Oxygen-regulated expression of the RNA-binding proteins RBM3 and CIRP by a HIF-1-independent mechanism

Sven Wellmann1, Christoph Bührer2*, Eva Moderegger1, Andrea Zelmer1, Renate Kirschner1, Petra Koehne2, Jun Fujita3 and Karl Seeger1

1Department of Pediatric Oncology/Hematology and the 2Department of Neonatology, Charité Campus Virchow-Klinikum, Medical University of Berlin, 13353 Berlin, Germany
2Department of Clinical Molecular Biology, Kyoto University, Kyoto 606-8507, Japan
*Author for correspondence (e-mail: christoph.buehrer@charite.de)

Summary
The transcriptional regulation of several dozen genes in response to low oxygen tension is mediated by hypoxia-inducible factor 1 (HIF-1), a heterodimeric protein composed of two subunits, HIF-1α and HIF-1β. In the HIF-1α-deficient human leukemic cell line, Z-33, exposed to mild (8% O2) or severe (1% O2) hypoxia, we found significant upregulation of two related heterogenous nuclear ribonucleoproteins, RNA-binding motif protein 3 (RBM3) and cold inducible RNA-binding protein (CIRP), which are highly conserved cold stress proteins with RNA-binding properties. Hypoxia also induced upregulation of RBM3 and CIRP in the murine HIF-1β-deficient cell line, Hepa-1 c4. In various HIF-1 competent cells, RBM3 and CIRP were induced by moderate hypothermia (32°C) but hypothermia was ineffective in increasing HIF-1α or vascular endothelial growth factor (VEGF), a known HIF-1 target. In contrast, iron chelators induced VEGF but not RBM3 or CIRP. The RBM3 and CIRP mRNA increase after hypoxia was inhibited by actinomycin-D, and in vitro nuclear run-on assays demonstrated specific increases in RBM3 and CIRP mRNA after hypoxia, which suggests that regulation takes place at the level of gene transcription. Hypoxia-induced RBM3 or CIRP transcription was inhibited by the respiratory chain inhibitors NaN3 and cyanide in a dose-dependent fashion. However, cells depleted of mitochondria were still able to upregulate RBM3 and CIRP in response to hypoxia. Thus, RBM3 and CIRP are adaptively expressed in response to hypoxia by a mechanism that involves neither HIF-1 nor mitochondria.

Key words: RBM3, CIRP, Hypoxia, HIF-1

Introduction
Hypoxia is a reduction in the normal level of tissue oxygen tension, which occurs in humans living in high altitude and commonly in a variety of acute and chronic vascular, pulmonary, and neoplastic diseases. Under hypoxic conditions, the transcription of a variety of genes involved in glucose metabolism, cell proliferation, angiogenesis, erythropoiesis and other adaptive responses increases (Harris, 2002). This gene regulation by hypoxia is widely dependent on activation of the transcriptional complex HIF-1 (Wang et al., 1995). HIF-1 is a heterodimer that consists of the hypoxic response factor HIF-1α and the constitutively expressed aryl hydrocarbon receptor nuclear translator (ARNT), also known as HIF-1β. ARNT also forms heterodimers with endothelial PAS protein 1 (EPAS1), which shows 48% sequence homology to HIF-1α (Wiesener et al., 1998). In the absence of oxygen, HIF-1α/ARNT heterodimers bind to hypoxia-response elements, thereby activating the expression of numerous hypoxia-response genes (Harris, 2002). In the presence of oxygen, HIF-1α is bound to the tumor suppressor von Hippel-Lindau protein (Maxwell et al., 1999). This interaction causes HIF-1α to become ubiquitylated and targeted to the proteasome for degradation.

The list of genes regulated in response to hypoxia by HIF-1 is ever increasing, and most research in hypoxia has concentrated on the HIF-1 pathway. The central role of HIF-1 is emphasized by its ubiquitous expression, hampering efforts to study alternative pathways regulating gene transcription in response to hypoxia. Serendipitously, we identified a human B-cell acute lymphoblastic leukemia cell line that failed to respond to hypoxia by expression of VEGF, a known HIF-1 target (Forsythe et al., 1996). Further investigations revealed this cell line to harbour a homozygous deletion for HIF-1α. Subsequent gene expression profiling showed that none of the HIF-1 inducible genes was upregulated under hypoxia whereas expression of two related RNA-binding proteins, RBM3 (Derry et al., 1995) and CIRP (Nishiyama et al., 1997), significantly increased in response to hypoxia.

Here we demonstrate that mRNA and protein levels of the two RNA-binding proteins, RBM3 and CIRP, increase in response to hypoxia by a mechanism not involving HIF-1. We further show that the oxygen threshold required for RBM3 and CIRP induction differs from that of HIF-1-regulated gene expression. Finally, we report that this novel mechanism of hypoxic adaptation is inhibited by the respiratory chain inhibitors NaN3 and cyanide but is also fully functional in the absence of mitochondria.
Materials and Methods

Cell culture

Human cell lines HeLa, Hep3B and REH were purchased from the German Collection of Microorganisms and Cell cultures (DSMZ, Braunschweig, Germany). The human acute lymphoblastic leukemia cell line Z-33 was a gift from Z. Estrov (MD Anderson Cancer Center, Houston, TX) (Estrov et al., 1996). Its karyotype was confirmed by comparative genomic hybridization (CGH), kindly performed by H. Tännies (Institute of Human Genetics, Charité Medical Center, Berlin) as described (Tännies et al., 2001). The murine hematoma line Heca-1 wt and the c4 mutant clone lacking ARNT function were a gift from P. Ratcliffe (Henry Wellcome Building for Genomic Medicine, University of Oxford, UK) (Wood et al., 1996).

