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

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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% O_2) or severe (1% O_2) 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 NaN_3 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 adaptatively 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 NaN_3 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. Tönnies (Institute of Human Genetics, Charité Medical Center, Berlin) as described (Tonnies et al., 2001). The murine hepatoma line Hepa-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 rho^0 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, Biochrom) and 1% penicillin/streptomycin (Biochrom) in a fully humidified incubator with room air and 5% CO_2. Media for mitochondria-depleted rho^0 cells was supplemented with pyruvate (1 mM) and uridine (50 μg/ml), as described previously (King and Attardi, 1996). Adherently growing cell lines were passaged 24 hours prior to experiments on to 25 cm^2 (1×10^6 cells) or 75 cm^2 (4×10^6) culture dishes to achieve near-confluence. Cells grown in suspension were used at 1.5×10^7 in 1.5 ml in 60 mm dishes. Culture medium was replaced from all cell lines by starving medium containing 1% fetal FCS at the start of each experiment. Normoxia was defined as 95% air and 5% CO_2. In hypoxia experiments, O_2 was tightly regulated at 1% or 8% as indicated, employing a humidified three gas regulated IG750 incubator (Jouan, Unterhaching, Germany). For hypothermia experiments, a humidified single-chamber incubator (Forma Scientific, Labotect, Göttingen, Germany) was used at 32°C. In addition, HIF-1α of cells kept in normoxia was targeted by incubation with the transition metal chelator 2,2′-dipyridyl, the iron chelator desferrioxamine (DFO) or cobalt chloride ($CoCl_2$) (Sigma-Aldrich, Taufkirchen, Germany), each used at a final concentration of 200 μM. Exposure to experimental conditions was for 24 hours unless otherwise indicated. After experiments, adherent cells were removed by scraping. After addition of ice-cold PBS, adherent cells or suspension cells were centrifuged at 2000 ***g*** for 5 minutes, and pellets were processed for protein or RNA extraction. To deplete HeLa cells from mitochondria, cells were grown in the presence of ethidium bromide (EB, Sigma) at concentrations of 50 ng/ml or 100 ng/ml, respectively, for 6 days, with addition of glucose (4.5 g/l, final concentration), pyruvate (110 μg/ml), and uridine (50 μg/ml) (Seidel-Rogol and Shadel, 2002).

Reagents

The following pharmacological inhibitors were purchased from Calbiochem-Novabiochem (Bad Soden, Germany): carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 2-thenoyltrifluoroacetone 4,4′-trifluoro-1-(2-thienyl)-1,3 butanedione (TTFA), nordihydroguairaretic acid (NDGA) and ebselen; or from Sigma-Aldrich: NaN_3, rotenone, antimycin, oligomycin, *N*-acetyl cystein (NAC), dithiothreitol (DTT), ascorbic acid, pyrrolidine dithiocarbamate (PDTC) and H_2O_2. Stock solution of inhibitors were prepared by dissolving them in ethanol, dimethylsulfoxide or water, as recommended by the manufacturers.

DNA analysis

Preparation of genomic DNA and PCR were performed as described previously (Taube et al., 1997). The following forward (F) and reverse (R) oligonucleotides (TIB Molbiol, Berlin, Germany) were used for detection of the HIF-1α gene (*HIF1A*):

F *HIF1A* exon-3: 5′-gtgatttggatattgaagatgaca; R *HIF1A* intron-3: 5′-agctcttaatatgtgtgcattttacc;

F *HIF1A* exon-12: 5′-tgttagctccctatatcccaatgg; R *HIF1A* exon-12: 5′-acttgcgctttcagggctt.

Markers for *HIF1A* flanking chromosomal regions were obtained from the Ensemble Human Genome browser (http://www.ensembl.org/Homo_sapiens/contigview). Markers located proximal to the *HIF1A* gene are:

D14S592: F 5′-ttccagagtatttgcttaagagg, R 5′-gcattgtgggatgaggtatg;

D14S1258: F 5′-tggggagtgagagagaggg, R 5′-gatatctgaaagttccatcc-ttcg.

