The gene encoding human retinoic acid-receptor-related orphan receptor α is a target for hypoxia-inducible factor 1

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Retinoic acid-receptor-related orphan receptor (ROR) α is a nuclear receptor involved in many pathophysiological processes such as cerebellar ataxia, inflammation, atherosclerosis and angiogenesis. In the present study we first demonstrate that hypoxia increases the amount of *Rora* transcripts in a wide panel of cell lines derived from diverse tissues. In addition, we identified a functional promoter sequence upstream of the first exon of the human *Rora* gene, spanning –487 and –45 from the translation initiation site of ROR α 1. When cloned in a luciferase reporter vector, this sequence allowed the efficient transcription of the luciferase gene in several cell lines. Interestingly, the activity of the *Rora* promoter was enhanced by hypoxia in HepG2 human hepatoma cells, and this effect was dependent on an HRE (hypoxia response element) spanning from –229 to –225. Using electrophoretic-mobility-shift assays, we showed

that HIF-1 (hypoxia-inducible factor 1), which plays a key role in the transcriptional response to hypoxia, bound to this HRE. Overexpression of HIF-1 α increased the activity of the *Rora* promoter through the HRE. Overexpression of a dominant-negative form of HIF-1 α producing transcriptionally inactive HIF-1 α /HIF-1 β dimers abolished hypoxic activation of the *Rora* promoter. This indicated that HIF-1 is involved in the response of ROR α to hypoxia. Taken together, our data reveal *Rora* as a new HIF-1 target gene. This illustrates, at the molecular level, the existence of cross-talk between signalling pathways mediated by HIF-1 and those mediated by nuclear receptors.

Key words: low oxygen tension, nuclear receptor, promoter, transcription.

INTRODUCTION

Reduced oxygen availability (hypoxia) is an important component of many physiological (e.g., embryonic development, angiogenesis) and pathological (e.g., organ ischaemia, cancer) processes. In response to hypoxia, the expression of a wide variety of genes, including those involved in angiogenesis, erythropoiesis and glycolysis, is altered. Undoubtedly, HIF-1 (hypoxia-inducible factor 1) plays a key role in the transcriptional control of gene expression in response to hypoxia (for reviews, see [1–3]. HIF-1 is a heterodimer composed of two subunits, HIF-1 α and HIF-1 β , both belonging to the Per-ARNT-Sim family of basic helix–loop–helix transcription factors [4] (Per and Sim are the *Drosophila* proteins Period and Single-minded, and ARNT is aryl hydrocarbon receptor nuclear translocator). HIF-1 β is a constitutive nuclear protein. By contrast, the HIF-1 α protein is maintained at a low level in normoxic cells through its rapid proteasomal degradation [5]. The O₂/Fe²⁺-dependent prolyl-hydroxylation of HIF1- α provides recognition for the polyubiquitination complex containing pVHL (von Hippel–Lindau protein) [6–8]. Recent elegant studies pointed to the PHD2 (HIF prolyl hydroxylase 2) as the key oxygen sensor which controls steady-state levels of HIF-1 α [9–11]. The stabilization of HIF-1 α in hypoxic cells allows its nuclear translocation and formation of the transcriptionally active HIF-1 α / β heterodimer that, upon binding to specific DNA sequences, stimulates the expression of target genes. Other signalling pathways co-operate with the HIF pathway to achieve a finely tuned response to hypoxia. This is well documented for the phosphoinositide 3-kinase and the mitogen-activated protein kinase pathways (for

a review, see [12]). Also, several studies, often based upon clinical observations, revealed the existence of cross-talk between signalling pathways mediated through nuclear receptors (e.g., androgen receptor, peroxisome-proliferator-activated receptors α and γ , glucocorticoid receptor) and those governing the response to hypoxia [13–19].

