Hypoxia Inhibits G\(_1\)/S Transition through Regulation of p27 Expression*

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Mammalian cellular responses to hypoxia include adaptive metabolic changes and a G\(_1\) cell cycle arrest. Although transcriptional regulation of metabolic genes by the hypoxia-induced transcription factor (HIF-1) has been established, the mechanism for the hypoxia-induced G\(_1\) arrest is not known. By using genetically defined primary wild-type murine embryo fibroblasts and those nullizygous for regulators of the G\(_1\)/S checkpoint, we observed that the retinoblastoma protein is essential for the G\(_1\)/S hypoxia-induced checkpoint, whereas p53 and p21 are not required. In addition, we found that the cyclin-dependent kinase inhibitor p27 is induced by hypoxia, thereby inhibiting CDK2 activity and forestalling S phase entry through retinoblastoma protein hypophosphorylation. Reduction or absence of p27 abrogated the hypoxia-induced G\(_1\) checkpoint, suggesting that it is a key regulator of G\(_1\)/S transition in hypoxic cells. Intriguingly, hypoxic induction of p27 appears to be transcriptional and through an HIF-1-independent region of its proximal promoter. This demonstration of the molecular mechanism of hypoxia-induced G\(_1\)/S regulation provides insight into a fundamental response of mammalian cells to low oxygen tension.

Cellular hypoxia is an environmental stress with important implications in developmental biology, normal physiology, and many pathological conditions, including cancer. Normal tissues display an oxygen gradient across a distance of 400 \(\mu\)m from a blood supply; tumors often have disordered and diminished vascularization, and hypoxia occurs in tumor tissue that is >100−200 \(\mu\)m away from a functional blood supply (1–3). Cells may adapt to hypoxia in numerous ways, including a transition from oxidative phosphorylation to glycolysis and neovascularization. Many of these metabolic responses are mediated by the transcription factor HIF-1, a heterodimer of a hypoxia-induced subunit HIF-1\(\alpha\) and a constitutive subunit HIF-1\(\beta\) (ARNT), which transactivates genes encoding several glycolytic enzymes as well as the vascular endothelial growth factor gene (4–7).

Cells may also respond to hypoxia by diminishing their proliferative rates. Both invasive and noninvasive studies of a variety of normal tissues and tumors suggest that hypoxic cells may be viable but nonproliferating (8–12). This low proliferative state may be related to the phenomenon of tumor dormancy, described in nonvascularized metastatic foci (13), and may also help explain why hypoxic tumors are relatively chemoresistant and radioresistant (14). Although some transformed cell lines undergo apoptosis in extreme hypoxia and an acidic environment, nontransformed hypoxic cells remain viable but arrest in G\(_1\) (15–17).

The best characterized molecular event necessary for the G\(_1\)/S phase transition is phosphorylation of the retinoblastoma protein (RB) by specific cyclin-dependent kinase (CDK)-cyclin complexes (18). CDK activity can be inhibited by cyclin-dependent kinase inhibitors (CDKIs), such as p27 and p21, which then promote RB hypophosphorylation. There is also evidence that CDKIs may promote a G\(_1\) arrest that is RB-independent (19, 20). Hypoxia-induced arrest is associated with hypophosphorylation of RB (16, 21) and, as opposed to hypoxia-induced apoptosis, appears to be independent of p53 induction (17, 22). A direct role for HIF-1\(\alpha\) in regulating the cell cycle in hypoxia has not been clearly demonstrated (6, 7), and the events leading to the hypoxic hypophosphorylation of RB, and indeed the very relevance of RB phosphorylation status in hypoxia-induced G\(_1\) arrest, have not been well delineated.

We therefore sought to characterize the molecular mechanisms responsible for hypoxia-induced growth arrest, and the role of HIF-1 in this response. Many previous studies exploring the effect of hypoxia on the cell cycle have been limited by the use of transformed and/or immortalized cell lines, which may have altered cell cycle regulators and/or other mutations (23). In this ambiguous genetic background, and without the ability to manipulate regulators of the G\(_1\)/S transition, conclusions regarding the significance of cell cycle regulators in hypoxia-induced G\(_1\) arrest have not been definitive. We elected to first identify cell cycle regulatory elements that are altered by hypoxia in immortalized fibroblasts, and then study the effect of hypoxia on the cell cycle of wild-type murine embryo fibroblasts (MEFs) and primary fibroblasts deficient in key regulators of the G\(_1\)/S checkpoint. We demonstrate that RB and p27 play important roles in the hypoxia-induced G\(_1\) arrest of primary...
fibroblasts. We then used immortalized fibroblasts for further molecular manipulation to show that hypoxia transcriptionally induces p27 and causes a G1 arrest in an HIF-1-independent manner. These observations suggest a molecular mechanism for hypoxia-induced cell cycle regulation.

