Rapid Communication

Hypoxia enhances human B19 erythrovirus gene expression in primary erythroid cells

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Abstract

Human B19 erythrovirus replicates in erythroid progenitors present in bone marrow and fetal tissues where partial oxygen tension is low. Here we show that infected human primary erythroid progenitor cells exposed to hypoxia (1% O_2) in vitro increase viral capsid protein synthesis, virus replication, and virus production. Hypoxia-inducible factor-1 (HIF-1), the main transcription factor involved in the cellular response to reduced oxygenation, is shown to bind an HIF binding site (HBS) located in the distal part of the B19 promoter region, but the precise mechanism involved in the oxygen-sensitive upregulation of viral gene expression remains to be elucidated.
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Introduction

Human B19 erythrovirus (B19), the only parvovirus clearly linked to human disease, is a small non-enveloped icosahedral virus. Its genome consists of a single-stranded linear DNA (5596 nucleotides) composed of an internal coding sequence flanked by inverted terminal repeats of 383 nucleotides each. P6, the only functionally active viral promoter, is located in the 5′ terminal region and regulates the synthesis of all nine viral transcripts that encode a multifunctional nonstructural protein (NS1), two structural capsid proteins (VP1 and VP2), and several small polypep-

tides of unknown functions (Morinet et al., 2000). The virus infects human erythroid progenitor cells in bone marrow and fetal hematopoietic tissues leading to inhibition of erythroid colony production. This cytotoxicity results in hypoplastic anemia in infected humans, sometimes leading to hydrops foetalis and severe erythroblastopenia in sensitive patients (Brown and Young, 1997). Given that oxygen concentration in bone marrow is low (Harrison et al., 2002; Ishikawa and Ito, 1988) and that fetal development occurs under low oxygen tension level conditions (Adelman et al., 1999; Gassmann et al., 1996; Iyer et al., 1998), we wondered if reduced oxygenation (hypoxia) could influence B19 gene expression and replication in vitro, thereby contributing to the viral tropism.

Results

Hypoxia enhances B19 parvovirus protein synthesis and replication

To analyze whether hypoxia influences B19 erythrovirus gene expression and replication, CD36+ human primary

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Abbreviations: B19, human B19 erythrovirus; HIF-1, hypoxia-inducible factor-1; HBS, HIF-1 DNA-binding site; ARNT, aryl hydrocarbon receptor nuclear translocator.

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erythroid cells were infected with purified B19 virus at a multiplicity of infection of 0.1 and then incubated at normoxic (20% O_2) or hypoxic (1% O_2) conditions. After 48 h of exposure, viral mRNA steady-state levels and protein levels were analyzed. As shown in Fig. 1A, hypoxia led to an about sevenfold increase of the viral transcripts coding for VP2, NS1, and small mRNAs, and to a fourfold increase of the viral transcripts coding for VP1. Immunoblot analysis revealed that capsid protein VP2, used as a representative viral protein, strongly accumulated within the CD36+ infected cells after exposure to hypoxia (Fig. 1B), clearly demonstrating that an oxygen-dependent pathway modulates viral gene expression. Similarly, hypoxia increased both NS1 protein amounts in B19 erythrovirus-infected cells as well as the number of NS1-expressing cells (Fig. 1C). As NS1 protein is highly implicated in parvovirus replication by site-specific DNA nicking within the origin of replication (Cotmore and Tattersall, 1996), we analyzed if reduced oxygen tension could also influence viral replication. A fourfold enhanced production of replicative forms was detected when CD36+ human primary erythroid cells were infected and incubated under hypoxic conditions for 48 h (Fig. 1D). This result was finally consolidated by the sevenfold increase of B19 infectious particles production when CD36+ human primary erythroid cells were incubated in hypoxic conditions (Table 1).

