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# Hypoxia-inducible Factor-1-dependent Overexpression of Myeloid Cell Factor-1 Protects Hypoxic Cells against *tert*-Butyl Hydroperoxide-induced Apoptosis*

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**Increased levels of Mcl-1 (myeloid cell factor-1) have been reported in several cancers, suggesting an important role played by Mcl-1 in cancer cell survival. Mcl-1 is an anti-apoptotic protein shown to delay or block apoptosis. In this work, using semiquantitative immunofluorescence, real-time PCR, and RNase protection assay, an increase in Mcl-1 expression was detected in hepatoma HepG2 cells incubated under hypoxia or in the presence of cobalt chloride. Through analysis of the Mcl-1 promoter sequence, a putative HIF-1 (hypoxia-inducible factor-1) binding site was identified. A Mcl-1 promoter fragment containing this hypoxia-responsive element was able to bind HIF-1 *in vitro*. It also induced hypoxia-dependent transcription of a luciferase reporter gene, which was suppressed by anti-HIF-1α short interfering RNA. Finally, overexpression of Mcl-1 protected HepG2 cells against apoptosis induced by *tert*-butyl hydroperoxide as shown by inhibition of caspase-3 activation and DNA fragmentation. All these data suggest a potential anti-apoptotic role of HIF-1 that could protect cells against apoptosis under hypoxia by overexpression of the Mcl-1 protein.**

As a result of oxygen deprivation (hypoxia), cells undergo different transcriptional adaptations that are in majority dependent on one key transcription factor, hypoxia-inducible factor-1 (HIF-1)[1] (for a review, see Ref. 1). HIF-1 is composed of two subunits belonging to the bHLH-PAS family: ARNT, which is constitutively expressed in the nucleus, and HIF-1α, which is regulated by hypoxia. In normoxia (20% oxygen), HIF-1α is hydroxylated on two prolines (residues 564 and 402) by an oxygen-dependent prolyl hydroxylase and on the asparagine 803 by one oxygen-dependent asparaginyl hydroxylase, FIH-1. Moreover, the lysine 532 is acetylated by the acetyl transferase ARD1. The two hydroxylated prolines and the acetylated lysine are recognized by the protein pVHL, which is part of a ubiquitin ligase complex, thus targeting the HIF-1α subunit for degradation by the proteasome (2–4). The hydroxylation on the asparagine prevents HIF-1α-cAMP-response element-binding protein-binding protein/p300 interaction (5). In low oxygen conditions, HIF-1α is no longer modified and is thus stabilized. HIF-1α then translocates into the nucleus where it dimerizes with ARNT. The active HIF-1 binds to its specific site called HRE (hypoxia-response element, (5′-(G/C/T)-(A/G)-**CGTG**-(C/G/A)-(G/T/C)-3′), present in the promoter of target genes. The products of these target genes (glucose transporter-1, vascular endothelial growth factor, and different glycolytic enzymes) allow the cell to adapt to the new conditions induced by hypoxia.

Besides the role played by HIF-1 in the adaptation to hypoxia, recent data describe a possible role for HIF-1 in the modulation of apoptosis. Indeed, HIF-1 has been reported to be involved in apoptosis in embryonic stem cells (6), because it is able to interact with p53 (7) (8). Moreover, different Bcl-2 pro-apoptotic members such as Nip3, Noxa, and other proteins involved in apoptosis such as RTP801 and HGTD-P have been described to be overexpressed under hypoxia (9–12). However, it has also been reported that hypoxia can protect cells against apoptotic cell death induced by different agents such as serum deprivation (7, 13) and incubation in the presence of anticancer drugs (fluorouracil and taxol) (14). Severe hypoxia was also reported to induce the expression of a member of the IAP (apoptosis inhibitor protein) family, IAP-2 (15). Erler *et al.* (16) also reported that hypoxia can down-regulate Bid and Bax via HIF-1-dependent and -independent mechanisms, respectively. Recently, Dong and Wang (17) described the overexpression of Bcl-$x_L$ in hypoxia-selected cells resistant to cell death. In one of our previous studies, we showed that physiological and chemical hypoxia protect HepG2 cells against *tert*-butyl hydroperoxide (*t*-BHP)- and serum deprivation-induced apoptosis (18). Moreover, some data demonstrate that constitutive expression of HIF-1α in pancreatic tumor cells protects these cells against apoptosis induced by hypoxic and glucose deprivation (19). A direct evidence of the anti-apoptotic action of HIF-1 was recently reported by using siRNA against HIF-1α (20). These contradictory data indicate that HIF-1 could display either a pro-apoptotic or an anti-apoptotic role. These inverse effects are probably related to the severity of the hypoxic conditions (21).

Myeloid cell factor-1 (Mcl-1) has also been shown to be over-

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[1] The abbreviations used are: HIF-1, hypoxia-inducible factor-1; HRE, hypoxia-response element; siRNA, short interfering RNA; Mcl-1, myeloid cell factor-1; MM, multiple myeloma; *t*-BHP, *tert*-butyl hydroperoxide; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; RPA, RNase protection assay; TBS, Tris-buffered saline; HA, hemagglutinin; BSA, bovine serum albumin; GLUT-1, glucose transporter 1; EPO, erythropoietin.


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expressed under hypoxia in polymorphonuclear leukocytes (22, 23). Mcl-1 belongs to the Bcl-2 family, which contains both anti- and pro-apoptotic proteins with, respectively, Bcl-2 and Bcl-$x_L$ acting as anti-apoptotic factors and Bad, Bax, Bid acting as pro-apoptotic proteins. Mcl-1 was originally isolated as an early gene from ML-1 myeloid leukemia cells during phorbol ester-induced differentiation (24). As described for other anti-apoptotic members of the Bcl-2 family, Mcl-1 was shown to delay or block apoptosis induced by c-Myc overexpression, growth factor withdrawal, and cytotoxic agents (25–27). Similar to the action of Bcl-2 and Bcl-$x_L$, Mcl-1 protects cells from apoptosis through blockage of cytochrome *c* release from the mitochondria (28). The inhibition of cytochrome *c* release could be attributed to the ability of Mcl-1 to interact with Bax (29). Recently, Mcl-1 was shown to complex with Bak in healthy cells and to prevent the oligomerization of Bak, blocking cytochrome *c* release from the mitochondria (30, 31). Interestingly, increased levels of Mcl-1 have been reported in several cancers, including ovarian and prostate cancers, B-cell chronic lymphocytic leukemia, multiple myeloma (MM), and large granular lymphocyte leukemia (32–37).

In this study, we showed that the hypoxia-induced overexpression of Mcl-1 in the HepG2 human hepatoma cell line is HIF-1-dependent. An active HIF-1 binding site was identified in the promoter of the gene coding for Mcl-1. Moreover, overexpression of HA-Mcl-1 was shown to protect HepG2 cells against *t*-BHP-induced apoptosis.

