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Cyclin Dependent Kinase Inhibitor $p27^{Kip1}$ Is Upregulated by Hypoxia Via an ARNT Dependent Pathway

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Abstract Expression of cyclin dependent kinase (Cdk) inhibitor $p27^{Kip1}$, which blocks cell cycle progression from G_1 to S phase, can be regulated via multiple mechanisms including transcription, protein degradation, and translation. Recently, it was shown that $p27^{Kip1}$ plays an important role in the cellular response to hypoxia. However, the mechanisms involved in the hypoxia-induced regulation of $p27^{Kip1}$ expression are still not clear. In this study, we compare the expression of $p27^{Kip1}$ in two related murine hepatoma cell lines, Hepa-1 and c4. Hepa-1 produces functional aryl hydrocarbon receptor nuclear translocator (ARNT). c4 cells are derived from Hepa-1, but are ARNT deficient. Interestingly, we observed cell line-dependent effects of hypoxia on the expression of $p27^{Kip1}$. The level of $p27^{Kip1}$ protein in Hepa-1 cells is enhanced by hypoxia, but is reduced by hypoxia in c4 cells. Further investigation demonstrated that hypoxia-induced, ARNT-mediated, transactivation of the $p27^{Kip1}$ gene in Hepa-1 cells is responsible for the increase in $p27^{Kip1}$ protein. Once c4 cells were stably transfected with the wild type ARNT gene, a hypoxia-induced increase in $p27^{Kip1}$ mRNA was observed and reduction of $p27^{Kip1}$ protein caused by hypoxia was blocked. Hence, our data indicate that ARNT is involved in transcriptional upregulation of the $p27^{Kip1}$ gene under hypoxic conditions. J. Cell. Biochem. 90: 548–560, 2003.

Key words: hypoxia; cell cyle; $p27^{Kip1}$; ARNT; HIF

In mammalian cells, molecular oxygen is involved in many important physiological reactions such as ATP synthesis. Cells respond in a variety of ways when subjected to low oxygen concentrations. These responses may include enhanced glycolysis, synthesis of factors that promote vascularization, cell cycle arrest, or apoptosis. The different responses to hypoxia are mediated by enhanced expression of a number of gene products that include erythropoietin [Semenza and Wang, 1992], vascular endothelial growth factor [Goldberg and Schneider, 1994; Levy et al., 1995], several glycolytic enzymes [Firth et al., 1994; Semenza et al., 1994], nitric oxide synthase [Melillo et al., 1997], and the transferrin receptor [Melillo et al., 1997; Bianchi et al., 1999; Tacchini et al., 1999]. The increase in expression of these gene products is regulated at the transcriptional level by hypoxia-inducible factor-1 (Hif-1). Hif-1 is a heterodimer composed of Hif-1α and aryl hydrocarbon receptor nuclear translocator (ARNT, also called Hif-1β) subunits and functions by binding to hypoxia response elements (HREs) within the regulatory regions of these genes [Wang et al., 1995]. Both Hif-1 subunits belong to the PAS family of basic helix-loop-helix transcription factors. In normoxic conditions, Hif-1α protein is rapidly degraded via the ubiquitin proteasome pathway. Upon exposure to hypoxia, Hif-1α protein is stabilized, dimerizes with ARNT, and stimulates transcription of its target genes [Huang et al., 1996; Salceda and Caro, 1997; Huang et al., 1998]. In

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contrast to Hif-1α, ARNT is a constitutively expressed nucleoprotein. In addition to interacting with Hif-1α, ARNT also forms heterodimers with other PAS family transcription factors. These include the aryl hydrocarbon receptor (AhR), which is involved in activation of xenobiotic responsive genes [Ko et al., 1996], and Sim, which plays a role in central midline and central nervous system development [Ema et al., 1996]. It has also been reported that ARNT can form homodimers to transactivate the expression of specific reporter genes [Antonsson et al., 1995; Sogawa et al., 1995; Swanson et al., 1995].

Most solid tumors are chronically subjected to hypoxic conditions [Dachs and Tozer, 2000]. Enhanced utilization of glycolysis and stimulation of angiogenesis in response to hypoxic conditions is thought to give tumor cells a growth advantage and facilitate progression of the disease [Dachs and Tozer, 2000]. Many tumors also overexpress HIF-1α and loss of either VHL or p53 tumor suppressor activity has been shown to lead to stabilization of the Hif-1α protein [Dachs and Tozer, 2000]. In addition, oncogenic signaling pathways can lead to enhanced expression of Hif-1α [Dachs and Tozer, 2000]. Thus, multiple mechanisms contribute to the ability of tumor cells to survive successfully in hypoxic conditions. It may thus be of particular importance to understand the role of hypoxia in the development of cancer.

The effect of low oxygen tension on cell proliferation is complex and appears to be cell type and oxygen concentration dependent. In a number of cases severe hypoxia or anoxia has been shown to promote G_1 cell cycle arrest [Krtolica et al., 1998; Gardner et al., 2001; Green et al., 2001] while in other cases moderate hypoxia (0.5–2% oxygen) has been shown to enhance cell proliferation [Green et al., 2001]. It is likely that cancer cells within a solid tumor are subjected to both severe and moderate levels of hypoxia depending on the level of vascularization and proximity of the cells to vascularized regions. Thus it is important to understand the mechanisms that contribute to cell cycle regulation under various hypoxic conditions.

Cell cycle arrest is regulated by multiple mechanisms including the presence or absence of Cdk inhibitors (CKIs). In particular, the CKI $p27^{Kip1}$ plays a critical role in control of the cellular transition from G1 to S phase. Downregulation of $p27^{Kip1}$ protein is required for S phase entry, whereas upregulation or forced overexpression of $p27^{Kip1}$ blocks cell cycle progression in G1 phase. Multiple mechanisms, including the rates of translation [Agrawal et al., 1996; Hengst and Reed, 1996; Millard et al., 1997; Miskimins et al., 2001], protein degradation [Pagano et al., 1995], and transcription of the $p27^{Kip1}$ gene [Kwon et al., 1997; Inoue et al., 1999; Kolluri et al., 1999; Dijkers et al., 2000; Servant et al., 2000; Hirano et al., 2001], regulate the levels of endogenous $p27^{Kip1}$ protein. A number of investigators have recently demonstrated that $p27^{Kip1}$ levels are enhanced by hypoxia [Krtolica et al., 1998; Krtolica et al., 1999; Gardner et al., 2001] and that for some cell types $p27^{Kip1}$ is essential for hypoxia-mediated cell cycle arrest [Gardner et al., 2001].