Mitochondria-depleted rho0 143B TK– cells resulting from the human osteosarcoma cell line, 143B TK–, were kindly provided by G. Hofhaus (Max-Planck-Institute, Frankfurt/Main, Germany). Z-33, REH and HeLa cells were grown in RPMI 1640 (Biochrom, Berlin, Germany). Hep3B, Hepa-1, 143B cells were grown in Dulbecco’s Modified Eagle’s medium (DMEM, Invitrogen, Karlsruhe, Germany) with 10% heat inactivated fetal calf serum (FCS, Biosan, Czech Republic) and 1% penicillin/streptomycin (Biochrom) in a fully humidified incubator (Jouan, Unterhaching, Germany). For hypothermia indicated, employing a humidified three gas regulated IG750 incubator (Jouan, Unterhaching, Germany). In hypoxia experiments, O2 was tightly regulated at 1% or 8% as required.

Gene expression profiling

Total RNA was extracted using TriReagent (Sigma-Aldrich) and subsequently purified employing the QiagenRNeasy kit (Qiagen, Hilden, Germany). RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). cDNA synthesis was performed from 9 μl (13.5 μg) of total RNA using a T-7 linked oligo-dT primer, and cRNA was then synthesized with biotinylated UTP and CTP; a detailed description is given elsewhere (Dürig et al., 2003). Fragmentation of cRNA, hybridization to Human Genome U133A oligonucleotide arrays (Affymetrix, Santa Clara, CA), washing and staining as well as scanning of the arrays in a GeneArray scanner (Agilent, Palo Alto, CA) were performed as recommended in the Affymetrix Gene Expression Analysis Technical Manual (Ludger Klein-Hitpass, Institute of Cell Biology, Medical Faculty, University of Essen, Germany). Signal intensities (MAS5 signal) and detection calls for statistical analysis were determined using the GeneChip 5.0 software (Affymetrix). A scaling across all probe sets of all four arrays to an average intensity of 1000 units was included to compensate for variations in the amount and quality of the cRNA samples and other experimental variables. Results show the mean signal intensities determined for the genes overexpressed in hypoxic versus normoxic cell lines, where a difference is significant at a p value less than 0.001.

Ribonuclease protection assay (RPA) and reverse-transcriptase-PCR (RT-PCR)

RPA for HIF1A and HIF-2α (EPAS1) were kindly performed by M. Wiesener (Department of Nephrology and Medical Intensive Care, Charité Medical Center, Berlin) as described (Maxwell et al., 1993), with parallel hybridization using 30 μg for HIF1A, 30 μg for EPAS1, and 1 μg for RNU6 (U6 small nuclear RNA). 32P-labeled riboprobes were generated using SP6 or T7 RNA polymerase. The templates used yielded protected fragments as follows: 221 bp for EPAS1 (nucleotides 2542 to 2762, accession no. U81984), 255 bp for HIF1A (nucleotides 764 to 1018, U22431), and 106 bp for RNU6 (nucleotides 1 to 107, X01366). After resolution on 8% polyacrylamide gels, quantification was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Signals for HIF1A mRNA and EPAS1 mRNA were normalized to a value of 100 for EPAS1 in Hep3B cells, allowing for the different number of labeled nucleotides in the two protected fragments. For RT-PCR, total RNA was extracted using the QiagenRNeasy kit (Qiagen), transcribed into cDNA by use of Superscript II Reverse Transcriptase (Invitrogen) and random primers.
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Nuclear run-on assay

Nuclei of HeLa cells were isolated, and run-on transcription experiments were performed by a method modified from Greenberg et al. (Greenberg and Bender, 1997). Briefly, 1x10^7 cells were collected by scraping and washed. After lysis for 12 minutes in ice-cold nuclear extraction buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 2.5 mM MgCl2 and 0.5% (v/v) Nonidet p-40) nuclei were isolated by centrifugation through 10% sucrose in nuclear extraction buffer. Pelleted nuclei were immediately forwarded to run-on transcription. During isolation, nuclear morphology was monitored by phase contrast microscopy. Cell membrane permeability was assessed by Trypan Blue exclusion. For nuclear run-on analysis, 50 µl of nuclei suspension were incubated for 40 minutes at 30°C in a total of 0.1 ml of reaction mixture containing 20 mM Tris-HCl, pH 8.0, 150 mM KCl, 5 mM MgCl2, 1 mM DTT, 0.5 mM each of CTP, ATP, UTP, GTP (Promega, Mannheim, Germany) and 20% glycerol. RNase inhibitor (Invitrogen) was included (20 U/vial) to prevent RNA degradation. The reaction was terminated by adding DNase I (10 U/vial) and proteinase K (200 µg/ml) (both purchased from Qiagen). Immediately before transcription a sample of each condition was removed. Total RNA before and after transcription was isolated, and RBM3, CIRP and B2M mRNA were quantitated using real-time RT-PCR, as described above. The extent of RBM3 and CIRP mRNA transcription was determined by subtracting the amount of RBM3 and CIRP mRNA standardized to B2M mRNA prior to transcription from the amounts post transcription. Data were normalized against the relative difference in expression of mRNA was analyzed with the U133A human cDNA expression array (Table 1B).

Protein extraction and immunoblot analysis

Cell protein extracts were quantified as described previously (Wiesener et al., 1998). For immunoblotting, proteins were separated by SDS-PAGE, blotted on Hybond-P PVDF (polyvinylidene fluoride) membranes (Amersham Biosciences, Freiburg, Germany), and stained with monoclonal anti-HIF-1α antibody (Transduction Laboratories, Lexington, KY), polyclonal anti-RBM3 antibody (Danno et al., 2000) or polyclonal anti-CIRP antibody (Nishiyama et al., 1997) as detailed previously. Bound antibodies were detected using secondary antibodies conjugated with horseradish peroxidase (for HIF-1α from DAKO, Ely, UK, and for RBM3 and CIRP from DPC Biermann, Bad Nauheim, Germany) and enhanced chemiluminescence systems (ECL, from Amersham Biosciences).