## EXPERIMENTAL PROCEDURES

*Cell Culture and Hypoxia Incubation*—Human hepatoma cells (HepG2) were maintained in culture in 75-$cm^2$ polystyrene flasks (Costar) with 15 ml of Dulbecco's modified Eagle's medium (DMEM) liquid containing 5 ml/500 ml Pen-Strep (BioWhittaker) and 10% fetal calf serum and incubated under an atmosphere of 5% $CO_2$. Monkey kidney fibroblast cells (COS-7) were maintained in culture in 75-$cm^2$ polystyrene flasks (Costar) with 15 ml of Dulbecco's high glucose containing 5 ml/500 ml Pen-Strep (BioWhittaker) and 10% fetal calf serum and incubated under an atmosphere of 5% $CO_2$. Hypoxia was achieved by replacing culture medium with $CO_2$-independent medium (Invitrogen) with or without 10% serum and incubating cells under 1% oxygen or in the presence of $CoCl_2$ (150 $\mu$M).

*Apoptosis Induction*—Apoptosis was induced using *t*-BHP (Merck) as described previously (18). Briefly, after 24 h of serum deprivation, *t*-BHP was added to cells at a final concentration of $5{\cdot}10^{-5}$ M in $CO_2$-independent medium during the incubation time under normoxia (20% $O_2$) or hypoxia.

*Extraction of RNA and cDNA Synthesis*—Preparation of total RNA was performed using an RNAgent total RNA isolation system kit (Promega) according to the manufacturer's instructions. cDNA was made from total RNA with a Superscript II reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions and using oligo(dT) primers.

*Real-time PCR*—After incubation (3, 8, or 16 h) under normoxia or hypoxia, total RNA was extracted and retrotranscribed into cDNA. The mRNA expression level was quantified by real-time PCR using the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Aldolase and Mcl-1 mRNA expression levels were quantified using the threshold cycle method. Values were then normalized to the relative amounts of the housekeeping gene, $\alpha$-tubulin. Each gene was amplified using the appropriate specific primers.

*Transfac 6.0 Software*—To find putative HRE sites in the human Mcl-1 promoter, Transfac 6.0 analysis software was used (www.gene-regulation.com/pub/databases.html#transfac).

*DNA Analysis by Flow Cytometry*—Flow cytometry DNA analysis was used to quantify the percentage of apoptotic cells. 1.2 million HepG2 cells/T75 (Costar) were transfected on day 1 with 3 $\mu$g of plasmid pCMV-HA or pCMV-HA-Mcl-1 and the transfection agent SuperFect (Quiagen) for 3 h. Fresh medium with serum was then added. On day 2, the medium was replaced by DMEM without serum for 24 h. On day 3, cells were incubated under normoxia or hypoxia and in the presence of *t*-BHP ($5{\cdot}10^{-5}$ M) for 4 h. Thereafter, cells were washed with cold PBS before being trypsinized. Cold PBS was then added, and cells were centrifuged twice for 5 min at 1200 rpm at 4 °C. The cell pellet was then resuspended in 300 $\mu$l of cold PBS before 700 $\mu$l of cold ethanol was added drop by drop on rocking cells. Cells were incubated at −20 °C for the night for permeabilization. Cells were then centrifuged for 10 min at 1800 rpm at 10 °C. After two washes with PBS + 10% serum and centrifugation for 10 min at 1800 rpm at 10 °C, cells were resuspended in PBS + 0.1% Tween 20 and RNase A (50 $\mu$g/ml) (Sigma) and incubated for 30 min at 37 °C. Finally, 800 $\mu$l of PBS + propidium iodide (20 $\mu$g/ml) was added to the cells before analysis for red fluorescence (relative DNA content) by flow cytometry.

Flow cytometric analysis was performed with a FACSsan (BD Biosciences Immunocytometry Systems). Doublets were excluded from the analysis by using the FL2 area/FL2 width gating method. 10,000 events were collected, and data were analyzed with the CELLQUEST software (BD Biosciences Immunocytometry Systems).

*RNase Protection Assay (RPA)*—RPA is a specific method to detect and quantitate mRNAs. This method is based on the hybridization of the target mRNA to a biotin-labeled antisense RNA probe. RNase treatment follows, resulting in degradation of single-stranded RNA and excess probe. The RNase-protected probes are purified and resolved on denaturing polyacrylamide gels and transferred to a positively charged nylon membrane. Immobilized protected RNA probes are cross-linked to the wet membrane by exposure to UV light. Finally, the signal is quantified using Streptavidin-horseradish peroxidase and enhanced chemiluminescent substrate. The level of each mRNA species in the original RNA sample is determined based on the intensity of the appropriately sized, protected probe fragment reported to the intensity of two housekeeping genes, L32 and glyceraldehyde-3-phosphate dehydrogenase. RPA was performed on total RNA using the RiboQuant ribonuclease protection assay kit (BD Biosciences) according to the manufacturer's instructions. The probes used in this study correspond to the hAPO-2b multiprobe template set (BD Biosciences). The control probe was Human Control RNA-2 (BD Biosciences).

*Mcl-1 cDNA Cloning*—The Mcl-1 cDNA was obtained by PCR from the RZPD clone IRALp962P054 and cloned in-frame with the hemagglutinin tag in the pCMV-HA vector (BD Biosciences) by double restriction with BglII and EcoRI. The sequence of the primers with BglII and EcoRI linkers designed on the basis of the sequence of the human Mcl-1 cDNA (GenBank® accession number BC017197) was: 5′-GGAATTCAAATGTTTGGCCTCAAAAGAAACG-3′ sense (EcoRI linker underlined), 5′-GAAGATCTCTATCTTATTAGATATGCCAAACC-3′ antisense (BglII linker underlined). The PCR consisted of 28 cycles of 30 s at 94 °C, 30 s at 55 °C, and 4 min at 72 °C. PCR was preceded by 5 min at 94 °C and followed by 10 min at 72 °C. After sequencing, it appears that a single mutation of the third nucleotide cytosine from the codon CGC coding for the amino acid arginine was replaced by adenosine. This mutation has no result on the sequence of the protein because the new codon is also coding for arginine.

*Promoter Isolation by PCR*—A fragment of 155 bp of the Mcl-1 promoter containing the putative HRE site was obtained by PCR using HepG2 genomic DNA (100 ng). This fragment including the nucleotides between −983 and −828 (in comparison with the +1 transcription initiation site) was amplified by PCR using the following primer sequences 5′-TCCCCCGGGACTTGAGGCCATGAGTTCGAGACCA-3′ sense, 5′-CCCAAGCTTCTCCACTTCCCACGTTCAGACGATT3′ antisense, flanked by SmaI and HindIII linkers (underlined) and designed on the basis of the sequence of the human Mcl-1 promoter (GenBank™ accession number AF147742). The PCR consisted of 28 cycles of 30 s at 94 °C, 30 s at 72.7 °C, and 1 min at 72 °C. The PCR was preceded by 2 min at 94 °C and followed by 10 min at 72 °C. This fragment was subcloned in a cloning vector by restriction with HindIII-SmaI. This construction was checked and confirmed by sequencing. The insert was then amplified by PCR with new primers: 5′-GGGGTACCGGGACTTGAGGCCATGAGTT-3′ sense (KpnI linker underlined), 5′-GAAGATCTTCTCCACTTCCCACGTTCAG-3′ antisense. The PCR consisted of 34 cycles of 30 s at 94 °C, 30 s at 56 °C, and 20 s at 72 °C. The PCR was preceded by 2 min at 94 °C and followed by 10 min at 72 °C. The fragment was then cloned in the pGL3 promoter (Promega) upstream of the SV40 promoter controlling the expression of the firefly luciferase gene by restriction with KpnI to obtain the pGL3–129bp containing the wild type Mcl-1 promoter fragment (129 bp long). This construction was checked and confirmed by sequencing.