In this study, hypoxia-induced changes in $p27^{Kip1}$ expression were investigated in two cell lines: Hepa-1, which produces functional ARNT, and its derivative c4, which expresses ARNT at very low levels due to a point mutation that leads to rapid turnover of the protein [Numayama-Tsuruta et al., 1997]. As shown in several other cell types, the levels of $p27^{Kip1}$ in Hepa-1 cells increase in response to hypoxia. However, in the ARNT-deficient c4 line hypoxic conditions lead to decreased expression of $p27^{Kip1}$. Hypoxia-mediated loss of $p27^{Kip1}$ expression in this line correlates with enhanced entry into S phase. The mechanisms leading to these changes in $p27^{Kip1}$ expression were investigated and indicate that ARNT plays a key role in the upregulation and maintenance of $p27^{Kip1}$ in hypoxic conditions.

MATERIALS AND METHODS

Cell Culture

Hepa-1, c4, Swiss/3T3, HTC, and H4-II-EC3 (EC3) cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. For normoxic conditions cells were maintained in an atmosphere of 5% CO_2 with the balance air. For hypoxic conditions, cells were incubated in a modular incubator chamber (Billups-Rothenberg) in an atmosphere of 1% O_2, 5% CO_2, and the balance N_2. Oxygen levels were measured using a Vacumed Fast Response O_2 analyzer and ranged from an initial value of 1–1.5% after 24 h.

Western Blotting

Cells were directly lysed in sample buffer [2.5% SDS, 2.5 mM Tris-Cl (pH 6.8), 100 mM dithiothreitol, 10% glycerol, 0.025% pyronine Y]. Samples were separated on a 10% SDS–polyacrylamide gel and transferred to Immobilon P membranes. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline [10 mM Tris-Cl (pH 7.5), 150 mM NaCl] containing 0.1% Tween-20 (TBS-T) for one hour at room temperature. Following incubation with the appropriate primary antibody for 1 h, the membrane was extensively washed in TBS-T. The membrane was then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for another hour. After extensively washing in TBS-T, proteins were detected using the SuperSignal chemiluminescence system (Pierce Chemical Co., Chester, UK). Anti-$p27^{Kip1}$ (K25020) and anti-ARNT were purchased from Transduction Laboratories (San Diego, CA). Anti-actin was purchased from Sigma Chemical (St. Louis, MO). Anti-Cdk2 and anti-mouse HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Metabolic labeling

For pulse labeling experiments, cells were incubated in normoxic or hypoxic conditions for 7 hr, washed three times with DMEM lacking Met and Cys, and then incubated in 4 ml of the same medium containing ^{35}S-labeled Met and Cys (150 μCi/ml; Tran^{35}S-label, ICN) for 1 hr in normoxic or hypoxic conditions. Cells were lysed as previously described [Wang et al., 2000]. Briefly, after washing with Dulbecco's phosphate-buffered saline (PBS), cells were harvested in 500 μl lysis buffer (PBS containing 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 0.1 U/ml aprotinin, 5 μg/ml leupeptin, and 0.1 mM sodium orthovanadate). The cell lysate was sonicated and then centrifuged to remove insoluble material. The protein concentration of the supernatant was estimated using the Bio-Rad protein assay. Equal amounts of protein from each sample were incubated with rabbit anti-$p27^{Kip1}$ polyclonal antibody (3 μg, Santa Cruz Biotechnology, sc-76) for 3 h at 4°C. Protein-A-conjugated agarose beads (30 μl) were added and the incubation continued for another 2 h. The beads were washed with lysis buffer five times and once with lysis buffer supplemented with 1 mg/ml Met and Cys. After the final wash, the supernatant was discarded and 60 μl SDS-sample buffer was added. After heating at 70°C for 5 min the sample was loaded on a 10% SDS–polyacrylamide gel.

For pulse-chase experiments, the cells were labeled as described above. After a 1 h pulse, the cells were washed three times with DMEM. Incubation in normoxic or hypoxic conditions was then continued for up to 6 h. After washing, cells were lysed and $p27^{Kip1}$ immunoprecipitation was performed as described above.

For determination of total protein synthesis, the cells were labeled and harvested as described in pulse labeling experiments. Equal amounts of protein from each sample were diluted to a final volume of 50 μl in lysis buffer. Protein was precipitated by addition of 500 μl cold 8% trichloroacetic acid (TCA). After centrifugation at full speed in a Marathon 16KM microcentrifuge at 4°C for 15 min, the pellet was washed with cold acetone and dried. Labeled protein was determined using a scintillation counter.

RNase Protection Assays

RNase protection assays were performed using the Hybspeed RPA kit (Ambion). A mouse $p27^{Kip1}$ cDNA cloned into pBluescript(SK+) (a generous gift of Tony Hunter) was linearized with Pst I and Ava II and transcribed in vitro in the presence of ^{32}P-labeled UTP using T3 RNA polymerase (MaxiScript kit, Ambion). For each assay 1×10^5 cpm of labeled probe was used. The protected RNA fragment was detected on a 5% denaturing polyacrylamide gel.

Transient and Stable Transfections

Transient cotransfection of the HRE reporter construct with CMV-β-Gal was performed using a total of 2 μg of DNA and GenePORTER Transfection Reagent (Gene Therapy Systems) following the manufacturer's protocol. Cells were plated in 35 mm dishes. One day post-transfection, the cells were incubated under normoxic or hypoxic conditions for an additional 24 h. Cotransfection of the $p27^{Kip1}$ promoter construct [Kwon et al., 1996] with phRL-TK (Promega) was performed under the culture conditions described by Gardner et al. [2001]. For these experiments, 3 μg of the $p27^{Kip1}$ promoter construct and 1 μg of the transfection control plasmid (phRL-TK) were transfected using GenePORTER.

For stable transfections, cells were cotransfected with CMV4-wtARNT plus pcDNA3.1-Hygro+ at a ratio of 20:1. Three days post-transfection, the cells were switched into medium containing 200 μg/ml hygromycin B. After selecting for two weeks, individual colonies were picked and expanded. Each stable line was tested for expression of the ARNT protein by Western blotting.

Reporter Gene Assays

For firefly luciferase assays, cells in 35 mm dishes were lysed in 200 μl 1× Reporter Lysis Buffer (Promega, Southampton, UK). The lysate was centrifuged at full speed in a Marathon 16KM microcentrifuge at 4°C for 15 min. The supernatant (100 μl) was incubated with an equal volume of SteadyGlo luciferase substrate (Promega) plus 1 μl of a mixture of $CaCl_2$ and $MgCl_2$ (100 mM each). Luciferase activity was determined using a TopCount luminometer (Packard Instruments).