Determination of cell viability

Apoptotic and necrotic cells were quantitated by use of the phosphatidyl serine detection kit (IQProducts, Groningen, Netherlands). Briefly, cells were sequentially incubated at 4°C for 20 minutes with fluorescein isothiocyanate (FITC)-conjugated Annexin V and for 5 minutes with propidium iodide as suggested by the manufacturer. Stained cells (10^4 per sample) were analyzed on a FACSCalibur flow cytometer with standard CellQuest software (BD Biosciences, Palo Alto, CA).

Measurement of intracellular ATP

Cellular ATP content was quantitated by luminometry of the luciferin firefly luciferase reaction using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the procedure recommended by the manufacturer.

Results

The B-cell acute lymphoblastic leukemia cell line, Z-33, lacks expression of HIF-1α owing to a homozygous microdeletion of the HIF-1α gene locus

When analyzed by RPA, no HIF1A signal could be detected in Z-33 cells, in contrast to human B-cell leukemia REH or hepatoma Hep3B cells (Fig. 1). No HIF1A signal was obtained after amplification of Z-33 genomic DNA using primers specific for the exon-3-intron3 boundary or exon-12, while expected signals were obtained with primers for the regions closely flanking HIF1A on chromosome 14 (Fig. 2). Thus, both HIF1A loci are deleted in Z-33 cells, preventing production of HIF-1α mRNA or protein (Figs 1, 3). CGH failed to detect any deletions within chromosome 14. When analyzed by RPA, EPAS1 expression was faint or absent in Z-33 or REH cells, respectively (Fig. 1). This finding was in line with the results from gene expression analysis with the U133A human cDNA expression array (Table 1B).

HIF-1α-deficient Z-33 cells increase expression of several genes in response to hypoxia

Z-33 cells were exposed to room air or 1% O2 for 8 hours, and the relative difference in expression of mRNA was analyzed by the U133A human cDNA expression array. Out of more than 15,000 genes analyzed, 9 genes fulfilled both criteria, genes overexpressed in hypoxic versus normoxic cells with P values <0.001 and absolute expression level over 800 units in hypoxia of Z-33 (Table 1A). Although expression of housekeeping genes, such as β-actin (ACTB), β2-microglobulin (B2M) and RPL13a, was virtually identical for hypoxia and normoxia, expression of several genes was found to be increased (Table 1B, Fig. 1). Among these, HIF1A homologue, EPAS1, was virtually identical for hypoxia and normoxia in both Z-33 and REH (Table 1C), whereas the HIF1A homologue, EPAS1, was virtually identical for hypoxia and normoxia in both Z-33 and REH (Table 1C). In contrast, HIF1A-dependent genes, such as WT1 (Wagner et al., 2003), RTP801 (Shoshani et al., 2002), BNIP3...
(Sowter et al., 2001), prolyl 4-hydroxylase α (P4HA1) (Takahashi et al., 2000), phosphofructo-2-kinase fructose-2,6-biphosphatase-3 (PFKFB3) (Minchenko et al., 2002), glucose transporter 3 (GLUT3) (O’Rourke et al., 1996), DEC-1/Stra13 (Miyazaki et al., 2002), and VEGF (Forsythe et al., 1996), were significantly overexpressed in response to hypoxia in HIF1A-competent REH cells but not in Z-33 cells.

The RNA-binding proteins RBM3 and CIRP are induced in response to hypoxia independently of HIF-1

The two related proteins RBM3 and CIRP were among the five most strongly expressed genes displaying increased mRNA transcription in response to hypoxia in both HIF1A-deficient Z-33 and HIF1A-competent REH cells (Table 1A). Therefore, we investigated RBM3 and CIRP regulation at the protein level. Western blot analysis showed strongly increased RBM3 expression in both leukemic cell lines, whereas CIRP increased moderately (Fig. 3A). Hypoxia increased RBM3 and CIRP mRNA (Fig. 3B) as well as protein expression (Fig. 3C) in Hepa-1 wildtype and ARNT-deficient Hepa-1 c4 mutant cells.

The dynamic range of oxygen tension differs between HIF-1α-dependent and HIF-1α-independent genes

Increased gene expression in response to hypoxia was further characterized in the two adherently growing human cancer cell lines, HeLa and Hep3B. Increased RBM3 and CIRP expression was equally induced by 8% and 1% oxygen but not by the iron chelator DFO (Fig. 4). In contrast, expression of VEGF, a known target of HIF, required 1% oxygen but did not increase when oxygen was lowered to only 8%. DFO was equally potent as 1% oxygen to induce VEGF transcription. Similarly, the transition metal chelator 2,2'-dipyridyl or cobalt chloride induced VEGF but not RBM3 or CIRP (data not shown). Decreasing the incubator temperature to 32°C induced RBM3 expression.

Fig. 1. Expression of genes encoding HIF-1α (HIF1A) and HIF-2α (EPAS1) in Z-33, REH and Hep3B cells. The human B-cell acute lymphoblastic leukemia cell lines Z-33 and REH as well as the hepatoblastoma cell line Hep3B (Hep) were cultured in normoxia. Ribonuclease protection analysis using 30 μg of total RNA revealed no expression of HIF1A RNA in Z-33 in contrast to REH and Hep3B. The latter depicted strong signal intensity for EPAS1 whereas very little EPAS1 signal was obtained in Z-33 or REH cells. Probing against U6 small nuclear RNA (RNU6) was used for loading control. Complete ribonuclease digestion of 32P-riboprobes in the absence of added RNA was demonstrated on the right lane (Con).