B19 promoter contains a putative HIF-1 binding site

Our data demonstrate that hypoxia induces an increase of B19 expression, replication, and virus production. As cell response to hypoxic stress is mainly mediated by hypoxia-inducible factor-1 (HIF-1) (for a review, see Hopfl et al., 2004), we explored if this transcription factor is involved in the viral upregulation observed. We first looked for a putative HIF-1 binding site (HBS) in the B19 genome. Analysis of the complete P6 promoter sequence revealed the presence of a single putative binding site (5′-GA**CGTG**CCA-3′) for HIF-1 294 bp upstream of the TATA box (Momoeda et al., 1994). This hypothetical HBS shares high similarity with the published mammalian HBS consensus sequence (Camenisch et al., 2001), and is conserved in various published B19 hairpin sequences (Fig. 2). The site is located in the distal part of the hairpin that contains many mismatches due to which only the "flop" conformation of the single-stranded genome contains the GA**CGTG**CCA (Deiss et al., 1990; Zhi et al., 2004).

Hypoxic upregulation of parvovirus B19 expression might involve HIF-1-dependent transcriptional activation

HIF-1 is a ubiquitously expressed heterodimeric transcription factor consisting of a hypoxia-regulated α-subunit and an oxygen-insensitive β-subunit (also known as aryl hydrocarbon receptor nuclear translocator, ARNT). Under hypoxic conditions, instantaneous stabilization of HIF-1α
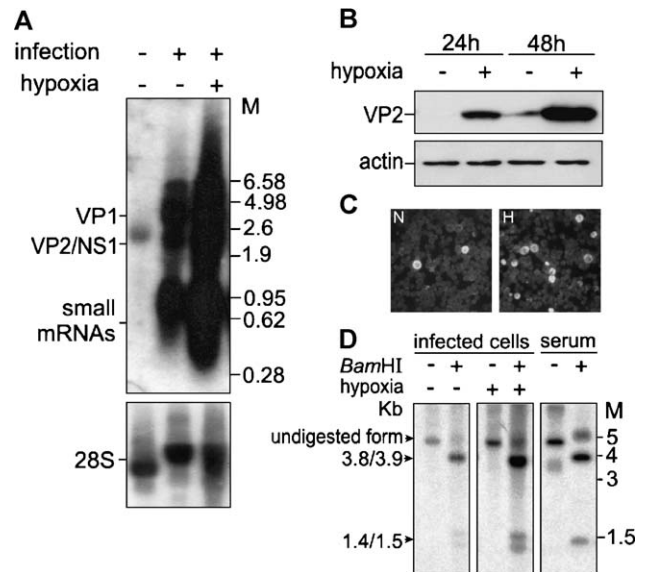

Fig. 1. Oxygen-regulated B19 erythrovirus expression in primary erythroid cells. (A) Northern blot analysis of total RNA extracted from CD36+ cells. Cells were infected under normoxia, and subsequently cultured under normoxic or hypoxic conditions for 48 h. Specific viral mRNA is indicated according to the currently accepted human B19 erythrovirus transcription map (Morinet et al., 2000). Hypoxia-mediated transcriptional induction of cellular aldolase, an oxygen-regulated gene (Semenza et al., 1996), served as a positive control for hypoxic exposure (data not shown). Radioactive signals were quantified by phosphorimaging. B19 mRNA signals were normalized to the signal obtained with a 28S cDNA probe, an oxygen-insensitive quantity control (Zhong and Simons, 1999). Activation factors were then calculated. Similar data were obtained in three independent experiments. M: RNA marker (single strand 0.28–6.58 kb ladder, Promega). (B) Immunoblot analysis of viral VP2 protein using whole-cell extracts prepared from CD36+ primary erythroid cells infected under normoxia, and cultured under normoxic or hypoxic conditions during 24 or 48 h. The signal obtained with an anti-actin antibody was used as a control for loading and transfer efficiency. Similar data were obtained in three independent experiments. (C) Detection of viral NS1 protein by indirect immunofluorescence analysis is shown. CD36+ cells were infected under normoxia and incubated during 24 h in normoxic (N) or hypoxic (H) conditions as mentioned above. Similar data were obtained in three independent experiments. (D) Southern blot analysis of viral replicative forms extracted from CD36+ cells infected under normoxia, and incubated under normoxic and hypoxic conditions for 48 h. To prove that the B19 DNA present in CD36+ cells (lanes 1 and 3) corresponds to replicative forms, viral DNA was digested with *Bam*HI restriction enzyme prior electrophoresis (lanes 2 and 4). The characteristic DNA doublets (1.4/1.5 and 3.8/3.9 kb) are indicated by arrows (Ozawa et al., 1986). Viral DNA extracted from infectious particles present in a high viremic serum does not form similar doublets and was simultaneously analyzed, emphasizing the replicative nature of the B19 DNA detected in lanes 5 and 6. Radioactive signals were quantified by phosphorimaging. Similar data were obtained in three independent experiments. M: DNA marker (1 kb ladder, Promega).