*Reporter Gene Assay*—HepG2 transfections were performed in 24-well plates (50,000 cells/well) with SuperFect reagent (Qiagen). 460 ng of pGL3 promoter vector or the pGL3–129bp containing 129 nucleotides from the human Mcl-1 promoter were co-transfected with 230 ng of pRL-SV40 (normalization; Promega) and with 2.3 $\mu$g of empty expression vector pCMV-Myc (Promega) in DMEM without serum. 24 h post-transfection, HepG2 cells were transfected with siRNA. Finally, before

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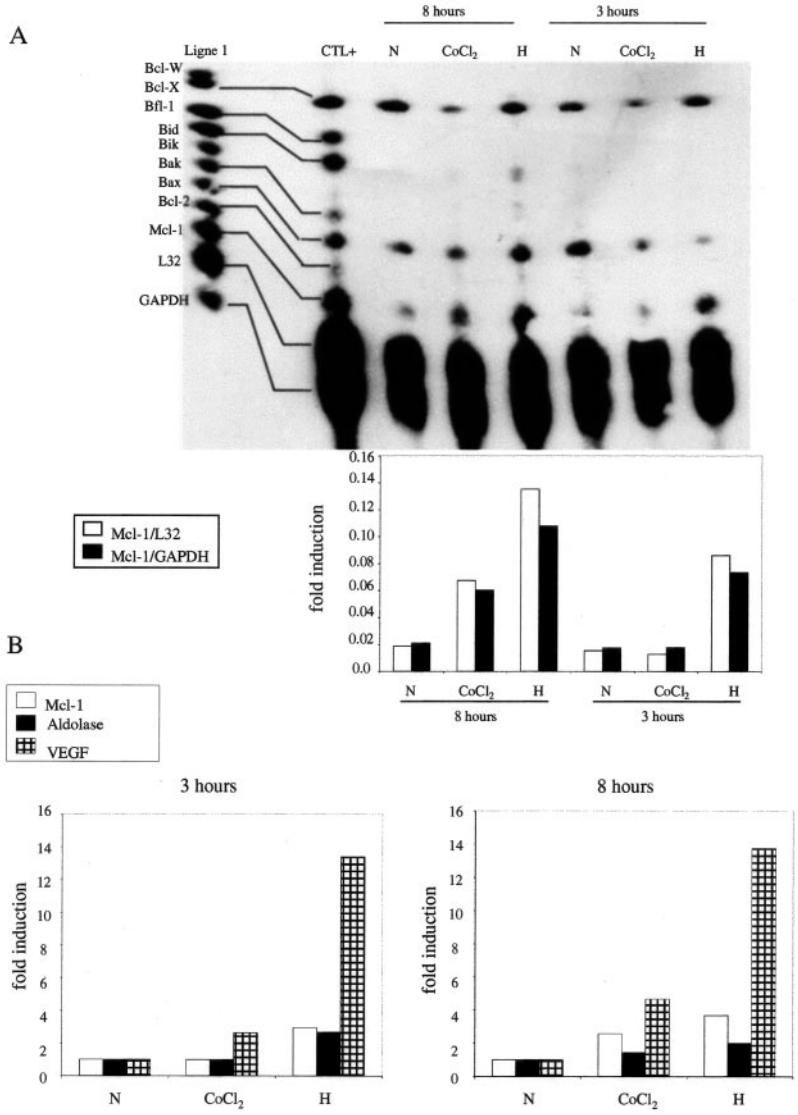


FIG. 1. **Mcl-1 overexpression under hypoxia.** *A*, RNase protection assay with total RNA extracted from cells incubated for 3 or 8 h under normoxia, hypoxia, or in the presence of $CoCl_2$. Total RNA was hybridized with the hAPO-2b multiprobe template, allowing the study of the expression of different anti- and pro-apoptotic mRNA. The -fold induction of the Mcl-1 mRNA is defined as the ratio between the intensity of Mcl-1 mRNA and the intensity of L32 or glyceraldehyde-3-phosphate dehydrogenase (housekeeping genes) mRNA. *B*, after 3 or 8 h of incubation under normoxia, hypoxia, or in the presence of $CoCl_2$, total RNA was extracted and retrotranscribed into cDNA. Real-time PCR was performed with specific primers for two HIF-1 target genes, aldolase and vascular endothelial growth factor, as well as for Mcl-1 and $\alpha$-tubulin. Results are expressed in -fold induction by comparison with the reference condition, normoxia. These experiments were performed as independent duplicates.

hypoxia incubation (16 h), medium was replaced by DMEM without serum for 8 h. After the incubation, the luciferase activity was measured and quantitated in a luminometer using the dual-luciferase reporter assay system (Promega). Experiments were performed in triplicate. Results are expressed as means of the ratio between the firefly luciferase activity and the *Renilla* luciferase activity.

*Colorimetric DNA Binding Assay for HIF-1*—HIF-1 DNA binding activity was measured using a competitive colorimetric assay (TransAM) developed in our laboratory (38) and sold by Active Motif. Assays were performed according to the manufacturer's instructions. For competition experiments, wild type and mutated oligonucleotides corresponding to the Mcl-1 HRE sequence were designed according to the sequence of the human Mcl-1 promoter (GenBank® accession number AF147742). The oligonucleotides W18 and M18 were designed on the basis of the EPO promoter (39). The oligonucleotides were introduced in variable -fold excess in binding buffer, prior to nuclear extract addition. Nuclear extracts were obtained from COS-7 cells incubated for 16 h in the presence of $CoCl_2$ in Dulbecco's high glucose medium without serum. HIF-1 binding to the HRE sequence linked to the well was revealed by anti-HIF-1$\alpha$ antibody (Transduction Laboratories) at a final dilution of 1/1000. A secondary horseradish peroxidase antibody against mouse IgG was used at a dilution of 1/1000 (Santa Cruz Biotechnology). The sequence of the oligonucleotides used (the HRE core is underlined and the HRE mutated nucleotides are indicated in bold) are W18, 5′-GCCCTACGTGCTGTCTCA-3′ sense, 5′-TGAGACAGCACGTAGGGC-3′ antisense; M18, 5′-GCCCTA**AAA**GCTGTCTCA-3′ sense, 5′-TGAGACAG**CTTT**TAGGGC-3′ antisense; Wmcl, 5′-TGGTGGCGCACGCCTGTA-3′ sense, 5′-TACAGGCGTGCGCCACCA-3′ antisense; Mmcl, 5′-TGGTGGCG**GAAT**CCTGTA-3′ sense, 5′-TACAGG**ATTC**CGCCACCA-3′ antisense; MmclA, 5′-TGGTGGCG**CTTT**CCTGTA-3′ sense, 5′-TACAGG**AAA**GCGCCACCA-3′ antisense; W25, 5′-ATGTGGTGGCGCACGCCTGTAATCC-3′ sense, 5′-GGATTACAGGCGTGCGCCACCACAT-3′ antisense; M25, 5′-ATGTGGTGGCG**GAAT**CCTGTAATCC-3′ sense, 5′-GGATTACAGG**ATTC**CGCCACCACAT-3′ antisense; M25A, 5′-ATGTGGTGGCG**CTTT**CCTGTAATCC-3′ sense, 5′-GGATTACAGG**AAA**GCGCCACCACAT-3′ antisense.