Fig. 2. Agarose gel electrophoresis of PCR products indicating homozygous deletion of the HIF-1α gene in Z-33. (A) Genomic PCR of DNA of Z-33 and REH was performed for the HIF-1α gene (HIF1A exon/intron-3, lane 3; HIF1A exon-12, lane 4) and HIF-1α flanking gene regions: proximal (prox) to the HIF-1α locus marker D14S592, lane 1 and D14S1258, lane 2; distal (dist) D14S1334, lane 5 and D14S183, lane 6; β-globin, lane 7; and 100-bp DNA size marker, lane M. Fig. 2B shows the positions of the different markers relative to the HIF-1α gene on the chromosome band 14 q23.1 and q23.2, numbered according to the lanes.
and CIRP expression similarly to hypoxia but had no effect on VEGF expression (Fig. 4). As a corollary, protein levels of RBM3 and CIRP increased in HeLa cells after 24 hours of hypothermia (32°C) or hypoxia (1% oxygen) but were unaffected by DFO (Fig. 5).

The increase of RBM3 and CIRP is sustained during prolonged hypoxia and requires de novo mRNA synthesis.

Kinetic analysis of RBM3 and CIRP protein content in HeLa cells showed that both proteins increased continuously during 24 hours of hypoxia (Fig. 6A). The increase of RBM3 and CIRP mRNA was abrogated in the presence of actinomycin-D (Fig. 6B). To demonstrate that the rise of RBM3 and CIRP mRNA in response to hypoxia was mediated by increased transcription, nuclear in vitro run-on assays were performed. Nuclei were isolated from control and hypoxic HeLa cells, and the RBM3/B2M and CIRP/B2M mRNA ratios were determined from samples taken before and after 40 minutes of in vitro transcription. De novo synthesis of RBM3 and CIRP mRNA normalized to B2M mRNA increased by more than threefold in response to hypoxia (Fig. 6C).

Reduced oxygen tension, not energy depletion, leads to RBM3 and CIRP expression

To investigate whether ATP depletion following hypoxia plays a role in inducing RBM3 and CIRP, we depleted cellular energy stores by reducing the glucose concentration in the medium and blocking the oxidative phosphorylation at various mitochondrial sites. Culturing cells in glucose-free medium for 24 hours decreased intracellular ATP concentrations to 37% of baseline while increasing RBM3 and CIRP expression 1.5-2-fold (data not shown). This effect was additive to that of hypoxia and paralleled changes observed for VEGF expression. However, NaN3 inhibited hypoxia-induced RBM3 and CIRP expression in a dose-dependent fashion. RBM3 expression after hypoxia was reduced by 50% at 5 mM NaN3 and was down to normoxia baseline levels at 10 mM NaN3 for 24 hours. Hypoxia-mediated CIRP induction was even more sensitive to NaN3, with expression reduced by 50% or down to normoxia baseline levels at 1 mM and 5 mM NaN3, respectively (Fig. 7A). In sharp contrast, NaN3 at 1-10 mM was strongly synergistic with hypoxia in inducing VEGF. Under normoxia, NaN3 up to 10 mM by itself only slightly reduced RBM3 and CIRP expression, whereas VEGF remained unchanged. The pan-respiratory chain uncoupler CCCP (15 μM) reduced ATP concentrations to 36% of baseline while simultaneously reducing hypoxia-mediated RBM3 and CIRP induction, inducing VEGF expression under normoxia and superinducing VEGF in response to hypoxia (Fig. 7A). Concentrations of NaN3 or CCCP that reduced overall cell viability by less than 50% (Fig. 7A,B) were able to elicit a robust increase in VEGF mRNA, similarly to hypoxia, while abolishing (NaN3) or diminishing (CCCP) hypoxia-mediated RBM3 and CIRP mRNA expression. A moderate reduction of hypoxia-mediated RBM3 and CIRP expression was observed with the mitochondrial inhibitors targeting complex I (rotenone, 1-5 μg/ml), complex II (TTFA, 20-100 μM), complex III (antimycin, 0.2-1 μg/ml), or oligomycin (10-50 μM), which downstream of complex IV blocks the H+ transporting ATP synthase and the Na+/K+ transporting ATPase (data not shown).

Hypoxia-induced RBM3 and CIRP expression does not involve changes of cellular redox status

Several drugs known to affect the cellular redox status were tested for their ability to influence RBM3 and CIRP expression. The antioxidants NAC (1-10 mM), DTT (0.5 mM), ascorbic acid (1-5 mM), PDTC (50-100 μM), NDGA (1-10 μM), or ebselen (10-100 μM) had only minimal effects on RBM3 or
CIRP expression under normoxic or hypoxic conditions (data not shown). H2O2 (1-10 mM) did not affect RBM3 or CIRP expression under normoxic conditions while increasing VEGF two-to threefold. Hypoxia-mediated RBM3 or CIRP expression was slightly reduced by H2O2 whereas hypoxia-mediated VEGF induction was enhanced (data not shown).