enables association with ARNT in the nucleus, and DNA-binding to the HBS either in a sense or an antisense orientation. This is followed by the recruitment of the CBP/p300 transcriptional co-activators and upregulation of hypoxia-dependent target genes, such as erythropoietin, aldolase, and vascular endothelial growth factor (VEGF) (Hofer et al., 2002). Therefore, the expression of both

Table 1
Increase of B19 infectious particles production in hypoxic conditions

Volume of supernatant (μl)	Normoxia	Hypoxia
500	12	89
50	0	7
5	0	0

B19-infected CD36+ cells were incubated under normoxic and hypoxic conditions. After 48 h exposure, supernatants were collected. Five, fifty, and five hundred microliters of these supernatants were used to infect fresh CD36+ cells in normoxic conditions. Infected cells were then immunostained with anti-VP2 antibody and counted, giving the titer of infectious B19 particles present in the initial supernatants. The experiment was repeated three times. Because the permissiveness of CD36+ cells depends of the human donor, the number of infected cells varies considerably from experiment to experiment; however, we found every times the same activation factor between cells infected in normoxic and hypoxic conditions using different lots of CD36+ cells. We show here a representative experiment. Normoxia: supernatant collected from CD36+ cells infected in normoxia. Hypoxia: supernatant collected from CD36+ cells infected in hypoxia.

proteins constituting the HIF-1 heterodimer was verified in primary erythroid cells exposed to low oxygen tension. Fig. 3A shows that hypoxic exposure leads to stabilization of HIF-1α in CD36+ human primary erythroid cells. As shown previously, under hypoxia, the oxygen-insensitive HIF-1β subunit (ARNT) tends to enter the nucleus as a heterodimer with HIF-1α (Chilov et al., 1999). This provides an explanation for the increased ARNT levels observed in the nuclear fraction under hypoxic conditions.

Subsequently, the HIF-1-binding properties to the putative viral promoter HBS were analyzed by electrophoretic mobility shift assays. Incubation of a B19-derived oligonucleotide with nuclear extracts isolated from CD36+ cells revealed the presence of a HIF-1/DNA-binding complex mainly formed with the hypoxic cell extracts (Fig. 3B). Competition experiments using unlabeled wild-type and mutated oligonucleotides as well as supershifts using specific anti-HIF-1α and anti-HIF-1β antibodies demonstrated the specificity of this HIF-1/DNA interaction (Fig. 3B). Finally, reporter gene analysis was performed to show the functional responsiveness of the predicted B19 promoter HBS. SV40 promoter-driven luciferase reporter gene constructs, containing either wild-type or mutant B19-derived HBS, were transfected into wild-type and HIF-1α-deficient CHO cell lines. Exposure to hypoxia led to a weak but reproducible increase of luciferase expression from the wild-type B19 and VEGF promoter HBS in wild-type CHO cells, but not in the HIF-1α-deficient CHO cell line (Fig. 3C). Similar results were found with desferrioxamin, an iron chelator, known to functionally stabilize HIF-1α (data not shown).