*Western Blotting*—HepG2 cells were transfected for 3 h with 3 $\mu$g of pCMV-HA or pCMV-HA-Mcl-1 using the SuperFect transfection reagent; fresh medium with serum was then added to the cells for 24 h. The following day, medium was replaced by medium without serum for 24 h. Cells were then scraped in 300 $\mu$l of lysis buffer (Tris 40 mM, pH 7.5, KCl 150 mM, EDTA 1 mM, Triton X-100 1%) containing a protease inhibitor mixture (Complete, added at a 1:25 dilution; Roche Applied Science) and phosphatase inhibitors ($NaVO_3$ 25 mM, PNPP 250 mM, $\alpha$-glycerophosphate 250 mM, and NaF 125 mM, at a 1:25 dilution). The lysate was then mixed 10 times on ice, centrifuged for 5 min at 15,000 rpm at 4 °C, and the supernatant was collected. Proteins were separated by SDS-PAGE on 15% acrylamide gel and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences). The membrane was blocked with TBS-Tween 5% fat milk (Gloria) for 1 h, followed by incubation for 2 h with the primary antibody in TBS-Tween 0.1% milk. After three washes of 5 min in TBS-Tween 0.1% milk, the incubation with the secondary antibody was performed for 30 min in TBS-Tween 0.1% milk, followed by three washes of 15 min in TBS-Tween. Finally, the membrane was revealed with ECL (Amersham Biosciences). Rabbit anti-Mcl-1 polyclonal antibody SC-819 (Santa Cruz Biotechnology) was used at 1:500 dilution. Monkey anti-rabbit IgG horseradish peroxidase-linked antibody (Amersham Biosciences) was

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used at 1:100,000 dilution as the secondary antibody.

The amount of proteins loaded on the gel was assayed with Bio-Rad protein assay according to the Bradford method. The revelation of the $\alpha$-tubulin with a mouse anti-$\alpha$-tubulin antibody (1:100,000 dilution; Sigma) allowed determination of the total amount of proteins loaded on the gel. The secondary antibody was a sheep anti-mouse IgG horseradish peroxidase-linked antibody (Amersham Biosciences) used at 1:100,000 dilution.

*Immunofluorescence*—For staining the protein Mcl-1, the following protocol was used: 50,000 HepG2 cells were seeded on glass cover slides in 24-well culture plates. The following day, the medium was replaced by medium with or without serum for 24 h prior to incubation in $CO_2$-independent medium for 3 h in normoxia, in the presence of $CoCl_2$ (150 $\mu$M), or in hypoxia. For co-staining hemagglutinin (HA) and active caspase-3, 50,000 HepG2 cells were seeded on glass cover slides in 24-well culture plates on day 0. On day 1, HepG2 cells were transfected with 3 $\mu$g of plasmid pCMV-HA or pCMV-HA-Mcl-1 and the transfection agent SuperFect for 3 h, and then fresh medium with serum was added to the cells. On day 2, medium was replaced by medium without serum for 24 h before incubation for 3 h in the presence or not of *t*-BHP under normoxia, in the presence of $CoCl_2$ (150 $\mu$M), or under hypoxia.

After hypoxia incubation, medium was removed and cells were fixed for 10 min with PBS containing 4% paraformaldehyde. Cells were permeabilized in PBS + 1% Triton X-100 and then washed 2 × 5 min in PBS followed by 30 min in PBS + 3% BSA (Sigma). The primary antibody was added in PBS + 3% BSA overnight at 4 °C in a wet room. The next day, cells were washed 3 × 10 min in PBS + 3% BSA before the secondary antibody was added in PBS + 3% BSA for 1 h in a wet room at room temperature. Cells were washed 3 × 10 min in PBS + 3% BSA. To visualize the nucleus, cells were then incubated for 35 min at room temperature in the presence of TO-PRO-3 (1:80 dilution in PBS + RNase 2 mg/ml; Molecular Probes). The coverslips were finally mounted in mowiol (Sigma) and observed with a confocal microscope TCS (Leica) using a constant photomultiplier. Rabbit anti-human Mcl-1 polyclonal antibody (Santa Cruz Biotechnology) (1:100 dilution), rat anti-hemagglutinin (Roche Applied Science) (1:100 dilution), and rabbit anti-human active caspase-3 antibody (Promega) (1:100 dilution) were used. Alexa Fluor 488 goat anti-rat IgG (H+L) conjugate (Molecular Probes), Alexa Fluor 488 goat anti-rabbit IgG (H+L) conjugate (Molecular Probes), and Alexa Fluor 568 goat anti-rabbit IgG (H+L) conjugate (Molecular Probes) were used at 1:1000 dilution.

*Short Interfering RNA Transfection*—The Silencer Validated siRNA anti-HIF-1$\alpha$ was obtained from Ambion. It targets exon 5 of the HIF-1$\alpha$ mRNA.

For transfection with anti-HIF-1$\alpha$ siRNA, the transfection agent siPORT Lipid (Ambion) was used. Briefly, 2 $\mu$l of siPORT Lipid was added to Opti-MEM medium (without serum and antibiotics) and incubated for 20 min at room temperature. Thereafter, siRNA diluted at 100 nM in Opti-MEM medium was mixed with diluted siPORT Lipid and incubated for 20 min at room temperature before being added for 4 h onto cells previously washed with Opti-MEM medium. After 4 h of transfection, fresh DMEM with medium and antibiotics was added directly to cells.

## RESULTS

*Hypoxia and $CoCl_2$ Increase the Expression of Mcl-1 mRNA and Protein*—Physiological hypoxia and chemical hypoxia (in the presence of $CoCl_2$) protect HepG2 cells against *t*-BHP- and serum deprivation-induced apoptosis (18). To investigate the mechanism by which hypoxia and $CoCl_2$ protected against apoptosis, we compared mRNA levels for different anti- or pro-apoptotic members of the Bcl-2 family in low and normal oxygen conditions. Total RNA was isolated from HepG2 cells incubated for 3 or 8 h under normoxia, hypoxia, or in the presence of $CoCl_2$. The mRNA expression for several pro- and anti-apoptotic proteins was assayed by ribonuclease protection assay (RPA) (Fig. 1*A*). Mcl-1 expression was induced by low oxygen concentration after 3 h of incubation. Using the same RNA extracts, cDNA was produced and quantified by real-time PCR. As already observed in the RPA experiment, Mcl-1 mRNA levels were increased by hypoxia or $CoCl_2$ (Fig. 1*B*). mRNAs coding for vascular endothelial growth factor and aldolase were used as positive control because the expression of these genes is known to be regulated by HIF-1 under hypoxia. An increase in the expression of both genes was indeed observed (Fig. 1*B*). The increase in the Mcl-1 mRNA was accompanied by an increase in the abundance of the Mcl-1 protein as shown by immunofluorescence studies using a Mcl-1-specific antibody (Fig. 2).

