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Activated pp60$^{c\text{-}Src}$ Leads to Elevated Hypoxia-inducible Factor (HIF)-1α Expression under Normoxia*

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Hypoxia-inducible factor (HIF)-1 is a master transcription factor, which up-regulates glycolysis, erythropoiesis, and angiogenesis under hypoxia. HIF-1α accumulates in normoxic tumor cells, leading to glycolysis under aerobic conditions. This phenomenon, known as the "Warburg effect," is caused by a yet unknown mechanism. Here we show that transformed cells that express constitutively active pp60$^{c\text{-}Src}$ (Src) express HIF-1α protein under normoxia, which results in the expression of multiple HIF-1α target genes. We show that this occurrence is due to an enhanced rate of HIF-1α protein synthesis and not due to reduced HIF-1α degradation. Furthermore, we show that the Src-induced increase in protein synthesis is due to the global increase in the rate of cap-dependent translation and does not involve inhibition of HIF-1α degradation.

Hypoxia-inducible factor (HIF)[1]-1 is a master transcription factor, which regulates oxygen homeostasis by inducing glycolysis, erythropoiesis, and angiogenesis (1). It has been suggested that pp60$^{c\text{-}Src}$ (Src) mediates the hypoxic induction of VEGF expression (2) and that the expression of v-Src enhances the expression of HIF-1α (3) which, in turn, up-regulates HIF-1 target genes.

In view of the presence of activated Src in many malignant tumors, such as colon (4), breast (5), and lung (6) cancers and in light of the observation that many of these tumors also express high levels of HIF-1α protein under normoxia, we examined the proposition that Src up-regulates HIF-1α and explored the molecular mechanism(s) by which Src may affect HIF-1α protein levels. Earlier reports have already pointed to a correlation between HIF-1α expression and Src activity. Studies have revealed hypoxia-induced activation of Src kinases (7) and increased levels of HIF-1α in v-Src transformed cells (3). It has also been shown that inhibition of c-Src expression by antisense expression reduces VEGF expression (8), although it has been argued that c-Src is not involved in inducing hypoxic-regulated genes (9). The well documented mechanism for HIF-1α regulation is by its stabilization under hypoxia and its rapid degradation via the ubiquitin-proteasome pathway under normoxia (10–12).

Here we report that HIF-1α protein accumulates due to elevated translation and not due to elevated mRNA levels or extended protein half-life. We show that the Src-induced increase in protein synthesis is not a HIF-1α-specific phenomenon, but is due to a general increase in cap-dependent translation.

MATERIALS AND METHODS

Cell Culture—CSH12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. HT29 and Saos-2 cells were grown in McCoy-5A medium supplemented with 10% fetal calf serum. All media were supplemented with penicillin and streptomycin. Saos-2 clones containing empty plasmid (pUSE(−), wild type c-Src (pUSE-WT Src), kinase-dead Src (K297R) (pUSE-KD Src) and active Src (Y529F) (pUSE-Active Src) were obtained by transfection of the above constructs (Upstate Biochemistry) into Saos-2 cells using FuGENE 6 (according to the manufacturer's instructions) and selection with 500 μg/ml G418. HeLa cells expressing HA-eIF4E were generated by transfection of HeLa cells with pCDNA-HA-eIF4E (a gift of Dr. Nahum Sonenberg, McGill University) and were grown in the presence of 500 μg/ml G418.

Src Kinase Inhibitor, Transient Transfections, and Reporter Assays—PP1, PP2 and PP3 were synthesized by Dr. Aviv Gazit as described (34). Transient transfections were performed with FuGENE 6 (according to the manufacturer's instructions) or polyethyleneimine (35)

Transcription from the HRE was measured using plasmid p-tk$_p$-luc, which contains five HRE sequences (17). Cells were seeded and 24 h later were transfected. Total DNA for transfection was 2.5 μg per well, comprising 1 μg of luciferase reporter constructs, 0.5 μg of β-galactosidase internal control vector and 1 μg of the Src construct, as indicated under "Results." In the experiments utilizing the Src inhibitor PP1, cells were transfected only with p-tk$_p$-luc and CMV-lac-Z plasmids; 24 h after transfection the medium was replaced with medium containing inhibitor for an additional 24 h, using PP1 concentrations as indicated in the results. In all the experiments cells were lysed 48 h after transfection with reporter lysis buffer (Promega) according to the manufacturer's instructions, and luciferase activity was measured and normalized to β-galactosidase activity. The reporter encoding secreted alkaline phosphatase and containing HIF-1α 5′-UTR was constructed by inserting 289 bp of HIF-1α 5′-UTR into plasmid pBKC/S in *Hin*dIII and *Xho*I sites. HIF-1α 5′-UTR was amplified using primers HIFI-5-R: 5′-GGTGAATCGGTCCCCGCGATG-3′ and HIFI-5-F: 5′-GTGCTGCCTCGTCTGAGGGGACA-3′.