Markers located distal from the *HIF1A* gene are:

D14S1334: F 5′-ttcaatccatggatgcagaa, R 5′-ggtcgtaggtgtgtggcttt

D14S183: F 5′-ggtgatttaaaggtgtgg, R 5′-gaaagatacagagatggg.

β-globin was used as control:

F β-globin: 5′-ctgacacaactgtgttcactagc, R β-globin: 5′-tattggtctcc-ttaaacctgtcttg.

Mitochondrial DNA was quantitated by real-time PCR according to a previously reported method (Chiu et al., 2003), *β-globin* was used as control.

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 cells, where a difference is significant at P values 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). ^{32}P-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

hexamers (Invitrogen) according to the manufacturer's recommendations. The cDNA was amplified by real-time PCR on the LightCycler as previously described (Wellmann et al., 2001) using the following forward (F) and reverse (R) oligonucleotides (TIB Molbiol, Berlin, Germany):

RBM3 human: F 5′-cttcagcagtttcggaccta, R 5′-accatccagagactct-ccgt

RBM3 mouse: F 5′-agctttgggcctatctctgagg, R 5′-cccatccagggactct-ccat

CIRP human: F 5′-caaagtacggacagatctctga, R 5′-cggatctgccgtcc-atcta

CIRP mouse: F 5′-ccaagtatgggcagatctccga, R 5′-ctgccgcccgtcca-cagact

VEGF (total VEGF) human: F 5′-ccctgatgagatcgagtacatctt, R 5′-cttgtcttgctctatctttctttggtct

VEGF mouse: F 5′-ttactgctgtacctccacc, R 5′-acaggacggcttgaagatg

β_2-microglobulin (*B2M*) human: F 5′-gatgagtatgcctgccgtgtg, R 5′-tccaatccaaatgcggcatct

ribosomal highly basic 23 kDa protein (RPL13a) mouse: F 5′-gcggatgaataccaaccc, R 5′-gtaggcttcagccgaacaac.

SYBR Green I was used as a fluorescent dye (Applied Biosystems, Foster City, CA, USA). Quantitation of mRNA expression was carried out by relating the PCR crossing point obtained from probe samples (automatically determined by the LightCycler software 3.3 in the second derivative maximum mode) to the appropriate plasmide calibration curve. Data were normalized against *B2M* RNA levels and, for experiments with Hepa-1 cells, against *RPL13a* RNA levels and are given as fold changes compared with the control experiments.

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, 1×10^7 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 $MgCl_2$ 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 Tryptan 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 reaction mixture containing 20 mM Tris-HCl, pH 8.0, 150 mM KCl, 5 mM $MgCl_2$, 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 are given as the ratio of copies of target gene mRNA to *B2M* mRNA copies.

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 (polyvinylidine 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% O_2 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 house keeping genes, such as β-actin (*ACTB*), β_2-microglobulin (*B2M*) and *RPL13a*, was virtually identical for hypoxia and normoxia in both Z-33 and REH (Table 1C), *HIF1A* was present in REH but not in Z-33, whereas the *HIF1A* homologue, *EPAS1*, was dimly expressed in Z-33 and absent in REH cells (Table 1B, Fig. 1). *ARNT* expression showed little variation in response to hypoxia 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*

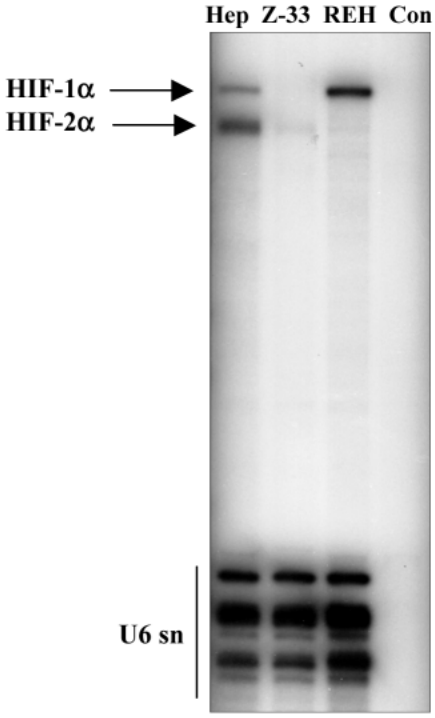

Fig. 1. Expression of genes encoding HIF-1α (*HIF1A*) and HIF-2α (*EPAS1*) in Z-33, REH and Hep3B cells. The human B-cell acute lymphoblastic leukemia cell lines Z-33 and REH as well as the hepatoblastoma cell line Hep3B (Hep) were cultured in normoxia. Ribonuclease protection analysis using 30 μg of 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 ^{32}P-riboprobes in the absence of added RNA was demonstrated on the right lane (Con).