Induction of RBM3 and CIRP in response to hypoxia also occurs in the absence of mitochondria. As the mitochondrial complex IV inhibitors NaN3 and CCCP abolished RBM3 and CIRP induction in response to hypoxia, we assessed the involvement of mitochondria in this hypoxia-response pathway. When HeLa cells were grown in the presence of EB at either 50 or 100 ng/ml, in medium enriched for glucose, pyruvate and uridine, for 6 days, mitochondrial DNA concentrations dropped to 15.3% or 3.9%, respectively, while no change was noted for baseline and hypoxia-induced RBM3 and CIRP transcripts (Fig. 8). In addition, baseline and hypoxia-induced

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**Table 1. Comparative gene expression analysis (normoxia versus hypoxia) of HIF-1α-deficient cells (Z-33) and HIF-1α-competent cells (REH)**

### A Transcripts significantly increased by hypoxia

| Gene or sequence name | GenBank accession no. | Z-33 | REH |
|-----------------------|-----------------------|------|-----|
| **RBM3** (RNA-binding motif protein 3) | NM_006743.1 | 8485 | 3649 | 2.3 | 5495 | 2036 | 2.7 |
| **C2orf97** (chromosome 20 open reading frame 97) | NM_021158.1 | 7005 | 2593 | 2.7 | 13816 | 8302 | 1.7 |
| **HLA-DPA1** (histocompatibility complex, class II, DPtr1) | NM_033554 | 5507 | 1967 | 2.8 | 7450 | 2480 | 3.0 |
| **CIRP** (cold inducible RNA-binding protein) | NM_001280.1 | 4948 | 1783 | 2.8 | 8170 | 3298 | 2.5 |
| **LTB** (lymphotoxin β) | NM_002341.1 | 1602 | 576 | 2.8 | 13068 | 8057 | 1.6 |

Clone 2.2H12 (Ndr protein kinase, SerThr kinase 38) | AF034187.1 | 1526 | 149 | 10.2 | 263 | 247 | 1.1 |
| **C20orf97** (chromosome 20 open reading frame 97) | NM_021158.1 | 7005 | 2593 | 2.7 | 13816 | 8302 | 1.7 |
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Table 1A shows genes fulfilling both criteria, overexpression in hypoxic versus normoxic Z-33 cells with P-values <0.001 and absolute gene expression in hypoxic Z-33 cells >800 units. The expression of these genes in REH is also given.

### B Effect of hypoxia on HIF-1α, EPAS1 and ARNT in Z-33 and REH

| Gene or sequence name | GenBank accession no. | Z-33 | REH |
|-----------------------|-----------------------|------|-----|
| **HIF1A** (hypoxia-inducible factor 1, α subunit) | NM_001530.1 | 33 | 62 | 0.5 | 7134 | 6351 | 1.1 |
| **EPAS1** (endothelial PAS domain protein 1) | NM_001430.1 | 78 | 64 | 1.2 | 26 | 82 | 0.3 |
| **ARNT** (aryl hydrocarbon receptor nuclear translocator) | NM_001668.1 | 333 | 606 | 0.5 | 458 | 371 | 1.2 |

### C Effect of hypoxia on various reference genes

| Gene or sequence name | GenBank accession no. | Z-33 | REH |
|-----------------------|-----------------------|------|-----|
| **ACTB** (β actin) | NM_001101.2 | 28433 | 29452 | 1.0 | 45102 | 40679 | 1.1 |
| **B2M** (β2-microglobulin) | NM_004048.1 | 36430 | 36616 | 1.0 | 45031 | 43295 | 1.0 |
| **RPL13A** (ribosomal protein L13a) | NM_012423.1 | 75066 | 65366 | 1.1 | 56125 | 57778 | 1.0 |

Both B-cell acute lymphoblastic cell lines, Z-33 and REH, were subjected to either hypoxia (Hy, 1% O2) or normoxia (No, 20% O2) for 8 hours.

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**Fig. 4.** Mild and severe hypoxia as well as hypothermia induce RBM3 and CIRP expression. HeLa and Hep3B cells were exposed to either normoxia (N), 1% oxygen (1%), 8% oxygen (8%), desferrioxamine (D) or 32°C-hypothermia (C) for 24 hours. RNA was isolated and subjected to real-time RT-PCR analysis. RBM3, CIRP and VEGF RNA levels were normalized to B2M RNA levels and are expressed as fold change in the different experiment samples compared with the corresponding normoxic samples (mean±s.e.m., n=3).
Oxygen-regulated expression of RBM3 and CIRP

Discussion

The present investigation demonstrates that the two structurally related RNA-binding proteins, RBM3 (Derry et al., 1995) and CIRP (Nishiyama et al., 1997), are upregulated in response to hypoxia. This regulation does not require HIF-1, as shown by upregulation of RBM3 and CIRP in HIF-1α- or ARNT (HIF-1β)-deficient cells, and the inability of HIF-1-targeting drugs to evoke an increase in RBM3 and CIRP. In contrast to HIF-1-regulated gene expression, RBM3 and CIRP levels increased under mild hypoxic conditions.

CIRP, also known as A18 hnRNP (Sheikh et al., 1997), and RBM3 belong to the hnRNP subgroup of RNA-binding proteins. While CIRP is expressed ubiquitously, RBM3 tissue distribution appears to be more restricted (Danno et al., 1997). Most notably, both RBM3 and CIRP belong to the small number of genes that show upregulation in response during mild hypothermia (32°C) but not by heat stress (Sonna et al., 2002). In addition, CIRP induction and translocation from the nucleus to the cytoplasm has also been observed after ultraviolet irradiation (Yang and Carrier, 2001).

RBM3 and CIRP are poorly characterized proteins that may participate in transcriptional and post-transcriptional events of gene expression. Target genes of CIRP identified after exposure to UV radiation include replication protein A and thioredoxin (Yang and Carrier, 2001). Possibly, CIRP plays a protective role against various stressors by stabilizing specific transcripts involved in cell survival. The RBM3 mRNA 5′ leader sequence contains a number of specialized sequences that allow initiation of translation independently of the methylated G nucleotide 5′-cap that is typically used by cells to tag an mRNA molecule for initiation of protein synthesis (Chappell et al., 2001). RBM3 has been identified in five genes to be highly involved in suppression of cell death in various cell lines (Kita et al., 2002). In addition, RBM3 was found to be involved in maintaining cytokine-dependent proliferation in the human premyeloid cell line, TF-1 (Baghdoyan et al., 2000).