ROR α (retinoic acid-receptor-related orphan receptor α ; NR1F1 in the unified nomenclature [20]) is a transcription factor belonging to the nuclear hormone receptor superfamily (for a review, see [21]). It is closely related to RAR α (retinoic acid receptor α) [22,23]. ROR α is a potent transcriptional activator that, most often, binds as a monomer to a RORE (ROR response element) composed of a 6-bp A/T-rich sequence immediately preceding a half-site core 5'-PuGGTCA-3' motif [23–26] (Pu = A or G). The *Rora* gene is located on human chromosome 15q21-q22 and on mouse chromosome 9 [27]. The *Rora* gene generates four isoforms (ROR α 1–4) that share common DNA and ligand-binding domains, but differ only by their N-terminal sequences encoded by first exons specific to each of the isoforms [22,23,28,29]. The *Rora* transcripts are widely distributed in different organs, including brain, heart, liver, testis and skin [22], suggesting that this receptor has physiological functions in several cell types. ROR α 4 is the most widespread isoform [22], whereas ROR α 1 is predominantly detected in a limited number of organs such as the cerebellum [30]. *In vivo* studies corroborated the role of ROR α in the regulation of diverse physiological and pathophysiological processes (for reviews, see [31,32]). Similar severe phenotypes are observed in mice where the *Rora* gene has been invalidated by homologous recombination [30,33] and in *staggerer* mice. In the latter, a spontaneous mutation in

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DN, dominant-negative; HIF, hypoxia-inducible factor; HRE, hypoxia response element; mut, mutated; IRNEM, Institut fédératif de recherche Necker Enfants-Malades; ROR, retinoic acid-receptor-related orphan receptor; RORE, ROR response element; RT-PCR, reverse-transcription PCR; wt, wild-type.

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the ligand-binding domain induces a frameshift that results in a protein truncated in its C-terminal part and which lacks any transcriptional activity [28,29]. These animals suffer from severe cerebellar ataxia caused by massive neurodegeneration of Purkinje cells [30,33,34]. Moreover, the phenotype of these mice revealed that ROR α is crucially involved in the susceptibility to atherosclerosis [35,36] and in regulating the inflammatory and immune responses [37,38], myogenesis [39], bone formation [40] and post-ischaemic angiogenesis [41]. Recent experiments identified cholesterol or a cholesterol derivative as a ligand for ROR α [42]. However, little is known regarding the stimuli and the transcription factors regulating *Rora* gene expression.

We had previously shown that hypoxia stimulates ROR α expression and transcriptional activity in hepatic cells [43]. In the present study we report that *Rora* expression was up-regulated by hypoxia in a wide panel of cell lines from different tissues and species. This strongly suggested that ROR α participates in the response to hypoxia. We also showed that ROR α is a new target for HIF-1 that acts upon binding to an hypoxia response element (HRE) that we fully characterized in the promoter region of the human *Rora* gene and which is conserved in the mouse.

EXPERIMENTAL

Cell culture and experimental conditions

All the cell lines (except PC12 and NT2) were cultured in a 1:1 mixture of DMEM (Dulbecco's modified Eagle's medium) and Ham's F12 media with GlutaMAXTM (Invitrogen), supplemented with 10 % (v/v) fetal bovine serum, 100 μ g/ml gentamycin and 2.5 μ g/ml fungizone (all from Invitrogen) as previously detailed for the HepG2 human hepatoma cells [A.T.C.C. (American Type Culture Collection, Manassas, VA, U.S.A.) HB-8065] [43]. PC12 cells were cultured in DMEM medium supplemented with 10 % (v/v) heat-treated horse serum, 5 % (v/v) heat-treated fetal bovine serum, 100 μ g/ml gentamycin and 2.5 μ g/ml fungizone. NT2 cells were cultured a 1:1 mixture of DMEM and Ham's F12 medium with GlutaMAXTM-I supplemented with 5 % (v/v) fetal bovine serum, 100 μ g/ml gentamycin, and 2.5 μ g/ml fungizone. Hypoxic treatment was performed for 24 h in sealed chambers, exactly as described in [44]. Final oxygen concentrations of 10, 2 and 0.1 % were used.