Immunoblotting—Cells were washed three times with PBS, then lysed with sample buffer (10% glycerol, 0.2 M Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 3% SDS) and boiled for 5 min. The whole cell lysate was then subjected to SDS-PAGE and transferred to nitrocellulose. The membranes were blocked in TBST (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 0.1% Triton X-100) containing 5% low fat (1%) milk for 30 min, followed by incubation for 1.5 h with primary antibody (as indi-

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[1] The abbreviations used are: HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor; HRE, HIF-1-responsive element; KD, kinase-dead; PI3K, phosphatidylinositol 3-kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MAPK, mitogen-activated protein kinase; HA, hemagglutinin; WT, wild type; ERK, extracellular signal-regulated kinase; IRES, internal ribosome entry site; SeAP, secreted alkaline phosphatase; UTR, untranslated region; PBS, phosphate-buffered saline; mAb, monoclonal antibody.

cated in the figure legends). Membranes were then washed extensively with TBST, and immunoreactive proteins were detected by incubation with either horseradish peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch 1:10000) for detection of monoclonal antibodies or horseradish peroxidase-conjugated anti-rabbit or anti-goat IgG (Jackson ImmunoResearch 1:10000) for detection of polyclonal antibodies. Proteins were visualized using enhanced chemiluminescence.

Northern Blotting—Cells were grown on 10-cm dishes and treated with PP1 for 2 h or 24 h as indicated in the results. RNA was prepared using Trizol reagent (Sigma), and 10 μg of total RNA were denatured and loaded on a 1% agarose gel containing 8% formaldehyde.

Following capillary blotting onto a nylon membrane, the RNA was UV-cross-linked (1200 j/m2), and the membrane stained with 0.1% methylene blue to verify equal loading and transfer. The blot was then hybridized overnight at 42 °C with ^{32}P-labeled DNA probe, prepared with the Rediprime kit (Amersham Biosciences). After two washes at 60 °C with 2× SSC, 1% SDS the blot was exposed to a MS-sensitive film (Kodak) or a phosphorimaging plate (Fuji).

HIF-1α Half-life Experiments—10^6 (HT29) or 5×10^5 (Saos-2) cells were seeded on 60-mm dishes, and 24 h later 100 μM cycloheximide was added for the length of time indicated in the results. Cells were then lysed with sample buffer and subjected to SDS-PAGE and Western blotting. The blots were probed with anti-HIF-1α (1:250 Transduction Laboratories), anti-actin (1:2000 Santa Cruz), and anti-c-Src (mAb 327) antibodies as indicated in the figure legends.

Accumulation Experiments—4×10^5 (HT29) and 2×10^5 (Saos-2) cells were seeded on 6-well plates and 24 h later were treated with PP1 (20 μM), PP2 (20 μM), PP3 (20 μM), LY294002 (20 μM, Calbiochem), PD98059 (50 μM, Calbiochem) wortmannin (200 nM, Sigma), and rapamycin (50 nM, Calbiochem) for 30 min. The medium was then replaced with medium containing ALLN (200 μM, Calbiochem) as well as the inhibitors mentioned above for an additional 2 h. Cells were then lysed with sample buffer.

^{35}S-labeling—For labeling of HA-HIF-1α, CSH12 cells were grown on 10-cm dishes (1×10^6 cells/dish) and were transfected with 0.5 μg of HA-HIF-1α expression plasmid, together with 5 μg of pUSE(−), (Upstate Biochemistry) using FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer's instructions. 24 h later cells were starved for methionine and cysteine for 1 h in the presence of the inhibitors indicated. Cells were then labeled with [^{35}S]methionine and [^{35}S]cysteine Promix (Amersham Biosciences), 100 μCi/ml for 45 min in the presence of 200 μM ALLN and the inhibitors. Cells were then washed with PBS and lysed with RIPA buffer (20 mM Hepes, pH 7.4, 125 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 5 mM NaF, 100 μM $NaVO_3$, 1 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 5 μg/ml leupeptin). After Bradford protein quantification, HA-HIF-1α was immunoprecipitated from lysates containing 0.8 mg of total protein, using anti-HA antibody mAb 12CA5 produced from hybridoma cells or Rat mAb 3F10 (Roche Molecular Biochemicals). 40 μl of protein G-Sepharose beads (Amersham Biosciences) per sample were incubated with the anti-HA antibodies for 1.5 h and after washes were incubated with lysates for 2 h. After additional washes the beads were mixed with 2× sample buffer and boiled for 5 min. After Western blotting and blocking, membranes were exposed to a phosphorimaging device (Fuji) or to film. For detection of HA-HIF-1α protein levels, membranes were probed with anti-HIF-1α antibody (Transduction Laboratories). For labeling of total protein, Saos-2 cells (3×10^5 cells/well) and HT29 cells (5×10^5 cells/well) were seeded on six-well plates (Nunc). 24 h later, cells were washed with PBS, and the medium was replaced with methionine- and cysteine-deficient medium containing the indicated inhibitors for 1 h. Medium was then replaced with medium containing 50 μCi/ml [^{35}S]Met/Cys Promix (Amersham Biosciences) and the indicated inhibitors. Cells were labeled for 10 min at 37 °C. Cells were then washed with PBS and lysed with sample buffer. Identical amounts of protein from the lysates of Saos-2 cells were run on 10% SDS-PAGE and exposed to film. To measure synthesis rates, lysates were loaded on Whatman No. 3MM paper squares. Paper squares were stained with Coomassie, and washed five times for 6 min with destain solution (20% methanol 7% acetic acid). Then the paper squares were dried, and radioactivity was counted with scintillation solution in a β-counter. In parallel, in another set of same samples, staining extracted from papers with 3% SDS and protein amounts were determined using a bovine serum albumin calibration curve, reading the absorbance at 590 nm.