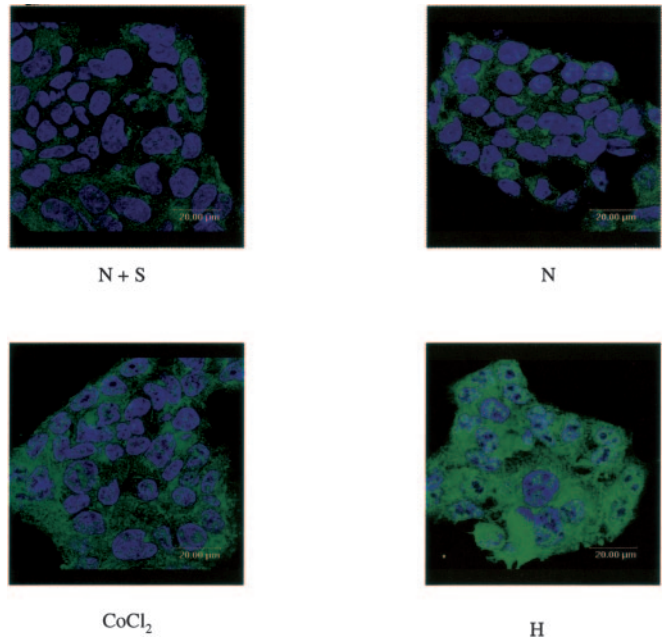


FIG. 2. **Immunofluorescence patterns of cells stained for the Mcl-1 protein.** HepG2 cells were incubated for 3 h under normoxia (*N*), hypoxia (*H*), or in the presence of $CoCl_2$ (150 $\mu$M) after a 24-h incubation in the presence (+*S*) or absence of serum. Mcl-1 protein was revealed by an anti-Mcl-1 antibody (*green*), and nuclei were stained with TO-PRO-3 (*blue*). Cells were observed in semiquantitative confocal microscopy.

*The Promoter of Mcl-1 Contains a Putative HRE Site*—On the basis of the above data and because Mcl-1 has been described to be overexpressed in polymorphonuclear leukocytes under hypoxia (22, 23), we investigated whether Mcl-1 gene expression could be regulated by HIF-1. The sequence of the Mcl-1 promoter, available in the human genomic databases (NCBI Map Viewer), was searched for putative HRE sites (40). A preliminary analysis of the Mcl-1 promoter was performed *in silico* using the Transfac website (www.gene-regulation.com/pub/databases.html#transfac). Seven putative HREs were identified. The comparison of the human Mcl-1 promoter sequence with that of the mouse Mcl-1 promoter allowed us to identify one conserved HRE. As shown in Fig. 3*A*, the Mcl-1 HRE sequence is similar to the HRE sequence identified in the mouse promoter of the glucose transporter GLUT-1, another HIF-1 target gene (41).

*HIF-1 Binds the HRE Site in the Human Mcl-1 Promoter in Vitro*—To investigate whether this putative HRE site could be recognized by the transcription factor HIF-1, two 18- (Wmcl) and 25- (W25) nucleotide-long probes were designed based on the HRE sequence present in the Mcl-1 promoter. Wmcl and W25 were tested on a competitive HIF-1-DNA binding assay. The HIF-1-DNA binding assay consists of a multiwell plate coated with DNA trappers containing the HRE motif present in the EPO enhancer. Nuclear extracts from COS-7 cells incubated in the presence or in the absence of cobalt chloride were loaded into the wells, allowing HIF-1 to bind to the EPO HRE. The bound fraction was then quantified using a colorimetric assay (Fig. 3*B*, *CTL*). In a competitive HIF-1-DNA binding assay, increasing concentrations of the free probe containing an HRE sequence were added to the well, preventing HIF-1 from binding to the trapper. As shown in Fig. 3*B*, the addition of an excess of free W18 probe containing a wild type EPO HRE (39) inhibited binding of HIF-1 to the trapper. When the free M18

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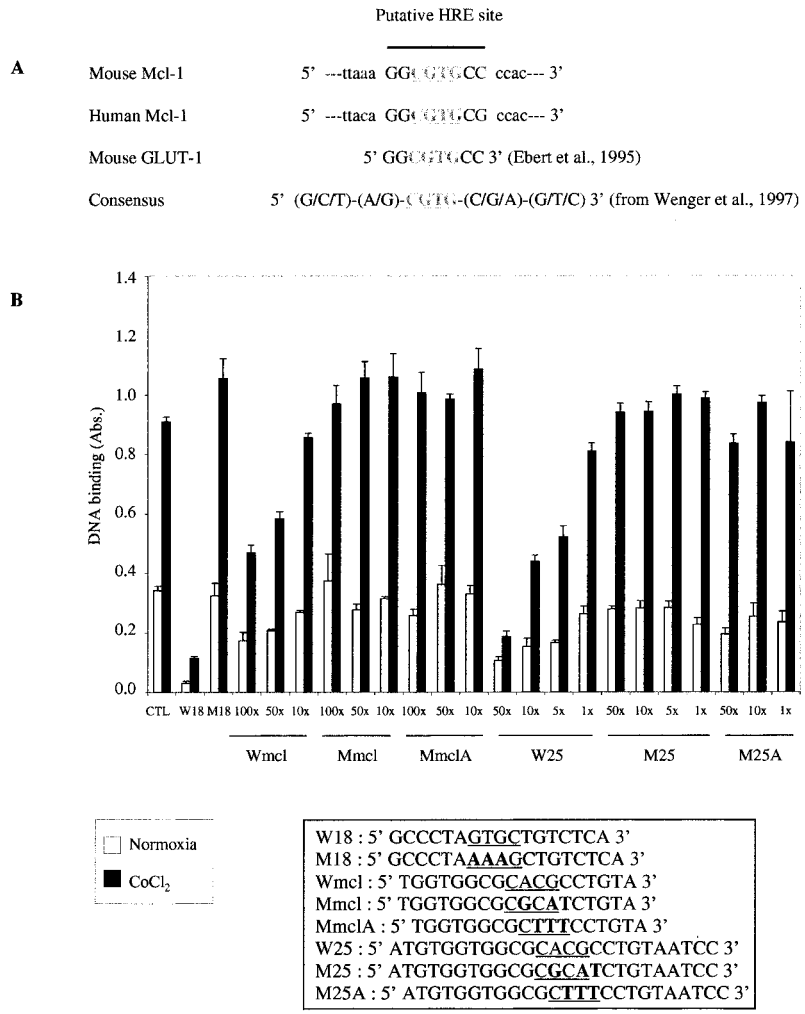


FIG. 3. **The Mcl-1 promoter contains an HRE site that binds HIF-1.** *A*, sequence alignments of the human and mouse Mcl-1 promoters. The HRE site present in the GLUT-1 gene promoter as well as the consensus HRE are indicated for comparison. *B*, analysis of HIF-1 binding to the putative Mcl-1 HRE using a colorimetric assay. The W18 and M18 probes were used as control. Wmcl and W25 were designed on the basis of the Mcl-1 promoter sequence and contain the HRE site. Mmcl, MmclA, and M25, M25A probes are mutants for the putative Mcl-1 HRE. Results are expressed as means ± 1 S.D. ($n$ = 3). This experiment was performed twice independently.

probe containing a mutated HRE binding site was added, HIF-1 still bound to the trapper. We then used both the Wmcl and W25 probes as free probes to compete with the HRE present in the trapper. Four additional probes, Mmcl, MmclA, and M25, M25A, corresponding, respectively, to mutated Wmcl and W25 were also tested. Nuclear extracts from COS-7 cells incubated in the presence or in the absence of cobalt chloride were incubated on plates coated with the HIF-1 trapper together with increasing concentrations of Wmcl, W25, or one of the mutated probes (Mmcl, MmclA, and M25, M25A), respectively. As shown in Fig. 3*B*, when increasing concentrations of Wmcl and W25 were added, a proportional decrease of HIF-1-DNA binding was observed. A 50-fold excess of the W25 probe induced a drastic decrease in HIF-1 binding on the trapper comparable with that observed with the probe W18 used as a positive control. An excess of 50-fold of the probe Wmcl induced a decrease of ~50% of the binding of HIF-1 to the DNA trapper. Conversely, the mutated probes Mmcl, MmclA, and M25, M25A did not affect the binding of HIF-1 to the trapper. These data showed that the transcription factor HIF-1 is able to specifically bind the HRE site identified in the promoter of the human MCL-1 gene.