Discussion

Our results clearly demonstrate that B19 erythrovirus protein expression and replication in human primary erythroid progenitors are upregulated by reduced oxygen sup-
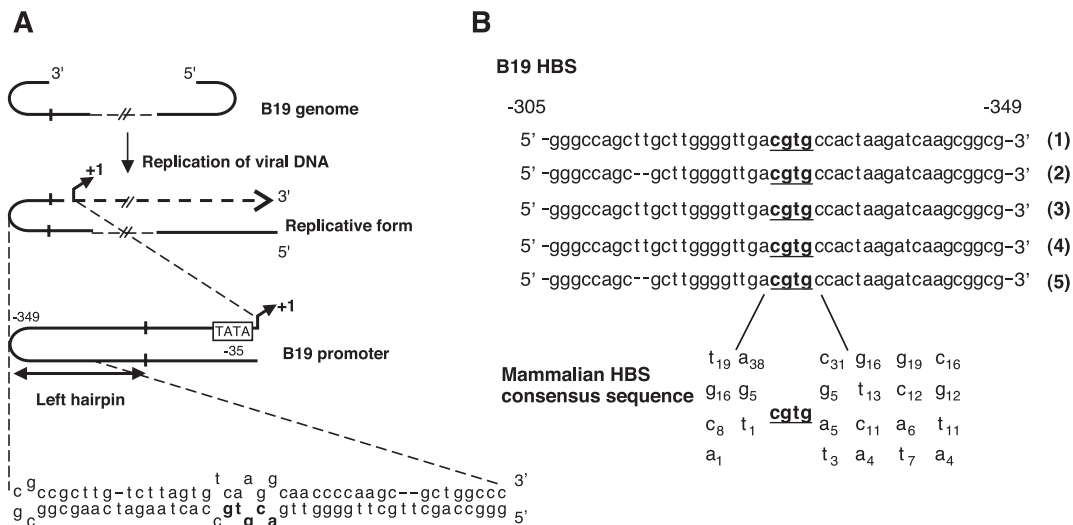