MATERIALS AND METHODS

Cell Culture and Hypoxic Induction—Rat1a fibroblasts, NIH-3T3 fibroblasts, Balb-3T3 fibroblasts, and mouse embryo fibroblasts (MEFs) null for RB, p53, p21 (obtained from Dr. Tyler Jacks (24)), and p27 (25) and their wild-type counterparts were cultured in Dulbecco's modified Eagle's medium containing 3.7 g/liter bicarbonate, 1.6 g/liter glucose, and supplemented with penicillin/streptomycin and 10% fetal calf serum (Life Technologies, Inc.). All MEFs were used after passage 14. Embryonal Stem (ES) cells were prepared and cultured in Dulbecco's modified Eagle's medium with 4.6 g/liter glucose supplemented with nonessential amino acids, insulin, monothioglycerol, serum, and HEPES as described (30). AIN4 cells were cultured in Improved MEM-Zinc Option supplemented with fetal calf serum, insulin, and hydrocoristine (26).

For all experiments, 1 × 10^5 cells were plated in 10-cm dishes and incubated in 20% O2 at 37°C for 24 h. The media were then changed and supplemented with 25 mM HEPES (pH 7.5). To render cells hypoxic, primary cultures were incubated in a modular incubator (B Chung, San Marcos, CA) flushed with 5% Ne, 5% CO2, and incubated at 37°C. This resulted in ∼0.1–0.5% O2 after several hours. After 32 h, cells were released from hypoxia and quickly scraped in ice-cold phosphate-buffered saline, and analyses were performed as described below. For GoC1 experiments, cells were incubated with GoC1 (200 μM) for 32 h prior to analysis.

Description of Plasmids and Transient Transfection—Each 10-cm dish was plated with 1 × 10^5 cells and 24 h later transfected with Lipofectin (Life Technologies, Inc.). After a 24-h incubation in 20% O2, cells were rendered hypoxic or incubated in 20% O2 for an additional 24 h prior to analysis. The HIF-1α expression vector, pCEP/HPF-1α, was described previously (5). For the cotransfection experiments, Rat1a fibroblasts were transfected with 10 μg of pCEP/HPF-1α (or pCEPs) and 1 μg of green fluorescent protein (GFP) expression plasmid (pEGFP-N1, CLONTECH). HIF-1 function was assessed by a construct containing a hypoxia-responsive element from the human erythropoietin gene subcloned into a SV40 promoter luciferase reporter (pGL2, Promega) (5). The murine p27 promoter and deletion constructs, subcloned into the pGL2 basic luciferase vector (Promega), were the generous gift of Dr. Sehng-Cai Lin (27) and the 249-602 deletion construct was created by digesting the 1.1-kb promoter with EcoQ1. The p53-responsive p21 promoter/luciferase reporter construct was obtained from Dr. Bert Vogelstein (28). Luciferase assays (Promega) were performed, and the data were normalized for total protein.

Manipulation of Cell Cycle Regulators—Balb-3T3 cells were plated at 2 × 10^5/10-cm plate and 24 h later transfected with 30 μg of pCAGG IRES-ZAG-reporter vector (Glen Research). After 5 h, media were added, and cells were incubated in 20% O2 or rendered hypoxic for an additional 32 h.

Recombinant adenoviruses containing full-length human p27 in the antisense direction and, as control, GFP alone were prepared using AdEasy method (29) and titered so that >90% of infected cells expressed GFP and no toxicity was noted. AIN4 cells, plated at 1 × 10^5/10-cm plate, were infected with virus for 6 h in the presence of 2% fetal calf serum, and 24 h later media were exchanged and exposed hypoxic or incubated in 20% O2 for 48 h.

Cell Cycle Analysis—After 32 h, cells were released from hypoxia and quickly scraped in ice-cold phosphate-buffered saline, washed, and suspended in a buffer containing sucrose and trisodium citrate. Samples were then incubated for 10 min consecutively with trypsin/Nonidet P-40/pspermine tetrahydrochloride, trypsin inhibitor/ribonuclease and propidium iodide (PI) as described (30). PI and forward light scattering were detected by using a Coulter EPICS 752 flow cytometer equipped with MDADS 11 software, version 1.6. Cell cycle distribution profiles were determined with a wave fitting program FLITE version 3.0 (Coulter).

To assess directly the rate of DNA synthesis, cells were exposed to media containing BrdUrd (10 μM) for 30 min. Cells were then trypsinized and removed from hypoxia. Nuclei were prepared and stained with a fluorescein isothiocyanate-labeled and BrdUrd antibody, and total DNA was stained with propidium iodide and analyzed as described (26). Wild-type MEF controls from RB, p53, and p27 wild-type animals all showed similar degrees of hypoxia-induced growth arrest and were averaged together.