(Sowter et al., 2001), prolyl 4-hydroxylase α (*P4HA1*) (Takahashi et al., 2000), phosphofructo-2-kinase fructose-2,6-biphosphophatase-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 cells 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 expresssion 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*

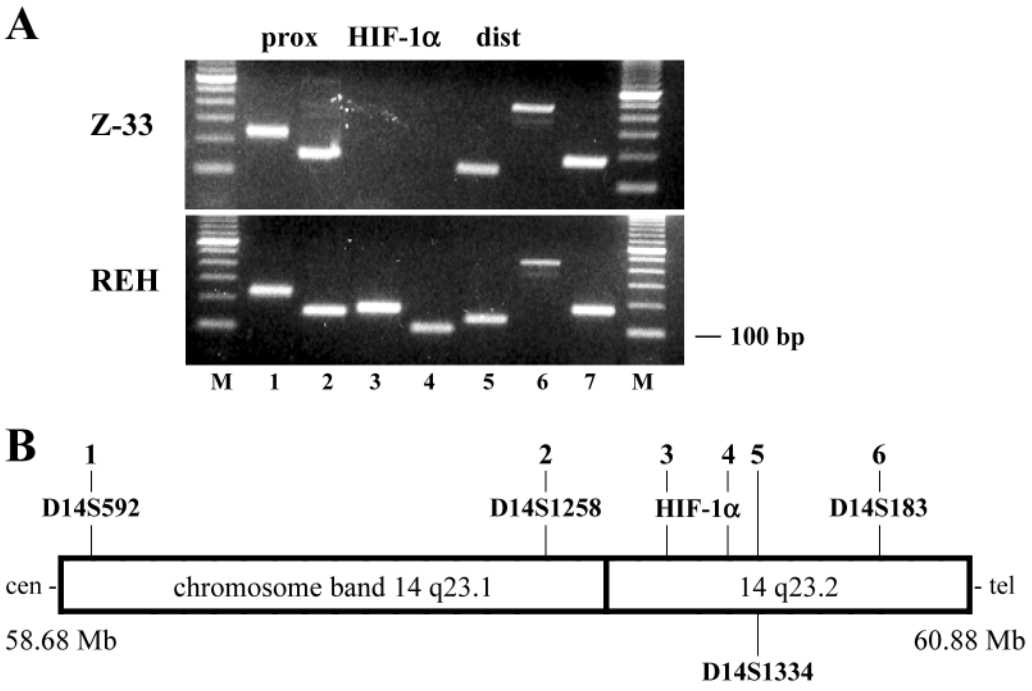

Fig. 2. Agarose gel electrophoresis of PCR products indicating homozygous deletion of the HIF-1α gene in Z-33. (A) Genomic PCR of DNA of Z-33 and REH was performed for the HIF-1α gene (*HIF1A* exon/intron-3, lane 3; *HIF1A* exon-12, lane 4) and HIF-1α flanking gene regions: proximal (prox) to the HIF-1α 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).