RESULTS

Src Kinase Activity Regulates the Expression of HIF-1 Target Genes—In the cell line CSH12 (NIH3T3 overexpressing the chimeric receptor, EGFR-HER-2 (13)) Src is constitutively active (14). We found that hypoxia-regulated genes such as PGK-1 and VEGF were more highly expressed under normoxia in CSH12 than in parental NIH3T3 cells (Fig. 1*A*). We also observed high levels of expression of these genes in cancer cell lines such as the colon cancer cell line HT29 (Fig. 1*B*) (15), and Saos-2 cells (data not shown), that possess high Src activity. Inhibition of Src kinase activity by the Src family kinase inhibitor, PP1 (16), resulted in a dose-dependent reduction in the mRNA levels of these hypoxia-regulated genes (Fig. 1*B*). The mRNA levels of HIF-1α, the transcription factor that regulates the expression of these genes, remained unchanged upon PP1 treatment (Fig. 1*B*). To further investigate the regulation of hypoxia-regulated gene by Src, we utilized a luciferase reporter-gene driven by a minimal promoter including five HIF-1-responsive elements (HRE) derived from the erythropoietin promoter (17). We found that the Src kinase inhibitor, PP1, inhibited the expression of the luciferase gene in HT29 and CSH12 cells (Fig. 1, *C* and *E*). When activated Src (Y529F) or wild type c-Src were co-transfected with the reporter, expression of the luciferase gene was induced under normoxic conditions, whereas kinase-dead (KD) Src (K297R) inhibited its expression (Fig. 1, *D* and *F*).

Src Regulates Levels of the HIF-1α Protein—Because the effect of the Src kinase inhibitor, PP1, may not be absolutely selective, we examined the effect of c-Src mutants on HIF-1α expression. We examined the levels of HIF-1α expression in Saos-2 cells stably expressing activated Src, wild type Src, or KD Src. As shown in Fig. 2*A*, Saos-2 cells expressing activated or overexpressing wild type Src robustly express high HIF-1α protein levels as compared with cells expressing empty plasmid or KD Src. Differences in protein levels were not due to different mRNA levels since they remained constant (Fig. 2*B*).

The established mechanisms for HIF-1α regulation are stabilization under hypoxia and degradation via the ubiquitin-proteasome pathway under normoxia (10). We therefore compared the half-life of HIF-1α protein in Saos-2 cells expressing the various forms of Src and in the presence or absence of Src inhibitor PP1 in HT29 cells. As shown in Fig. 2, *C* and *D*, the half-life of HIF-1α is similar in all clones and in the presence or absence of PP1 and is very short ($t_{1/2}$ ~5 min), confirming earlier findings (11).

Involvement of the PI3K and the MEK1 Pathways in the Synthesis of HIF-1α—To further investigate whether Src kinase inhibition affects HIF-1α degradation or synthesis and to examine the contribution of the Ras/MAPK and the PI3K/mTOR pathways downstream of Src, we blocked the proteasomal degradation of HIF-1α in HT29 cells using the proteasome inhibitors PI-1 or ALLN (Fig. 3*A*). Inhibition of the proteasome degradative pathway resulted in the accumulation of the HIF-1α protein. This accumulation was completely blocked in the presence of PP1. HIF-1α accumulation was prevented also by the MEK1 inhibitor PD98059, the PI3K inhibitor LY294002 and wortmannin, and to a lesser degree by the inhibitor of mTOR, rapamycin, in Saos-2 cells (Fig. 3, *B* and *C*). PP2, a Src kinase inhibitor that is very similar to PP1, inhibited the accumulation of HIF-1α in a similar fashion to PP1, while PP3, that is similar in structure but does not inhibit Src kinases (18), did not prevent HIF-1α accumulation (Fig. 3*C*). These results suggest that Src signaling is required to up-regulate HIF-1α protein synthesis in a manner independent of protein stabilization. In corroboration of these findings we observed that the accumulation of HA-HIF-1α in the presence of proteasome in-