Immunoblotting and Immunoprecipitation—Cells were released from 32 h of hypoxia, quickly washed with ice-cold phosphate-buffered saline, and then resuspended in solution containing 1% SDS and boiled. Protein was quantitated by the BCA method (Pierce), and equal amounts of total cellular protein were resolved by SDS-polyacrylamide gel electrophoresis and subjected to immunoblot analysis (26). RB antibody was obtained from PharMingen, and cyclin A, D, and E were from Upstate Biotechnology, Inc.; for immunoblots, p27, p53, and p21 were obtained from Santa Cruz Biotechnology, and HIF1α was from Novus Biologicals. Coomassie Blue staining of total cellular protein confirmed equal protein loading. For immunoprecipitation (IP) experiments, cyclin A (H432) and CDK2 (M2) antibodies were obtained from Santa Cruz Biotechnology, and cyclin E antibody (Ab1) was obtained from NeoMarkers. 100 μl of protein A-Sepharose beads (Amersham Pharmacia Biotech) were loaded with 20–40 μg of antibodies in IP buffer (50 mM Tris-HCl (pH 7.4), 250 mM NaCl, 0.5 mM NaVO4, 20 μM β-glycerophosphate, 15 mM phosphatase substrate p-nitrophenyl phosphate, protease inhibitor Complete tablets (Roche Molecular Biochemicals), and 0.1% Nonidet P-40). Lysates from cells subjected to hypoxic or nonhypoxic culture conditions for 32 h were lysed with IP buffer, sonicated, and solubilized for 30 min at 4°C, and protein concentration was determined using the Bio-Rad protein assay. Washed protein A beads were incubated with 300 μl of lysate for 2 h at 4°C, and the beads were then washed three times with IP buffer. Histone kinase assays were performed by incubating the beads in buffer containing 50 mM Tris (pH 7.4), 1 mM CaCl2, 5 mM MgCl2, 0.5 mM NaVO4, 20 μM β-glycerophosphate, 15 mM phosphatase substrate p-nitrophenyl phosphate, protease inhibitor Complete tablets (Roche Molecular Biochemicals), 50 μM ATP, 0.2 μg of purified histone H1 (Roche Molecular Biochemicals), and 10 μCi of [γ-32P]ATP at 30°C for 30 min. The reaction was stopped by the addition of 50 μl of 2× Laemmli buffer and heating for 95°C for 5 min. The products were separated by SDS-polyacrylamide gel electrophoresis, and autoradiography was performed. To assess the amount of CDK2 protein in the lysates, CDK2 was immunoprecipitated with a goat anti-CDK2 antibody, and the blot was probed with a rabbit anti-CDK2 antibody.

RNA Analysis—After 28 h of hypoxic or nonhypoxic conditions, medium was changed to contain 0.5 μg/ml actinomycin D (Sigma), a concentration at which no toxicity was seen after 12 h of incubation. Total RNA was harvested at 0, 30, 120, and 240 min after actinomycin D addition under continued hypoxic or nonhypoxic conditions using Trizol (Life Technologies, Inc.) and the supplier's protocol. 15 μg of RNA per lane was subjected to electrophoresis in 1% agarose-formaldehyde gels, transferred to nylon membranes, and hybridized with a p27 cDNA probe. Autoradiographic signals were quantitated with a PhosphoImager and normalized to 18 S RNA.

RESULTS

Hypoxia Induces a G1 Cell Cycle Arrest in Immortalized Fibroblasts and Is Associated with Hypoxia-mediated Changes in G1 Regulators—Since contact inhibition and serum withdrawal are well documented to result in G1 cell cycle arrest (31–34), all experiments were performed at low (<50%) confluency in the presence of fresh, serum-containing, buffered media. We initially studied a well characterized, easily manipulated, immortalized fibroblast cell line to identify key cell cycle components that could be further investigated. When Rat1a fibroblasts were exposed to hypoxia for approximately two doubling times (32 h), propidium iodide staining and flow cytometric analysis of cell cycle distribution revealed a significant (p < 0.005) G1 arrest (Table 1). To assess directly the rate of DNA synthesis, fibroblasts were rendered hypoxic for 30 h, transferred to a large hypoxic atmospheric chamber (PlasLabs), and BrdUrd labeling was performed as described (26). Hypoxic cells incorporated significantly less BrdUrd (44 ± 0.1% in normoxia versus 24 ± 0.3% in hypoxia) confirming a decrease in S phase under hypoxic conditions.

We then sought to identify further components of the G1 checkpoint that respond to hypoxia and might contribute to cell cycle arrest. RB phosphorylation is promoted by CDK2 and CDK4 when they complex with cyclins E, A, and D. A G1 arrest
occurs when these CDK activities are inhibited by the CDKIs p21, p27, or p16. After two doubling times in either normoxia or hypoxia, Rat1a fibroblasts were collected, and the expression of several proteins important for the G1-S phase transition was examined. As previously reported in other immortalized cell lines (16, 21, 35–36), hypoxia induced hypophosphorylation of RB in Rat1a cells, a hallmark of G1 arrest in normal cells (Fig. 1A). This reduction in RB phosphorylation was associated with a decrease in CDK2 activity (Fig. 1A). Despite this decrease in kinase activity, the amount of immunoprecipitated CDK2 protein was unaffected by hypoxia (Fig. 1A), indicating that a modifier of CDK activity (e.g. cyclin E, A, p21, and/or p27) is altered by hypoxia. Although p53 may be induced in hypoxia by an HIF-1α-dependent manner (37, 38), we observed only a minimal increase in p53 and actually observed a decrease in protein lysates of hypoxic cells revealed a decrease in cyclin E and decreased BrdUrd uptake (Fig. 2A) when rendered hypoxic. The extent of this hypoxia-induced arrest was most apparent when wild-type MEFs were synchronized with 32 h of serum withdrawal. When re-stimu-

luted with serum, MEFs incubated in 20% O2 showed a dramatic (>60%) percentage of S phase at 16 h, whereas serum-stimulated hypoxic MEFs increased S phase only minimally (<12%) throughout the 32-h experiment (Fig. 2B). These observations indicate that hypoxia inhibits G1/S transition in normal primary fibroblasts.