*Hypoxia-induced Mcl-1 Transcription Is HIF-1-dependent—In vitro* binding of HIF-1 to the HRE site present in the promoter of the human MCL-1 gene does not prove, however, that this site is functional *in vivo*. To test this hypothesis, we investigated the effect of anti-HIF-1$\alpha$ siRNA on the Mcl-1 mRNA level by real-time PCR (Fig. 4). The anti-HIF-1$\alpha$ siRNA inhibited the Mcl-1 hypoxia-induced transcription (50% inhibition). The HIF-1 target gene aldolase was used as a positive control to demonstrate the specificity of the siRNA, and 80% inhibition was obtained.

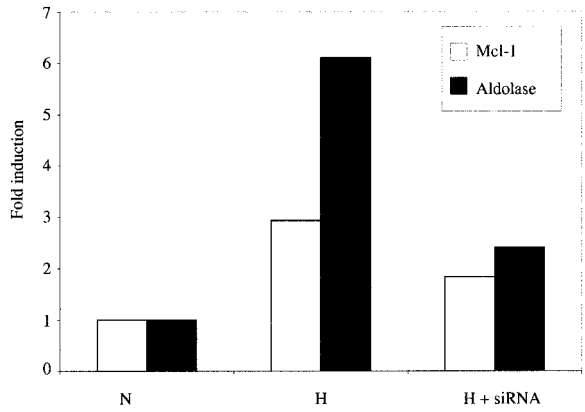


FIG. 4. **HIF-1 is responsible for the hypoxia-induced Mcl-1 overexpression.** After 16 h of incubation under normoxia or hypoxia, total ARN was extracted and retrotranscribed into cDNA. Aldolase, Mcl-1, $\alpha$-tubulin cDNA were amplified by real-time PCR. Aldolase is a HIF-1 target gene used as control for hypoxia; signal normalization was obtained with $\alpha$-tubulin, a housekeeping gene. Results are expressed in -fold induction by comparison with the reference condition, normoxia. This experiment was performed twice independently.

To confirm these data, we cloned a 129-bp Mcl-1 promoter fragment containing the putative HRE site into the pGL3-promoter vector upstream of the SV40 promoter controlling the

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expression of a luciferase reporter gene (Luc+). This construction, named pGL3–129bp, was transiently transfected in HepG2 cells and tested in a reporter assay. Background luciferase activity was measured using cells transfected with the empty pGL3. Thereafter, the luciferase activity was measured with the pGL3–129bp under normoxia and hypoxia (Fig. 5). A 2-fold increase of luciferase activity was measured under normoxia with the pGL3–129bp revealing basal activity of this fragment of the promoter. This luciferase activity was further increased under hypoxia. To confirm that the putative HRE site is indeed involved in the hypoxia-induced transcription of the luciferase reporter gene, anti-HIF-1$\alpha$ siRNA was used. Anti-HIF-1$\alpha$ siRNA completely inhibited pGL3–129bp luciferase activity under hypoxia (Fig. 5). In parallel, HIF-1 activity was controlled using a pGL3-SV40/6HRE reporter vector containing an artificial promoter with six copies of the EPO HRE cis-element cloned upstream of the firefly luciferase, and the expected results were obtained (data not shown).

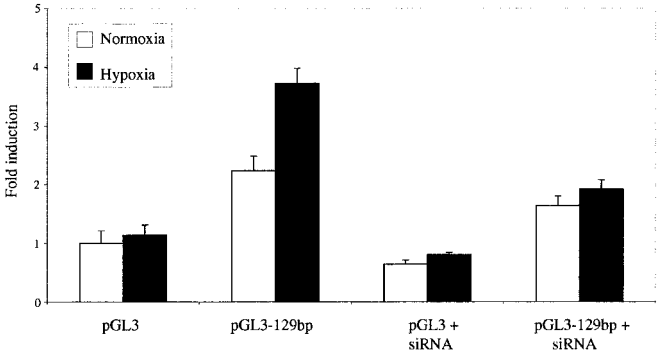


FIG. 5. **The HRE site of the Mcl-1 promoter induces HIF-1-dependent expression of luciferase inhibited by anti-HIF-1$\alpha$ siRNA.** HepG2 cells were first co-transfected with empty pGL3 or pGL3–129bp and pRL, together with pCMV-Myc before being transfected with anti-HIF-1$\alpha$ siRNA. HepG2 cells were then incubated for 16 h under normoxia or hypoxia, and luciferase activity was measured. Results are expressed as means ± 1 S.D. ($n$ = 3).

Together, these results indicated that an HRE site, which is specifically recognized by HIF-1, is active in the MCL-1 gene promoter. HIF-1 binds this specific site and allows the transactivation of the Mcl-1 promoter in a luciferase reporter system.

*Overexpression of Mcl-1 Protects HepG2 Cells against tert-Butyl Hydroperoxide-induced Apoptosis*—We previously observed that hypoxia and cobalt chloride were able to protect HepG2 cells against *t*-BHP-induced apoptosis (18). The molecular pathways involved in protection against oxidative stress under hypoxia are not understood. Because Mcl-1 has been described as an anti-apoptotic member of the Bcl-2 family, we sought to understand whether Mcl-1 could be a regulator of cell survival in response to hypoxia. HepG2 cells were transiently transfected with an expression vector containing the full-length Mcl-1 cDNA in-frame with an HA tag (pHA-Mcl-1). The expression of HA-Mcl-1 was checked by Western blotting (Fig. 6*A*). Transfected HepG2 cells were subjected to oxidative stress using *t*-BHP; apoptotic cells, containing low DNA content, were detected by fluorescence-activated cell sorter. HepG2 cells were transfected with the plasmid pHA-Mcl-1 or with the empty plasmid (pHA) before being serum-deprived for 24 h and incubated in the presence of *t*-BHP ($5{\cdot}10^{-5}$ M) for 4 h under normoxia. HepG2 cells were permeabilized with ethanol, and DNA was stained with propidium iodide. The apoptotic cells, containing low DNA content, were then counted using flow cytometry. Fig. 6*B* shows the proportion of the cells dying by apoptosis. Serum deprivation induced by itself a 10% increase in

