A dominant-negative isoform lacking exons 11 and 12 of the human hypoxia-inducible factor-1 α gene

Yang-Sook CHUN*, Eunjoo CHOI*, Tae-You KIM†, Myung-Suk KIM* and Jong-Wan PARK*¹

*Department of Pharmacology, Seoul National University, College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-799, South Korea

and †Department of Internal Medicine, Heart Research Institute, BK21 Human Life Sciences, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-799, South Korea

Hypoxia-inducible factor-1 α (HIF-1 α), a member of the transcription family characterized by a basic helix-loop-helix (bHLH) domain and a PAS domain, regulates the transcription of hypoxia-inducible genes involved in erythropoiesis, vascular remodelling and glucose/energy metabolism. It contains bHLH/PAS domains in the N-terminal half, and a nuclear localization signal (NLS) and two transactivation domains (TADs) in the C-terminal half. It also has an oxygen-dependent degradation (ODD) domain, which is required to degrade HIF-1 α protein by the ubiquitin–proteasome pathway. In this study, we identified a new alternatively spliced variant of human HIF-1 α mRNA, which lacked both exons 11 and 12, producing a frame shift and giving a shorter form of HIF-1 α . In the corresponding protein, a part of the ODD domain, both TADs and the C-terminal NLS motif were missing. Expression of endogenous HIF-1 α variant protein was identified using immunoprecipitation and immuno-

blotting methods. The expressed HIF-1 α variant exhibited neither the activity of transactivation nor hypoxia-induced nuclear translocation. In contrast with HIF-1 α , the variant was strikingly stable in normoxic conditions and not up-regulated to such an extent by hypoxia, cobalt ions or desferrioxamine. It was also demonstrated that the HIF-1 α variant competed with endogenous HIF-1 α and suppressed HIF-1 activity, resulting in the down-regulation of mRNA expression of hypoxia-inducible genes. The association of the variant and arylhydrocarbon receptor nuclear translocator in the cytoplasm may be related to the inhibition of HIF-1 activity. It is assumed that this isoform preserves the balance between aerobic and anaerobic metabolism by counteracting the overaction of HIF-1 α .

Key words: arylhydrocarbon receptor nuclear translocator, hypoxia, hypoxia-inducible gene, RNA splicing.

INTRODUCTION

Mammalian cells up-regulate the expression of hypoxic genes in order to survive in hypoxic conditions. The hypoxia-induced proteins are involved in glucose/energy metabolism, vascular development/remodelling, vasodilation and erythropoiesis [1,2]. The hypoxic induction of these genes depends on a basic helix-loop-helix (bHLH) transcription factor, hypoxia-inducible factor 1 (HIF-1), which is activated by hypoxia and binds to a consensus sequence (5'-RCGTG-3') in the hypoxia-response elements [3,4]. HIF-1 is composed of HIF-1 α and arylhydrocarbon receptor nuclear translocator (ARNT). They both belong to the PAS family of bHLH transcription factors [5]. Normally, HIF-1 activity depends on the amount of HIF-1 α protein, which is increased markedly by hypoxia, whereas ARNT protein is present constitutively regardless of oxygen tension. Under normoxic conditions, the *de novo*-synthesized HIF-1 α protein is immediately degraded by the ubiquitin–proteasome pathway. However, under hypoxic conditions, it escapes from the degradation process and imports into the nucleus, where it co-dimerizes with ARNT. It binds to the hypoxia-response elements of hypoxia-inducible genes and transactivates these genes [6,7].

HIF-1 α is an 826-amino-acid protein that is unique to HIF-1, whereas ARNT is a common transcription factor to be dimerized with the arylhydrocarbon receptor and other bHLH/PAS proteins [5]. The N-terminal half of HIF-1 α contains the bHLH and PAS domains, which are essential for dimerization and DNA

binding [8]. The C-terminal half contains two transactivation domains (TADs), which are localized to amino acid residues 531–575 for N-terminal TAD and 786–826 for C-terminal TAD [9]. Two nuclear localization signals (NLSs) are localized in the N-terminal (amino acids 17–74) and C-terminal (amino acids 718–721) regions. Although both NLSs have been identified as determining the nuclear localization of HIF-1 α , the C-terminal NLS motif is considered to be more important in the nuclear translocation induced by hypoxia [10]. HIF-1 α contains a unique oxygen-dependent degradation (ODD) domain, which is localized to amino acid residues 401–603 [6]. The whole ODD domain might be required for the ubiquitin/proteasome-dependent degradation of HIF-1 α , because HIF-1 α remains stable even under normoxic conditions when part of the ODD domain is deleted. Recently, the subdomains in the ODD domain have been analysed intensively in an attempt to understand the mechanism of oxygen-dependent regulation of HIF-1 α stability. Under normoxic conditions, von Hippel-Lindau tumour suppressor protein (pVHL), one of multiprotein ubiquitin E3 ligase complex, mediates the ubiquitylation of HIF-1 α , which becomes the target of proteasomal proteolysis [11]. In this process, the N-terminal (380–417) and the C-terminal (556–572) sites of the ODD domain are minimal subdomains interacting with pVHL, and the hydroxylation of two proline residues (Pro-402 and Pro-564) in these subdomains determines pVHL binding to HIF-1 α [12–14]. However, under hypoxic conditions, the proline hydroxylation is inhibited and then HIF-1 α becomes stable.

Abbreviations used: bHLH, basic helix-loop-helix; HIF-1, hypoxia-inducible factor 1; ARNT, arylhydrocarbon receptor nuclear translocator; AINT, ARNT-interacting protein; TAD, transactivation domain; NLS, nuclear localization signal; ODD, oxygen-dependent degradation; pVHL, von Hippel-Lindau tumour suppressor protein; RT-PCR, reverse transcriptase PCR; EPO, erythropoietin; VEGF, vascular endothelial growth factor; HA, haemagglutinin; EMSA, electrophoretic mobility-shift assay; CBZ-LLL, *N*-carbobenzoxyl-Leu-Leu-Leu-al; EPAS1, endothelial PAS domain protein 1.

¹ To whom correspondence should be addressed (e-mail parkjw@plaza.snu.ac.kr).