RNA analysis

Total RNA was isolated using TRIZOLTM LS reagent (Invitrogen). Total RNA was reverse-transcribed and subsequently amplified by PCR using an iCycler thermal cycler (Bio-Rad, Marnes La Coquette, France) exactly as described in [43]. Real-time quantitative PCR was carried out with the LightCycler Instrument using the LightCycler FastStart DNA Master SYBR Green I kit for detection of PCR products (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's guidelines. Final concentrations of MgCl₂ and primers were 3 mM and 0.5 μ M respectively. Sequences of primers used to simultaneously amplify all the *Rora* transcripts or to specifically amplify the *Rora1* and the *Rora4* transcripts have been described [43]. The amount of β -actin transcripts was evaluated as a control using the β -actin forward primer 5'-TGACCCAGATCATGTTTGAGACC-3' and the β -actin reverse primer 5'-GGATGTCCACGTCACACTTCATG-3' (fragment size 513 bp).

Vectors, transient transfection and luciferase assay

The DNA sequences from the human *Rora* gene were obtained from the UCSC genome browser database (<http://genome.ucsc.edu/cgi-bin/hgGateway>) using the June 2002 freeze. Fragments covering region -3051 to -45 and -487 to -45 relative to the translation initiation site of ROR α 1 were obtained by PCR using the hROR α -3051 primer (5'-CGATACGCGTGGGAAATGAGAGCTGTAGTTCAGG-3') for the hROR α -3051/-45 sequence, the hROR α -487 primer (5'-GTACACGCGTCTCAGAGTAGGCACGGAATCATC-3') for the hROR α -487/-45 sequence and the common hROR α -45 primer (5'-TGAGAGATCTCCCTCTATCTTCTGTTTTCAGAGC-3'). PCR amplification was carried out using Platinum *Pfx* DNA polymerase (Invitrogen). PCR amplification products were then cloned in the *Bgl*III-*Mlu*I restriction sites in the polylinker region of the promoterless pGL3-basic luciferase reporter vector (pGL3-Luc) from Promega. The constructions containing the -3051/-45 and -487/-45 sequences are referred to as p(-3051)Rora-Luc and p(-487)Rora-Luc respectively. Site-directed mutagenesis of the putative HRE located between nucleotides -229 and -225 from the translation initiation site of the ROR α 1 isoform (C⁻²³⁷GCA-CACACACGTCACCCAGA⁻²¹⁶ $\rightarrow$ C⁻²³⁷GCACACACTTTTCCACCCAGA⁻²¹⁶) was done using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The pcDNA3-HIF1 α , pcDNA3-HIF1 β and pcDNA3-HIF1 α DN expression vectors, allowing expression of HIF1- α , HIF1- β and HIF1- α -DN, a DN (dominant-negative) form of HIF1- α , respectively, have been described [45,46]. The pcDNA3 empty vector was from Invitrogen.

Transient transfection experiments were done using the calcium phosphate method, and luciferase activity was assayed 48 h later as described [47]. For hypoxic treatment, in order to optimize reproducibility, each calcium phosphate/DNA precipitate was split into two equal parts that were spread over two plates of cells. At 24 h after transfection, one plate was incubated under hypoxia while the other was incubated under normoxia as a control. Luciferase activities were measured 24 h later. Activities corresponding to cells cultured under hypoxia were expressed relative to those of control cells cultured under normoxia. For co-transfection experiments, care was taken to keep constant the total amount of transfected DNA using the empty pcDNA3 vector.

Electrophoretic-mobility-shift assays

Unlabelled HIF-1 α and HIF-1 β proteins were obtained *in vitro* from the pcDNA3-HIF1 α and pcDNA3-HIF1 β vectors using the TNT-T7 Quick coupled transcription/translation kit (Promega). Aliquots (1 μ l) of the programmed lysates were incubated at room temperature in a 15 μ l reaction mixture containing 15 mM Tris/HCl, pH 7.5, 50 mM KCl, 15 mM NaCl, 1 mM MgCl₂, 0.03 mM EDTA, 5 mM dithiothreitol, 7 % (v/v) glycerol, 50 ng of poly(dI-dC), and 150 ng of a mix of unspecific single-stranded oligonucleotides. A 0.3 ng (10 000 c.p.m.) portion of the double-stranded oligonucleotide, labelled by fill-in with the klenow polymerase and [α -³²P]dATP (3000 Ci/mmol; Amersham), was added 5 min later. Binding was then allowed to proceed for 15 min at room temperature. For competition experiments, double-stranded oligonucleotides were added simultaneously with the labelled probe. Electrophoresis was performed at 200 V for 2 h 20 min at 15 °C in 0.25 $\times$ TBE buffer (1 $\times$ TBE is 45 mM Tris/borate/1 mM EDTA) on a native 5%-(w/v)-polyacrylamide gel that had been pre-run for 2 h under the same conditions. The gels were then fixed for 15 min in 20 % (v/v) ethanol/10 % (v/v) acetic acid, dried, and submitted to autoradiography.