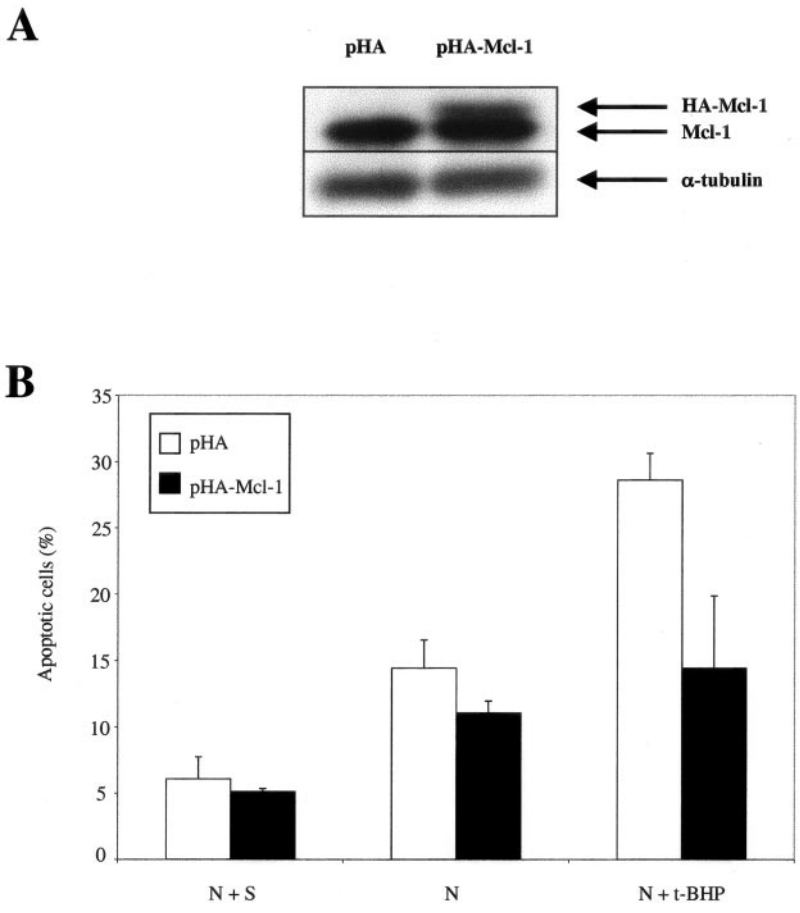


FIG. 6. **Overexpression of HA-Mcl-1 protected HepG2 cells against *t*-BHP-induced apoptosis.** *A*, total cell extracts were analyzed by Western blotting with a rabbit anti-human Mcl-1 antibody. Mcl-1 protein (37 kDa) and HA-Mcl-1 protein are indicated by *arrows*. $\alpha$-tubulin Western blotting was performed to assess for the total amount of proteins loaded on the gel. *B*, detection of apoptotic HepG2 cells by flow cytometry after 24 h of serum deprivation followed by 4 h of incubation in the presence of *t*-BHP ($5{\cdot}10^{-5}$ M) under normoxia. Cells were transfected with 3 $\mu$M empty pCMV-HA or pCMV-HA-Mcl-1 before being incubated for 24 h in the absence of serum and, thereafter, 4 h in the presence or not of *t*-BHP ($5{\cdot}10^{-5}$ M) under normoxia. DNA was stained with propidium iodide, and 3,500 cells/sample have been counted. Results are expressed as means ± 1 S.D. ($n$ = 3).

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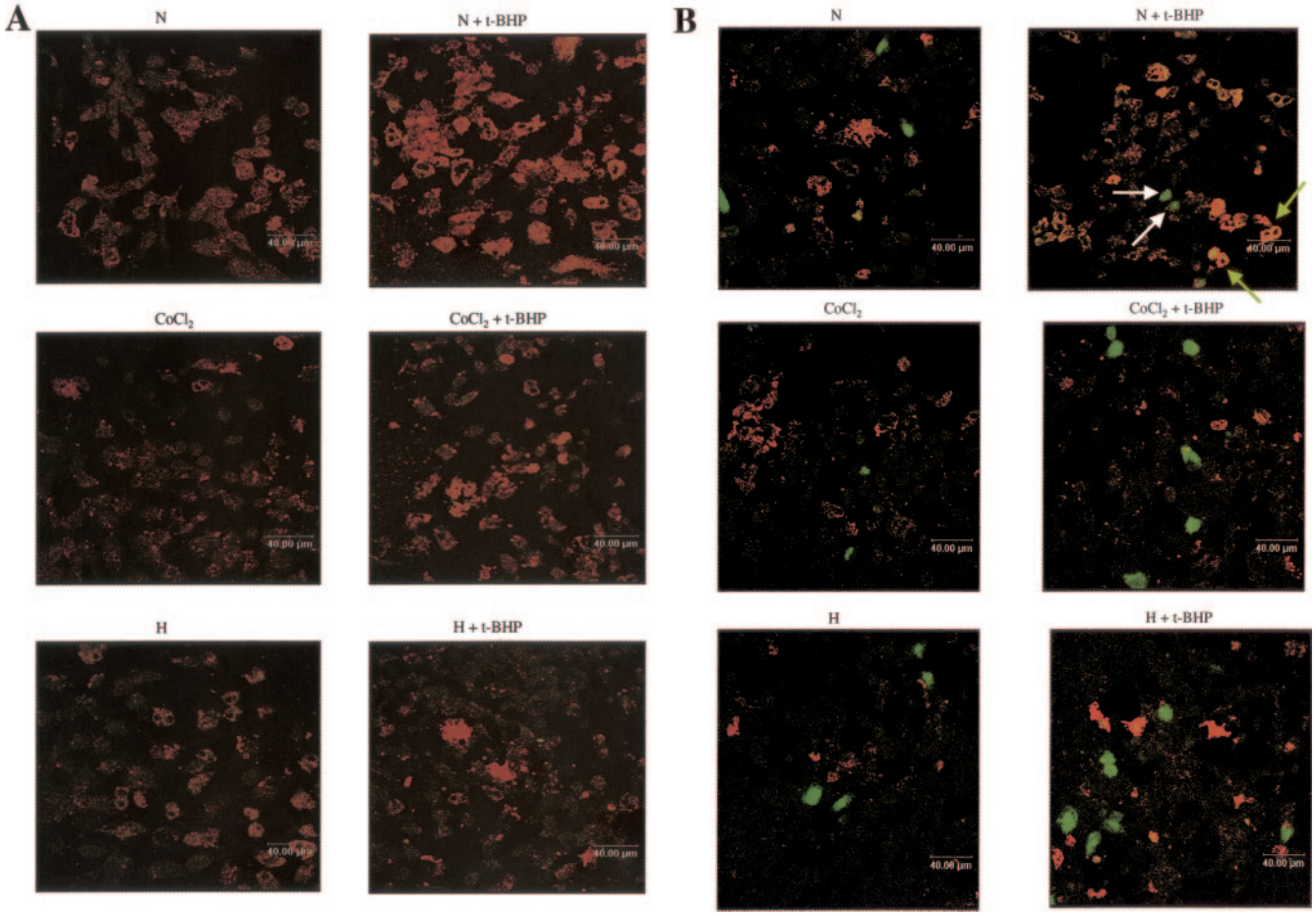


FIG. 7. **Immunofluorescence staining for the HA-tagged Mcl-1 protein and for active caspase-3 in HepG2 cells incubated for 3 h under normoxia (*N*), hypoxia (*H*), or in the presence of $CoCl_2$ (150 $\mu$M).** Cells were transfected with 3 $\mu$M empty pCMV-HA or pCMV-HA-Mcl-1 before being incubated for 24 h in the absence of serum and, thereafter, 3 h in the presence or not of *t*-BHP ($5{\cdot}10^{-5}$ M) under normoxia, hypoxia (*H*), or in the presence of $CoCl_2$ (150 $\mu$M). *A*, HepG2 cells transfected with empty pCMV-HA. *B*, HepG2 cells transfected with pCMV-HA-Mcl-1. Hemagglutinin tag was revealed by antibody anti-hemagglutinin (*green*); active caspase-3 was revealed by anti-active caspase-3 (*red*). Cells were observed in confocal microscopy. *White arrows*, pCMV-HA-Mcl-1-transfected cells; *yellow arrows*, active caspase-3-positive cells.

apoptosis. When *t*-BHP was added, the number of apoptotic cells reached 25%. The expression of Mcl-1 weakly protected cells from serum deprivation but induced a reduction of >50% of the apoptosis induced by *t*-BHP.