Several alternatively spliced variants of HIF-1 α mRNA have been previously found in mouse, rat and human cells. Two splicing events in mouse cells have been described with alternative translation start sites [15] and alternative splicing within exon 10 [16]. The alternative splicing in rat cells occurred at the N-terminal end of HIF-1 α [17]. In human cells, two splicing events were described with alternative splicing between exons 1 and 2 and also between exons 13 and 14 [18]. The former variant had an additional 3 bp at the junction of exons 1 and 2 without a frame shift. The latter had lost exon 14, resulting in a frame shift and an immediate termination codon. Because the latter had most of the essential domains of HIF-1 α conserved, it showed oxygen-tension-dependent regulation and the transactivation by the vascular endothelial growth factor (VEGF) promoter.

In this study, we identified a new splicing variant of HIF-1 α mRNA in human cells. The new variant differs from the HIF-1 α mRNA by lacking exons 11 and 12, which produces a frame shift and introduces a shorter form of HIF-1 α . The deduced protein contains bHLH/PAS domains and the N-terminal NLS motif, but is deprived of a part of the ODD domain, both the TADs and the C-terminal NLS motif. The expressed HIF-1 α isoform exhibited neither transactivation activity nor hypoxia-induced nuclear translocation. In contrast with HIF-1 α , the expressed protein was strikingly stable under normoxic conditions and was up-regulated by hypoxia, cobalt ions and desferrioxamine to a smaller degree. We also demonstrated that the HIF-1 α isoform competed with endogenous HIF-1 α and suppressed HIF-1 activity, resulting in the down-regulation of mRNA expression of hypoxia-inducible genes.

EXPERIMENTAL

Cell culture

Most cancer cell lines were obtained from the A.T.C.C., but the SNU-601 cell line, a stomach cancer cell line, was obtained from the Korean Cell Line Bank (Seoul, South Korea). Hep3B cells were cultured in α -modified Eagle's medium (Gibco BRL). HEK-293, HeLa and SNU-601, and the murine cell lines NIH 3T3 and CT-26, were cultured in Dulbecco's modified Eagle's medium (Gibco BRL). Both culture media were supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL), 100 units/ml penicillin and 100 μ g/ml streptomycin in a humidified atmosphere containing 5% CO₂ at 37 °C. Oxygen tensions in the incubator (model 9108MS2; Vision Sci Co., Seoul, South Korea) were either 140 mmHg (20% O₂, v/v; normoxia) or 7 mmHg (1% O₂, v/v; hypoxia).

Cloning and sequencing of a human HIF-1 α cDNA isoform

An isoform of HIF-1 α cDNA was cloned by reverse transcriptase PCR (RT-PCR). RNAs extracted from HEK-293 cells were reverse transcribed using the avian myeloblastosis virus reverse transcriptase system (Promega). This template was amplified using five pairs of PCR primers designed as shown in Figure 1(A). The nucleotide sequences of the primers are summarized in Table 1. The resulting PCR products of the HIF-1 α cDNAs were extracted from agarose gels and cloned using a pCR2.1-TOPO cloning kit (Invitrogen). The plasmids containing the isoform were amplified, purified and analysed for DNA sequencing.

RT-PCR of mRNAs for HIF-1 α and hypoxia-inducible genes

For identification and quantification of the HIF-1 α variant mRNA and the mRNAs of hypoxia-inducible genes, a highly sensitive, semi-quantitative RT-PCR was performed as described

previously [19]. Total RNAs were isolated from the cultured cells using Trizol (Gibco BRL). After verifying their qualities on a 1% denaturing agarose gel, 1 μ g of total RNA was added to a 50 μ l RT-PCR reaction mixture containing 5 μ Ci of [α -³²P]dCTP and 250 nM primers. The RT-PCR was completed in a reaction involving one cycle of reverse transcription at 48 °C for 1 h and 18 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and elongation at 68 °C for 2 min. The resulting PCR fragments (5 μ l) were electrophoresed on a 4% polyacrylamide gel at 120 V in 0.3 $\times$ TBE buffer (where 1 $\times$ TBE is 45 mM Tris/borate/1 mM EDTA) at 4 °C, and dried gels were autoradiographed. The nucleotide sequences of the primers are summarized in Table 1.

Expression plasmids and transfection

A haemagglutinin (HA)-tagged HIF-1 α variant expression plasmid was made using a PCR-based mutagenesis kit (Stratagene). The mutagenesis with specific oligonucleotides (sense, 5'-AACT-ACAGTTCCAGAGGAAGAACTAA-3'; antisense, 5'-CTCA-GGTGAACCTTTGTCTAGT-3') was employed to delete nucleotides derived from exons 11 and 12. The fidelity of the PCR and the identity of the construct were confirmed by sequencing the insert. For transient transfection, about 40% of the confluent HEK-293 cells in 60 mm cell-culture dishes were transfected with 4 μ g of pcDNA3, pcDNA3-HIF-1 α or pcDNA3-HIF-1 α variant using the calcium phosphate method as described previously [20].

Reporter assays

The erythropoietin (EPO)- and VEGF-enhancer-driven luciferase reporter genes were constructed as described previously [20]. The synthetic DNAs encoding HIF-1-binding enhancer regions (EPO, 5'-GGTACCGGCCCTACGTGCTGTCTCACACAGCCTGTCTGACCTCTCGACCTACCGGCCAGATCT-3'; VEGF, 5'-GTACCCACAGTGCATACGTGGGCTCCAAACAGGTCTCTTGATC-3') were inserted into the pGL3 promoter plasmid (Promega). To assay HIF-1 activity, HEK-293 cells were co-transfected with 5 μ g each of luciferase reporter gene and the plasmid cytomegalovirus- β -gal for each 100 mm dish, using the calcium phosphate method. Transfected cells were split into nine aliquots and incubated for 42 h. After stabilizing, the cells were incubated for 16 h in 20% or 1% O₂ (normoxia or hypoxia, respectively). Cells were lysed and assayed for luciferase activity using a Biocounter M1500 luminometer (Lumac). β -Galactosidase assays were performed for normalization of transfection efficiency.

Preparations of nuclear and cytosolic proteins

Cells were cooled quickly by placing the plates on ice and were then washed twice in ice-cold PBS. Scraped cells were centrifuged at 1000 *g* for 5 min at 4 °C and then washed twice with PBS. They were resuspended in a lysis buffer containing 0.2% Nonidet P-40, and the nuclear and cytosolic proteins were extracted as described previously [20].