Statistical analysis

Results are expressed as means $\pm$ S.E.M. for the number of experiments indicated in each case. Statistical analysis was carried

Figure 1 Both the *Rora1* and *Rora4* mRNA levels are increased under hypoxic conditions

HepG2 cells and COS-7 cells were cultured for 24 h under normoxia (20 % O_2) or hypoxia (10, 2, or 0.1 % O_2). (A and B) Total RNA was analysed for *Rora* mRNA (all isoforms) by quantitative RT-PCR. β -Actin mRNA was monitored as a control. Results are expressed relative to the values at 20 % O_2 and are given as means $\pm$ S.E.M. ($n=3$). Significant differences ($P < 0.01$) from 20 % O_2 controls are indicated by **. (C) Total RNA from COS-7 cells was analysed for *Rora1* mRNA (35 cycles), *Rora4* mRNA (30 cycles) by semi-quantitative RT-PCR. Amplification of β -actin mRNA (17 cycles) was performed as a control. The PCR controls (without cDNA) are shown (lanes -). The photographs are representative of those obtained after at least three independent experiments.

out using the ANOVA test [48]. Differences were considered to be significant at $P < 0.01$.

RESULTS AND DISCUSSION

In order to determine whether the stimulatory effect of hypoxia on *Rora* gene expression is a general phenomenon, we analysed the effect of hypoxia on the amount of *Rora* transcripts in various cell types. As evidenced by our real-time quantitative RT- (reverse-transcription) PCR experiments, there were 15–25-fold more *Rora* transcripts in HepG2 human hepatoma cells (Figure 1A) and COS-7 monkey kidney cells (Figure 1B) cultured for 24 h under hypoxia (2 or 0.1 % O_2) than in control cells cultured under normoxia (20 % O_2). This effect was specific, since the level of β -actin-gene transcripts was not affected by the hypoxic treatment (Figure 1). The amount of *Rora* transcripts was also specifically increased by hypoxia in all the other cell lines analysed, namely Hep3B human hepatoma cells, Caco-2 human colorectal adenocarcinoma cells, T47D human breast-carcinoma cells, HeLa human cervical-adenocarcinoma cells, PC12 rat pheochromocytoma cells and NT2 human teratocarcinoma cells (results not shown). These results strongly suggested that the stimulatory effect of hypoxia on *Rora* gene expression is a general phenomenon that is dependent neither on the cell type nor on

the species. In agreement with our results, others have reported a transient up-regulation of the *Rora* transcripts by hypoxia in human aortic smooth-muscle cells and human endothelial cells [49]. The *Rora* transcripts are also transiently up-regulated *in vivo* by ischaemia in the mouse hindlimb [41]. We next evaluated whether the expression of specific ROR α isoforms is up-regulated by hypoxia. In COS-7 cells in which ROR α 1 and ROR α 4 isoforms are detected, the amount of both the *Rora1* and *Rora4* transcripts was increased under hypoxia (2 or 0.1 % O_2 , 24 h), as compared with normoxia (20 % O_2 , 24 h) (Figure 1C). We concluded that the expression of both the ROR α 1 and ROR α 4 isoforms is up-regulated by hypoxia.