The same lysate was used for determination of β-galactosidase (β-Gal) activity. For each sample, 5 μl of the cell lysate was assayed using the Galacto-Light Plus chemiluminescence system (Tropix) following the manufacturer's protocol. Activity was determined using a TopCount luminometer (Packard Instruments).

Dual luciferase assays were performed according to the manufacturer's protocol using the Dual-Glo assay system (Promega) and 25 μl of cell extract.

Labeling of S-Phase Cells With Bromodeoxyuridine (BrdU)

After hypoxic treatment of cells for 23 h, BrdU was added directly into the medium. Incubation under normoxic or hypoxic conditions was continued for another hour. The cells were then fixed and cells that had entered S-phase were detected using the BrdU Labeling and Detection Kit II (Roche, Indianapolis, IN) following the protocol provided by the manufacturer. Random fields were viewed under a microscope and the number of labeled and unlabeled cells was determined. At least 5 separate fields were analyzed for each plate.

Lactate Dehydrogenase (LDH) Activity Assays

After cells were treated with hypoxia for 24 h, cells were lysed in 1× PBS containing 0.1% Triton X-100. LDH activity was measured by monitoring the decrease in A_{340} over three minutes due to the production of NAD during the reduction of pyruvate.

RESULTS

Effect of Hypoxia on the Levels of $p27^{Kip1}$ Protein

One cellular response to hypoxia is cell cycle arrest in G_1 which, in some cases, has been demonstrated to require $p27^{Kip1}$ [Gardner et al., 2001b]. We investigated hypoxia-induced alterations in $p27^{Kip1}$ protein levels in several cell lines including Swiss/3T3, HTC, EC3, Hepa-1, and c4 (Fig. 1A). Swiss/3T3 is an immortal fibroblast cell line. Hepa-1 and c4 are mouse hepatoma cells. HTC and EC3 are rat hepatoma cells. Consistent with the previous reports using other cell lines [Krtolica et al., 1998; Krtolica et al., 1999; Gardner et al., 2001b], the levels of $p27^{Kip1}$ protein in Swiss/3T3, Hepa-1, HTC, and EC3 increased after hypoxia treatment for 24 hr. Surprisingly, there was a significant decrease in $p27^{Kip1}$ protein in c4 cells by the same treatment (Fig. 1A). The c4 cell line was derived from Hepa-1 [Hankinson, 1979; Hankinson, 1983; Probst et al., 1993]. The only known difference between the two lines is that c4 expresses very low levels of ARNT (see Fig. 1C). This is due to a Gly to Asp mutation which makes the protein unstable [Numayama-Tsuruta et al., 1997]. Thus the ARNT deficiency in c4 cells correlates with decreased $p27^{Kip1}$ protein levels in hypoxia-treated c4 cells.

We further examined the time course of hypoxia-induced changes in $p27^{Kip1}$ levels in the Hepa-1 and c4 cell lines. Cells were treated with hypoxia for up to 24 h and western blotting was performed to assess $p27^{Kip1}$ protein levels. In Hepa-1, no significant change in $p27^{Kip1}$ was observed by 4 h. Elevated $p27^{Kip1}$ was first detected after 8 h of hypoxia (Fig. 1B) and continued to increase out to 24 h. Over a similar time course, $p27^{Kip1}$ levels were significantly decreased in hypoxic c4 cells (Fig. 1C). These results indicate that the ARNT deficiency in c4 cells results in reduction of $p27^{Kip1}$ protein levels.

It is known that downregulation of $p27^{Kip1}$ protein levels is required for entry into S-phase, whereas increased or forced expression of $p27^{Kip1}$ blocks cell cycle progression in G_1. Therefore, we determined the percentage of S-phase cells in Hepa-1 and c4 cells under hypoxic

