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Study of the hypoxia-dependent regulation of human *CYGB* gene

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Abstract

Cytoglobin (CYGB) is ubiquitously expressed in all tissues and has been characterized as a respiratory protein in connective tissues. CYGB is up-regulated during hypoxia, implicating its function in maintaining the homeostasis redox of the cell. Here, we study the underlying molecular mechanisms by which hypoxia regulates human *CYGB* gene expression. When cells were subjected to hypoxia, the expression of endogenous CYGB was up-regulated ~1.7-fold in BEAS-2B cells ($p \leqslant 0.05$) and ~1.6-fold in HeLa cells ($p \leqslant 0.05$). Dual-luciferase assays and site directed mutagenesis showed the presence of hypoxia responsive elements (HREs) at positions −141, −144 and −448 that were essential for activation of CYGB expression under hypoxic conditions. The binding of hypoxia inducible factor (HIF-1) protein to the HREs was confirmed by gel shift and chromatin immunoprecipitation (ChIP) assays. These results indicate that HRE motifs are directly involved in the activation of the CYGB transcription under hypoxia.
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In the vertebrates, five types of globin have been identified so far, namely hemoglobin (HB), myoglobin (MB), neuroglobin (NGB), cytoglobin (CYGB) and globin X (GbX) [1–4]. Hemoglobin (HB) and myoglobin (MB) are well known pentacoordinated globin proteins that have been examined extensively [5–7]. Neuroglobin (NGB) and cytoglobin (CYGB) are new members of the globin superfamily, both of which are hexacoordinated globins discovered recently in human and other vertebrates. NGB is mainly expressed in the cytoplasm of neurons in the central and peripheral nervous systems [8], whereas CYGB is detected in the nucleus as well as in the cytoplasm of many types of tissues [9–11]. 'Globin X' (GbX) as the fifth type of vertebrate globin, is only found in fish and amphibians [12]. Although it is well know that all of the globin members are able to transport and store oxygen, to sustain the oxidative metabolism in cells [5,9,13,14], the physiological function of CYGB is still unclear.

CYGB is found in all tissues analyzed so far, suggesting a general function of this ubiquitously expressed protein [10,15,16]. The strict conservation of CYGB sequence among mouse, rat and human suggested an important function in metabolism [9,16]. Studies indicated that CYGB might act as scavenger of reactive oxygen species (ROS). Xu et al. [17] reported that overexpression of CYGB protected hepatic stellate cells (HSCs) against oxidative stress *in vitro*. Using real-time quantitative RT-PCR, Schmidt et al. [11] and Fordel et al. [15,18] showed that CYGB was up-regulated upon hypoxic conditions *in vitro* and *in vivo*. However, the detailed molecular mechanisms by which hypoxia regulates human *CYGB* gene expression is still unclear.

In this study, we demonstrate an increase of CYGB expression under hypoxia as compared to normoxia. We also report that the HRE motifs at positions −141, −144 and −448 are essential for up-regulation of CYGB expression under hypoxia conditions.

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Materials and methods

Cell culture. BEAS-2B, a transformed human bronchial epithelial cell line, and HeLa, a human cervix carcinoma cell line, were purchased from the American Type Culture Collection (ATCC). Cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum (HyClone) at 37 °C. Hypoxia conditions were established by placing the cells in a tissue culture chamber (Billups-Rothenberg, Inc., US) at reduced oxygen content (1% O_2, 5% CO_2 and 94% N_2).

Plasmids DNA. Cloning of the substitution mutants were constructed by PCR based site directed mutagenesis from human CYGB gene promoter plasmid pGL3 (−1113) [19]. In Table 1, primers were listed for mutagenesis of the HRE motifs in the promoter region of the human *CYGB* gene.

Luciferase assay. To determine whether the putative HREs found within the CYGB promoter region was indeed responsible for the transcription activity under hypoxia, the prepared mutant constructs were transfected into BEAS-2B and HeLa cells. The plasmids were transfected into the cells as described previously [19]. The cells were cultured for 24 h after transfection under hypoxia (1% O_2) and normoxia (21% O_2) conditions and then harvested. Luciferase activity was measured and calculated as described previously [19].

Western blotting analysis. BEAS-2B and HeLa cells were exposed to hypoxia (1% O_2) for 1 and 3 h, with reference to the normoxia (21% O_2) maintained cells as control. Cells were lysed and the Western blot analyses for HIF-1α protein were performed by using anti-HIF-1α monoclonal antibody (Santa Cruz Biotechnology). The signal was visualized with the ECL (enhanced chemiluminescence system) according to the manufacturer's instruction (PIERCE).

Electrophoretic mobility shift assay (EMSA). Electrophoretic mobility shift assays were carried out with nuclear extracts from BEAS-2B cells, which had been grown under hypoxia (1% O_2) and normoxia (21% O_2) conditions, respectively. Nuclear extract preparation and DNA binding reaction were performed according to the procedures mentioned previous [19]. Double-stranded DNA probes were obtained by annealing the individually synthesized single-stranded oligonucleotides shown in Table 2.