Fig. 2. Left part of B19 genome contains a putative HBS. (A). Localization of the putative HBS in the sequence of the left B19 hairpin. "Flop" single-stranded B19 genomes are converted during viral replication into double-stranded genomes that contain the HBS 294 bp upstream of the TATA box in an antisense orientation. The HBS exists only in B19 single-stranded genomes in the "flop" conformation of the "bubble" of the hairpin (according to Deiss et al., 1990). Nucleotide positions of the TATA box and the extremity of the left hairpin are shown according to the complete P6 promoter sequence described by Momoeda et al. (1994). (B). Conservation of the putative B19 HBS with mammalian consensus sequence. B19 sequences contain a motif strongly resembling the mammalian HBS consensus (core and flanking sequences) (Camenisch et al., 2001). Nucleotide positions of the B19 hairpin sequences are shown according to the complete P6 promoter sequence described by Momoeda et al. (1994). The core sequence of cellular and viral HBS is shown in bold. This sequence was found in all published B19 left hairpin sequences [(3) Deiss et al., 1990; (2) Gallinella G. and Venturolli S., 1999 Genbank access number: AF162273; (4) Kakkola, Hedman, and Soderlund-Venermo, with permission; (1) Momoeda et al., 1994; (5) Zhi et al., 2004].
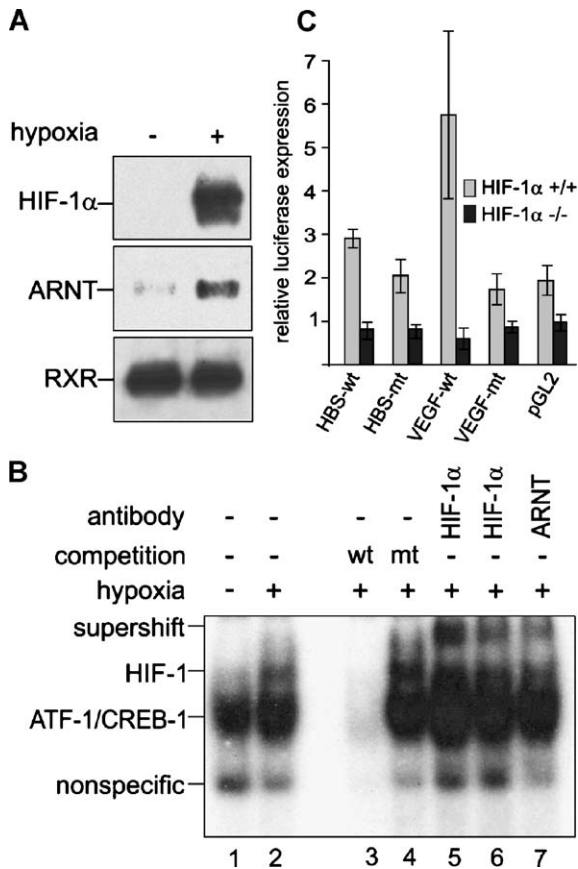
Fig. 3. Evidence that hypoxic upregulation of B19 erythrovirus expression involves HIF-1. (A) Immunoblot analysis of HIF-1α and ARNT (the β subunit of HIF-1) expression in nuclear extracts prepared from noninfected CD36+ cells grown under normoxic or hypoxic conditions during 30 h. The signal obtained with an anti-RXR antibody was used as a control for loading and transfer efficiency. (B) DNA-binding assays of nuclear extracts prepared from noninfected CD36+ cells grown under normoxic or hypoxic conditions during 30 h. Possible HIF-1 DNA-binding activity was analyzed in nuclear extracts using a ^{32}P-radiolabeled probe containing the HBS of the B19 promoter (lanes 1 and 2). Competition experiments were performed using a 50-fold excess of probe containing the wild-type B19 HBS (ACGTG) (lane 3) or with a probe containing a mutated HBS (TTTTG) (lane 4). Specific HIF-1 DNA binding was confirmed by supershift analysis using two different anti-HIF-1α antibodies (lanes 5 and 6) and an anti-ARNT antibody (lane 7). The cellular HBS can also be recognized by the oxygen-insensitive transcription complex ATF-1/CREB (Kvietikova et al., 1995). This complex can bind constitutively to the putative HBS present in B19 P6 promoter, and supershift experiments were performed to verify its specificity (data not shown). (C) Luciferase reporter gene assays of transiently transfected wild-type (C4.5) or HIF-1α-deficient (Ka13) CHO cells cultured under normoxic and hypoxic conditions for 48 h. An SV40 promoter-driven luciferase reporter gene construct contained either the wild-type or the mutant B19 promoter-derived HBS. A corresponding luciferase expression plasmid, containing the VEGF promoter-derived HBS, served as positive control. A cotransfected β-galactosidase expression vector was used as an internal control for transfection efficiency and to correct the luciferase expression. On the vertical axis, the ratio of corrected luciferase expression is represented as obtained under hypoxic versus normoxic conditions. Mean ± standard error of the mean of three independent transfections is shown.

ply (Fig. 1). Hypoxia leads to higher proportion of cells expressing viral proteins (Fig. 1C). This could be due to a higher proportion of infected cells, and also a higher level of expression in each cells leading to its visualization in immunofluorescence analysis. In our model, erythroid cells are infected in normoxic conditions, meaning that virus-receptor interaction and virus internalization occurred at 20% oxygen. This was done to avoid the possibility that hypoxia could act on the attachment or internalization phase. It has also been shown that the expression of the cellular P antigen, the main B19 receptor, is not regulated by hypoxia at the surface of KU812F cells (P. Caillet-Fauquet et al., in press). However, we cannot completely exclude a possible influence of hypoxia on the migration of viral capsids through the cytoplasm and their entry into the nucleus.