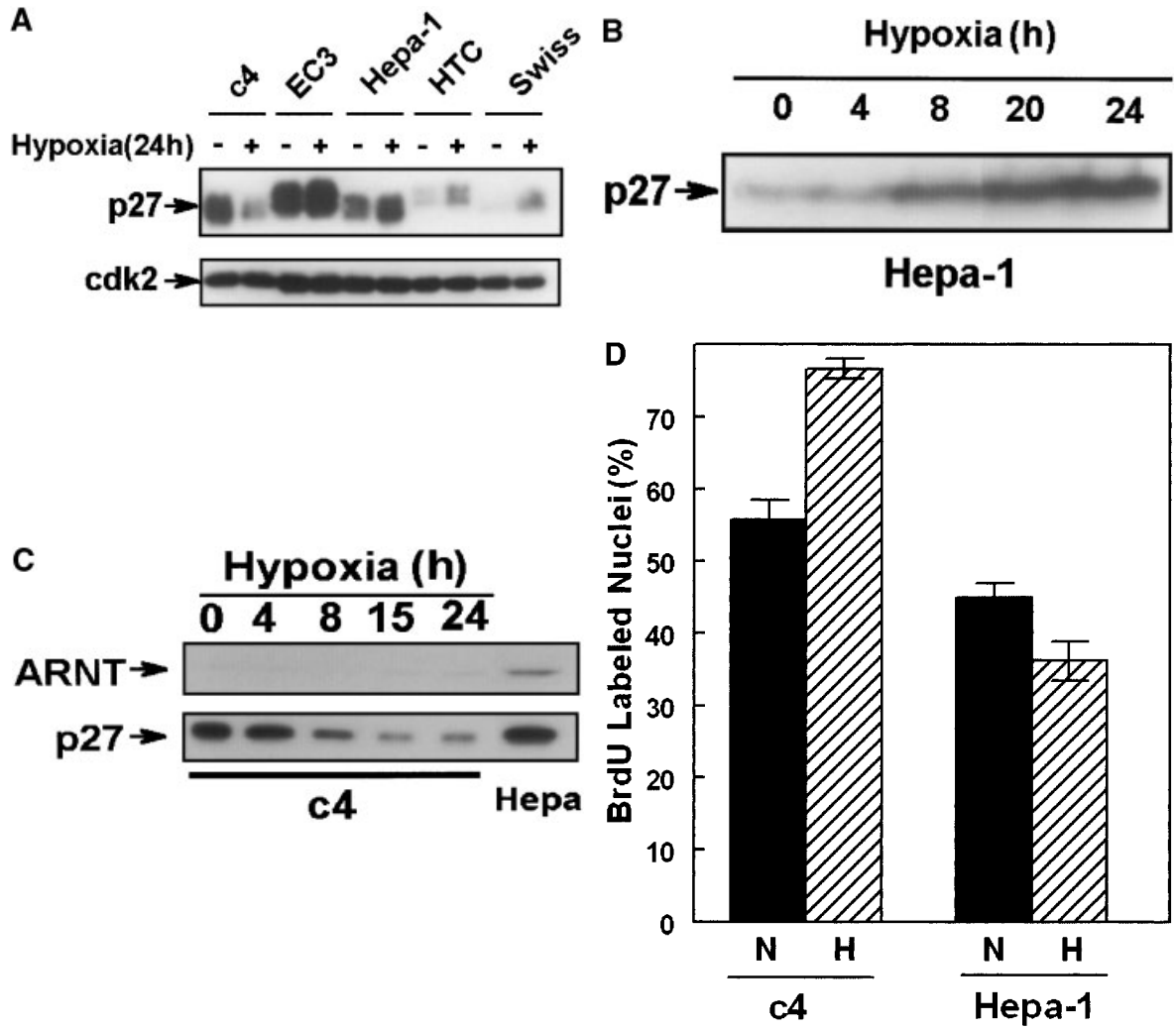

Fig. 1. Hypoxia-mediated changes in the level of $p27^{Kip1}$ protein. **A**: EC3, HTC, Swiss/3T3, Hepa-1, and c4 cells were incubated under normoxic or hypoxic conditions for 24 hours. The expression of $p27^{Kip1}$ and Cdk2 were then detected by Western blotting. Hepa-1 **(B)** and c4 **(C)** cells were incubated under hypoxic conditions for the indicated times and $p27^{Kip1}$ protein or ARNT was detected by Western blotting. In all experiments, the same amount of protein was loaded per lane. In (C), the levels of ARNT and $p27^{Kip1}$ in normoxic Hepa-1 cells was used as a comparison to those in c4 cells. **D**: Hepa-1 and c4 cells were incubated under normoxic (N) or hypoxic (H) conditions for 23 h and then labeled with BrdU for 1 h. The number of labeled cells (mean ± SEM) is shown.

conditions using a BrdU-labeling method. In Hepa-1 cells, hypoxia treatment caused a slight decrease in the percentage of BrdU-labeled nuclei. In contrast, the same treatment produced a significant increase in the percentage of BrdU labeled nuclei in c4 cells (Fig. 1D). The inverse correlation between the level of $p27^{Kip1}$ protein and number of cells in S-phase in response to hypoxia treatment implies that $p27^{Kip1}$ may play an important role in hypoxia-induced changes in cell cycle progression.

Effect of Hypoxia on $p27^{Kip1}$ Synthesis and Turnover

To define the key steps in the hypoxia-induced increase in $p27^{Kip1}$ levels in Hepa-1 cells and the downregulation of $p27^{Kip1}$ in c4 cells, pulse labeling and pulse-chase experiments were performed to determine the rates of $p27^{Kip1}$ protein synthesis and degradation. In Hepa-1 cells incubation under hypoxic conditions for 8 h leads to an increase in $p27^{Kip1}$ protein synthesis

compared to normoxic cells (Fig. 2A, compare 0 h time points). In contrast, hypoxia caused a decrease in $p27^{Kip1}$ synthesis in c4 cells (Fig. 2B). In Hepa-1 cells (Fig. 2A), there also appears to be somewhat of a decrease in $p27^{Kip1}$ turnover after hypoxic treatment with an estimated increase in half-life of about 2 h (from ~2.1 h in normoxic cells to 4.1 h in hypoxic cells; Fig. 2A). In c4 cells (Fig. 2B), there is no difference in $p27^{Kip1}$ turnover after exposure to hypoxic conditions ($t_{1/2} \sim 2.1$ h in normoxic cells and ~2.0 in hypoxic cells). Thus the loss of $p27^{Kip1}$ expression in c4 cells in response to hypoxia does not involve enhanced degradation of the protein.

The effect of hypoxia on the global level of protein synthesis was also estimated for both cell lines. At various times after incubation under hypoxic conditions the cells were pulse labeled and harvested as described above. Radioactivity in the TCA insoluble fraction was then used as an estimate of total protein synthesis. Consistent with previous findings [Heacock and Sutherland, 1990; Amellem and Pettersen, 1991], the overall rate of protein synthesis declined in both Hepa-1 and c4 cells under hypoxic conditions (Fig. 2C).

Hypoxia Enhances the Levels of $p27^{Kip1}$ mRNA in Hepa-1 But Not c4 Cells

To determine if the observed changes in $p27^{Kip1}$ synthesis reflect increased levels of $p27^{Kip1}$ mRNA, cells were exposed to hypoxia over a 24 h time course and RNase protection assays were performed. In Hepa-1 cells $p27^{Kip1}$ mRNA levels increased within 4 h of hypoxia treatment and remained elevated for up to 24 h (Fig. 3A). However, no change in $p27^{Kip1}$ mRNA levels was observed in c4 in response to hypoxia (Fig. 3A).

The possibility that mRNA stabilization contributes to the accumulation of $p27^{Kip1}$ mRNA in hypoxic Hepa-1 cells was assessed by measuring the rate of decay of $p27^{Kip1}$ mRNA following the addition of actinomycin D. There was very little difference in the rate of decay between treated and untreated cells over a 3-h time period (Fig. 3B). In fact, when normalized using actin mRNA levels, the $p27^{Kip1}$ mRNA appears to be slightly more stable in normoxic conditions than hypoxic conditions (Fig. 3C). Thus, the observed increase in $p27^{Kip1}$ mRNA in Hepa-1 cells is most likely due to transcriptional activation of the $p27^{Kip1}$ gene.

Wild-Type ARNT Rescues the Hypoxia-Induced Decrease in $p27^{Kip1}$ Protein in c4 Cells

Since the only known difference between Hepa-1 and c4 cells is the level of active ARNT, it is likely that ARNT plays an essential role in hypoxia-induced activation of $p27^{Kip1}$ expression. To directly test this, wild-type ARNT was stably transfected into c4 cells to determine if it could reverse the decrease in $p27^{Kip1}$ that occurs under hypoxic conditions. Two c4 derived cell lines (named c4wt62 and c4wt63) that stably express ARNT protein as determined by western blotting were selected for further analysis (Fig. 4). It is known that the expression of the lactate dehydrogenase-A (LDH-A) gene is activated by hypoxia through an Hif-1α/ARNT-dependent pathway. To further characterize the activity of stably transfected wild type ARNT, both stable cell lines were treated with hypoxia for 24 h and the endogenous LDH activity was then analyzed. In contrast to the parental c4 cells, in which LDH activity is not responsive to hypoxia, LDH activity in both stably transfected cell lines is induced. However, the level of induction is somewhat reduced compared to that observed in Hepa-1 cells (Fig. 4A).