Fig. 5. Mild hypoxia as well as hypothermia increase RBM3 and CIRP protein but not HIF-1α, whereas desferrioxamine (DFO) does not affect RBM3 and CIRP protein. HeLa cells were cultured in parallel in normoxia (N), 8% oxygen (8%), DFO or 32°C-hypothermia (cold) for 24 hours. Whole-cell lysates were isolated and subjected to western blot analysis for RBM3, CIRP and HIF-1α. To control sample loading and transfer, the blots were stripped and reprobed for β-actin.

Fig. 6. Hypoxia induces a persistent increase in RBM3 and CIRP protein levels via de novo mRNA synthesis. (A) HeLa cells were cultured in parallel in normoxia or 1% oxygen as indicated. Whole-cell lysates were isolated and subjected to western blot analysis for RBM3 and CIRP. To control sample loading and transfer, the blots were stripped and reprobed for β-actin. (B) HeLa cells were cultured in parallel in normoxia or 1% oxygen exposed to actinomycin-D for 24 hours. RNA was isolated and subjected to real-time RT-PCR analysis. RBM3 and CIRP mRNA levels were normalized to β2-microglobulin (B2M) mRNA levels and are expressed as fold change in the different experiment samples compared with the corresponding normoxic samples (mean±s.e.m., n=3). (C) HeLa cells were cultured in parallel in normoxia or 1% oxygen as indicated for 24 hours. Nuclei were isolated, and in vitro transcription was allowed to resume for 40 minutes. RNA was isolated before and after in vitro transcription and subjected to real-time RT-PCR analysis. The extent of RBM3 and CIRP mRNA transcription was determined by subtracting the amount of RBM3 and CIRP mRNA standardized to B2M prior to transcription from the amounts post transcription (mean±s.e.m., n=3). Results are given as a ratio of copies of target gene (RBM3 or CIRP) to copies of the reference gene (B2M).
Thus, the increased intracellular levels of RBM3 and CIRP in response to hypoxia may contribute to the physiological changes enabling cell integrity and survival under conditions of reduced oxygen supply.

The mechanism involved in increasing RBM3 and CIRP mRNA and protein synthesis in response to hypoxia appears to involve enhanced transcription of the genes, as shown by inhibition by actinomycin-D and direct nuclear in vitro run-on assays. Glucose deprivation was able to induce RBM3 and CIRP, but only to levels approximating less than 50% of those seen with hypoxia. Low oxygen tension, but not energy depletion, appears to be the critical event resulting in RBM3 and CIRP induction, as inhibition of the respiratory chain was not able to induce RBM3 and CIRP. On the contrary, CCCP, which depletes cellular energy stores by uncoupling electron transfer of mitochondrial complex I, II, III and IV, diminished the induction of RBM3 and CIRP seen after hypoxia or hypothermia. RBM3 and CIRP induction was totally blocked by NaN3, which more specifically targets complex IV (cytochrome C oxidase) by binding to its heme moiety (Palmer, 1993). In contrast, hypoxia-induced RBM3 and CIRP induction was only moderately reduced by drugs targeting the complex I, II or III of the mitochondrial respiratory chain. However, reduction of mitochondrial DNA to 10% or 3% by culture with ethidium bromide did not alter the hypoxic induction of RBM3 and CIRP, and the hypoxic inducibility of RBM3 and CIRP was comparable in osteosarcoma cells devoid of mitochondria and wild-type osteosarcoma cells, arguing against a role for mitochondria in mediating the RBM3/CIRP response to hypoxia. As NaN3 and CCCP display strong affinity for heme (Palmer, 1993), we propose that the O2 sensor governing hypoxic expression of RBM3 and CIRP involves a heme-containing protein (Rodgers, 1999).

Requirements for RBM3 and CIRP induction by hypoxia differ in several points from those described for HIF-1-

Fig. 7. The mitochondrial inhibitors NaN3 and CCCP inhibit RBM3 and CIRP induction by hypoxia but superinduce VEGF expression by hypoxia. HeLa cells were cultured in parallel in normoxia or 1% oxygen as indicated for 24 hours. Different concentrations of either NaN3 or CCCP were added as indicated. (A) RNA was isolated and subjected to real-time RT-PCR analysis. RBM3, CIRP and VEGF RNA levels were normalized to B2M RNA levels and are expressed as fold change in the different experiment samples compared with the corresponding normoxic samples (means±s.e.m., n=3). To control for cell viability, cells were harvested and incubated with annexin V and propidium iodide. Cell viability is given as the percentage of cells negative for both annexin V and propidium iodide staining (means±s.e.m., n=3). (B) Annexin V reactivity and propidium iodide uptake of control HeLa cells (left dot blot), HeLa cells exposed to 5 mM NaN3 (center dot blot) or HeLa cells exposed to 20 µM CCCP (right dot blot) for 24 hours.
Oxygen-regulated expression of RBM3 and CIRP

Fig. 8. Induction of RBM3 and CIRP in response to hypoxia does not require mitochondria. mtDNA was depleted from HeLa cells with EB in two different concentrations for 6 days as indicated. Subsequently cells were cultured in parallel in normoxia or 1% oxygen as indicated for 24 hours. Parental (WT) and rho0 143B cells, which are devoid of mtDNA, were used as control. Total DNA and RNA was isolated. The upper diagram shows the relative percentage (amount relative to the untreated control cell lines) of mtDNA, assessed by quantitative real-time PCR (see Materials and Methods). RNA was subjected to real-time RT-PCR analysis; RBM3, CIRP and VEGF RNA levels were quantitated, normalized to B2M RNA levels and are expressed as fold change in the different experiment samples compared with the corresponding normoxic samples (mean±s.e.m., n=3).