Chromatin immunoprecipitation (ChIP) assay. BEAS-2B and HeLa cells were exposed to hypoxia (1% O_2) for 1 and 3 h, respectively, using normoxia (21% O_2) maintained cells as control. 1% formaldehyde was added to the culture medium and incubated for 10 min to cross-link histones to DNA. ChIP assays were carried out with a commercial kit (Upstate Biotechnology) according to the manufacture's manual. The extracted DNA was used for PCR using primers: HIF-448(ChIP) forward: 5′-AGTTCCCCGCCGCGGGAAGGG-TCCG-3′ and reverse: 5′-GC CGCCCGCCCACCCGCAG-GCCACG-3′ and HIF-144/-141 (CHIP) forward: 5′-TTTAAACATTTTCCAGCAGACCACA-3′ and reverse 5′-TGCTCGGCGGCGGC-GGTGGCGGGGC-3′. The PCR (30 cycles) was performed on a PTC-200TM Programmable Thermal Controller (MJ Research, Inc.) and the conditions of the PCR program were as follows: the samples were first heated at 95 °C for 3 min, and then 30 PCR cycles were applied: 95 °C for 15 s, 59 °C for 15 s, 72 °C for 15 s, the samples were further held at 72 °C for 10 min and cooled down to 15 °C.

Reverse transcription real-time PCR. Total RNA was isolated from cultured cells by the use of TRIzol® reagent (Invitrogen) according to the manufacturer's protocol. First-strand cDNA synthesis was performed with 2 μg of total RNA using oligo$(dT)_{18–20}$ primers and Superscript II reverse transcriptase (Invitrogen). 1% of the cDNA products were used for quantitative real-time PCR amplification with SYBR® Green PCR Master Mix (Applied Biosystems, Inc.). To normalize the input cDNA amount in each set of real-time PCR, the house keeping gene *β-actin* was chosen as an internal control. Primers (listed in Table 3) were designed to determine the expression level of CYGB, VEGF and β-actin under hypoxia as well as normoxia. The PCRs were carried out on iCycler Real-time PCR detection system (Bio-Rad) under the following conditions (40 cycles): DNA denaturation (15 s) at 95 °C, primer annealing (15 s) at 61 °C and extension of double-stranded DNA at 72 °C (15 s). SYBR Green analyses were followed by dissociation curves in a temperature range from 55 to 90 °C to analyze the specificity of the amplification reactions. Quantification was performed by dividing the mean value of expression of the hypoxia samples through that of the normoxia samples.

Table 1
Oligonucleotides used for mutagenesis of HRE motifs

Oligonucleotides	Sequence (5′–3′)
Common forward primer (CF)	CCGACGCGTGCCCTTGGTGGCGGCTGAGAT
Common reverse primer (CR)	CGAAGCTTCAAGCCCAGCCCGGCTTTGCTC
h-CYGB-pGL3 (Δ −448)	(F) GCGCCGAGCGACCGCCCATTCCTCCC
	(R) CGGTCGCTCGGCGCTGGGCGGCGCG
h-CYGB-pGL3 (Δ −144)	(F) GGCGCGCGAAGACACACGCTCCCTC
	(R) GTGTGTCTTCGCGCGCCGGGTGTGT
h-CYGB-pGL3 (Δ −141)	(F) AGACACGGGCTCCCTCCCTCCGCGC
	(R) AGGGAGCCCGTGTCTGTGCGCGCCG

Results and discussion

Identification of the putative HRE motifs in the human CYGB gene promoter region

Analysis of the human CYGB promoter region showed that there are hypoxia responsive elements (HREs) in the 5′ UTR region of the *CYGB* gene, which suggests that it may have an oxygen-dependent regulation. There are two hypoxia inducible factors (HIF-1) binding sites at −141 and −448, and one erythropoietin (EPO) binding site at −144 (Fig. 1A). Potential transcriptional binding sites of HREs are identified using the programs MatInspector 7.4 (http://www.genomatix.com) and Transcription Element Search System (TESS http://www.cbil.upenn.edu/tess/).

Transcriptional regulation of the CYGB gene promoter by hypoxia

To determine whether the putative HRE motifs found within the CYGB promoter were indeed responsible for the transcriptional activation under hypoxia, specific muta-

Table 2
Oligonucleotides used for gel shift assays on the HRE motifs binding

Oligonucleotides	Sense (5′–3′)	Antisense (5′–3′)
h-cygb(E)-448	CCAGCGCCGCGTGACCGCCCA	TGGGCGGTCACGCGGCGCTGG
h-cygb(E)-448Muta	CCAGCGCCGAGCGACCGCCCA	TGGGCGGTCGCTCGGCGCTGG
h-cygb(E)-144/141	CGCGCACAGACACACGCTCCC	GGGAGCGTGTGTCTGTGCGCG
h-cygb(E)-144Muta	CGCGCGAAGACACACGCTCCC	GGGAGCGTGTGTCTTCGCGCG
h-cygb(E)-141Muta	CGCGCACAGACACGGGCTCCC	GGGAGCCCGTGTCTGTGCGCG
Consensus-HIF	CCGTGGAGACGTGCGCGTCCT	AGGACGCGCACGTCTCCACGG

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