These stable cells lines were further tested for their ability to express a luciferase reporter construct driven by a multimerized HRE. In parental c4 cells, expression of this construct declined after 24 h of hypoxia, similar to the change in $p27^{Kip1}$ expression. However, stable expression of ARNT in c4 cells restored the ability of hypoxia to enhance activity of luciferase from the HRE reporter gene to levels similar to that observed in Hepa-1 cells (Fig. 4B). It is known that the Hif-1α/ARNT heterodimer is required for hypoxia-induced activation of both LDH and the HRE reporter gene construct. Therefore, our data indicate that the wild-type ARNT expressed in c4wt62 and c4wt63 cells is functional and suggest that it is able to heterodimerize with Hif-1α to transactivate hypoxia-responsive genes.

The c4-derived lines expressing wild-type ARNT were also examined for $p27^{Kip1}$ expression following treatment with hypoxia for 24 hours (Fig. 4C). In contrast to the parental c4 line, there was no hypoxia-mediated decrease in $p27^{Kip1}$ in the ARNT transfected lines. However, $p27^{Kip1}$ levels did not increase to the same extent as observed in Hepa-1. One

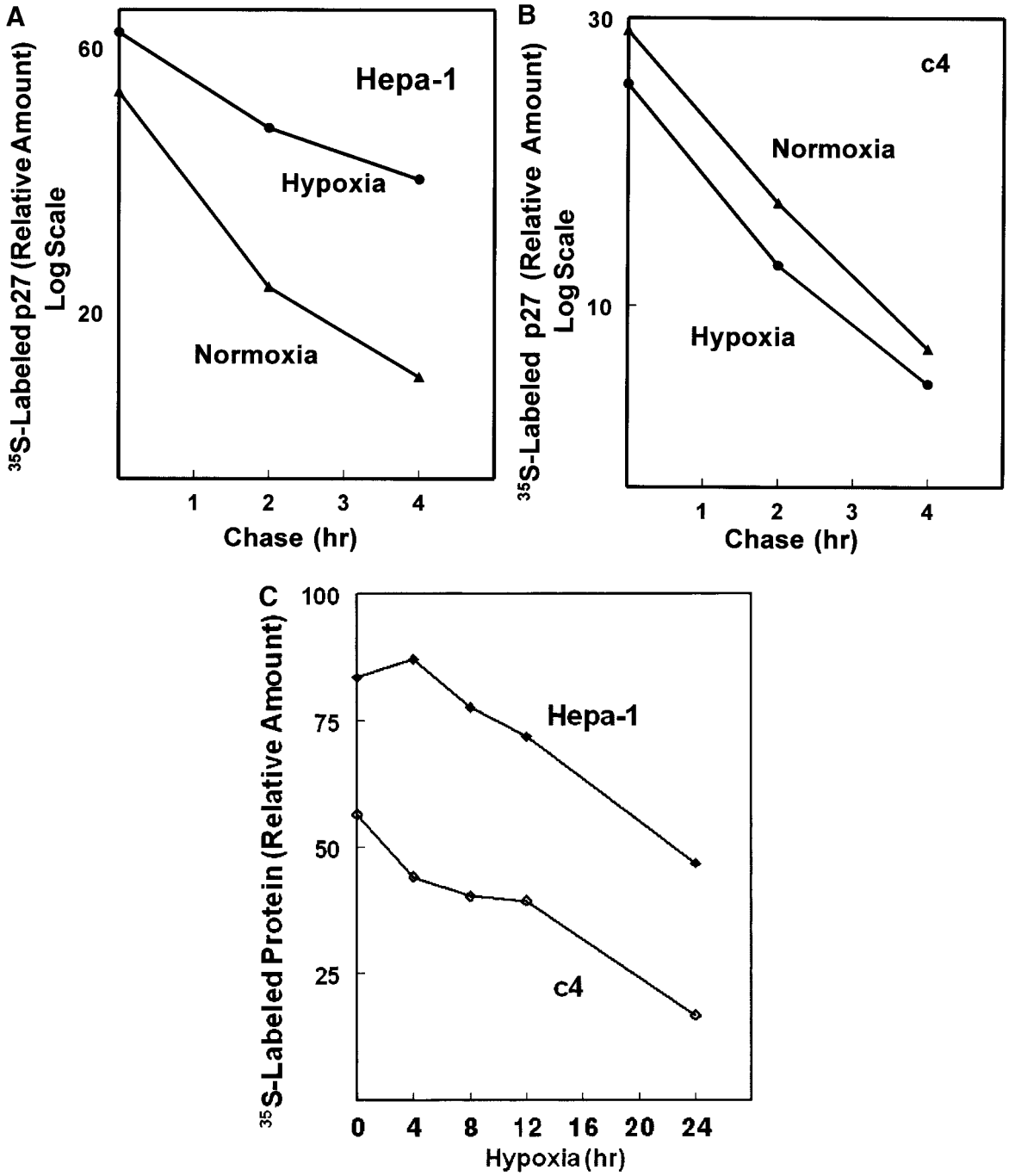

Fig. 2. Synthesis and degradation of $p27^{Kip1}$ protein in Hepa-1 **(A)** and c4 **(B)** cells. Hepa-1 or c4 cells were incubated under normoxic or hypoxic conditions for 7 h. The cells were then pulse labeled with [^{35}S]Met and [^{35}S]Cys for 1 h followed by a chase for 2, 4, or 6 h under the same conditions in the absence of labeled amino acids. $p27^{Kip1}$ was immunoprecipitated from the cell extracts, separated by SDS–PAGE, and the levels estimated by densitometry of the autoradiograph. **C**: Hypoxia represses general protein synthesis. Hepa-1 or c4 cells were exposed to hypoxia for up to 24 h. For each time point, cells were labeled with [^{35}S]Met and [^{35}S]Cys and the incubation was continued for another hour. The same amount of total cellular protein from the cell lysate was precipitated by the addition of cold TCA. Newly synthesized total protein in the TCA-insoluble material was counted in a liquid scintillation counter.