We thank Michael Wiesener for helpful discussion and RPA analysis, and Lucia Badiali for flow cytometry measurements. This study was supported by grants from the Madelaine Schickedanz Kinderkrebs-Stiftung, Fürth, Germany (to S.W.) and from the BMBF National Genome Research Network, Bonn, Germany (to K.S.).

References

Baghdoyan, S., Dubreuil, P., Eberle, F. and Gomez, S. (2000). Capture of cytokine-responsive genes (NACA and RBM3) using a gene trap approach. Blood 95, 3750-3757.

Chappell, S. A., Owens, G. C. and Mauro, V. P. (2001). A 5' leader of Rbm3, a cold stress-induced mRNA, mediates internal initiation of translation with increased efficiency under conditions of mild hypothermia. J. Biol. Chem. 276, 36917-36922.

Chiu, R. W., Chan, L. Y., Lam, N. Y., Tsui, N. B., Ng, E. K., Rainer, T. H. and Lo, Y. M. (2003). Quantitative analysis of circulating mitochondrion DNA in plasma. Clin. Chem. 49, 719-726.

Danno, S., Itoh, K., Matsuda, T. and Fujita, J. (2000). Decreased expression of mouse Rbm3, a cold-shock protein, in Sertoli cells of cryptorchid testis. Am. J. Pathol. 156, 1685-1692.

Danno, S., Nishiyama, H., Higashitsuji, H., Yokoi, H., Xue, J. H., Itoh, K., Matsuda, T. and Fujita, J. (1997). Increased transcript level of RBM3, a member of the glycine-rich RNA-binding protein family, in human cells in response to cold stress. Biochem. Biophys. Res. Commun. 236, 804-807.

Derry, J. M., Kerns, J. A. and Francke, U. (1995). RBM3, a novel human gene in Xp11.23 with a putative RNA-binding domain. Hum. Mol. Genet. 4, 2307-2311.

Dong, Z., Venkatachalam, A. M., Wang, J., Patel, Y., Saikumar, P., Semenza, G. L., Force, T. and Nishiyama, J. (2001). Up-regulation of apoptosis inhibitory protein IAP-2 by hypoxia. Hif-1-independent mechanisms. J. Biol. Chem. 276, 18702-18709.

Dürig, J., Näckel, H., Hüttemann, A., Kruse, E., Höltter, T., Halfmeyer, K., Führer, A., Rudolph, R., Kalhorni, N., Nusch, A. et al. (2003). Expression of ribosomal and translation-associated genes is correlated with a favorable clinical course in chronic lymphocytic leukemia. Blood 101, 2748-2755.

Estrov, Z., Talpaz, M., Ku, S., Harris, D., Lappushin, R., Koller, C. A., Hirsh-Ginsberg, C., Huh, Y., Yee, G. and Kurzrock, R. (1996). Molecular and biologic characterization of a newly established Philadelphia-positive acute lymphoblastic leukemia cell line (Z-33) with an autocrine response to GM-CSF. Leukemia 10, 1534-1543.

Forsythe, J. A., Jiang, B. H., Iyer, N. V., Agani, F., Leung, S. W., Koos, R. D. and Semenza, G. L. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol. Cell Biol. 16, 4604-4613.

Greenberg, M. E. and Bender, T. P. (1997). Identification of Newly Transcribed RNA. Harvard, MA: John Wiley & Sons.

Harris, A. L. (2002). Hypoxia – a key regulatory factor in tumour growth. Nat. Rev. Cancer 2, 38-47.

King, M. P. and Attardi, G. (1996). Isolation of human cell lines lacking mitochondrial DNA. Methods Enzymol. 264, 304-313.

Kita, H., Carmichael, J., Swartz, J., Muro, S., Wyttenbach, A., Matsubara, K., Rubinstein, D. C. and Kato, K. (2002). Modulation of polyglutamine-dependent mechanisms. First, iron chelators or divalent transition metal ions replacing ferrous iron from the HIF-prolyl hydroxylase were ineffective in inducing RBM3 or CIRP, while inducing HIF-1 or HIF-1-regulated genes such as VEGF. Second, the threshold of oxygen tension evoking gene transcription was found to be equivalent to 8% oxygen for CIRP and RBM3, as opposed to 1% oxygen for HIF-1. Third, RBM3 and CIRP induction by hypoxia was abolished by CCCP or NaN3 whereas both mitochondrial inhibitors strongly superinduced hypoxia-mediated VEGF induction.

HIF-1-independent induction of gene expression has also been described for inhibitor of apoptosis protein 2 (IAP-2) (Dong et al., 2001). IAP-2 expression is not induced by iron chelators or divalent transition metal ions, glucose deprivation, or pharmacological inhibition of mitochondrial respiration. However, IAP-2 induction was observed only after virtually complete lack of oxygen. Whereas RBM3 and CIRP, as well as HIF-1-regulated genes, required several hours of hypoxia, IAP-2 expression occurred within the first hour of oxygen depletion. Thus we propose that there are at least three different mechanisms enabling mammalian cells to respond to hypoxia in a graded fashion. Moderate hypoxia induces transcription of RBM3 and CIRP, more pronounced hypoxia leads to the stabilization of HIF-1 that subsequently upregulates the transcription of a variety number of genes such as VEGF or erythropoietin, and severe anaoxia induces IAP-2.
induced cell death by genes identified by expression profiling. *Hum. Mol. Genet.* **11**, 2279-2287.