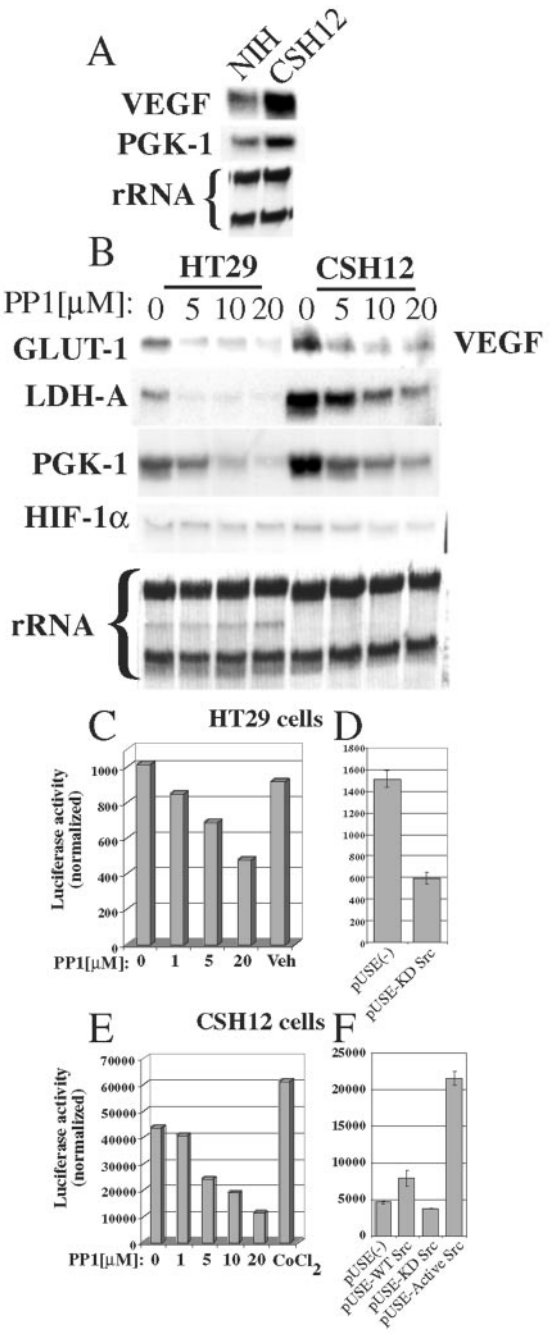

FIG. 1. **Src kinase activity regulates the mRNA levels of hypoxia-regulated genes and the transcriptional activity of HIF-1α.** *A*, the levels of VEGF and PGK1 mRNA transcripts in NIH3T3 and CSH12 cells (NIH3T3 overexpressing EGFR-HER-2 chimeric receptor and possessing constitutive Src activity). *B*, the levels of mRNA transcripts of HIF-1α, Glut-1, PGK-1, LDH-A, or VEGF in CSH12 cells and HT29 cells. HT29 and CSH12 cells were seeded and treated with PP1 at the indicated concentrations (see "Material and Methods"). *C* and *E*, 3×10^5 (HT29) or 8×10^4 (CSH12) cells were seeded on six-well plates (Nunc) and 24 h later were co-transfected with the HRE reporter plasmid (p-tk$_p$-luc), and the CMV-lac-Z plasmid as an internal control. 24 h after transfection the medium was replaced with medium containing PP1 at the concentrations indicated for an additional 24 h. Cells were then lysed and luciferase activity was measured using a luciferase kit (Promega). Luciferase activity was normalized to β-galactosidase activity. The vehicle (*Veh*) in which PP1 was dissolved was 0.02% Me_2SO, 0.4% ethanol. Cobalt chloride ($CoCl_2$) is known to stabilize HIF-1α and to induce HRE. *D* and *F*, cells were co-transfected with p-tk$_p$-luc and the CMV-lac-Z plasmids together with Src constructs (empty plasmid (*pUSE (−)*, wild type c-Src (*pUSE-WT Src*), kinase-dead Src (K297R) (*pUSE-KD Src*), or active Src (Y529F) (*pUSE-Active Src*)) as indicated in the figure and luciferase and β-galactosidase activity was measured 48 h after transfection.

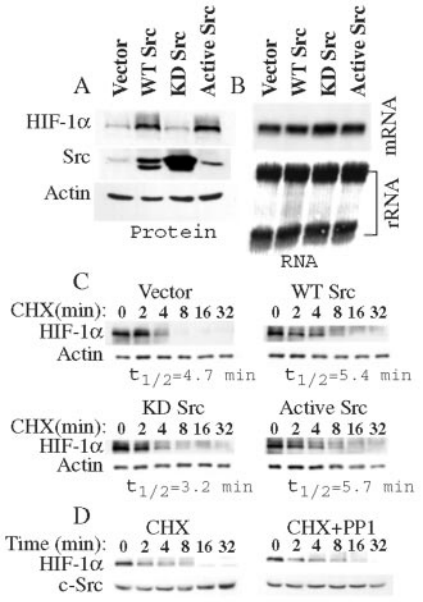

FIG. 2. **Expression of active c-Src elevates the protein levels of HIF-1α with no effect on its mRNA levels or protein stability.** *A*, Saos-2 clones stably expressing empty plasmid (*pUSE*(-)), wild type c-Src (*pUSE-WT Src*), kinase-dead Src (K297R) (*pUSE-KD Src*), or active Src (Y529F) (*pUSE-Active Src*) were seeded on six-well plates (2×10^5 cells) or 10-cm dishes (2×10^6 cells). Cells were lysed with sample buffer, and after Western blotting the blot was probed with antibodies against HIF-1α (1:250 Transduction Laboratories), c-Src (mAb 327), and actin (1:2000 Santa Cruz). *B*, total RNA from the Saos-2 clones (10 μg) was subjected to Northern blotting. The blot was hybridized with a HIF-1α probe. To verify equal loading and transfer, the levels of ribosomal RNA were detected by methylene blue. *C*, Saos-2 clones were incubated in the presence of 100 μM cycloheximide (*CHX*) for the indicated times. Cells were then lysed with sample buffer, and after Western blotting the blot was probed with antibodies to HIF-1α and actin as in *A*. *D*, HT29 cells were grown on 60-mm dishes (10^6), and 24 h later 100 μM cycloheximide was added for the indicated times in the presence or absence of 20 μM PP1. Cells were then lysed with sample buffer and subjected to SDS-PAGE and Western blotting. The blots were probed as in *A*. Calculation of $t_{1/2}$ was performed after quantification of HIF-1α levels normalized to actin levels by the NIH-IMAGE program and was done by the "Regression" program (Blackwell Scientific Software, Osney Mead, Oxford, UK).

hibitors in CSH12 cells was also blocked by co-transfected KD Src, but not by WT Src, active Src, or empty plasmid (Fig. 3*D*). Because Src did not affect the mRNA levels or the half-life of HIF-1α, (Figs. 1 and 2), although inhibition of Src by PP1 or KD Src blocked the accumulation of HIF-1α, we concluded that Src affected the synthesis of HIF-1α.