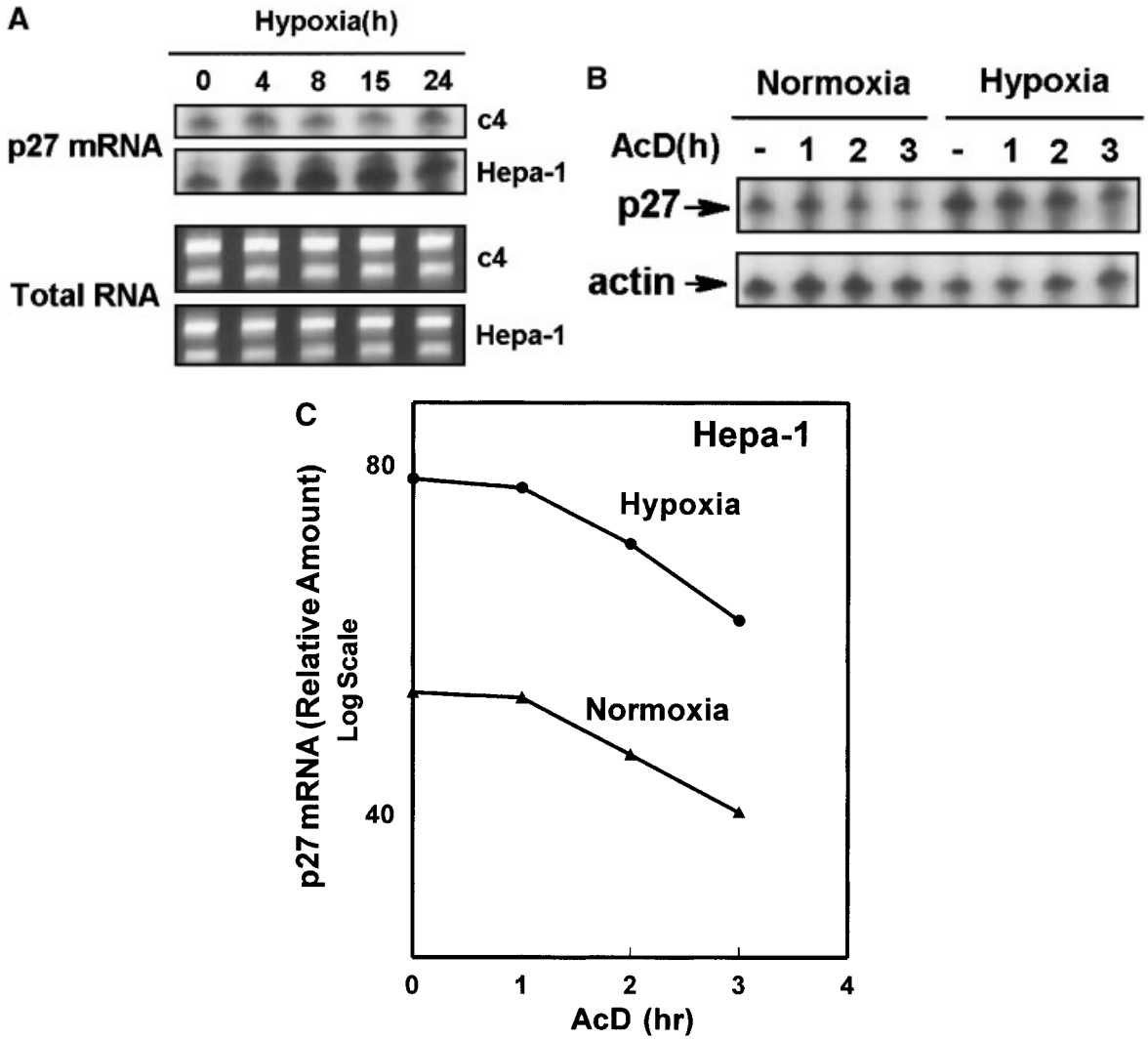

Fig. 3. **A**: Hypoxic treatment enhances the levels of p27^{Kip1} mRNA in Hepa-1 cells but has no effect on the levels of p27^{Kip1} mRNA in c4 cells. Total cellular RNA was isolated from Hepa-1 or c4 cells exposed to hypoxia for the times indicated. RNase protection assays were performed to detect p27^{Kip1} mRNA. Total RNA from the same samples is shown as a loading control. **B**: The rate of p27^{Kip1} mRNA decay is not altered by hypoxia in Hepa-1 cells. Hepa-1 cells were grown under hypoxic condition for 21 h. Actinomycin D (5 μg/ml) was then added into the medium and the incubation was continued for 1, 2, or 3 h under hypoxic or normoxic conditions prior to RNase protection analysis. The level of actin mRNA was used as a control. **C**: Plot of densitometric scan of data in panel B. The levels of p27^{Kip1} mRNA were normalized relative to actin mRNA levels.

observation of potential relevance is that hypoxia also leads to increased levels of ARNT in Hepa-1 cells (Fig. 4C). We have observed a similar induction of ARNT by hypoxia in mouse fibroblasts (not shown). However, in the transfected cells, in which ARNT is expressed under the control of the CMV promoter, there is no increase in the level of ARNT in response to hypoxia. In these stably transfected cell lines, the levels of ARNT are similar to those found in Hepa-1 in normoxic conditions. A second possibility is that the mutant ARNT, although it is rapidly degraded, has a dominant interfering effect on the exogenously expressed ARNT.

As with Hepa-1 cells, the level of p27^{Kip1} mRNA was found to increase in the c4wt62 stable cell line after 24 h in hypoxic conditions (Fig. 4D). This further demonstrates the critical role of ARNT in modulating levels of functional p27^{Kip1} protein in hypoxia treated cells.

Hypoxia Enhances the Activity of the p27^{Kip1} Promoter

Gardner et al. [2001] demonstrated that the mouse p27^{Kip1} promoter is responsive to