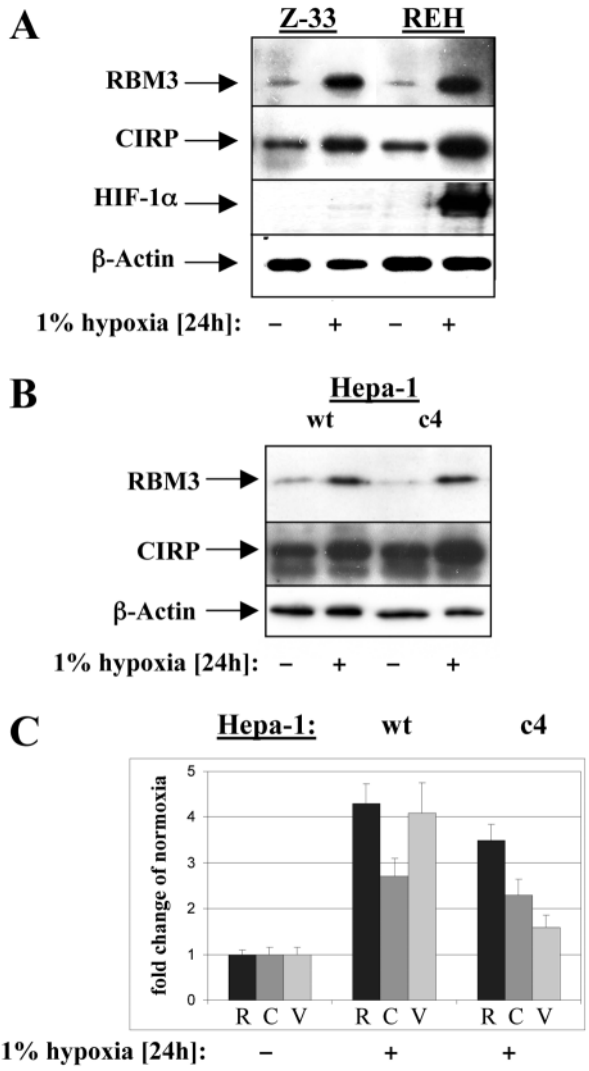

Fig. 3. Hypoxia induces RBM3 and CIRP in HIF-1α-deficient Z-33 cells as well as in murine ARNT-deficient Hepa-1 c4 cells. Z-33, REH, Hepa-1 wt and c4 cells were cultured in parallel in normoxia or 1% oxygen for 24 hours. (A,B) Whole-cell lysates were isolated and subjected to western blot analysis for RBM3, CIRP and HIF-1α in Z-33 and REH cells. To control sample loading and transfer, the blots were stripped and reprobed for β-actin. (C) RNA was isolated and subjected to real-time RT-PCR analysis. *RBM3* (R), *CIRP* (C), and *VEGF* (V) RNA levels were normalized to *RPL13a* 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).

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, NaN_3 inhibited hypoxia-induced *RBM3* and *CIRP* expression in a dose-dependent fashion. *RBM3* expression after hypoxia was reduced by 50% at 5 mM NaN_3 and was down to normoxia baseline levels at 10 mM NaN_3 for 24 hours. Hypoxia-mediated *CIRP* induction was even more sensitive to NaN_3, with expression reduced by 50% or down to normoxia baseline levels at 1 mM and 5 mM NaN_3, respectively (Fig. 7A). In sharp contrast, NaN_3 at 1-10 mM was strongly synergistic with hypoxia in inducing *VEGF*. Under normoxia, NaN_3 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 NaN_3 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 (NaN_3) 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

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* Hy	*Z-33* No	*Z-33* Fold change	*REH* Hy	*REH* No	*REH* Fold change
		Absolute expression			Absolute expression		
RBM3 (RNA-binding motif protein 3)	NM_006743.1	8485	3649	2.3	5495	2036	2.7
C20orf97 (chromosome 20 open reading frame 97)	NM_021158.1	7005	2593	2.7	13816	8302	1.7
HLA-DPA1 (histocompatibility complex, class II, DPα1)	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
PTP4A3 (protein tyrosine phosphatase type IVA, 3)	NM_007079.1	1483	567	2.6	1776	1149	1.5
est:7o16e03.x1	BF431618	1265	647	2.0	1331	604	2.2
CASP8 (caspase 8)	NM_001228.1	830	349	2.4	880	514	1.7

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-1a, EPAS1 and ARNT in Z-33 and 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

ACTB (β actin)	NM_001101.2	28433	29452	1.0	45102	40679	1.1
B2M (β_2-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% O_2) or normoxia (No, 20% O_2) for 8 hours.