Electrophoretic mobility-shift assay (EMSA)

The oligonucleotide probe used in the gel shift assay for HIF-1 consisted of the sequence 5'-ACCGGCCCTACGTGCTGTCTCAC-3'. The ³²P-labelled double-stranded probe was prepared

Figure 1 Identification of a human HIF-1 α mRNA isoform

Total RNA was extracted from HEK-293 cells cultured under normoxic conditions. **(A)** Five pairs of PCR primers were used to analyse the whole structure of HIF-1 α RNA and the resulting PCR products were named as indicated. Numbered boxes represent exons and HIF-1 α domain organization is shown above the boxes. **(B)** HIF-1 α and its isoform RNAs were amplified and identified using a highly sensitive RT-PCR method with [α - 32 P]dCTP, and analysed by electrophoresis and autoradiography, as described in the Experimental section. The molecular size of each PCR product is indicated in parentheses. The smaller PCR products (607 bp in RT-PCR 3 and 261 bp in RT-PCR 4) were cloned and analysed as described in the Experimental section. The 607 bp cDNA was identified as a novel splicing variant but the 261 bp cDNA was the HIF-1 α ⁵¹⁶ variant reported previously [18]. **(C)** A pair of PCR primers (RT-PCR 5) was designed to examine the expression levels of the novel variant in various human and murine cancer cell lines, as represented in **(A)**. The upper major bands represent the wild-type HIF-1 α mRNA and the lower minor bands represent the variant. **(D)** The relative amounts of the mRNAs were calculated by an equation: (c.p.m. of HIF-1 α variant/c.p.m. of wild-type HIF-1 α) $\times$ (705/148). The results presented in **(B)**–**(D)** are representative of three separate experiments.

Table 1 Nucleotide sequences of the primers used in RT-PCR

LDH A, lactate dehydrogenase A.

Primer name	Strand	Nucleotide sequence (5' $\rightarrow$ 3')
RT-PCR 1	Sense	AACGACAAGAAAAGATAAGTTCT
	Antisense	GTTGGTACTGTTGGTATCATA
RT-PCR 2	Sense	AATGTGAACAAATACATGGGATTA
	Antisense	CTCAGGTGAACCTTTGTCTAGT
RT-PCR 3	Sense	TATGATACCAACAGTAACCAA
	Antisense	ACCATCATGTTCCATTTTTCG
RT-PCR 4	Sense	CCTGATGCTTTAACTTTTGCTG
	Antisense	TCAGTTAACTTGATCCAAACGCTC
RT-PCR 5	Sense	CCCCAGATTGAGGATCAGACA
	Antisense	ACCATCATGTTCCATTTTTC
VEGF	Sense	AACCTTCTGCTGTCTTGG
	Antisense	TTTGGTCTGCATTACAT
Enolase 1	Sense	AAGAACTGAACGTCACAGA
	Antisense	GATCTTCGATAGACACCACT
Aldolase A	Sense	GTCATCCTCTTCCATGAGAC
	Antisense	AGGTAGATGTGGTGGTCACT
LDH A	Sense	CTTTTCCTTAGAACACCAAA
	Antisense	TTGAGTTTGATCACCTCAT
β -Actin	Sense	AAGAGAGGCATCCTCACCT
	Antisense	ATCTCTTGCTCGAAGTCCAG

and EMSA was performed as described previously [20]. DNA–protein binding reactions were carried out for 20 min at 4 °C and run on 5% non-denaturing polyacrylamide gels. For supershift

analysis, 1 μ l of a rabbit HIF-1 α antiserum was added to the completed EMSA reaction mixture and incubated for 2 h at 4 °C prior to loading.

Immunoblotting and immunoprecipitation

For immunoblotting of the HIF-1 α and its variant protein, 20 μ g of cell extract was separated on SDS/6% and 10% polyacrylamide gels, respectively, and transferred to an Immobilon-P membrane (Millipore). Immobilized proteins were incubated overnight at 4 °C with a rabbit anti-HIF-1 α antibody, diluted 1:1000, with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TTBS). Horseradish peroxidase-conjugated anti-rabbit antiserum (Amersham Bioscience) was used as a secondary antibody (1:5000 dilution in 5% skimmed milk in TTBS). After extensive washing with TTBS, the complexes were visualized using Enhanced Chemiluminescence Plus (Amersham Bioscience). HIF-1 antiserum was generated in rabbits against a bacterially expressed fragment encompassing amino acids 418–698 of human HIF-1 α as described previously [19]. For immunoprecipitation of endogenous HIF-1 α variant protein, HEK-293 cells were treated with 130 μ M desferrioxamine for 8 h or 10 μ M *N*-carbobenzoxyl-Leu-Leu-Leu-al (CBZ-LLL) for 18 h. The cell lysates were incubated with the rabbit HIF-1 α antiserum or a preimmune rabbit serum, followed by incubation with Protein A–Sepharose beads (Amersham Bioscience). After washing, the immunocomplexes were eluted by boiling for 3 min in SDS sample buffer containing 10 mM dithiothreitol, and then subjected to SDS/PAGE. Immunoblotting using a rat anti-HIF-1 α antibody was performed as

described previously [20]. For co-immunoprecipitation of HIF-1 α variant and ARNT, cells were co-transfected with the HA-HIF-1 α variant and ARNT expression plasmids. After 48 h of incubation, the cytosolic proteins were prepared as described previously [20]. The HA-tagged HIF-1 α variant was immunoprecipitated with a rabbit anti-HA antibody (Santa Cruz Biotechnology) and then the co-immunoprecipitation of ARNT was identified with a goat anti-ARNT antibody (Santa Cruz Biotechnology) using Western blotting.

Immunofluorescence microscopy

HEK-293 cells transfected with pcDNA3-HA-HIF-1 α variant were subjected to hypoxia for 4 h, and fixed with 4 % paraformaldehyde in PBS at room temperature for 15 min. The cells were washed three times with 100 mM glycine in PBS, permeabilized with 0.1 % Triton X-100 in PBS for 1 min and washed three times with PBS. After blocking non-specific binding with 5 % normal goat serum in PBS for 1 h, the cells were incubated overnight at 4 °C with the rabbit anti-HIF-1 α antibody (1:1000) or the rabbit anti-HA antibody (1:250), purchased from Santa Cruz Biotechnology. The primary antibodies were stained by incubation with FITC-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories) at room temperature for 1 h. After washing in PBS, the cells were analysed by confocal laser scanning microscopy (Bio-Rad MRC1024).