Maxwell, P. H., Pugh, C. W., Ratcliffe, P. J. (1993). Inducible operation of the erythropoietin 3' enhancer in multiple cell lines: evidence for a widespread oxygen-sensing mechanism. *Proc. Natl. Acad. Sci. USA* **90**, 2423-2427.

Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R. and Ratcliffe, P. J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* **399**, 271-275.

Minchenko, A., Leshchinsky, I., Opentanova, I., Sang, N., Srinivas, V., Armstead, V. and Caro, J. (2002). Hypoxia-inducible factor-1-mediated expression of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) gene. Its possible role in the Warburg effect. *J. Biol. Chem.* **277**, 6183-6187.

Miyazaki, K., Kawamoto, T., Tanimoto, K., Nishiyama, M., Honda, H. and Kato, Y. (2002). Identification of functional hypoxia response elements in the promoter region of the DEC1 and DEC2 genes. *J. Biol. Chem.* **277**, 47014-47021.

Nishiyama, H., Itoh, K., Kaneko, Y., Kishishita, M., Yoshida, O. and Fujita, J. (1997). A glycine-rich RNA-binding protein mediating cold-inducible suppression of mammalian cell growth. *J. Cell Biol.* **137**, 899-908.

O’Rourke, J. F., Pugh, C. W., Bartlett, S. M. and Ratcliffe, P. J. (1996). Identification of hypoxically inducible mRNAs in HeLa cells using differential-display PCR. Role of hypoxia-inducible factor-1. *Eur. J. Biochem.* **241**, 403-410.

Palmer, G. (1993). Current issues in the chemistry of cytochrome c oxidase. *J. Bioenerg. Biomembr.* **25**, 145-151.

Rodgers, K. R. (1999). Heme-based sensors in biological systems. *Curr. Opin. Chem. Biol.* **3**, 158-167.

Seidel-Rogol, B. L. and Shadel, G. S. (2002). Modulation of mitochondrial transcription in response to mtDNA deletion and repletion in HeLa cells. *Nucleic Acids Res.* **30**, 1929-1934.

Sheikh, M. S., Carrier, F., Papathanasiou, M. A., Hollander, M. C., Zhan, Q., Yu, K. and Fornace, A. J., Jr (1997). Identification of several human homologs of hamster DNA damage-inducible transcripts. Cloning and characterization of a novel UV-inducible cDNA that codes for a putative RNA-binding protein. *J. Biol. Chem.* **272**, 26720-26726.

Shoshani, T., Faerman, A., Mett, I., Zelin, E., Tenne, T., Gorodin, S., Moshel, Y., Elbaz, S., Budanov, A., Chajut, A. et al. (2002). Identification of a novel hypoxia-inducible factor 1-responsive gene, RTP801, involved in apoptosis. *Mol. Cell. Biol.* **22**, 2283-2293.

Sonna, L. A., Fujita, J., Gaffin, S. L. and Lilly, C. M. (2002). Invited review: Effects of heat and cold stress on mammalian gene expression. *J. Appl. Physiol.* **92**, 1725-1742.

Sowter, H. M., Ratcliffe, P. J., Watson, P., Greenberg, A. H. and Harris, A. L. (2001). HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors. *Cancer Res.* **61**, 6669-6673.

Takahashi, Y., Takahashi, S., Shiga, Y., Yoshimi, T. and Miura, T. (2000). Hypoxic induction of prolly 4-hydroxylase alpha (I) in cultured cells. *J. Biol. Chem.* **275**, 14139-14146.

Taube, T., Seeger, K., Beyermann, B., Hanel, C., Duda, S., Linderkamp, C. and Henze, G. (1997). Multiplex PCR for simultaneous detection of the most frequent T cell receptor-delta gene rearrangements in childhood ALL. *Leukemia* **11**, 1978-1982.

Tonnis, H., Stumm, M., Wegner, R. D., Chudoba, I., Kalscheuer, V. and Neitzel, H. (2001). Comparative genomic hybridization based strategy for the analysis of different chromosome imbalances detected in conventional cytogenetic diagnostics. *Cytogenet. Cell Genet.* **93**, 188-194.

Wagner, K. D., Wagner, N., Wellmann, S., Schley, G., Bondke, A., Theres, H. and Scholz, H. (2000). Oxygen-regulated expression of the Wilms’ tumor suppressor Wt1 involves hypoxia-inducible factor-1 (HIF-1). *FASEB J.* **14**, 1364-1368.

Wang, G. L., Jiang, B. H., Rue, E. A. and Semenza, G. L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc. Natl. Acad. Sci. USA* **92**, 5510-5514.

Wellmann, S., Taube, T., Paal, K., Graf, V. E. H., Geilen, W., Seifert, G., Eckert, C., Henze, G. and Seeger, K. (2001). Specific reverse transcription-PCR quantification of vascular endothelial growth factor (VEGF) splice variants by LightCycler technology. *Clin. Chem.* **47**, 654-660.

Wiesener, M. S., Turley, H., Allen, W. E., Willam, C., Eckardt, K. U., Talks, K. L., Wood, S. M., Gatter, K. C., Harris, A. L., Pugh, C. W. et al. (1998). Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. *Blood* **92**, 2260-2268.

Wood, S. M., Gleadle, J. M., Pugh, C. W., Hankinson, O. and Ratcliffe, P. J. (1996). The role of the aryl hydrocarbon receptor nuclear translocator (ARNT) in hypoxic induction of gene expression. Studies in ARNT-deficient cells. *J. Biol. Chem.* **271**, 15117-15123.

Yang, C. and Carrier, F. (2001). The UV-inducible RNA-binding protein A18 (A18 hnRNP) plays a protective role in the genotoxic stress response. *J. Biol. Chem.* **276**, 47277-47284.