Since nothing was known about the *cis*- and *trans*-acting elements controlling the transcription of the *Rora* gene, we first checked whether DNA fragments located upstream from the first exon of the human *Rora* gene, which encodes the 5' part of the *Rora1* transcript, contain a promoter activity. To this aim, we cloned the genomic sequences located between nucleotides –3051 and –45, and between nucleotides –487 and –45 upstream from the translation initiation site of ROR α 1 in the promoter-less pGL3-Luc reporter vector. Transient transfection experiments in HepG2 cells showed that both the p(–3051)*Rora*-Luc and the p(–487)*Rora*-Luc constructs have a much higher transcriptional activity (about 20- and 50-fold respectively) than that of the promoter-less pGL3-Luc vector (Figure 2A). This indicated that the –487-to–45 *Rora* genomic sequence contains a promoter active in HepG2 human hepatoma cells. Similar results were obtained with other cell types, such as HeLa, COS-7, and CHO (Chinese-hamster ovary) cells (results not shown). This indicated that the *Rora* promoter is active in cells of widespread origin. This result fits very well with those from the computer-assisted analysis we performed to search for putative promoters in the human *Rora* gene using the PromoterInspector program (Genomatix Software GmbH, Munich, Germany). It showed that the region between nucleotides –295 and –64 from the translation initiation site of ROR α 1 has strong characteristics common to promoters lacking the classical TATA box. Interestingly, similar data were obtained when analysing the corresponding region of the *Rora* gene in mouse chromosome 9. Taken together, these data indicated that a functional promoter for the *Rora* gene lies in the proximal upstream region of exon 1 in man and probably in the mouse.

To gain further insight into the mechanisms by which hypoxia up-regulates *Rora* gene expression, we tested the effect of hypoxia on the transcriptional activity of the p(–487)*Rora*-Luc construct in HepG2 cells, which are widely used as a convenient cellular model for analysing the effects of hypoxia on gene expression. Hypoxic treatment (2 % O_2) resulted in a 2.5-fold enhancement of the activity of the p(–487)*Rora*-Luc construct, as compared with the normoxic (20 % O_2) condition (Figure 2B). This indicated that the effect of hypoxia on ROR α expression is, at least in part, transcriptional and that the –487-to–45 *Rora* promoter region is involved in this regulation. This prompted us to carefully analyse the *Rora* promoter region for a putative HRE. We identified a putative HRE sequence (CACGT) between nucleotides –229 and –225 relative to the translation initiation site of ROR α 1. We mutated (mut) the –229-to–225 HRE in three crucial nucleotides (CTTTT) to obtain the pmut(–487)*Rora*-Luc construct. This mutation did not alter the basal activity of the –487-to–45 *Rora* promoter, because luciferase activity of the pmut(–487)*Rora*-Luc vector was about 1.18-fold that of the p(–487)*Rora*-Luc vector. We found that the –229-to–225 HRE mediated the response of the *Rora* promoter to hypoxia. Indeed, the stimulatory effect of hypoxia on the activity of p(–487)*Rora*-Luc was found dependent on the integrity

Figure 2 A functional promoter up-regulated by hypoxia lies in upstream region close to the first exon of the human *Rora* gene

(A) HepG2 cells cultured in 12-well plates were transfected with 500 ng/well of the p(-3051)*Rora*-Luc reporter vector or with 500 ng/well of the p(-487)*Rora*-Luc reporter vector, which allow expression of the luciferase gene under the control of the human genomic sequences between nucleotides -3051 and -45, or between nucleotides -487 and -45, from the *Rora*1 translation initiation site respectively, or with 500 ng/well of the promoter-less pGL3-Luc control vector. The luciferase activity of the p(-3051)*Rora*-Luc and p(-487)*Rora*-Luc vectors is expressed relative to that of the promoter-less pGL3-Luc vector. Results are given as means $\pm$ S.E.M. ($n=12$). Significant differences ($P<0.01$) from the luciferase activity of the pGL3-Luc vector are indicated by **. (B) HepG2 cells cultured in 60-mm-diameter glass dishes were transfected with 5 μ g of the p(-487)*Rora*-Luc vector or with 5 μ g of the pmut(-487)*Rora*-Luc vector, which bears three point mutations in the putative HRE located between nucleotides -229 and -225 from the *Rora*1 translation initiation site. The luciferase activities of the reporter vectors in cells cultured for 24 h under hypoxia (2% O_2) are expressed relative to their respective activities in control cells cultured under normoxia (20% O_2). Results are given as means $\pm$ S.E.M. ($n=6$). Significant differences ($P<0.01$) from the luciferase activity of the reporter vectors in cells cultured under normoxia (20% O_2) are indicated by **.

of the -229-to--225 HRE, because the pmut(-487)*Rora*-Luc construct was no longer sensitive to hypoxia (Figure 2B).