Several of the changes that we (Fig. 1A) and others (16, 21, 35, 36) noted in immortalized cells were not apparent in primary cell lines. Specifically, we noted only mild decreases in cyclin E and cyclin A in MEFs (Fig. 1C and data not shown). Also, in contrast to Rat1a cells, we noted minimal kinase activities associated with CDK4 and cyclin E in both hypoxic and normoxic MEFs (data not shown). However, similar to the immortalized cells that underwent a G1 arrest in hypoxia, we noted that hypoxia increased p27, decreased CDK2 activity, and led to a hypophosphorylation of RB in hypoxic MEFs (Fig. 1C and Fig. 3, B and C). These data suggest that these changes may be key in regulating hypoxia-induced growth arrest.

Isogenic p27 and RB-deficient MEFs Demonstrate the Importance of These Proteins in Hypoxia-induced G1 Arrest—To better assess the significance of RB hypophosphorylation and p27 induction in hypoxia, MEFs deficient in G1 cell cycle regulators were subjected to 32 h of hypoxia. Previous studies have shown that hypoxia-induced growth arrest occurs in transformed cells with mutant or null p53 status (17, 22). Consistent with these reports, MEFs null for p53 or p21 arrested in hypoxia to the same degree as wild-type cells (Table I and Fig. 2A). Therefore, neither p53 nor p21 is required for hypoxia-induced G1 arrest. However, as compared with these cells, the ability of RB-null MEFs to arrest in G1 in response to hypoxia was markedly diminished (Table I and Fig. 2A), indicating that RB participates in this arrest.

Although the decrease in cyclin E in wild-type MEFs was less dramatic than that in immortalized fibroblasts (Fig. 1, A and C), either a decrease in cyclin E and/or an increase in p27 could contribute to diminished CDK2 activity leading to RB hypophosphorylation in hypoxia. Previous studies have shown that p27 induction is necessary for the growth arrest observed in Balb-3T3 fibroblasts subjected to serum withdrawal (33). To determine the extent that p27 induction is responsible for hypoxia-induced growth arrest, p27 null MEFs were rendered hypoxic for two doubling times. There was no significant change in cell cycle profile or incorporation of BrdUrd (Table I and Fig. 2A) despite hypoxia, indicating that p27 is necessary for hypoxia-induced G1 arrest. The contribution of p27 to hypoxia-induced G1 arrest was also apparent when CDK2 activity and RB phosphorylation status were examined in p27 null fibroblasts rendered hypoxic (Fig. 3, B and C). The base-line RB phosphorylation appears enhanced in the p27 null fibroblasts. Whereas hypoxia led to a 42% decrease in CDK2 activity as well as RB hypophosphorylation in wild-type MEF cells, these changes were not evident in p27 null fibroblasts.

Could it be argued that the increase of p27 in hypoxic wild-type MEFs is a secondary effect of cell cycle arrest and not a mediator of hypoxia-induced G1 arrest. However, p27 expression was also induced in hypoxic yet cycling RB null MEFs (Fig. 1C), which indicates that the induction of p27 is a direct effect of hypoxia rather than a secondary feature of RB hypophosphorylation and/or G1 cell cycle arrest. We also observed that cyclin E decreased in the hypoxic RB null MEFs, although the absolute levels of cyclin E in both hypoxic and normoxic RB null MEFs were greater than those in wild-type MEFs (Fig. 1C). Therefore, we cannot exclude the possibility that the resistance of RB null cells to hypoxia-induced arrest may be due, in part, to increased cyclin E, which in turn could then titrate hypoxia-induced p27.