RESULTS

Identification of an alternatively spliced variant of human HIF-1 α mRNA

Five pairs of PCR primers were designed to identify the splicing of HIF-1 α mRNA. Total RNAs extracted from the HEK-293 cells were reverse transcribed and amplified with specific primers. The RT-PCR products incorporated by [α - 32 P]dCTP were electrophoresed and the dried gel was autoradiographed. Two distinct DNA bands of different sizes were observed reproducibly for both the RT-PCR 3 and RT-PCR 4 primers (Figure 1B, lanes 3 and 4). These PCR products were cloned and sequenced as described in the Experimental section. The lower PCR fragment in RT-PCR 3 was identified as having lost both exons 11 and 12 and as having been formed by a direct connection between exons 10 and 13, representing a new variant of HIF-1 α cDNA. The lower PCR fragment in RT-PCR 4 was identified as having lost exon 14, which is identical with a variant reported previously [18]. To confirm that the variant cDNA had no other splicing site than exons 11 and 12, the structures of HIF-1 α cDNAs were analysed further using the other specific primers (RT-PCR 1 and 2). No other splicing was found between exons 1 and 10 (Figure 1B, lanes 1 and 2). These results indicated that the new splicing HIF-1 α variant loses exons 11 and 12 without additional splicing at any other exons.

Figure 2 Schematic representation of the cloned HIF-1 α isoform

The exons 10 and 13 were joined directly, which generated a new reading frame in the 13th exon. This alternative splicing introduced four new amino acids and an immediate termination codon. It translates into a 516-amino-acid polypeptide (HIF-1 α ⁵¹⁶), losing the ODD, TAD and the NLS domains. UTR, untranslated region; A and B, PAS A and PAS B subdomains respectively.

Figure 3 Identification and characterization of HIF-1 α^{516} protein

(A) HEK-293 cells were incubated under normoxic conditions (N) or were treated with 130 μ M desferrioxamine (Dfx) for 8 h or 10 μ M CBZ-LLL (Pi) for 18 h. HIF-1 α^{516} protein was immunoprecipitated with a rabbit anti-HIF-1 α antibody (Rabbit α HIF) or a preimmune rabbit serum (Pre), followed by Western blotting with a rat anti-HIF-1 α antibody. (B) HEK-293 cells were transfected with 4 μ g of pcDNA3 (pcDNA) or 4 μ g of pcDNA3-HA-tagged HIF-1 α^{516} (pHIF-1 α^{516}), using the calcium phosphate method. The transfected cells were subjected to the normoxia, hypoxia (H), 130 μ M desferrioxamine or 75 μ M CoCl₂ (Co) for 8 h, or 10 μ M CBZ-LLL (Pi) for 18 h. The total cell lysates (50 μ g of protein) were analysed using Western blotting with anti-HA antibody. (C, D) Cells were transfected with 1 μ g of pcDNA3-HA-tagged HIF-1 α^{516} (C) or with 4 μ g of pcDNA-HIF-1 α (pHIF-1 α) (D), and then were treated as indicated. (E–G) Cells were homogenized to isolate the cytosolic (Cy) and nuclear (Nu) proteins. In each fraction extracted from the cells cultured under normoxic or hypoxic conditions, the expression level of endogenous wild-type HIF-1 α (E) or HIF-1 α^{516} (F) was analysed with the rabbit anti-HIF-1 α antibody. HIF-1 α^{516} was re-examined with anti-HA antibody (G). (H) To analyse subcellular localization of HIF-1 α and HIF-1 α^{516} , HEK-293 cells (upper panels) or pHA-HIF-1 α^{516} -transfected cells (lower panels) were subjected to 8 h of hypoxia, and then incubated with rabbit anti-HIF-1 α antibody or anti-HA antibody, respectively, followed by a FITC-conjugated goat anti-rabbit antibody. The corresponding transmission images are shown in the right-hand panels.

To compare the mRNA levels of HIF-1 α and the variant, semi-quantitative RT-PCR using [α -³²P]dCTP and autoradiography was performed as described in the Experimental section. The HIF-1 α variant was expressed ubiquitously in all human cell lines tested, whereas its expression was much lower in murine cell lines (Figure 1C). For quantification of these PCR fragments, each radioactive band was cut out from the dried gel and the radioactivities were measured using a β -counter. The relative amounts of variant mRNA are represented in Figure 1(D) and were assessed by dividing the c.p.m. value of the variant fragment by that of the corresponding HIF-1 α fragment. The approximate molar ratio of the variant to HIF-1 α was calculated using the following equation: (c.p.m. of HIF-1 α variant/c.p.m. of wild-type HIF-1 α) $\times$ (705/148) (705 and 148 represent the numbers of nucleotides present in the wild-type and variant PCR products, respectively). The mean expression level of the variant mRNA in human cell lines was approx. 7.5% of that of HIF-1 α . However, the expression levels of the variant were much lower in the mouse

cell lines, which suggests that the variant was expressed differently among species.

The structure of HIF-1 α cDNA isoforms

The HIF-1 α variant cDNA was cloned and sequenced as described in the Experimental section, and its structure is summarized in Figure 2. The cloned cDNA was identical with the HIF-1 α cDNA except for a 557 bp deletion in the middle. Reference to HIF-1 α genomic organization [21] showed that the cloned cDNA was derived from an alternatively spliced mRNA that lacks both exons 11 and 12 of the HIF-1 α gene (Figure 2). In this mRNA, exons 10 and 13 were joined, generating a new reading frame after the site of the deletion and an immediate termination codon in exon 13. Consequently, this alternative splicing introduces four new amino acids, Asn-Tyr-Ser-Ser, as a new C-terminus following Glu⁵¹² of the wild-type 826-amino acid HIF-1 α protein. This novel form of HIF-1 α mRNA translates

Figure 4 Effect of HIF-1α⁵¹⁶ on HIF-1 activity

Luciferase reporter plasmids containing the EPO (A) and VEGF (B) enhancers were co-transfected with pHIF-1α and pHIF-1α⁵¹⁶ in HEK-293 cells. After 16 h of hypoxia, the luciferase activity of the reporter gene was measured. Each bar, in arbitrary units, represents the mean ± S.D. from six experiments. (C) HIF-1 was extracted from the nuclei of the transfected cells subjected to normoxia (N) or 8 h of hypoxia (H), and analysed by EMSA. For supershift analysis, each 1 μl of rabbit HIF-1α antiserum was added to the completed EMSA reaction mixture containing the nuclear extract of hypoxic pcDNA-transfected cells. HIF-1 binding (HIF-1), non-specific binding (NS) and supershifted bands (SS) are indicated.

into a 516-amino-acid polypeptide, which we designated HIF-1α⁵¹⁶. Compared with HIF-1α, HIF-1α⁵¹⁶ conserves both bHLH and PAS domains, which are essential for the binding with ARNT. However, it loses the C-terminal pVHL-interacting subdomain (amino acids 556–572) in the ODD domain, TAD and NLS motif. Based on its structure, it is expected to be regulated partly by oxygen tension and to exhibit neither transactivation activity nor hypoxia-induced translocation into the nucleus.