To test whether the HIF-1 α /HIF-1 β heterodimer binds to the HRE identified within the *Rora* promoter, we used electrophoretic-mobility-shift assays with *in-vitro*-synthesized HIF-1 α and -1 β proteins. A specific DNA-protein complex with a retarded migration was formed exclusively when both the HIF-1 α - and HIF-1 β -programmed lysates were incubated together with the 32 P-labelled probe covering the wt (wild-type) *Rora* HRE (Figure 3, lanes 4 and 13). The binding of the HIF-1 α /HIF-1 β heterodimer to the probe was specific, since it was competed for by an excess of the unlabelled wt-*Rora*-HRE oligonucleotide (Figure 3, lanes 5–8), but not with the mut-*Rora*-HRE mutated oligonucleotide (Figure 3, lanes 9–12). In the same manner, the binding of the HIF-1 α /HIF-1 β heterodimer to the wt-*Rora*-HRE probe was no longer detected when competition was done with an excess of the wt-*Epo*-HRE oligonucleotide [50], which bears the well-characterized HIF-binding site of the erythropoietin (*Epo*) gene enhancer (Figure 3, lane 14), but not with the mut-*Epo*-HRE mutated oligonucleotide (Figure 3, lane 15). These

Figure 3 HIF-1 α binds specifically to the -229-to--225 HRE of the *Rora* promoter

Radiolabelled wt-*Rora*-HRE probe was incubated with 1 μ l of HIF-1 α programmed lysate (lane 2), 1 μ l of HIF-1 β programmed lysate (lane 3), or both (lanes 4–15). Unprogrammed reticulocyte lysate was used as a control (lane 1) and to keep the total volume of lysate at 2 μ l (lanes 2 and 3). Competition assays were carried out by incubating the radiolabelled wt-*Rora*-HRE probe with the HIF-1 α and HIF-1 β programmed lysates in the presence of unlabelled wt-*Rora*-HRE (lanes 5–8) or mut-*Rora*-HRE (lanes 9–12) double-stranded oligonucleotides at a 10-, 25-, 50- or 100-fold molar excess. A 100-fold molar excess of the wt-*Epo*-HRE (lane 14) or mut-*Epo*-HRE (lane 15) double-stranded oligonucleotides was also used. The arrow points to the specific HIF-1-probe complexes. The asterisk (*) indicates an unrelated complex formed with proteins present in the unprogrammed lysate. Sequences of the oligonucleotides used in the electrophoretic-mobility-shift assays are represented in the upper part of the Figure. The sequence of the HREs is written in **bold**, the point mutations are underlined, and the nucleotides written in lower case do not belong to the *Rora* or the *Epo* sequence and were added for convenience.

data demonstrated that HIF-1 binds to the functionally active HRE present in the *Rora* promoter.

To determine whether HIF-1 transactivates the *Rora* promoter, we performed co-transfection experiments in HepG2 cells. Overexpression of the HIF-1 α protein alone, or in combination with the HIF-1 β protein, resulted in an approx. 10-fold stimulation of the activity of the p(-487)*Rora*-Luc vector. By contrast, that of the pmut(-487)*Rora*-Luc vector, in which the HRE is mutated, was not altered (Figure 4). This strongly suggested that HIF-1 is involved in the upregulation of the *Rora* promoter activity by hypoxia.