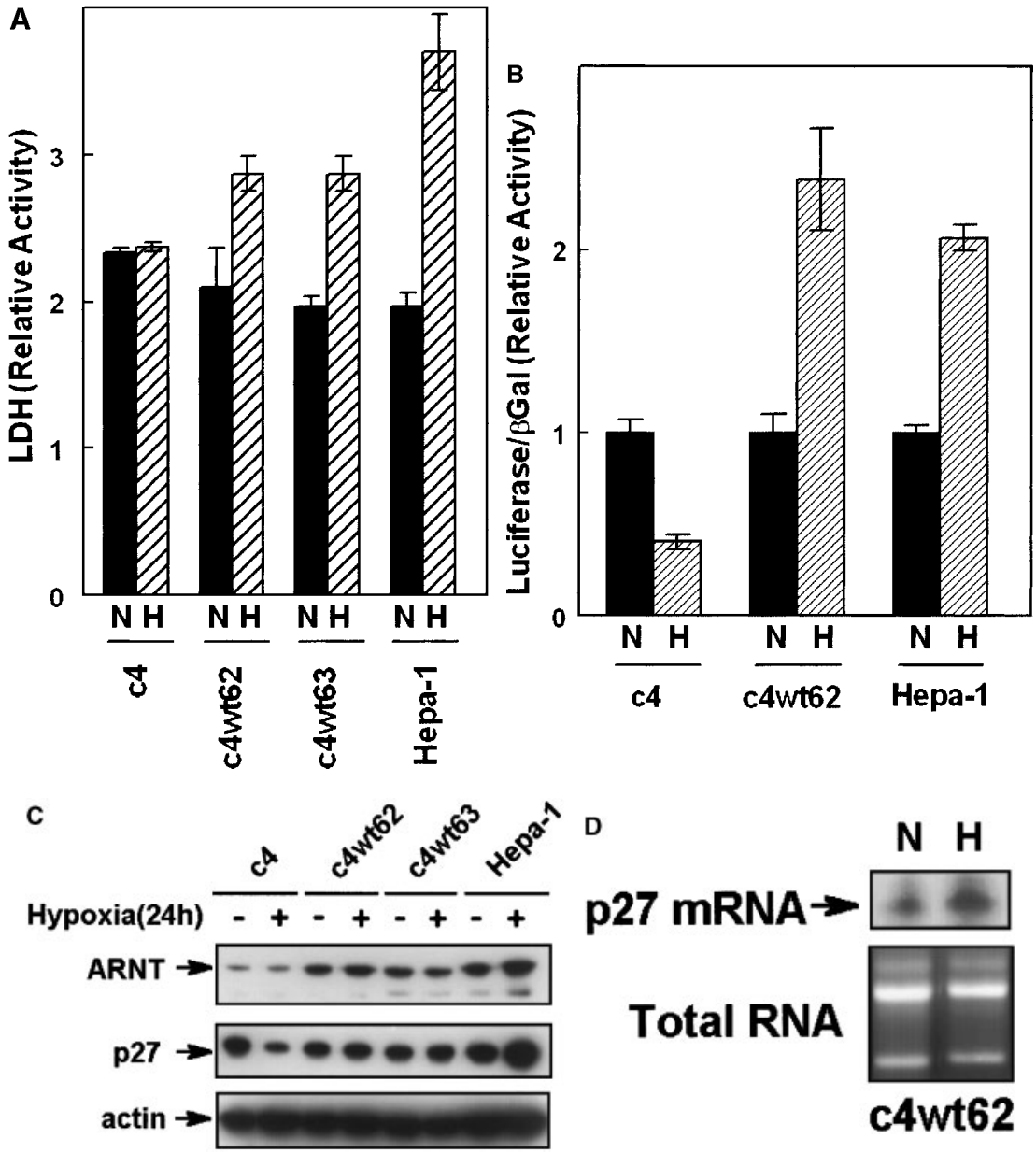

Fig. 4. Wild type ARNT is involved in the hypoxia-induced increase in $p27^{Kip1}$ transcription. **A**: c4 cells were stably transfected with wild-type ARNT. Lines derived from two independent colonies (c4wt62 and c4wt63) were determined to express wild type ARNT at levels similar to Hepa-1 cells (see C). These two stably transfected cell lines, c4, and Hepa-1 were exposed to normoxic (N) or hypoxic (H) conditions for 24 h and then LDH activity assays were performed. The ratio of LDH activity to protein concentration is shown. **B**: c4, c4wt62, and Hepa-1 cells were transiently transfected with an HRE/luciferase reporter gene together with a β-Gal expression construct. Transfected cells were treated with hypoxia for 24 h and then luciferase and β-Gal activities were analyzed. The ratio (the average value of triplicate samples ± SD) of luciferase to β-Gal activity is shown. **C**: c4, c4wt62, c4wt63, and Hepa-1 cells were treated with hypoxia for 24 h followed by Western blotting to analyze the levels of $p27^{Kip1}$ protein and ARNT. The level of actin protein was used as control. **D**: After exposure to normoxia (N) or hypoxia (H) for 24 h, the mRNA from c4wt62 was isolated and the level of $p27^{Kip1}$ mRNA determined using RNase protection assays.

hypoxia in NIH 3T3 cells. They observed a 2.8-fold induction of promoter activity that required both proximal and distal regions of the $p27^{Kip1}$ 5′ flanking regions. To verify this effect, NIH 3T3 cells were transfected with a luciferase reporter construct driven by $p27^{Kip1}$ promoter sequences extending 690 nucleotides upstream of the major transcription start site (Fig. 5). The level

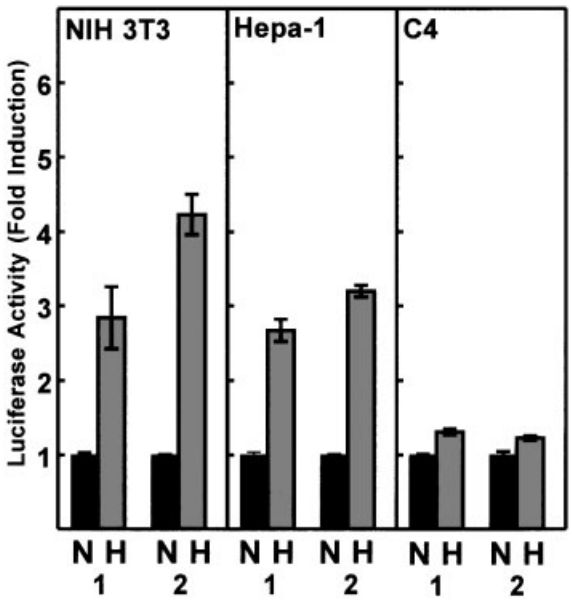

Fig. 5. Hypoxia induced activation of the p27^{Kip1} promoter. NIH 3T3, Hepa-1 or C4 cells were transiently cotransfected with a p27^{Kip1} promoter/firefly luciferase construct (containing 690 nucleotides upstream of the major transcriptional start site) and a TK promoter/Renilla luciferase construct and then subjected to normoxic (N) or hypoxic (H) conditions. The data was normalized by dividing firefly luciferase activity by Renilla luciferase activity. The results of two independent experiments are shown. Each transfection was performed in triplicate and the error bars represent the standard error of the mean.

of induction by hypoxia was very similar to that obtained by Gardner et al. [2001]. When the same experiment was performed in Hepa-1 cells hypoxia also enhanced expression from the p27^{kip1} promoter to a similar level as that observed in 3T3 cells. However, there was only a very slight increase in promoter activity in C4 cells, supporting the conclusion that ARNT is necessary for the response.