To confirm these observations, apoptotic cells were detected by immunofluorescence using an antibody raised against the active form of caspase-3. The cells transfected with an empty pHA vector did not show any staining for the HA tag (Fig. 7*A*). In the presence of *t*-BHP ($5{\cdot}10^{-5}$ M), apoptosis was detected by an increase in the abundance of the active form of caspase-3 (Alexa fluorochrome 568, *red*) (Fig. 7, *A* and B). The caspase-3 active form was decreased when the cells were incubated with cobalt chloride or under hypoxia, as shown by a fainter staining with the anti-caspase-3 antibody (Fig. 7, *A* and *B*). In cells overexpressing Mcl-1 (stained in *green*, Alexa fluorochrome 488), we did not detect the active form of caspase-3 (Fig. 7*B*). Cells labeled for active caspase-3 (red cells) showed no staining for HA-Mcl-1, suggesting that the presence of HA-Mcl-1 protects cells against *t*-BHP-induced apoptosis. Together, these data indicate that overexpression of Mcl-1 protects HepG2 cells against *t*-BHP-induced apoptosis.

## DISCUSSION

Besides the role played by HIF-1 in the adaptation to hypoxia, recent data describe a possible role for HIF-1 in the modulation of apoptosis. On one hand, hypoxia has been described as able to induce the transcription of different Bcl-2 pro-apoptotic members such as Nip3, Noxa, and other proteins involved in apoptosis, including RTP801 and HGTD-P (9–12). On the other hand, different studies also indicate that hypoxia and, in some case HIF-1, can protect cells against apoptosis induced by pro-apoptotic agents (13, 14, 18–20). Until now, the mechanisms explaining how hypoxia and HIF-1 could mediate this protection against apoptosis remained unclear. An interesting report by Dong and Wang (17) recently described the overexpression of Bcl-$x_L$ in hypoxia-selected cells resistant to cell death.

In one of our previous studies, we showed that hypoxia and $CoCl_2$ protected HepG2 cells from *t*-BHP-induced apoptosis. To elucidate how this protection could be mediated, we investigated the expression of different anti- and pro-apoptotic proteins under hypoxia. By RNase protection assay, real-time PCR, and semiquantitative immunofluorescence, we observed an increase in Mcl-1 expression under hypoxia (Figs. 1 and 2). A putative HRE site was identified in the Mcl-1 promoter at position −894 (Fig. 3*A*). This putative HRE site was shown to sustain specific HIF-1 binding (Fig. 3*B*) and to induce HIF-1-dependent transcription of a luciferase reporter gene (Fig. 5). Mcl-1 overexpression under hypoxia has already been reported in polymorphonuclear leukocytes, but no data were available on the mechanism responsible for this overexpression (22, 23). Mcl-1 expression was shown to be regulated by different transcription factors, including serum-response factor, Elk-1, Sp1, NF$\kappa$B, and cAMP-response element-binding protein under

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other conditions (27, 42, 43). The present data are the first demonstration that the human Mcl-1 promoter contains an active HRE site.

To investigate whether the hypoxia-induced expression of Mcl-1 could protect HepG2 cells against *t*-BHP-induced apoptosis, the effect of Mcl-1 overexpression was investigated. HepG2 cells were transfected either with the pHA-Mcl-1 plasmid or with the empty plasmid (pHA) before being serum-deprived for 24 h and incubated in the presence of *t*-BHP. The overexpression of Mcl-1 was shown to reduce *t*-BHP-induced apoptosis (Figs. 6 and 7). Together, these data underline the important role played by Mcl-1 in protecting cells against apoptosis in hypoxic conditions.

These observations may have implications regarding the role played by HIF-1 in cancer development. During tumor growth, the center area becomes hypoxic because of poor access to blood vessels capable of delivering oxygen (44, 45). Immunohistochemical analyses using monoclonal antibodies revealed that HIF-1$\alpha$ is overexpressed in many human cancers (46–48). Furthermore, HIF-1$\alpha$ expression was shown to be correlated with tumor grade in gliomas (46), and significant association between HIF-1$\alpha$ overexpression and patient mortality have been shown in breast, cervix, oropharynx, ovary, and endometrial cancers (for a review, see Ref. 1). In addition, clinical studies show that, in many cases, invasive growth and metastatic spread are associated with the degree of tumor hypoxia (49, 50). HIF-1 plays an important role in solid tumor protection against hypoxia and nutrient deprivation *in vivo* by promoting angiogenesis, glycolysis, and ATP production even under normal oxygen tension (the Warburg effect) (51–53). Moreover, HIF-1 supports tumor growth and survival by inducing the expression of growth factors such as insulin-like growth factor-2 and transforming growth factor-$\alpha$ (54, 55). The expression of survival factors and anti-apoptotic proteins could thus be additional mechanisms by which HIF-1 contributes to tumor growth and survival. Its regulation of Mcl-1 expression, as described here, would thus represent one more such mechanism. Indeed, HIF-1 has been described as having anti-apoptotic action: overexpression of HIF-1 target genes coding for GLUT-1 and vascular endothelial growth factor protects cells against hypoxia- and serum deprivation-induced apoptosis, respectively (13, 56). Moreover, its constitutive expression renders pancreatic tumor cells resistant to apoptosis induced by hypoxia and glucose deprivation (19). Conversely, the use of a dominant negative HIF-1$\alpha$ sensitizes these cells to apoptosis and growth inhibition induced by hypoxia and glucose deprivation. It also reduces the expression of GLUT-1 and glucose uptake in the tumor tissues and consequently *in vivo* tumorigenicity (57).

Mcl-1 has been shown to be overexpressed in several cancers including ovarian cancer, leukemia, prostate cancers, B-cell chronic lymphocytic leukemia, multiple myeloma (MM), and large granular lymphocyte leukemia (32–37). Mcl-1 seems to be an important survival factor for MM cells. Indeed, inhibition of Mcl-1 by Mcl-1 antisense oligonucleotide led to caspase activation and eventually to apoptosis, whereas overexpression of Mcl-1 protected MM cells from actinomycin D-induced apoptosis. These data suggest that MM cells have to maintain a high level of Mcl-1 protein to prevent the spontaneous induction of apoptosis (36). Another study underlines the key role of Mcl-1 in survival of human myeloblastic leukemia U937 cells: Mcl-1 antisense treatment results in a rapid entry of these cells into apoptosis (58). The results described here bring for the first time a molecular mechanism for Mcl-1 overexpression in cancer cells.

In conclusion, HIF-1-regulated expression of Mcl-1 could provide a mechanism by which HIF-1 increases tumor growth and resistance to cancer therapy.

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