Src Elevates HIF-1α by Enhancement of Cap-dependent Translation—We next measured the incorporation of [^{35}S]methionine into transfected HA-HIF-1α in CSH12 cells. We found that PP1 and LY294002 had strong inhibitory effects, whereas PD98059 had no effect on HIF-1α synthesis in CSH12 cells (Fig. 4*A*).

Because both the PI3K pathway and the ERK pathway are involved in cap-dependent translation initiation (19–21) and both are downstream to activated Src, we examined the effects of active Src, KD Src, and PP1 on cap-dependent translation initiation activity. We utilized a bi-cistronic construct composed of a luciferase reporter gene followed by an internal ribosome entry site (IRES) sequence with secreted alkaline phosphatase (SeAP) as a reporter (Fig. 4*B*). We measured effects on luciferase activity (cap-dependent translation) relative to effects on SeAP activity (IRES-dependent translation). We found that active Src increased cap-dependent translation, whereas kinase-dead Src and PP1 inhibited cap-dependent

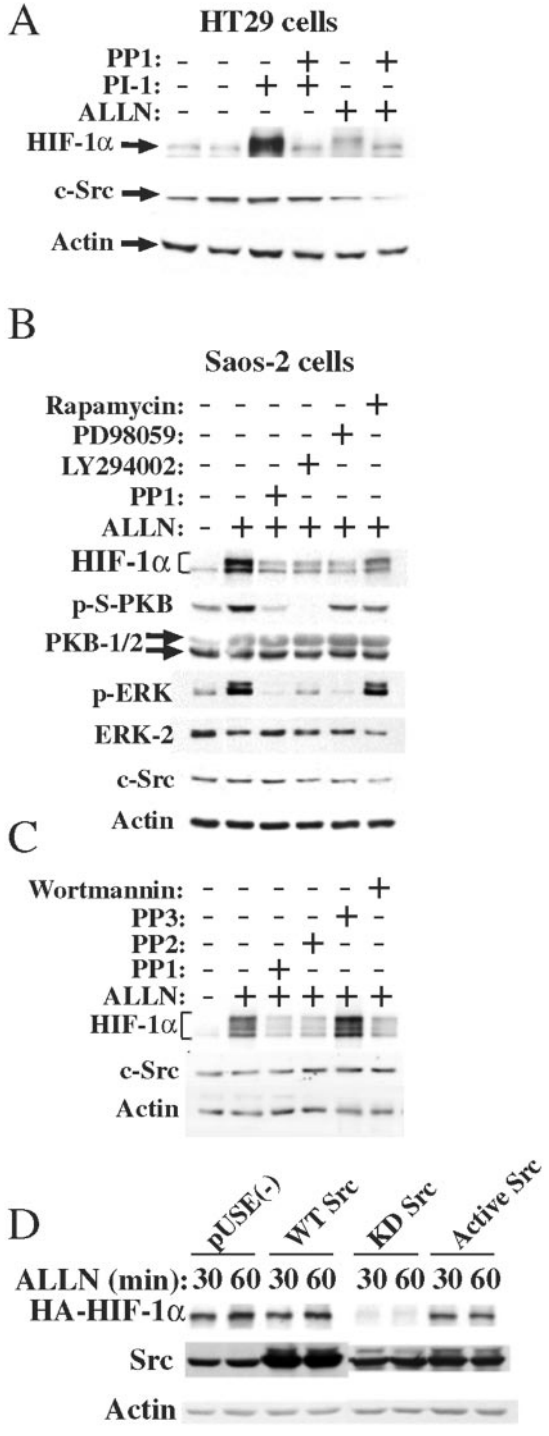

FIG. 3. **Src, MEK1, mTOR, and PI3-kinase inhibitors block the accumulation of HIF-1α protein.** *A*, HT29 cells were seeded on six-well plates (Nunc) (4×10^5 cells/well). After 24 h, the medium was replaced with medium containing the inhibitor (20 μM PP1) for 30 min and then with medium containing the inhibitor and proteasome inhibitors (100 μM PI-1 (Proteasome Inhibitor-1) or 200 μM ALLN (Calbiochem)) for an additional 2 h. Cells were then lysed with sample buffer and after Western blotting were probed as in Fig. 2. *B*, Saos-2 cells were seeded on six-well plates (Nunc) (2×10^5 cells/well). After 24 h, the medium was replaced with medium containing the inhibitor (20 μM PP1, 20 μM LY294002, 50 μM PD98059, or 50 nM rapamycin) for 30 min and then with medium containing the inhibitors and the proteasome inhibitor ALLN (200 μM) (Calbiochem) for an additional 2 h. Cells were then lysed with sample buffer, and after Western blotting the blot was probed with antibodies against HIF-1α (1:250 Transduction Laboratories), c-Src (mAb 327) and actin (1:2000 Santa Cruz), phosphorylated ERK (1:10000 Sigma), phospho-serine-Protein Kinase B (1:1000 NEB), ERK-2 (1:5000 Santa Cruz) and Akt-1, 2 (*PKB*) (1:1000 Santa Cruz). *C*, Saos-2 cells were seeded and treated with PP1, PP2, or PP3 (20 μM) or with wortmannin (200 nM) as in *B*. *D*, CSH12 cells were grown on six-well plates and co-transfected with 25 ng of HA-HIF-1α expression plasmid together with 1 μg of the Src plasmids as indicated. 24 h later, the medium was replaced with medium containing 200 μM ALLN for 30 or 60 min as indicated. Cells were then lysed with sample buffer, and after Western blotting the membranes were probed with anti-HIF-1α, anti-Src, and anti-actin as in *A*.