Oxygen tension has been previously reported as a factor that may affect viral multiplication (Ebbesen and Zachar, 1998). The recently reported study on human herpesvirus 8 (HHV8) demonstrates that HIF-1, the main cellular hypoxia-responsive transcription factor, is directly implicated in the activation of HHV8 transcription and replication in chronically infected cells (Haque et al., 2003). As we detected the presence of an HBS in the B19 promoter (Fig. 2), it was studied whether HIF-1 could also be implicated in the transcriptional activation of B19 expression leading to higher viral protein synthesis. Hypoxic erythroid cells express high level of HIF-1α that can bind the viral HBS found in the B19 promoter (Figs. 3A and B). Nevertheless, the discrepancy between the high increase of viral mRNA steady-state levels (Fig. 1A) and the quite low induction levels of luciferase expression (Fig. 3C) was surprising. To our opinion, this could be explained in the following three ways. First, the intracellular environment is different between the infection and the transfection models (CD36 vs. CHO cells); however, as it is extremely difficult to transfect primary erythroid cells (data not shown), reporter gene expression had to be studied in CHO cell lines. Second, the P6 promoter may harbor additional binding sites for other oxygen-sensitive factors as shown for the endothelin-1 (ET-1) promoter (Camenisch et al., 2001; Yamashita et al., 2001). Previous studies have shown that the proximal part of the B19 promoter can adopt a conformation that could favor the interaction between distantly binding factors leading to optimal transcriptional activation (Gareus et al., 1998). Additional experiments with a complete B19 infectious clone should be helpful to understand the involvement of HIF-1α and relatives in the transcriptional activation of B19 expression under low oxygen tension levels. Third, as described for several cellular genes, hypoxia could also increase viral mRNA stability. However, the extreme toxicity of the actinomycin D and cycloheximide treatments in the primary erythroid cells did not allow us to study eventual changes in B19 mRNA half time.

In our model, hypoxia repeatedly reduced the number of CD36+ erythroid cells after 24 and 48 h of incubation (data

not shown). Previous reports showed that oxygen supply has an impact on cell maturation and differentiation. Particularly, low oxygen tension levels enhance BFU-E and CFU-E in vitro (Cipolleschi et al., 1997; Koller et al., 1992) maintaining erythroid progenitors in a high B19-sensitive stage (Takahashi et al., 1990). Oxygen tension measurements and mathematical models showed that bone marrow contains low level and that proliferation of erythroid progenitors occurs in hypoxic conditions (Chow et al., 2001; Harrison et al., 2002; Ishikawa and Ito, 1988). As a consequence, we can speculate that hypoxia, by maintaining cells in undifferentiated stages, could possibly help B19 expression and replication, leading to high-grade viremia especially in patients producing high numbers of erythroid progenitor cells (Brown and Young, 1997). Similarly, the high fetal sensitivity to B19 infection could be explained, in addition to the high proportion of erythroid progenitors, by the hypoxic development of fetal tissues (Adelman et al., 1999; Iyer et al., 1998).

Taken together, our data show that the hypoxia enhances significantly the expression and the replication of human B19 erythrovirus in vitro. The low oxygen tension levels measured in several tissues could contribute to B19 replication in sensitive cells in vivo. The mechanisms implicated in this oxygen-upregulated gene expression are probably complex but should be explored in more detail to elucidate the sharp erythroid tropism of B19 erythrovirus. Actually, few infection models are available for in vitro B19 replication, and the production of infectious particles is very low. Transfection of cells with the very recently obtained infectious clone (Zhi et al., 2004) in hypoxic conditions should be very useful to produce high reproducible viral titers, either wild type or recombinant, and study in depth B19 biology.