Expression of HIF-1α⁵¹⁶ protein in HEK-293 cells

To identify endogenous HIF-1α⁵¹⁶ protein, immunoprecipitation of the protein was performed using two kinds of anti-HIF-1α antibody, raised in rabbit and rat. A broad band was observed in the immunoprecipitate samples pulled down by the anti-HIF-1α antibody, whereas no immunopositive band was detected in the sample treated with the preimmune serum. The intensity of the immunopositive band somehow increased in desferrioxamine-treated cells and increased markedly in cells treated with a proteasome inhibitor, CBZ-LLL, as shown in Figure 3(A). The molecular size of the immunopositive protein was slightly less than that of the expressed HA-tagged HIF-1α⁵¹⁶ protein. This difference in molecular size was expected because HA-tagged HIF-1α⁵¹⁶ has the extra sequence associated with HA and the hinge peptides. The immunopositive protein might be endogenous HIF-1α⁵¹⁶ protein because its molecular size, protein band pattern and inducibility by desferrioxamine and CBZ-LLL were similar to those of expressed HIF-1α⁵¹⁶ shown in Figures 3(B) and 3(C). However, a direct comparison of the expression levels of endogenous HIF-1α⁵¹⁶ and wild-type HIF-1α is impossible because the antibodies used, made from an approx. 250-amino-acid peptide including exons 11 and 12, had lower antigenicity to HIF-1α⁵¹⁶ compared with wild-type HIF-1α.

To characterize HIF-1α⁵¹⁶ protein, we transfected HEK-293 cells with 4 μg of the HA-tagged HIF-1α⁵¹⁶ expression plasmid. On an SDS/polyacrylamide gel the protein band was somewhat broad, and its molecular mass was analysed as being between 60 and 75 kDa (Figure 3A). This molecular mass is higher than the theoretical molecular mass (58.5 kDa) calculated using the compute pI/Mw program in ExPASy Proteomics tools, which suggests that the variant protein, like HIF-1α, may have been

modified after translation. The HIF-1α⁵¹⁶ protein was present even under normoxic conditions. The HIF-1α⁵¹⁶ protein was induced mildly by CBZ-LLL (a proteasome inhibitor), hypoxia, desferrioxamine (an iron chelator) and cobalt ions (Figure 3A), but its fold induction was much lower than that of HIF-1α protein [19,20]. Since the mechanism of HIF-1α degradation could be saturated by overexpressing HIF-1α⁵¹⁶, as in the case of overexpressed wild-type HIF-1α [22], we reduced the dose of HIF-1α⁵¹⁶ plasmid to 1 μg (Figure 3C). The fold induction of HIF-1α⁵¹⁶ by hypoxia and cobalt was still lower than that of wild-type HIF-1α (Figure 3D). However, HIF-1α⁵¹⁶ was induced significantly by treatment with desferrioxamine. This HIF-1α⁵¹⁶ induction by desferrioxamine seems to be related to the remaining the N-terminal pVHL-interacting subdomain (amino acids 380–417) in the ODD domain of HIF-1α⁵¹⁶, but the role of the N-terminal subdomain in this desferrioxamine effect remains to be investigated.

To study the subcellular localization of HIF-1α⁵¹⁶, we measured HIF-1α and HIF-1α⁵¹⁶ in the cytosolic and nuclear fractions. HIF-1α⁵¹⁶ protein was present dominantly in the cytosolic fraction (Figures 3F and 3G), whereas HIF-α protein was present predominantly in the nucleus (Figure 3E). To confirm the subcellular localization of HIF-1α⁵¹⁶, we performed the immunocytochemistry, as shown in Figure 3(H). HIF-1α⁵¹⁶ was localized to the cytoplasm, whereas wild-type HIF-1α was localized to the nucleus. These properties are a result of the loss of the ODD domain and NLS motif, as expected from its protein structure, shown in Figure 2.

Inhibitory effect of HIF-1α⁵¹⁶ on HIF-1 activity

Since HIF-1α⁵¹⁶ protein has the same structure as HIF-1α in part, but lacks transcription-factor function, we considered the possibility that it works like a dominant-negative mutant of HIF-1α. To test this hypothesis, the effects of HIF-1α⁵¹⁶ on the expression of reporter genes, which are regulated by HIF-1, were examined. The transfected cells were co-transfected with reporter plasmids containing either EPO- or VEGF-enhancer elements fused to the luciferase gene. The luciferase activities of the EPO-enhancer reporter (Figure 4A) and VEGF-enhancer reporter (Figure 4B) increased in hypoxic pcDNA-transfected cells. To examine whether HIF-1α⁵¹⁶ competes with HIF-1α, increasing amounts

Figure 5 mRNA expression of hypoxia-inducible genes

mRNAs were isolated from pcDNA- or pHIF-1 α^{516} -transfected cells subjected to normoxia (N) or 16 h of hypoxia (H), and analysed by semi-quantitative RT-PCR. The results presented in each panel are representative of three separate experiments. LDH A, lactate dehydrogenase A; PGK 1, phosphoglycerate kinase 1.

of pHIF-1 α^{516} were co-transfected into pHIF-1 α -transfected cells with EPO or VEGF-enhancer reporter plasmids. The overexpressed HIF-1 α exaggerated the hypoxic responses of the reporters, but the hypoxic responses were diminished markedly by HIF-1 α^{516} in a dose-dependent manner (Figures 4A and 4B). These results suggest that HIF-1 α^{516} competes with HIF-1 α in terms of HIF-1 activity in a manner similar to a dominant-negative mutant.