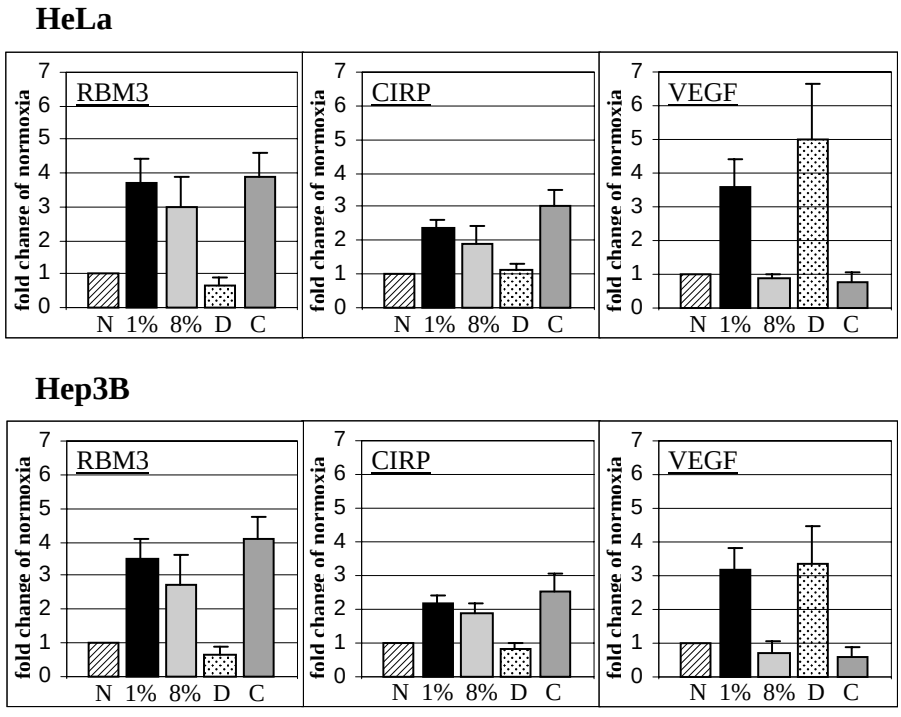

Fig. 4. Mild and severe hypoxia as well as hypothermia induce *RBM3* and *CIRP* expression. HeLa and Hep3B cells were exposed to either normoxia (N), 1% oxygen (1%), 8% oxygen (8%), desferrioxamine (D) or 32°C-hypothermia (C) for 24 hours. RNA was isolated and subjected to real-time RT-PCR analysis. *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).

CIRP expression under normoxic or hypoxic conditions (data not shown). H_2O_2 (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 H_2O_2 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 NaN_3 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

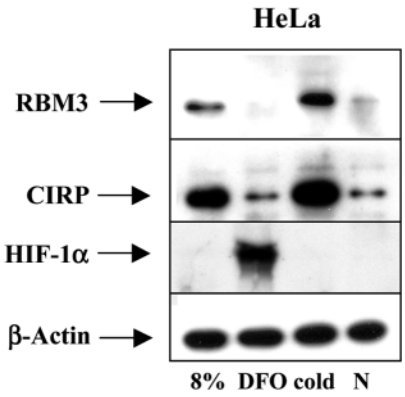

Fig. 5. Mild hypoxia as well as hypothermia increase RBM3 and CIRP protein but not HIF-1α, whereas desferrioxamine (DFO) does not affect RBM3 and CIRP protein. HeLa cells were cultured in parallel in normoxia (N), 8% oxygen (8%), DFO or 32°C-hypothermia (cold) for 24 hours. Whole-cell lysates were isolated 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.

RBM3 and *CIRP* transcripts did not differ between 143B TK$^-$ (mitochondria$^+$) and rho^0 (mitochondria$^-$) osteosarcoma cells.

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).

