A new HIF-1 alpha variant induced by zinc ion suppresses HIF-1-mediated hypoxic responses

Yang-Sook Chun[1], Eunjoo Choi[1], Eun-Jin Yeo[1], Jong Ho Lee[2], Myung-Suk Kim[1] and Jong-Wan Park[1,*]

[1]Department of Pharmacology and Heart Research Institute, BK21 Human Life Sciences, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-799, Korea
[2]Institute of Animal Science and Technology, College of Agriculture and Life Sciences, Seoul National University, Suwon 441-744, Korea
*Author for correspondence (e-mail: parkjw@plaza.snu.ac.kr)

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SUMMARY

The expressions of hypoxia-inducible genes are upregulated by hypoxia-inducible factor 1 (HIF-1), which is a heterodimer of HIF-1α and HIF-1β/ARNT (aryl hydrocarbon receptor nuclear transporter). Under hypoxic conditions, HIF-1α becomes stabilized and both HIF-1α and ARNT are translocated into the nucleus and codimerized, binding to the HIF-1 consensus sequence and transactivating hypoxia-inducible genes. Other than hypoxia, cobalt and nickel, which can substitute for iron in the ferroprotein, induce the stabilization of HIF-1α and the activation of HIF-1. We found previously that, although zinc, another example of a metal substitute for iron, stabilized HIF-1α, it suppressed the formation of HIF-1 by blocking the nuclear translocation of ARNT. Here, we identify a new spliced variant of human HIF-1α that is induced by zinc. The isoform lacks the 12th exon, which produced a frame-shift and gave a shorter form of HIF-1α (557 amino acids), designated HIF-1αZ (HIF-1α induced by Zn). This moiety was found to inhibit HIF-1 activity and reduce mRNA expressions of the hypoxia-inducible genes. It blocked the nuclear translocation of ARNT but not that of endogenous HIF-1α, and was associated with ARNT in the cytosol. These results suggest that HIF-1αZ functions as a dominant-negative isoform of HIF-1 by sequestering ARNT in the cytosol. In addition, the generation of HIF-1αZ seems to be responsible for the inhibitory effects of the zinc ion on HIF-1-mediated hypoxic responses, because the expressed HIF-1αZ behaved in the same manner as zinc in terms of inhibited HIF-1 activity and ARNT translocation.

Key words: HIF-1α, ARNT, Zinc ion, Dominant-negative isoform

INTRODUCTION

Under hypoxic conditions, mammalian cells induce hypoxia-inducible genes such as erythropoietin (EPO), vascular endothelial growth factor (VEGF), various glycolytic enzymes and glucose transporter 1 (Semenza, 2000a). The expression of these genes is regulated by HIF-1, a basic helix-loop-helix (bHLH) transcription factor, which is a heterodimer of HIF-1α and HIF-1β (aryl hydrocarbon receptor nuclear transporter (ARNT)) subunits (Semenza et al., 1991; Wang et al., 1995). At the protein level, HIF-1α protein is markedly increased by hypoxia, whereas ARNT protein is constitutively present regardless of oxygen tension. Under normoxia, HIF-1α is remarkably unstable and degraded through the ubiquitin-proteasome pathway. However, under hypoxia it becomes stable and accumulates to be dimerized with ARNT, to bind with the consensus sequence (5′-RCGTG-3′) and transactivate the hypoxia-inducible genes (Huang et al., 1996; Kallio et al., 1999).