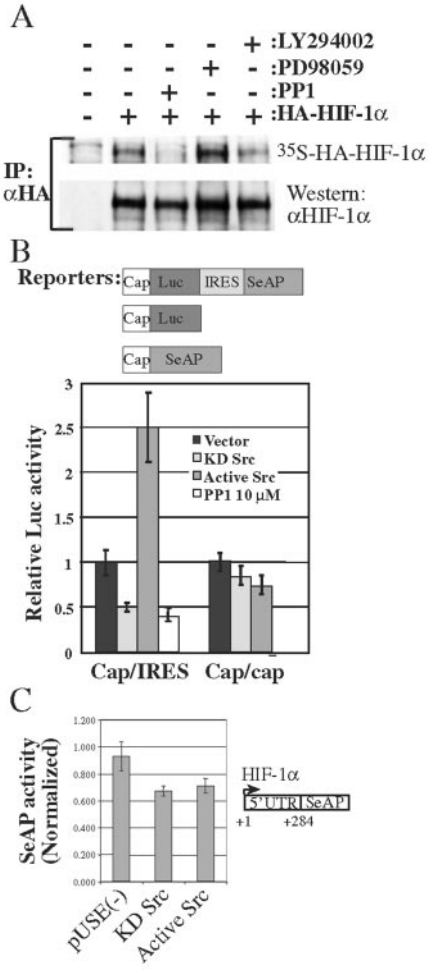

FIG. 4. **Src kinase activity regulates the synthesis of HIF-1α by a cap-dependent mechanism in CSH12 cells.** *A*, CSH12 cells were grown on 10-cm dishes and were co-transfected with HA-HIF-1α expression plasmid (0.5 μg) and pUSE(−) plasmid (5 μg). After 24 h the medium was replaced with methionine- and cysteine-deficient medium for 1 h. 100 μCi/ml of [^{35}S]Met/Cys Promix (Amersham Biosciences) was then added for 1 h in the presence of 200 μM ALLN, which prevents degradation of HA-HIF-1α. HA-HIF-1α was immunoprecipitated as described under "Materials and Methods." After Western blotting, the membrane was exposed for HA-HIF-1α radioactivity and probed as in Fig. 3 for HA-HIF-1α protein levels. *B*, CSH12 cells were seeded on 24-well plates (Nunc). 24 h later, cells were co-transfected with 0.5 μg of Src constructs as indicated and 0.1 μg of luc-IRES-SeAP plasmid (see "Materials and Methods"), or luc and SeAP on separate plasmids. PP1 was added 24 h following transfection. 24 h after addition of PP1, cells were lysed with reporter lysis buffer (Promega), and luciferase and SeAP activities were measured. *C*, the HIF-1α 5′-UTR-SeAP construct was co-transfected as described under "Materials and Methods" with the indicated Src constructs.

translation (Fig. 4*B*). The two reporter genes were also co-transfected as mono-cistronic constructs, namely as single reporters subject to cap-dependent initiation (Fig. 4*B*). Active Src and KD Src affected the cap-SeAP and cap-luc in a similar fashion, thus the ratio of luc to SeAP activities remained the same (Fig. 4*B*), confirming that the effects of Src constructs on translation initiation were independent of the nature of the

reporter used. Because it was asserted recently that the 5′-untranslated region (5′-UTR) of HIF-1α affects its synthesis (22), we cloned the 5′-UTR of HIF-1α (+1 to +284) upstream of the SeAP reporter gene and measured the effects of Src on the reporter activity, as depicted in Fig. 4*C*. We found no significant effect on the reporter activity by the different Src constructs. To examine whether the Src effect on protein synthesis is HIF-1α-specific or whether it affects global protein synthesis, we measured the incorporation of [^{35}S]methionine into Saos-2 and HT29 cells in the absence or presence of Src kinase inhibitors and inhibitors of its downstream signaling pathways. PP1 and PP2 inhibited protein synthesis in Saos-2 and HT29 cells, while PP3, which is similar in structure but does not inhibit Src (18), did not (Fig. 5, *A* and *B*). Inhibition of PI3K by LY294002 and wortmannin or inhibition of its downstream target mTOR by rapamycin also inhibited protein synthesis, and so did the MEK1 inhibitor PD98059. Because the availability of the cap-binding protein eIF4E is the rate-limiting step in the machinery of cap-dependent translation (23), we expressed eIF4E in HeLa cells and examined its effect on the levels of HIF-1α protein. As seen in Fig. 5*C*, expression of eIF4E elevated the levels of HIF-1α.

Inhibition of Src and Its Downstream Pathways Reduces Lactate Production—One of the hallmarks of advanced human tumors is their capacity to conduct efficient glycolysis in the presence of oxygen, nullifying the Pasteur effect and converting pyruvate to lactate by means of lactate dehydrogenase A (24, 25), which is under the transcriptional control of HIF-1α. We show that lactate accumulation was markedly reduced in the presence of Src, PI3K, MEK1, and mTOR inhibitors (PP1, LY294002, PD98059, and rapamycin, respectively), and 100 μM cycloheximide (Fig. 6*A*). Moreover, the rate of production of lactic acid was higher in CSH12 than in parental NIH3T3 cells, which most probably reflects the differences in glycolytic enzyme mRNA levels (Fig. 1*A*).