To evaluate directly the involvement of HIF-1 in mediating the effect of hypoxia on the *Rora* promoter, we used the

Figure 4 HIF-1 stimulates the activity of the *Rora* promoter through the -229-to--225 HRE

HepG2 cells cultured in 12-well plates were transfected with 500 ng/well of the p(-487)Rora-Luc or with 500 ng/well of the pmut(-487)Rora-Luc reporter vector, together with 333 ng/well of the pcDNA3-HIF1 α expression vector, and/or with 33 ng/well of the pcDNA3-HIF1 β expression vector, or with the pcDNA3 control expression vector. The total amount of transfected DNA was kept constant at 866 ng by adding the pcDNA3 insertless vector. The luciferase activities in the presence of HIF-1 are expressed relative to those in the absence of HIF-1 expression vector. Results are given as means $\pm$ S.E.M. ($n = 12$). Significant differences ($P < 0.01$) from the luciferase activity of the reporter vectors in the absence of HIF-1 expression vector are indicated by **.

pcDNA3-HIF1 α DN construct, which encodes a DN form of HIF-1 α lacking the N-terminal and the C-terminal transactivation domains. HIF-1 α -DN heterodimerizes with HIF-1 β and this leads to the formation of a non-functional HIF-1 dimer. In co-transfection experiments, expression of the HIF-1 α -DN protein abolished the stimulatory effect of hypoxia on the p(-487)Rora-Luc vector. This effect was specific, since the activity of the pmut(-487)Rora-Luc vector was not altered by the expression of the HIF-1 α -DN protein (Figure 5).

Taken together, these results showed that the stimulatory effect of hypoxia on *Rora* gene transcription is mediated by HIF-1 through the -229-to--225 HRE in the promoter. Interestingly, a sequence identical with the human HRE lies in the corresponding region of the *Rora* gene in the mouse. This conservation between the two species further underscores the importance of this regulatory sequence in the control of *Rora* gene expression.

In conclusion, we clearly demonstrate that hypoxia up-regulates expression of the *Rora* gene in a wide panel of cell types. These findings bring substantial information on the regulation of ROR α with potentially interesting physiological and pathological consequences. One may hypothesize that this widely expressed nuclear receptor probably participates in the response to the hypoxic stress by regulating a subset of genes. Characterization of these genes is obviously an exciting challenge. ROR α is known to possess anti-inflammatory properties [38] and to play some role in post-ischaemic angiogenesis [41]. We now show that *Rora* is a hypoxia-responsive gene. Inflammation, hypoxia and angiogenesis are the basis of major pathophysiological processes such as atherosclerosis and cancerogenesis. The protective role of ROR α against atherosclerosis is well known [35,36]. It would not be surprising if ROR α also plays some part in the carcinogenic process. However, further work is required to explore this route. Interestingly, our study reveals *Rora* as a new HIF-1 target gene. This result fits well with those from a very recent study indicating that the Nur77 orphan nuclear receptor is also a direct target

Figure 5 A DN form of HIF-1 α abolishes the stimulatory effect of hypoxia on the *Rora* promoter

HepG2 cells cultured in 60-mm-diameter glass dishes were transfected with 5 μ g of the p(-487)Rora-Luc or 5 μ g of the pmut(-487)Rora-Luc reporter vector, without or with 500 ng of the pcDNA3-HIF1 α DN expression vector, while keeping the total amount of transfected DNA constant to 5.5 μ g by adding the pcDNA3 insertless vector. The luciferase activities of the reporter vectors in cells cultured for 24 h under hypoxia (2% O₂) are expressed relative to their respective activities in control cells cultured under normoxia (20% O₂). Results are given as means $\pm$ S.E.M. ($n = 6$). Significant differences ($P < 0.01$) from the luciferase activity of the reporter vectors in normoxia are indicated by **.

for HIF-1 [51]. These results allow deciphering, at the molecular level, one aspect of the cross-talk between the signalling pathways used to sense and respond to hypoxia and those mediated by nuclear receptors. This is an important step in the extensive characterization of the cross-talk between these two signalling pathways, which may be useful to finely tune both the response to hypoxia and to hormones/nutrients. Such an approach may ultimately allow one to develop new strategies for the efficient treatment of many critical health problems.

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