| Table I | Effects of hypoxia on the cell cycle profiles of various cell lines |
|---------|---------------------------------------------------------------|
|         | G1 % of cells | S % of cells | G2/M % of cells |
| Rat1a   | 45 ± 0.7      | 40 ± 0.9     | 15 ± 0.6       |
| Rat1a hypoxia | 60 ± 1.4      | 29 ± 1.5     | 11 ± 0.6       |
| Rat1a CoCl2 | 47 ± 2.3      | 36 ± 2.3     | 17 ± 0.3       |
| Rat1a/PCEP4 | 58 ± 0.1      | 36 ± 0.1     | 6 ± 0.2        |
| Rat1a/HIF1 | 59 ± 0.1      | 35 ± 0.1     | 6 ± 0.7        |
| Wild-type MEF | 47 ± 1.3      | 36 ± 1.0     | 18 ± 2.0       |
| Wild-type MEF hypoxia | 58 ± 0.6      | 25 ± 2.8     | 17 ± 2.4       |
| MEF Rb+/− | 47 ± 1.3      | 27 ± 1.7     | 23 ± 1.1       |
| MEF Rb+/− hypoxia | 50 ± 0.6      | 25 ± 1.8     | 23 ± 0.9       |
| MEF p53+/− | 36 ± 0.3      | 52 ± 1.5     | 13 ± 1.5       |
| MEF p53+/− hypoxia | 44 ± 2.0      | 44 ± 2.0     | 12 ± 0.0       |
| MEF p21+/− | 50 ± 3.5      | 37 ± 4.0     | 13 ± 0.0       |
| MEF p21+/− hypoxia | 66 ± 1.5      | 22 ± 1.0     | 12 ± 0.0       |
| MEF p27+/− | 54 ± 3.0      | 29 ± 1.0     | 17 ± 1.0       |
| MEF p27+/− hypoxia | 55 ± 2.0      | 20 ± 1.2     | 23 ± 1.0       |
Transient Decreases in p27 Also Abrogate the Hypoxia-induced G1 Arrest—Because a total absence of p27 may result in abnormalities of cyclin expression and CDK-cyclin complex formation (39, 40), we assessed whether a transient decrease in p27 would also diminish the hypoxia-induced G1 checkpoint. p27 antisense and missense phosphorothioate oligonucleotides containing both the “G-clamp” and 5-methylcytosine analogues (41) were transfected with cytofectin under conditions that have been shown to result in >90% efficiency of oligonucleotide delivery to the nucleus (42). Whereas hypoxia led to a G1 arrest and p27 induction in nontransfected Balb-3T3 cells (data not shown) and missense transfected cells, the antisense-treated cells had markedly less p27 induction with hypoxia (Fig. 3D) and failed to arrest in G1 (Table II). Antisense treatment also diminished RB hypophosphorylation (data not shown), again indicating that the hypoxic induction of p27 is an upstream event of RB hypophosphorylation. To determine whether our finding of a p27-mediated hypoxia-induced G1 arrest may be generalized to other cell types, immortalized human mammary epithelial cells (A1N4) were infected with a full-length antisense p27-expressing adenovirus. Whereas A1N4 cells infected with control adenovirus expressing GFP arrested in hypoxia, AIN4 cells treated with the antisense p27 virus showed a decrease in inducible p27 and cell cycle progression despite hypoxia (Table II and Fig. 3D). These observations further support the hypothesis that an increase in p27 is required for hypoxia-induced cell cycle arrest.

Hypoxia-induced G1 Arrest Affects the CDK2-Cyclin A Complex—CDK2 is activated when complexed with either cyclin E or cyclin A; both of these complexes are inhibited by p27 (39). Hypoxia led to an increase in the amount of p27 associated with CDK2 in wild-type MEFs (Fig. 3A) as has been reported in immortalized cell lines (21). We confirmed that radiolabeled in vitro translated cyclin E and cyclin A associate with CDK2 present in MEF lysates (data not shown). Despite the fact that our protocol immunoprecipitated in vitro translated cyclin E, the CDK2 activity associated with immunoprecipitated cyclin E was minimal in both wild-type and p27 null MEFs, and hypoxia decreased this minimal cyclin E/CDK2 activity in both wild-type and p27 null cells (data not shown). Additionally, immunodepletion of cyclin E had a negligible effect on total CDK2 activity in these MEFs (data not shown). In contrast,
when CDK2- and cyclin A-associated kinase activities were measured from the same lysates, >90% of total CDK2 activity was recovered in cyclin A-associated complexes (Fig. 3B). Cyclin A/CDK2 activity decreased in hypoxic wild-type MEFs but was unchanged in p27 null cells. Although cyclin A is a target of E2F (43), is directly inhibited by hypophosphorylated RB (44), and has also been reported to be diminished by hypoxia in immortalized kidney cells (36), we did not observe cyclin A levels to change appreciably with hypoxia in either wild-type or p27 null MEFs (data not shown). Therefore, our data suggest that the predominant active CDK2 complex in MEFs is CDK2-cyclin A and that increased p27 associated with this complex in hypoxia is responsible for inhibiting CDK2 activity and inhibiting exit from G1.

**Hypoxia Induces p27 Transcription via an HIF-1-independent Hypoxia-responsive Proximal Promoter Region**—Because of the importance of p27 inhibition of CDK2-cyclin A complexes in hypoxia-induced G1 arrest, we sought to characterize better the mechanism of p27 induction in hypoxia. Although the regulation of p27 during the cell cycle and tumor progression is thought to occur through translational and post-translational mechanisms (45, 46), we observed a unique hypoxic regulation of p27 at the RNA level. When Rat1a fibroblasts were exposed to hypoxia for 32 h, p27 RNA levels increased 3-fold (Fig. 4A). When cells were treated with the RNA synthesis inhibitor actinomycin D in either hypoxia or normoxia, the p27 mRNA half-life did not increase in hypoxia (220 min in hypoxia versus 260 min in normoxia) (Fig. 4). These observations suggest that hypoxia induces p27 at the transcriptional level, which contrasts with the increased stability of some mRNAs, including vascular endothelial growth factor, in hypoxia (47).