DISCUSSION

Expression of several oncogenes has previously been found to increase activation of the HIF-1α pathway, but no molecular mechanism has been reported. H-Ras transformation has been reported to increase the level of HIF-1α protein and to induce expression of HIF-1α target genes in both normoxia and hypoxia via PI3K (26, 27). Similarly, expression of v-Src increases HIF-1α protein levels and target gene activation. It has been claimed that the effect of v-Src is transcriptional, thus involving the increase in HIF-1α mRNA (3). p42/p44 mitogen-activated protein kinase (Erk^{MAPK}) has been implicated in HIF-1α phosphorylation, and activation of Erk^{MAPK} has been shown to promote transcriptional activation by HIF-1α (28, 29). In these studies, as in our own (Figs. 2 and 3), the activation of HIF-1α did not appear to involve increased protein stability. We find that active Src induces the synthesis of the HIF-1α protein (Fig. 4) and as a consequence, of its target genes (Fig. 1), with no effect on HIF-1α mRNA levels (Figs. 1 and 2).

In this study we show that the elevated levels of HIF-1α under normoxia, in cells which possess persistently active Src, results from enhanced cap-dependent protein translation (Fig. 4*B* and Fig. 5), mediated by the PI3K-mTOR pathway and, depending on the cell line, also by the Erk^{MAPK} pathway (Figs. 3–5). It is likely that HIF-1α expression is up-regulated by a similar mechanism in other tissues where the activity of protein tyrosine kinases other than Src are enhanced (17, 22, 30, 31). Thus, the elevation in the protein level of HIF-1α that occurs at normal oxygen levels is the outcome of an elevated rate of global protein synthesis rather than reduced rates of HIF-1α degradation (Figs. 2–5). Interestingly, 23 years ago Carroll *et al.* (32) demonstrated that in chicken cells infected with temperature-sensitive v-Src, the v-Src induces aerobic glycolysis (the "Warburg effect") at permissive temperatures, while the cells revert to normal metabolism upon shift to the

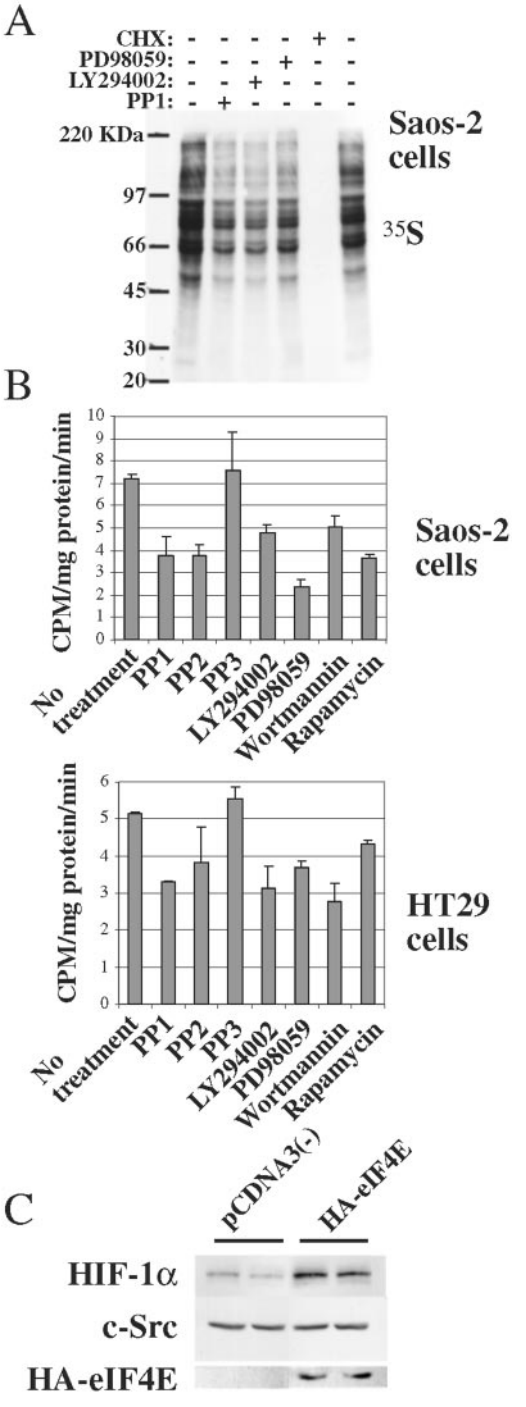

FIG. 5. **Inhibition of Src kinase, PI3K/mTOR or Ras/MAPK pathways reduces protein synthesis rate.** *A*, Saos-2 cells (3×10^5 cells/well) were metabolically labeled with [^{35}S]Met/Cys as described under "Materials and Methods." Equal amounts of protein from each sample were separated on 10% SDS-PAGE and exposed to film. An identical experiment was performed with HT29 cells (not shown). *B*, Saos-2 (3×10^5 cells/well) and HT29 (5×10^5 cells/well) were metabolically labeled with [^{35}S]Met/Cys as described under "Materials and Methods." Protein synthesis rate was calculated as described under "Materials and Methods." *C*, HeLa cells expressing empty plasmid (pCDNA3) or HA-eIF4E were seeded (2×10^5), and 24 h later were lysed with sample buffer. After Western blotting, the membrane was probed with antibodies against HIF-1α and c-Src (as in Fig. 3), and anti-HA tag (1:1000, Roche Molecular Biochemicals).

