularly EGF plus hypoxia led to an increase in function. Therefore, serum-starved A549 cells were exposed to EGF under normoxic and hypoxic conditions and then stimulated with varying concentrations of CXCL12 to measure the chemotactic potential of the cells (Fig. 8). As described previously (compare Fig. 1D and Fig. 8), hypoxia strongly up-regulated chemotaxis in response to CXCL12. Remarkably, the combination of hypoxia and EGF promoted an even greater increase in A549 chemotaxis. In addition, under normoxic conditions pretreatment with EGF alone mediated a significant increase in migration in response to CXCL12 when compared with the normoxic control (Fig. 8). Moreover, this response was maximal at a lower concentration of CXCL12, suggesting that EGF sensitizes the cells in response to CXCL12. Thus, the growth factor EGF is capable of up-regulating CXCR4 expression and chemotactic potential in NSCLC cells via a pathway that involves PI 3-kinase, AKT, mTOR, and HIF-1α.

**PI 3-Kinase Also Regulates CXCL12-induced Chemotaxis in NSCLC Cells**—Because CXCR4 is an important component of the metastatic pathway in non-small cell lung cancer, we wanted to determine whether the PI 3-kinase pathway modulated the chemotactic behavior of NSCLC cells in response to its cognate ligand, CXCL12. Therefore, serum-starved A549 cells were pretreated with either LY294002 (20 μM) or wortmannin (100 nm) for 2 h and then stimulated for a further 6 h in the presence of CXCL12 (30 ng/ml). Our data revealed that
both PI 3-kinase inhibitors strongly inhibited CXCL12-induced chemotaxis, whereas the MEK1/2 inhibitor, UO126, had no effect on the CXCR4/CXCL12 chemotactic axis (Fig. 9 and data not shown). This suggests that the PI 3-kinase signaling pathway is capable of regulating both the expression of CXCR4 and the CXCR4/CXCL12 chemotactic axis in non-small cell lung cancer cells.

**DISCUSSION**

This study provides to our knowledge the first indication that both hypoxia and the EGF regulate expression of CXCR4 on non-small cell lung cancer cells. Moreover, EGFR activation in the presence of hypoxia further augments CXCR4 expression. Having identified two key physiological signals that regulate CXCR4 expression, we then examined the molecular signaling pathways involved. The EGFR is a receptor-tyrosine kinase that activates PI 3-kinase and subsequent downstream targets including AKT and mTOR. In the presence of either PI 3-kinase inhibitors or mTOR inhibitors we found that we could block both activation of HIF-1α and increases in CXCR4 expression. We further showed that HIF-1 directly transactivates CXCR4 gene expression. Finally, inhibitors of PI 3-kinase also prevent chemotaxis of NSCLC cells in response to CXCL12, the cognate ligand for CXCR4. Thus, the PI 3-kinase pathway abrogates increased expression of CXCR4 induced by hypoxia and the EGFR and of CXCL12-mediated chemotaxis (see Fig. 8 for an illustration of these pathways).

HIF-1α is constitutively expressed in most cells, but it normally undergoes a post-translational modification that targets it for degradation by the 26S proteasome (14–16). The key regulatory molecule involved in this process is the tumor suppressor gene, VHL (22, 23). This protein moiety is the recognition component of the E3 ubiquitin-protein ligase, and it is the VHL-mediated ubiquitination of HIF-1α that targets the transcription factor for degradation. Hypoxia prevents VHL from binding to HIF-1α, thus stabilizing the expression of this transcription factor. Active HIF-1 then regulates a host of genes involved in cellular processes such as proliferation, survival, glucose metabolism, and angiogenesis (14, 16, 18, 21). Our data and that of two other recent studies (10, 36) now suggest that HIF-1 also regulates CXCR4-mediated metastasis. In particular, Staller et al. (10) have shown that a common mutation in clear cell renal carcinoma is loss (or functional inactivation) of VHL, resulting in persistent activation of HIF-1 and a dramatic up-regulation of CXCR4 expression. We have demonstrated an analogous pathway in NSCLC cells that regulates CXCR4 expression. Here, hypoxia functionally inactivates VHL, albeit temporarily, and facilitates accumulation of HIF-1α and increased CXCR4 transcription. Interestingly, little is known about the regulation of the VHL gene itself despite the fact that the promoter was cloned 10 years ago (44, 45). A better understanding of the regulatory elements governing the expression of this important tumor suppressor gene may yield vital clues in the ongoing search for effective therapeutics to abrogate the aggressive metastasis associated with NSCLC.

Although permanent functional inactivation of VHL is not a common phenotype in NSCLC, overexpression of EGFR is strongly correlated with disease progression in squamous carcinomas, large cell and adenocarcinomas. Furthermore, we have demonstrated for the first time that under ambient oxygen tension the EGF/EGFR biological axis activates the HIF-1α
transcription factor, and this in turn up-regulates CXCR4 expression and function. The underlying biochemistry associated with this phenomenon involves activation of the PI 3-kinase/PTEN/AKT/mTOR pathway. Indeed, overexpression of wild type PTEN effectively inhibits up-regulation of CXCR4 expression. In similar studies Zundel et al. (26) reported that overexpression of wild type PTEN in glioblastoma cells that lacked a functional PTEN ablated hypoxia and insulin-like growth factor induction of HIF-1-regulated genes. Further evidence in support of the notion that growth factors such as EGF stabilize and activate HIF-1α is provided by a study in prostate cancer cells where EGF-mediated HIF-1α activation resulted in the induction of VEGF gene expression, a gene known to be under the control of HIF-1 (38). Other signaling molecules including tumor necrosis factor-α and interleukin-1β have also been shown to activate HIF-1α expression, although in these studies it has been suggested that the cytokines act indirectly through NF-κB (32, 33).

Indeed, the role of VHL in the growth factor and cytokine-mediated activation of HIF-1α remains unclear. Jung et al. (33) have indicated that an unknown tumor necrosis factor and NF-κB-regulated factor that interferes with VHL binding to HIF-1α is involved. Moreover, ligation of the EGFR also activates the mitogen-activated protein kinase pathway, which appears to share at least some of the downstream elements that are found in the PI 3-kinase signaling pathway, including p70 S6 kinase and the eukaryotic translation initiation factor, 4E-BP1 (14, 43). It is again unclear at this time what role, if any, the mitogen-activated protein kinases play in EGF/EGFR-dependent expression of CXCR4. Thus, in future studies we will examine the potential contribution of both the NF-κB and mitogen-activated protein kinase signal transduction pathways to EGFR-mediated activation of HIF-1α.

In addition to binding EGF, the EGFR also binds transforming growth factor-α, a gene whose expression is regulated by HIF-1 (46). This observation introduces the intriguing possibility that an autocrine signaling pathway may develop in the malignant progression of NSCLC cells involving activation of HIF-1 (either via the EGFR or via hypoxia), persistent up-regulation of transforming growth factor-α expression, and chronic activation of the EGFR. This in turn would lead to the continuous expression of CXCR4 and ultimately the genesis of a highly metastatic tumor cell.

Taken together, our data identify several key signaling molecules necessary for the development of a metastatic phenotype in NSCLC. These include EGFR, PI 3-kinase/PTEN, mTor, HIF-1α/VHL, and CXCR4. The chemokine receptor, CXCR4, represents the final common mediator of these pathways and, therefore, provides an attractive therapeutic target for treatment of not only NSCLC but also other highly metastatic cancers such as breast and kidney.

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