The best described hypoxia-induced transcription factor is HIF-1. HIF-1 has also been reported to have a role in cellular proliferation and is overexpressed in some tumors (6, 7, 48). We thus examined the role of HIF-1 in hypoxia-induced growth arrest and, more specifically, on p27 induction. Rat1a fibroblasts were cotransfected with an HIF1α expression vector and GFP expression plasmid in a 10:1 w/w ratio, and 48 h later cell cycle analysis was performed on GFP-positive cells. In addition, Rat1a fibroblasts were treated for 32 h with 200 μM CoCl2, a known inducer of HIF-1α (5). For both conditions HIF-1α expression was assayed by protein immunoblot, and HIF-1 activity was measured from the same lysates, equal loading (input). B, cyclin A and CDK2 histone kinase (HK) activity in wild-type MEFs and p27 null MEFs. MEFs were normoxic or rendered for 32 h, and kinase activity associated with immunoprecipitated cyclin A and CDK2 was assayed. The bottom panel displays kinase activity of wild-type and p27 null MEFs in both normoxia and after 32 h of hypoxia (dark bars). Hypoxic values reflect mean percent ± S.E. of normoxic values, all averaged from three separate experiments. Kinase activities of normoxic p27 null lysates were normalized to normoxic wild-type levels. C, phosphorylation status of RB in hypoxic MEFs. Cell lysates from normoxic and hypoxic wild-type and p27 null MEFs were harvested. and RB was immunoblotted. PP represents the hyperphosphorylated forms of RB, and P represents the hypophosphorylated forms of RB. Coomassie Blue staining of total cellular protein confirms equal protein loading. D, p27 antisense oligonucleotides and p27 antisense adenovirus decrease the hypoxia-induced accumulation of p27 and hypoxia-induced G1 arrest. Balb-3T3 cells were transfected with 30 nM p27 antisense or missense. A1N4 cells were infected with either full-length human p27 antisense virus or, as control, GFP-expressing adenovirus. After a brief recovery period, cells were exposed to 32 (Balb-3T3) or 48 h (A1N4) of hypoxia and compared with cells incubated in normoxia. Coomassie Blue staining of total cellular protein confirms equal protein loading.

**TABLE II**

|       | G1  | S   | G1/M |
|-------|-----|-----|------|
| **B** |     |     |      |
| Balb-3T3 |     |     |      |
| p27 missense | 44.0 ± 2.0 | 37.3 ± 2.0 | 18.7 ± 0.6 |
| p27 missense hypoxia | 51.7 ± 1.2 | 32.4 ± 1.3 | 15.9 ± 0.6 |
| p27 antisense | 40.4 ± 2.1 | 41.1 ± 1.1 | 18.5 ± 2.9 |
| p27 antisense hypoxia | 39.6 ± 1.1 | 38.3 ± 2.2 | 21.9 ± 2.5 |
| A1N4 |     |     |      |
| GFP control | 74 ± 0.4 | 16 ± 0.1 | 9 ± 0.5 |
| GFP hypoxia | 82 ± 1.6 | 9 ± 0.3 | 9 ± 0.5 |
| AS p27 | 35 ± 0.3 | 36 ± 1.9 | 29 ± 3.5 |
| AS p27 hypoxia | 34 ± 1.7 | 45 ± 1.8 | 22 ± 3.6 |
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Further arrest cells in G1 (p = 0.26, Table I). Similarly, the CoCl2-induced expression of functional HIF-1α in normoxic cells did not alter the cell cycle profile of control treated cells as assessed by both propidium iodide staining (Table I) and by BrdUrd uptake (41 ± 0.1% in CoCl2-treated cells versus 44 ± 0.1% in control cells). These data indicate that HIF-1 transactivation is not sufficient for hypoxia-induced cell growth arrest.

Recent studies on the proliferative capacity of HIF-1α-deficient ES cells have been contradictory. Whereas one study suggested that ES cells lacking HIF-1α do not have a lower S fraction in hypoxia than in normoxia (6), another reported that HIF-1α--null ES cells proliferate more slowly in hypoxia as compared with normoxia (7). When we subjected subconfluent ES cells (prepared and cultured as described (7)) to 32 h of hypoxia, similar degrees of G1 arrest were observed in wild-type cells (G1 29 ± 0.1% in normoxia versus 34 ± 0.1% in hypoxia) and in HIF-1α-null ES cells (G1 26 ± 0.1% in normoxia versus 31 ± 0.6% in hypoxia). Although aspects of cell cycle regulation may be significantly different in ES cells as compared with somatic cells (49, 50), these observations suggest that, at least in ES cells, HIF-1α is not necessary for hypoxia-induced growth arrest.