Inhibitory effect of HIF-1 α^{516} on HIF-1 binding to the hypoxia-response element

To examine whether HIF-1 α^{516} inhibits the DNA binding of endogenous HIF-1 to the hypoxia-response element, nuclear extracts from HEK-293 cells transfected with either pcDNA or pHIF-1 α^{516} were incubated with the EMSA probe and electrophoresed. HIF-1 binding was increased greatly by hypoxia in the pcDNA-transfected cells and HIF-1 α^{516} inhibited this binding activity by 70 % (Figure 4C). The lower bands (Figure 4C, NS) represent the constitutive binding and non-specific binding. Super-shift by anti-HIF-1 α antibody (Figure 4C, SS) indicated that the upper band represents the HIF-1–DNA complex. From these results, we can postulate two possible mechanisms of HIF-1 α^{516} -induced suppression of endogenous HIF-1 activity: HIF-1 α^{516} may inhibit formation of the HIF-1 complex or it may weaken the DNA-binding affinity of endogenous HIF-1 to DNA. However, the second possibility is excluded here because HIF-1 α^{516} conserved the domains for ARNT binding and DNA binding. Moreover, since HIF-1 α^{516} is not translocated to the nucleus, it is unlikely that it affects the behaviour of HIF-1 located in the nucleus. Therefore, it is suggested that HIF-1 α^{516} inhibits the formation of the HIF-1 complex, but in what manner does this inhibition take place? HIF-1 α^{516} may inhibit HIF-1 formation before HIF-1 α and ARNT migrate from the cytosol to the nucleus.

Suppression of the hypoxic induction of HIF-1 α -regulated genes

To confirm further that HIF-1 α^{516} functions like a dominant-negative mutant in response to hypoxia, we examined the mRNA

Figure 6 Association of HIF-1 α^{516} and ARNT

(A) Cells transfected with pcDNA or pHA-HIF-1 α^{516} were incubated under hypoxic conditions for 8 h. The nuclear levels of ARNT protein were measured in the nuclear extracts by Western blotting with a goat anti-ARNT antibody. (B) Cells transfected with pARNT and pcDNA or pHA-HIF-1 α^{516} were incubated under hypoxic conditions for 8 h. HA-HIF-1 α^{516} protein in the cytosolic fraction was immunoprecipitated (IP) by the rabbit anti-HA antibody (α HA), and followed by Western blotting with the goat anti-ARNT antibody.

levels of hypoxia-inducible genes such as VEGF, lactate dehydrogenase A (LDH A), enolase 1, aldolase A and phosphoglycerate kinase 1 (PGK 1) by semi-quantitative RT-PCR. In pcDNA-transfected cells exposed to hypoxia, all mRNAs of hypoxia-inducible genes were increased markedly compared with the normoxic control. In pHIF-1 α^{516} -transfected cells subjected to hypoxia, however, the hypoxic induction of these genes was reduced (Figure 5). This result indicates that HIF-1 α^{516} competes with HIF-1 α in terms of HIF-1 formation and activity, consequently suppressing the hypoxic induction of HIF-1-regulated genes.

Association of HIF-1 α^{516} and ARNT

How does HIF-1 α^{516} inhibit endogenous HIF-1 activity? We thought it possible that HIF-1 α^{516} associates with ARNT before the formation of the wild-type HIF-1 α and ARNT complex in the nucleus. Our results demonstrated that the amount of ARNT protein in the nuclear extract was reduced by the overexpression of HIF-1 α^{516} (Figure 6A). However, the reduction of nuclear ARNT by HIF-1 α^{516} was not complete. Some untransfected cells seemed to remain and to show nuclear ARNT. To examine whether HIF-1 α^{516} associates with ARNT, immunoprecipitation of HA-tagged HIF-1 α^{516} was performed in the cytoplasmic fraction extracted from hypoxic cells using anti-HA

antibody, followed by Western blotting with anti-ARNT antibody. As shown in Figure 6(B), ARNT was co-precipitated with HIF-1 α ⁵¹⁶. The lower band appears to be a short form of ARNT protein, resulting from proteolytic cleavage. These results suggest that ARNT is present in the cytoplasm and is physically associated with HIF-1 α ⁵¹⁶. The association of HIF-1 α ⁵¹⁶ and ARNT in the cytoplasm may cause the reduction of nuclear ARNT and may be related to HIF-1 α ⁵¹⁶-induced suppression of HIF-1 activity and the expression of hypoxia-inducible genes.

DISCUSSION

In this report, we identified a new alternatively spliced variant of HIF-1 α mRNA. This variant, HIF-1 α ⁵¹⁶, was expressed in all human cell lines tested, such as HEK-293, Hep3B, HeLa and SNU-601, but only negligibly in two murine cell lines. The variant differs from wild-type HIF-1 α (HIF-1 α ⁸²⁶) by lacking both exons 11 and 12, which introduces a frame shift resulting in a shorter form of HIF-1 α , which was designated HIF-1 α ⁵¹⁶. HIF-1 α ⁵¹⁶ was expressed stably even under hypoxic conditions, but was not translocated into the nucleus. The expressed HIF-1 α ⁵¹⁶ inhibited HIF-1 activity and reduced mRNA expression of the hypoxia-inducible genes that are regulated by HIF-1. HIF-1 α ⁵¹⁶ competed with endogenous and expressed wild-type HIF-1 α , inhibiting the hypoxic activation of HIF-1. To our knowledge, this is the first report describing the presence of an endogenous HIF-1 α isoform functioning like a dominant-negative mutant. HIF-1 α ⁵¹⁶ could represent another useful tool in molecular biology as a dominant-negative isoform capable of suppressing cellular responses to hypoxia.