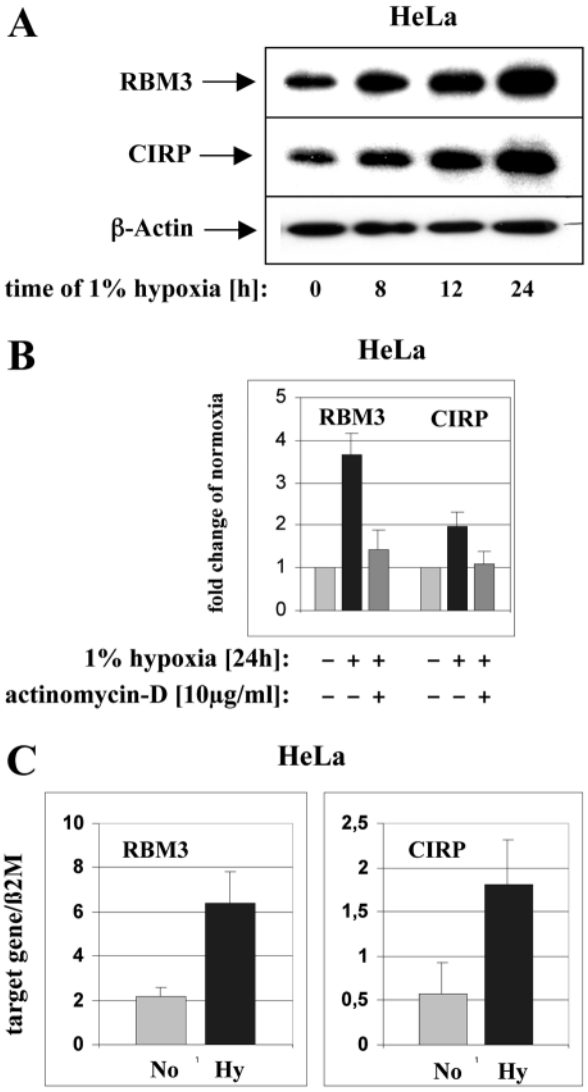

Fig. 6. Hypoxia induces a persistent increase in RBM3 and CIRP protein levels via de novo mRNA synthesis. (A) HeLa cells were cultured in parallel in normoxia or 1% oxygen as indicated. Whole-cell lysates were isolated and subjected to western blot analysis for RBM3 and CIRP. To control sample loading 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 β_2-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*).