Consistent with our finding that HIF-1 transactivation does not contribute to the hypoxia-induced G1 arrest, induction of HIF-1 in normoxic cells did not increase p27 expression (Fig. 1B). We thus sought to determine whether the p27 promoter contains a hypoxia-responsive region. NIH-3T3 cells, which arrest in G1 and induce p27 when hypoxic (data not shown), were transiently transfected with various fragments of the p27 promoter linked to a luciferase reporter and subsequently subjected to 24 h of hypoxia. Hypoxia increased the transcriptional activity of a 1.1-kb fragment of the p27 promoter almost 3-fold relative to normoxia (Fig. 4B). Consistent with our observation that p21 is not induced by hypoxia, transient transfections with a p53-responsive p21 promoter-luciferase reporter construct did not show an increase in luciferase activity in hypoxic cells (data not shown). This lack of activity also argues against nonspecific activation of G1 CDK1 promoters when cells are arrested in G1. Induction of the p27 promoter was dependent on DNA sequences located 1133 to 602 bp 5’ to the transcription start site. Although it is necessary for the hypoxic response of the p27 promoter, this 531-bp region is not sufficient, since it alone subcloned 5’ to an SV40 promoter-luciferase construct did not result in a hypoxia-induced increase in luciferase activity above that of the SV40 promoter alone (data not shown). Deletional analysis indicated that the 531-bp region, when joined with more proximal promoter sequences, is sufficient for hypoxic induction of luciferase activity (1.1 D249–602 in Fig. 4B). Cotransfection of the 1.1-kb p27 promoter and an HIF-1α expression vector did not result in a significant increase in luciferase activity, even in hypoxia where HIF-1 transactivation of the erythropoietin promoter increased nearly 10-fold over base line (data not shown).

DISCUSSION

Cellular responses to hypoxia are fundamental adaptive changes that are required for normal mammalian physiology and may be exploited by cancer cells for a proliferative/survival advantage. Although the understanding of hypoxia-induced cellular metabolic changes is rapidly emerging, our understanding of the effects of oxygen deprivation on the control of the cell cycle is still rudimentary. In this study, we have observed that hypoxia induces an accumulation of cells in G1. This was best demonstrated when cells were synchronized prior to being rendered hypoxic. As we and others (24) have noted, even after prolonged serum withdrawal ~25% of MEFs continue to incorporate thymidine. The observation that cells in other stages of the cell cycle are also present after prolonged incubation in hypoxia suggests several possibilities. One is that some individual cells may escape G1 arrest. Puring the external environment of oxygen may not necessarily lead to equivalent amounts of intracellular hypoxia, and cell cycle regulation may vary with the extent of hypoxia. Indeed, truly anoxic cells show an immediate and dramatic decrease in BrdUrd incorporation, possibly through a mechanism other than the one we observed at more moderate (~0.1%) levels of hypoxia. It is also possible that other stages of the cell cycle are also delayed in hypoxia. We observed a decrease in S phase in all cell lines that exhibited a hypoxia-induced G1 arrest (Table I); this hypoxia-induced decrease in S phase was greatly diminished or absent in RB null cells, in p27 null cells, or when p27 was transiently reduced with antisense techniques (Table II and Fig. 3D). A hypoxia-induced G1 delay has been reported in some transformed cell lines (23), and indeed when hypoxic cells were manipulated to escape their G1 arrest with antisense p27 oligonucleotides, a small accumulation in G2 was noted (Table II). Our data, however, are most consistent with the activation of a hypoxia-induced G1/S checkpoint.

Although several studies have demonstrated changes in cell cycle regulatory proteins associated with hypoxia-induced growth arrest, mechanistic insight was not forthcoming from these reports. Tumor cells lines with altered G1 checkpoints have also been used in several studies. For example, previous studies have revealed that hypoxia-induced arrest neither occurs in osteosarcoma cell lines lacking functional RB nor in cells infected with HPV-E7 or E1a, in which RB is inactivated.
A G₁ arrest, however, does occur in transformed and immortalized cells with mutant or null p53 status (17, 22). The use of tumor cell lines provides some insight into cellular responses to hypoxia, but it is unclear if these cells have other abnormalities in their cell cycle regulatory mechanisms (51). Indeed we have noted subtle differences in the effects of hypoxia on immortalized fibroblasts and early MEFs. The immortalization process often leads to an inactivation of p53, RB, and/or p16/p19/ARF (52). Additionally, immortalized rat fibroblasts have been found to have a methylated p21 promoter that may not be responsive to p53 transactivation (53). We observed that immortalized fibroblasts have a greater reduction of cyclin E and cyclin A in response to hypoxia, when compared with wild-type MEFs (Fig. 1, A and C, and data not shown). Additionally, CDK2/cyclin E kinase activity was higher in immortalized rat fibroblasts than in MEFs, where it was negligible (data not shown). However, consistent changes in both immortalized and primary fibroblasts rendered hypoxic included an increase in p27, a decrease in CDK2 activity, and RB hypophosphorylation.

By using genetically defined primary fibroblasts, we demonstrate that hypoxia-induced G₁ arrest requires a functional RB but not p53 or p21. Indeed, neither the protein levels nor the promoter of the CDKI p21, which is transcriptionally regulated by p53, is induced under our hypoxic conditions. MEFs null for p53 failed to arrest in hypoxia. The importance of p27 induction near the G₁/S transition does not delay S entry (55). We noted that the predominant cyclin associated with CDK2 activity during G₁/S transition is cyclin A, which may also play a role in titrating induced p27, decreasing CDK activity, and promoting entry into S phase even in hypoxia. However, the observation that cyclin E decreased minimally in wild-type MEFs, where a significant decrease in CDK2 activity was noted, and the fact that CDK2 activity changed minimally in hypoxic p27 null MEFs, argues for a predominant role of p27 induction in hypoxia-induced G₁ arrest. Thus, our data strongly suggest that p27 induces a G₁ arrest in hypoxia by modulating RB, although we cannot exclude the additional presence of an RB-independent mechanism or an additional contribution from the high levels of cyclin E noted in p27 null cells.