Materials and methods

Cell culture and infection

CD36+ primary erythroid cells were obtained as previously described (Freyssinier et al., 1999). Normoxic CD36+ cells were infected with 20% sucrose cushion-purified infectious B19 erythrovirus particles at a multiplicity of infection of 0.1 as described (Sol et al., 1999). Briefly, cells were incubated with the viral suspension under normoxic conditions at 4 °C during 1 h and subsequently transferred to 37 °C for 2 h; cells were then carefully washed three times with sterile PBS to eliminate the viral inoculum and subsequently incubated either in normoxia or hypoxia. For transfection studies, C4.5 and Ka13 cells were cultured as described (Wood et al., 1998). CD36 and CHO cells were grown at 37 °C in a conventional incubator at pO_2 of 140 mm Hg (20% O_2 v/v, normoxia) or in hermetic boxes (Modular Incubator Chamber, Billups-Rothenberg Inc.) in which an hypoxic atmosphere was introduced: 7 mm Hg (1% O_2 v/v, hypoxia).

Protein analysis

Immunofluorescence and immunoblot analysis were carried out as described using rabbit anti-NS1 and VP2-specific polyclonal antibodies (Pallier et al., 1997). Nuclear extracts were prepared as previously described (Sol et al., 1999). HIF-1 was detected by immunoblot analysis using specific antibodies against HIF-1α (Transduction Laboratories) and ARNT (a kind gift from O. Bernard).

Infectious particles count

To determine viral titers, CD36+ cells were infected under normoxia with various volumes of viral suspensions provided from infected CD36+ cells grown either under normoxic or hypoxic conditions. After 48 h of exposure to normoxic condition, 2.10^5 CD36+ cells were immunostained using an anti-VP2 specific monoclonal antibody (Chemicon).

Southern and Northern analysis

Viral replicative forms were isolated by performing a Hirt extraction from 10^7 infected CD36+ cells cultured either in normoxia or hypoxia, and analyzed using a ^{32}P-radiolabeled B19 genomic sequence as a probe according to Ozawa et al. (1986).

Total RNA was extracted at 4 °C from 10^6 CD36+ infected cells cultured either in normoxia or hypoxia, and analyzed as previously described (Pallier et al., 1997). ^{32}P-radiolabeled B19 (nucleotides 1813–2451, Shade et al., 1986) and aldolase (a kind gift from O. Bernard) were used as probes. In order to correct for loading and blotting efficiency, B19 mRNA signals were normalized to the signal obtained with a 28S cDNA probe (Ambion).

Radioactive signals were quantified by phosphorimaging (Molecular Imager System GS-525, Biorad).

Electrophoretic mobility shift assays

DNA-binding assays were carried out as described (Chilov et al., 1999; Forsythe et al., 1996). A double-stranded oligonucleotide (5′-TGATCTTAGTGGCACGTCAACC-CCA-3′) containing the HBS of the B19 promoter was used as a probe. An oligonucleotide containing the mutated HBS (5′-TGATCTTAGTGGCTTTTCAACCCCA-3′) was used for competition experiments. Specific HIF-1 DNA binding was confirmed by supershift analysis using two anti-HIF-1α (from Novus Biologicals and NeoMarkers) and one anti-ARNT antibodies (Santa Cruz Biotechnology).

Transfection and reporter gene assays

An oligonucleotide containing either the wild-type (5′-GTCTTAGTGGCACGTCAACCCCAA-3′) or the mutant (5′-GTCTTAGTGGCTTTTCAACCCCAA-3′) B19 pro-

moter-derived HBS was cloned in an antisense orientation into a *Mlu*I–*Xho*I-opened pGL2-promoter vector (Promega). A corresponding luciferase expression plasmid, containing the VEGF promoter-derived HBS (Forsythe et al., 1996), served as positive control. Wild-type (C4.5) or HIF-1α-deficient (Ka13) CHO cells were transiently transfected with Lipofectin according to the manufacturer's recommendations (Invitrogen). After 48 h exposure to normoxia or hypoxia, luciferase detection and correction of transfection efficiency by β-galactosidase expression (Roche) were carried out as described (Semenza et al., 1996). The reporter gene transfection experiments were performed independently at least three times in duplicate.

Acknowledgments

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