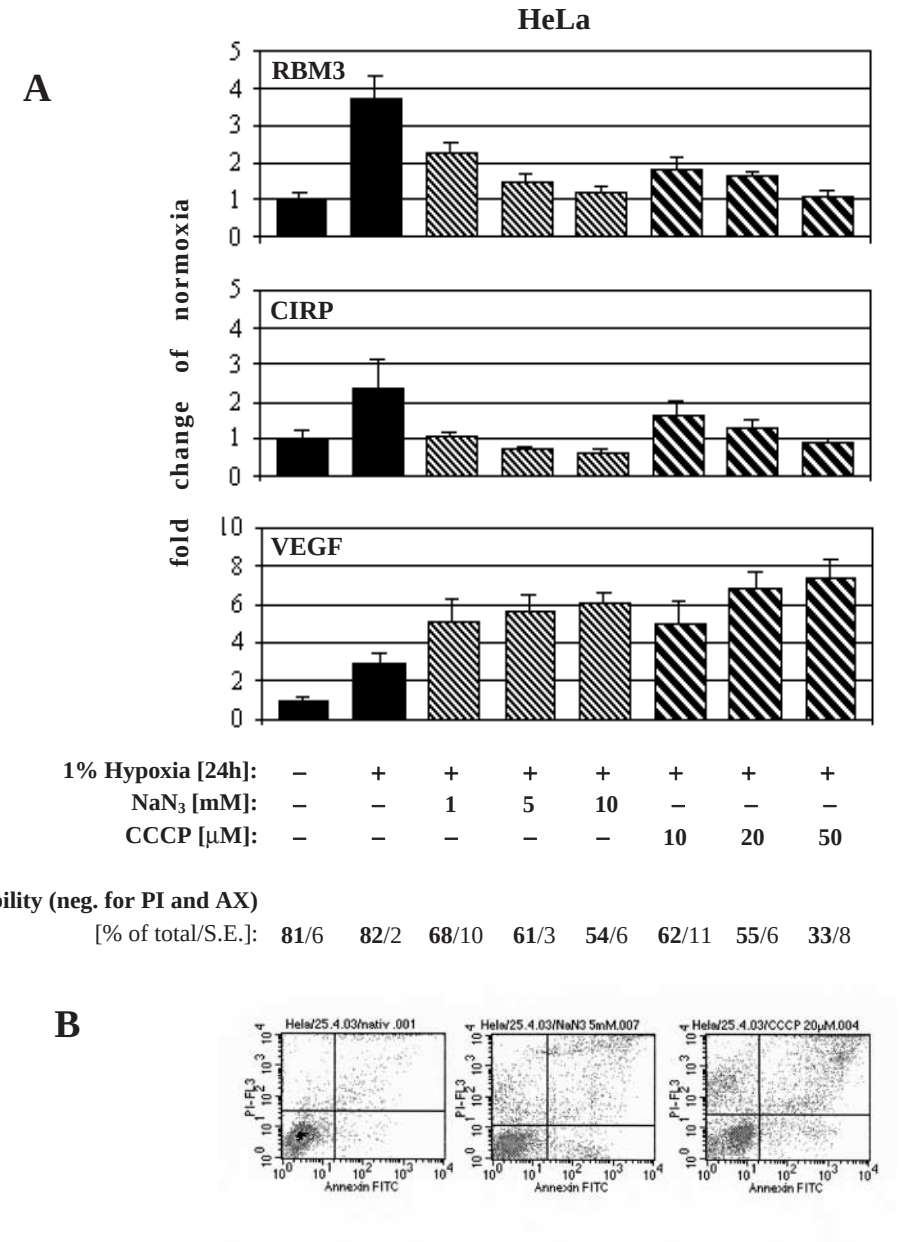

Fig. 7. The mitochondrial inhibitors NaN_3 and CCCP inhibit *RBM3* and *CIRP* induction by hypoxia but superinduce *VEGF* expression by hypoxia. HeLa cells were cultured in parallel in normoxia or 1% oxygen as indicated for 24 hours. Different concentrations of either NaN_3 or CCCP were added as indicated. (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 (mean±s.e.m., *n*=3). To control for cell viability, cells were harvested and incubated with annexin V and propidiun iodide. Cell viability is given as the percentage of cells negative for both annexin V and propidium iodide staining (mean±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 NaN_3 (center dot blot) or HeLa cells exposed to 20 μM CCCP (right dot blot) for 24 hours.

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 NaN_3, 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 NaN_3 and CCCP display strong affinity for heme (Palmer, 1993), we propose that the O_2 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-

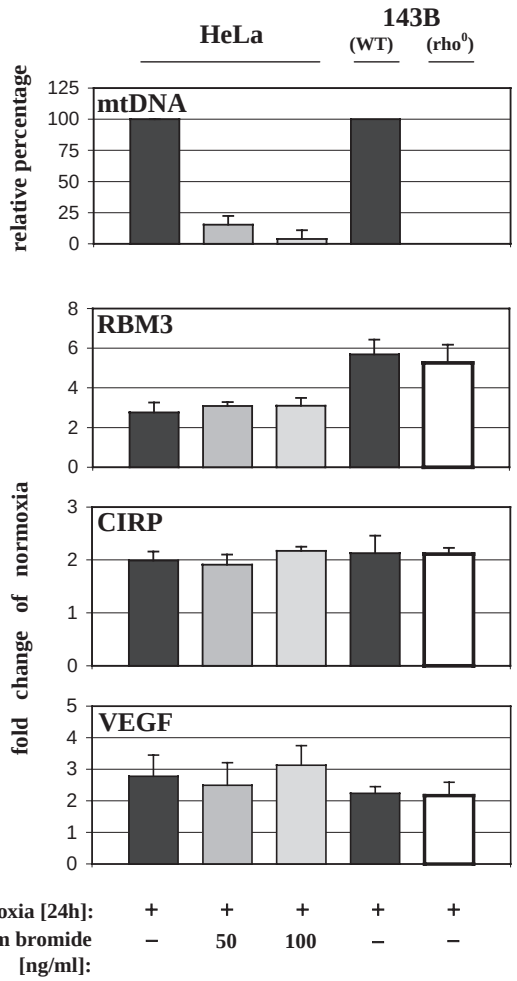

Fig. 8. Induction of *RBM3* and *CIRP* in response to hypoxia does not require mitochondria. mtDNA was depleted from HeLa cells with EB in two different concentrations for 6 days as indicated. Subsequently cells were cultured in parallel in normoxia or 1% oxygen as indicated for 24 hours. Parental (WT) and rho^0 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).

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 NaN_3 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 anoxia induces IAP-2.

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