Two alternatively spliced variants of human HIF-1 α mRNA have been identified recently [18]. One of them translates a shorter form, composed of 736 amino acids (HIF-1 α ⁷³⁶), which only has exon 14 missing, resulting in a frame shift and an immediate termination of translation. Since it conserves the whole of the ODD domain, it is well regulated by oxygen tension. Deletion of one of the two TADs causes the lower transactivation activity of HIF-1 α ⁷³⁶, which results in competition with overexpressed HIF-1 α . However, the overexpressed HIF-1 α ⁷³⁶ markedly increases the hypoxic induction of reporter activity by up to 5-fold, compared with the hypoxic induction by endogenous HIF-1 α . Although this HIF-1 α ⁷³⁶ is comparable with our HIF-1 α ⁵¹⁶ in terms of mRNA and protein structures, there are big differences between them. HIF-1 α ⁵¹⁶ lacks ODD, both TADs and NLS because of the deletion of exons 11 and 12. Therefore, HIF-1 α ⁵¹⁶ was more stable under normoxia and did not transactivate the EPO and VEGF promoters at all. Although it is uncertain whether HIF-1 α ⁷³⁶ works as a transcription factor or as a dominant-negative isoform, HIF-1 α ⁵¹⁶ was demonstrated to work as a dominant-negative isoform to inhibit endogenous HIF-1 α , as shown in Figures 4 and 5. The results also suggest that HIF-1 α ⁵¹⁶ can be employed as a molecule to suppress HIF-1-mediated hypoxic responses.

Endothelial PAS domain protein 1 (EPAS1) is another bHLH/PAS transcription factor exhibiting high homology with HIF-1 α and which is expressed preferentially in vascular endothelial cells. It also dimerizes with ARNT and then binds to the hypoxia-response element as HIF-1 α does [23,24]. Maemura et al. [25] found that a truncated EPAS1 (EPAS1⁴⁸⁵) lacking TAD functions as a dominant-negative mutant of EPAS1⁸⁷⁰ and also competes with HIF-1 α in a VEGF promoter assay. Unlike HIF-1 α ⁵¹⁶, EPAS1⁴⁸⁵ is not a natural product but an artificially constructed one. No natural variant of EPAS1 capable of working like a

dominant-negative mutant has yet been found. However, because the protein structure of EPAS1⁴⁸⁵ is comparable with that of HIF-1 α ⁵¹⁶, it is a suitable model for comparative investigation into the function of HIF-1 α ⁵¹⁶. The expressed EPAS1⁴⁸⁵ showed similar effects on VEGF promoter activity and VEGF mRNA induction to those of HIF-1 α ⁵¹⁶. It also competed with HIF-1 α proteins, resulting in the inhibition of HIF-1 activity. Therefore, it is speculated that overexpressed EPAS1⁴⁸⁵ may bind with ARNT, a common partner of HIF-1 α and EPAS1, exhausting the unbound ARNT available for endogenous EPAS1 or HIF-1 α . If so, the HIF-1 α ⁵¹⁶ possibly interacts with ARNT before the dimerization of HIF-1 α and ARNT, and then interferes with the formation of functional HIF-1 complex.

How can cytosolic HIF-1 α ⁵¹⁶ interfere with the nuclear events of DNA binding and gene transactivation of HIF-1? To be able to bind to ARNT, HIF-1 α ⁵¹⁶ possibly interferes with the formation of HIF-1. Actually, a novel ARNT-interacting protein (AINT) has been reported to interfere with the formation of HIF-1 by sequestering ARNT in the cytoplasm [26]. AINT is a cytosolic protein having the property of physical association with ARNT. When the cytosolic protein was overexpressed, nuclear ARNT disappeared in the nucleus and was detected in the cytoplasm. Since HIF-1 α ⁵¹⁶ also has comparable properties in terms of cytoplasmic localization and association with ARNT, it is possible that HIF-1 α ⁵¹⁶, like AINT, inhibits nuclear translocation of ARNT. Recently, nuclear proteins other than ARNT have been reported to be sequestered in the cytoplasm by cytosolic proteins. For example, the nuclear translocation of β -catenin and Fe65 are inhibited by cadherins [27] and β -amyloid precursor protein [28], respectively. These cytosolic proteins function as cytoplasmic anchoring sites, regulating the activities of the transcription factors. Based on this information, it is speculated that HIF-1 α ⁵¹⁶ as a dominant-negative isoform inhibits the formation of HIF-1 by sequestering ARNT in the cytoplasm.

HIF-1 α ⁵¹⁶ is not the only gene that generates dominant-negative splice variants. Recently, other dominant-negative isoforms have been reported, including a caspase-9 variant missing the catalytic site [29], a STAT6 variant lacking the SH2 domain [30], a vitamin D receptor isoform lacking the ligand-binding domain [31] and an oestrogen receptor isoform lacking the DNA-binding domain [32]. In general, these naturally occurring dominant-negative isoforms lose the critical domain(s) required for the functioning of the wild-type protein. HIF-1 α ⁵¹⁶ shares this common property as one of the dominant-negative isoforms. These isoforms are considered to maintain the metabolic balance by counteracting the overactions of their wild-type forms and may be ascribed to genetic or metabolic diseases. In this aspect, it is assumed that HIF-1 α ⁵¹⁶ may participate in the regulation of hypoxic gene expression as a counterpart of HIF-1 α . To further address the physiological or pathological significance of the variant, a new method needs to be developed to specifically block its synthesis.

This work was supported by a Korean Research Foundation Grant (no. KRF-01-015-FP0096).

REFERENCES

- 1 Semenza, G. L. (2000) HIF-1 and human disease: one highly involved factor. *Genes Dev.* **14**, 1983–1991.
- 2 Ratcliffe, P. J., O'Rourke, J. F., Maxwell, P. H. and Pugh, C. W. (1998) Oxygen sensing, hypoxia-inducible factor-1 and the regulation of mammalian gene expression. *J. Exp. Biol.* **201**, 1153–1162.
- 3 Semenza, G. L., Neefelt, M. K., Chi, S. M. and Antonarakis, S. E. (1991) Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5680–5684.