DISCUSSION

The data presented here identify ARNT as an important factor in increasing p27^{Kip1} levels. This conclusion is supported by data showing that p27^{Kip1} is elevated in hypoxic Hepa-1 cells while in c4 cells, a derivative of Hepa-1 that expresses very low levels of ARNT, p27^{Kip1} expression declines under hypoxic conditions. Furthermore, stable transfection of wild-type ARNT into c4 cells prevented the downregulation of p27^{Kip1} in response to hypoxia.

BrdU labeling experiments suggest that the ARNT-mediated increase in p27^{Kip1} levels plays a critical role in hypoxia-induced inhibition of cell cycle progression. The response of c4 cells to hypoxia in terms of cell proliferation, like the change in p27^{Kip1} levels, was opposite to that of Hepa-1. Thus, enhanced entry of hypoxic c4 cells into S-phase correlates with lack of ARNT and downregulation of p27^{Kip1}. This is consistent with other recently reported findings. The data of Krtolica et al. [1998, 1999] indicate that hypoxia-induced upregulation of p27^{Kip1}, through inhibition of Cdk activity, is involved in accumulation of hypophosphorylated pRB leading to cell cycle arrest. In addition, Gardner et al. [2001], using cells derived from knockout mice, demonstrated that both p27^{Kip1} and pRB are necessary for hypoxia-induced cell cycle arrest. In contrast to these findings, Green et al. [2001] showed that immortalized mouse embryo fibroblasts from p27^{Kip1} knockout mice arrest in G_1 after exposure to hypoxic conditions. However, they showed that p27^{Kip1} can affect the rate of cell cycle re-entry following reoxygenation. The reason for these diverse observations is unknown but may be related to the cell type used, the specific complement of HLH-PAS domain proteins present in these cell types, or differences in the method of hypoxia treatment.

Cellular p27^{Kip1} levels are responsive to numerous signals and stimuli and are regulated most often at the level of protein stability or translational efficiency. However, several reports indicate that p27^{Kip1} gene expression is also regulated at the transcriptional level [Kwon et al., 1997; Inoue et al., 1999; Kolluri et al., 1999; Dijkers et al., 2000; Servant et al., 2000; Hirano et al., 2001]. Pulse-labeling experiments showed that the rate of p27^{Kip1} protein synthesis after hypoxic treatment is enhanced in Hepa-1 cells and reduced in c4 cells. In Hepa-1 cells increased synthesis correlates with an increase in the level of p27^{Kip1} mRNA. However, in c4 there is no change in p27^{Kip1} mRNA levels even after a 24 h hypoxic treatment. Our data (Fig. 2C) and that of others [Heacock and Sutherland, 1990; Amellem and Pettersen, 1991] show that there is a global decline in protein synthesis in hypoxic cells. Therefore, it appears that the increase of p27^{Kip1} mRNA in Hepa-1 may compensate for the decrease in global protein synthesis. In contrast, in c4 cells p27^{Kip1} is downregulated due to the normally short half-life of p27^{Kip1} protein, the inability to elevate p27^{Kip1} mRNA levels, and a general decline in protein synthesis under

hypoxic conditions. The essential role of ARNT in this process is indicated by the fact that in c4 cells stably transfected with wild-type ARNT the downregulation of $p27^{Kip1}$ protein is prevented and this correlates with enhanced $p27^{Kip1}$ mRNA levels after hypoxic treatment. A similar mechanism may also explain the hypoxia-mediated decrease in luciferase activity from the HRE reporter gene in c4 cells (see Fig. 4C). Without ARNT, the HRE cannot be activated by hypoxia. The global repression of protein synthesis, coupled with the short half-life of the luciferase protein, leads to the observed loss of luciferase activity in c4 cells.

The increase in $p27^{Kip1}$ mRNA in hypoxic Hepa-1 cells appears to result from transcriptional activation of the $p27^{Kip1}$ gene since the rate of $p27^{Kip1}$ mRNA decay is not changed under hypoxic conditions. This is consistent with the results of Gardner et al. [2001] using Rat1A fibroblasts. Both Gardner et al. [2001] and Carmeliet et al. [1998] reported that the hypoxia-induced increase in $p27^{Kip1}$ mRNA is independent of Hif-1α. Gardner et al. [2001] showed that, in NIH3T3 cells, hypoxia activates the $p27^{Kip1}$ gene promoter through a specific region upstream of the transcriptional start site. They reported that the hypoxia responsive region resides somewhere within the region between 1,133 and 602 bp 5′ to the transcription start site. We were able to reproduce the results of Gardner et al. using a construct containing 690 nucleotides of the $p27^{kip1}$ 5′ flanking sequence. We were also able to show a similar response in Hepa-1 cells but failed to observe an increase in $p27^{Kip1}$ promoter activity in response to hypoxia in c4 cells. In contrast to the results of Gardner et al. [2001] and Carmeliet et al. [1998], Goda et al. [2003] reported that enhanced expression of $p27^{Kip1}$ and cell cycle arrest during hypoxia is Hif-1α-dependent. They suggested that the contradictory finding may be due to diffences in methodology or the various cell types used. Although ARNT is the dimerization partner for Hif-1α, ARNT is able heterodimerize with many other HLH-PAS domain factors. ARNT can also form functional homodimers. In this regard, it is interesting to note that we have observed a hypoxia-mediated increase in the level of ARNT itself in several cell lines (e.g., see Fig. 4C). Thus it is possible that ARNT functions independent of Hif-1α in stimulating expression of the $p27^{Kip1}$ gene. The nature of the ARNT complex required for hypoxia-mediated enhancement of $p27^{Kip1}$ levels remains to be investigated.

In summary, ARNT is required for the hypoxia-induced transactivation of the $p27^{Kip1}$ gene in Hepa-1 cells and in mediating cell cycle arrest. In the absence of ARNT protein, the level of $p27^{Kip1}$ in hypoxic cells is downregulated in parallel with global suppression of protein synthesis. ARNT is necessary for activation of $p27^{Kip1}$ gene transcription in response to hypoxia but the precise mechanism by which ARNT functions, including its dimerization partner and the *cis* regulatory elements involved, remain to be identified. Understanding this mechanism is of importance, considering the significant role of hypoxia in tumor cell growth and metastasis.

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