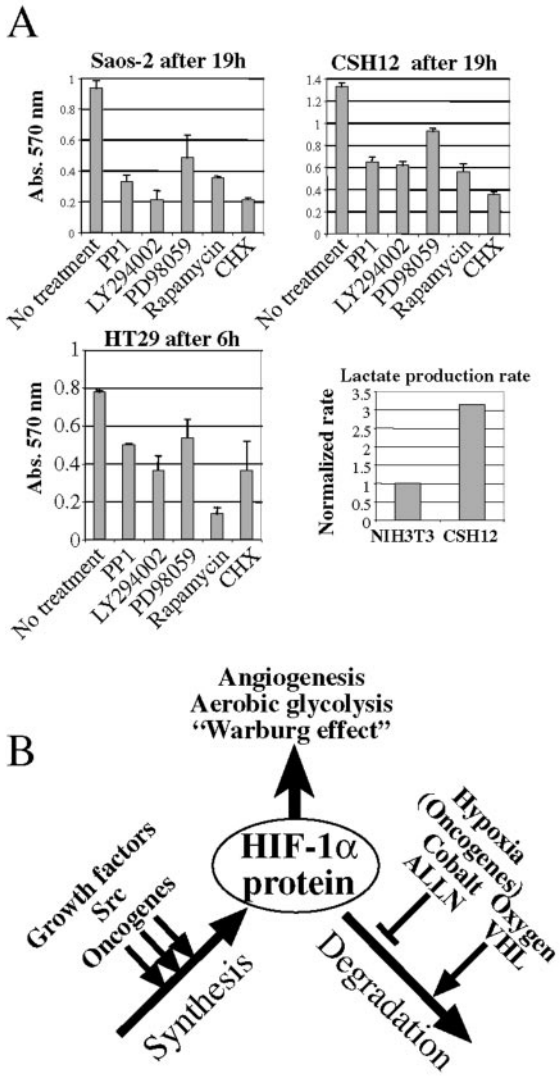

FIG. 6. **Inhibition of Src kinase, PI3K/mTOR, or Ras/MAPK pathways reduces the production of lactic acid.** *A*, CSH12, Saos-2, and HT29 cells were grown on six-well plates, and the amounts of lactic acid in the medium were measured using a lactate detection kit (Sigma). Levels were measured at 6 and 19 h after medium was replaced by medium with dialyzed serum containing the kinase inhibitors as indicated. CSH12 and NIH3T3 cells were grown as in *A*, and the rate of lactate production was calculated from the slope of the accumulation curve over 2, 4, and 6 h. *B*, the steady state level of HIF-1α protein is determined by the balance between the rates of degradation and synthesis. Any protein or oncogene that can enhance HIF-1α translation or stimuli that inhibit its degradation will elevate its protein levels. The outcome will be elevated glycolysis and angiogenesis.

non-permissive temperatures. Our study provides a biochemical mechanism for this classic observation. Moreover, inhibition of protein synthesis in chicken cells resulted in the reversal of the Warburg effect in a fashion similar to our study, which shows that inhibition of Src activity or inhibition of protein synthesis leads to reduced lactate production (Fig. 6*A*).

Taken together, our results suggest that Src activation brings about the up-regulation of cap-dependent translation, dramatically affecting the level of HIF-1α. The translational control of HIF-1α was also implicated in a recent study by Laughner *et al.* (22) who offered a different molecular explanation. These authors showed that HER-2/neu activation elevates HIF-1α levels by enhancing HIF-1α translation through the PI3K-PKB-mTOR pathway. Laughner *et al.* claimed that the enhancement was HIF-1α-specific and was achieved through the structure of the 5′-UTR of HIF-1α mRNA (22). The discrepancy between our study and the study of Laughner *et al.* may be due to different experimental conditions. The exogenous Heregulin added by Laughner *et al.* might have affected other signaling pathways. In that study Src activity was not measured. We expressed Src mutants in cells and thus measured only the direct effect of Src. We also used a slightly different 5′-UTR sequence (containing only the 5′-UTR from +1 to +284 without any sequence from the promoter of HIF-1α), which could lead to differences in the response of the reporter. Our experiments clearly show that the HIF-1α 5′-UTR is not involved in the enhancement of translation by Src (Fig. 4*C*) and that the enhancement of HIF-1α translation results from Src-induced up-regulation of the cap-dependent translational machinery (Figs. 4*B* and Fig. 5). In support of this assertion, we show that the expression of the central component of the cap-dependent translation machinery, namely the cap-binding protein eIF4E, elevated the levels of HIF-1α in HeLa cells (Fig. 5*C*). Moreover, as seen in Fig. 5, *A* and *B*, inhibition of Src kinase and its downstream pathways (*i.e.* PI3K-mTOR and MEK1-ERK) resulted in the inhibition of total protein synthesis in Saos-2 and HT29 cells. Furthermore, Src activity affects levels and synthesis of other short-lived proteins by the same mechanism as for HIF-1α.[2] Our results also provide a biochemical mechanism for the similar HIF-1α up-regulation by other PTKs (17, 22, 30, 31).

In summary, we suggest that the enhanced translation of HIF-1α should lead to a metabolic shift to aerobic glycolysis (Fig. 6*B*) (25). The molecular mechanism we describe most probably accounts for the strong expression of HIF-1α in many advanced tumors under normoxia (33) and leads to the onset of the Warburg effect, which is a hallmark of advanced cancers (Fig. 6*B*).

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