We noted that the predominant cyclin associated with CDK2 in both unsynchronized, cycling MEFs, as well as in hypoxic arrested wild-type MEFs, was cyclin A and not cyclin E. Cyclin E/CDK2 collaborates with cyclin D-dependent kinases to complete RB phosphorylation. Inhibition of cyclin E-associated CDK2 activity during G₁ inhibits entry into S phase, whereas inhibition near the G₁/S transition does not delay S entry (55). Cyclin A is necessary for G₂ and S phase progression, is up-regulated by E2F, and is repressed by hypophosphorylated RB (43, 44). However, cyclin A-associated CDK2 activity is also vital for the G₁/S transition. Microinjection of anti-cyclin A antibodies inhibits entry into S phase (56), and cyclin A can complex with CDK2 to phosphorylate RB (57). p27 can bind to and inhibit CDK2/cyclin A activity (57, 58), and increases in cyclin A activity found in late G₁ do not correlate with increased cyclin A levels but rather decreased binding of p27 to the CDK2-cyclin A complex (57). These observations, combined with our data, suggest that in mid to late G₁ CDK2-cyclin E and CDK2-cyclin A complexes are sequentially activated, and when cells are hypoxic, p27 inhibits cyclin A/CDK2 activity and RB phosphorylation and prevents entry into S phase (Fig. 5).

Although it might be argued that the hypoxia-induced increase in p27 protein levels is simply a secondary effect of G₁ arrest, our observations that changes in both proteins also occur in RB null cells, which continue to cycle in hypoxia, suggest that they are primary effects of hypoxia. The regulation of p27 during the cell cycle typically occurs at the protein level, regulated by translational control and ubiquitination (45, 46). However, our experiments revealed a hypoxia-induced elevation of the p27 transcript that has an unaltered half-life in hypoxia. These results are consistent with a transcriptional activation of p27 by hypoxia. The existence of a physiological role for the transcriptional regulation of p27 is supported by the recent finding that dioxins can bind to the Ah receptor and increase transcription of p27 in hepatoma cells and thymocytes (59). Although the Ah receptor is closely related to HIF-1, we found that functional HIF-1 could not induce growth arrest, activate the p27 promoter, or elevate p27 expression. Although regulation of the cell cycle in somatic cells and ES cells may not be directly comparable (49, 50), we found no abrogation of the hypoxia-induced growth arrest in HIF-1α null ES cells. In addition, p27 mRNA increases in hypoxic HIF-1α-null ES cells (6). In aggregate, these studies suggest that p27 is induced by hypoxia at the transcriptional level independent of HIF-1, and the hypoxia-induced G₁ arrest is also independent of HIF-1. Although we have not formally excluded the other well characterized hypoxia-inducible transcription factor, endothelial periodic acid-Schiff protein-1 (EPAS-1/HLF/HIF-2α) (60), this factor is also induced by CoCl₂ (61), a condition in which we noted no cell cycle arrest.

The p27 CDKI appears to play an important role in the response of cells to their environment. Induction of p27 expression is responsible for the growth arrest seen in serum-deprived fibroblasts (33). p27 protein is also induced by E-cadherin when transformed cells are grown in spheroid culture and is responsible for the growth arrest and chemotherpay resistance seen under these conditions (34, 42). Our data suggest that hypoxia is another example of environmental control of p27. In the potentially damaging environment of hypoxia, elevation of p27 inhibits DNA replication and may prevent the
inappropriate proliferation of genetically damaged cells.

Two of the most common genetic abnormalities found in human tumors are mutations or inactivation of the p53 and RB pathways (62). p53 expression in transformed hypoxic cells, possibly promoted by HIF-1 (37), leads to apoptosis and thus selects for cells with mutant p53 (15). Our data suggest that these cells will still be hypoproliferative in the background of a functional RB. Further studies will be required to determine whether other RB-related proteins such as p107 or p130 may also participate in the cellular response to hypoxia. Hypoxic proliferation of tumor cells could occur through inactivation of RB or, as shown by our studies, a decrease in inducible p27. Although p27 is not one of the many cell cycle regulators known to be commonly deleted in human cancers, its importance in neoplastic cells to escape this G1 arrest, and to determine modulation of p27 expression accounts for the ability of some neoplastic cells to escape this G1 arrest, and to determine whether modulation of p27 expression may alter chemosensitivity or radioresistance of hypoxic tumors.

Acknowledgments—We appreciate the comments from anonymous reviewers, which have significantly contributed to the quality of the paper. We thank John Flook for fluorescence-activated cell sorter analysis and Andrew Koff and members of the Dang and Semenza labs for paper. We thank J. Flook for fluorescence-activated cell sorter analysis and our reviewers, which have significantly contributed to the quality of the paper. We thank J. Flook for fluorescence-activated cell sorter analysis and our reviewers, which have significantly contributed to the quality of the paper.

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