- 4 Wenger, R. H. and Gassmann, M. (1997) Oxygen(es) and the hypoxia-inducible factor-1. *Biol. Chem.* **378**, 609–616
- 5 Wang, G. L., Jiang, B. H., Rue, E. A. and Semenza, G. L. (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 5510–5514
- 6 Huang, L. E., Gu, J., Schau, M. and Bunn, H. F. (1998) Regulation of hypoxia-inducible factor 1 α is mediated by an O₂-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 7987–7992
- 7 Kallio, P. J., Wilson, W. J., O'Brien, S., Makino, Y. and Poellinger, L. (1999) Regulation of the hypoxia-inducible transcription factor 1 α by the ubiquitin-proteasome pathway. *J. Biol. Chem.* **274**, 6519–6525
- 8 Jiang, B. H., Rue, E., Wang, G. L., Roe, R. and Semenza, G. L. (1996) Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. *J. Biol. Chem.* **271**, 17771–17778
- 9 Jiang, B. H., Zheng, J. Z., Leung, S. W., Roe, R. and Semenza, G. L. (1997) Transactivation and inhibitory domains of hypoxia-inducible factor 1 α . Modulation of transcriptional activity by oxygen tension. *J. Biol. Chem.* **272**, 19253–19260
- 10 Kallio, P. J., Okamoto, K., O'Brien, S., Carrero, P., Makino, Y., Tanaka, H. and Poellinger, L. (1998) Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1 α . *EMBO J.* **17**, 6573–6586
- 11 Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R. and Ratcliffe, P. J. (1999) The tumor suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature (London)* **399**, 271–275
- 12 Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S. and Kaelin, Jr, W. G. (2001) HIF1 α targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science* **292**, 464–468
- 13 Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J. et al. (2001) Targeting of HIF-1 α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science* **292**, 468–472
- 14 Masson, N., Willam, C., Maxwell, P. H., Pugh, C. W. and Ratcliffe, P. J. (2001) Independent function of two destruction domains in hypoxia-inducible factor-1 α chains activated by prolyl hydroxylation. *EMBO J.* **20**, 5197–5206
- 15 Wenger, R. H., Rolfs, A., Kvietikova, I., Spielmann, P., Zimmermann, D. R. and Gassmann, M. (1997) The mouse gene for hypoxia-inducible factor-1 α -genomic organization, expression and characterization of an alternative first exon and 5' flanking sequence. *Eur. J. Biochem.* **246**, 155–165
- 16 Luo, G., Gu, Y. Z., Jain, S., Chan, W. K., Carr, K. M., Hogenesch, J. B. and Bradfield, C. A. (1997) Molecular characterization of the murine HIF-1 α locus. *Gene Expr.* **6**, 287–299
- 17 Drutel, G., Kathmann, M., Heron, A., Gros, C., Mace, S., Schwartz, J. C. and Arrang, J. M. (2000) Two splice variants of the hypoxia-inducible factor HIF-1 α as potential dimerization partners of ARNT2 in neurons. *Eur. J. Neurosci.* **12**, 3701–3708
- 18 Gothie, E., Richard, D. E., Berra, E., Pages, G. and Pouyssegur, J. (2000) Identification of alternative spliced variants of human hypoxia-inducible factor-1 α . *J. Biol. Chem.* **275**, 6922–6927
- 19 Chun, Y. S., Choi, E., Kim, G. T., Lee, M. J., Lee, S. E., Kim, M. S. and Park, J. W. (2000) Zinc induces the accumulation of hypoxia-inducible factor (HIF)-1 α , but inhibits the nuclear translocation of HIF-1 β , causing HIF-1 inactivation. *Biochem. Biophys. Res. Commun.* **268**, 652–656
- 20 Chun, Y. S., Choi, E., Kim, G. T., Choi, H., Kim, C. H., Lee, M. J., Kim, M. S. and Park, J. W. (2000) Cadmium blocks hypoxia-inducible factor (HIF)-1-mediated response to hypoxia by stimulating the proteasome-dependent degradation of HIF-1 α . *Eur. J. Biochem.* **267**, 4198–4204
- 21 Iyer, N. V., Leung, S. W. and Semenza, G. L. (1998) The human hypoxia-inducible factor 1 α gene: HIF1A structure and evolutionary conservation. *Genomics* **52**, 159–165
- 22 Tanimoto, K., Makino, Y., Pereira, T. and Poellinger, L. (2000) Mechanism of regulation of the hypoxia-inducible factor-1 α by the von Hippel-Lindau tumor suppressor protein. *EMBO J.* **19**, 4298–4309
- 23 Tian, H., McKnight, S. L. and Russell, D. W. (1997) Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev.* **11**, 72–82
- 24 O'Rourke, J. F., Tian, Y. M., Ratcliffe, P. J. and Pugh, C. W. (1999) Oxygen-regulated and transactivating domains in endothelial PAS protein 1: comparison with hypoxia-inducible factor-1 α . *J. Biol. Chem.* **274**, 2060–2071
- 25 Maemura, K., Hsieh, C. M., Jain, M. K., Fukumoto, S., Layne, M. D., Liu, Y., Kourembanas, S., Yet, S. F., Perrella, M. A. and Lee, M. E. (1999) Generation of a dominant-negative mutant of endothelial PAS domain protein 1 by deletion of a potent C-terminal transactivation domain. *J. Biol. Chem.* **274**, 31565–31570
- 26 Sadek, C. M., Jalaguier, S., Feeney, E. P., Aitola, M., Dandimopoulos, A. E., Pelto-Huikko, M. and Gustafsson, J. A. (2000) Isolation and characterization of AINT: a novel ARNT interacting protein expressed during murine embryonic development. *Mech. Dev.* **97**, 13–26
- 27 Fagotto, F., Funayama, N., Gluck, U. and Gumbiner, B. M. (1996) Binding to cadherins antagonizes the signaling activity of beta-catenin during axis formation in *Xenopus*. *J. Cell Biol.* **132**, 1105–1114
- 28 Minopoli, G., de Candia, P., Bonetti, A., Faraonio, R., Zambrano, N. and Russo, T. (2001) The beta-amyloid precursor protein functions as a cytosolic anchoring site that prevents Fe65 nuclear translocation. *J. Biol. Chem.* **276**, 6545–6550
- 29 Seol, D. W. and Billiar, T. R. (1999) A caspase-9 variant missing the catalytic site is an endogenous inhibitor of apoptosis. *J. Biol. Chem.* **274**, 2072–2076
- 30 Patel, B. K. R., Pierce, J. H. and LaRochelle, W. J. (1998) Regulation of interleukin 4-mediated signaling by naturally occurring dominant negative and attenuated forms of human Stat6. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 172–177
- 31 Ebihara, K., Masuhiro, Y., Kitamoto, T., Suzawa, M., Uematsu, Y., Yoshizawa, T., Ono, T., Harada, H., Matsuda, K., Hasegawa, T. et al. (1996) Intron retention generates a novel isoform of the murine vitamin D receptor that acts in a dominant negative way on the vitamin D signaling pathway. *Mol. Cell Biol.* **16**, 3393–3400
- 32 Wang, Y. and Miksicek, R. J. (1991) Identification of a dominant negative form of the human estrogen receptor. *Mol. Endocrinol.* **5**, 1707–1715

Received 23 July 2001/29 October 2001; accepted 30 November 2001