HIF-1α, an 826 amino acid protein, is unique to HIF-1, whereas ARNT is a common partner and is able to dimerize with the aryl hydrocarbon receptor and other bHLH-PAS proteins (Wang et al., 1995). The bHLH and PAS domains comprise the N-terminal halves of both HIF-1α and ARNT, which are required for dimerization and DNA binding (Jiang et al., 1996). The C-terminal half of both proteins is required for transactivation. In the case of HIF-1α, its transactivation domains are localized to two amino acid residues 531-575 (N-terminal TAD) and 786-826 (C-terminal TAD), which are separated by an inhibitory domain (Jiang et al., 1997). Two nuclear localization signals (NLSs) are localized to the N-terminal (amino acids 17-74) and the C-terminal parts (amino acids 718-721). The C-terminal NLS motif of HIF-1α plays a crucial role in mediating hypoxia-inducible nuclear import of the protein, whereas the N-terminal NLS motif may be less important (Kallio et al., 1998). In addition, HIF-1α contains an oxygen-dependent degradation (ODD) domain, which is localized to amino acid residues 401-603 (Huang et al., 1998). The ODD domain is suggested to control HIF-1α degradation by the ubiquitin-proteasome pathway because its deletion makes HIF-1α stable even under normoxic conditions (Huang et al., 1998) (see the HIF-1α structure in Fig. 2).

Other than hypoxia, transition metal ions such as cobalt and nickel stabilize HIF-1α protein under normoxic conditions and induce HIF-1 activity and the expression of its downstream hypoxia-inducible genes (Bunn and Poyton, 1996). By contrast, other metal ions such as zinc (Chun et al., 2000a) and cadmium (Chun et al., 2000b) suppress HIF-1 activity and the expression of the hypoxia-inducible genes. Interestingly, although zinc, like cobalt and nickel, strongly stabilizes HIF-1α protein, it inhibits the dimerization of HIF-1 by blocking the nuclear translocation of ARNT in hypoxic cells, which is

likely to be responsible for the zinc-induced suppression of HIF-1 (Chun et al., 2000a). However, the actual mechanism responsible for the effect of zinc on ARNT translocation has not been elucidated.

Here, we identify for the first time a new alternative splicing variant of human *HIF-1α* that is induced by zinc treatment. The difference between the new variant and wild-type *HIF-1α* is its lack of the 12th exon, which produces a frame-shift and a shorter form of *HIF-1α*. In the corresponding protein, the bHLH-PAS structure and the N-terminal NLS motif are conserved, but a part of the ODD domain, both the TADs and the C-terminal NLS motif are removed. The expressed HIF-1α variant loses transactivation activity and hypoxia-inducible nuclear import, as might be expected from its protein structure. We have also demonstrated that the HIF-1α variant functions as a dominant-negative isoform and inhibits HIF-1-regulated gene induction under hypoxic conditions.

MATERIALS AND METHODS

Cell culture

HEK 293 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO/BRL), supplemented with 10% heat-inactivated fetal calf serum (GIBCO/BRL), 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. Oxygen tension in the incubator (Vision Sci Co., model 9108MS2, Seoul, Korea) was either 140 mm Hg (20% O_2, v/v, normoxia) or 7 mm Hg (1% O_2, v/v, hypoxia).

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

The full length *HIF-1α* cDNA isoform was determined by RT-PCR. RNAs were extracted from HEK 293 cells treated with 500 μM $ZnCl_2$ for 4 hours and reverse-transcribed using the avian myeloblastosis virus reverse transcriptase system (Promega). This template was amplified using two primers located 24 bases upstream from the start codon and at the stop codon of the human *HIF-1α* coding sequence (sense, 5-AGACATCGCGGGGACCGATT-3; antisense, 5-TCAG-TTAACTTGATCCAAAGCTCT-3). The PCR products of the full-length *HIF-1α* cDNAs were cloned using a pCR2.1-TOPO cloning kit (Invitrogen). To select a colony of *E. coli* transformed with the full-length *HIF-1α* cDNA isoform, colony PCRs using the specific primers (5-CCCCAGATTCAGGATCAGACA-3 and 5-CCATCATG-TTCCATTTTTCGC-3) were performed. The plasmid containing the full-length *HIF-1α* cDNA isoform was amplified and purified, and the insert DNA sequence analyzed.

Expression plasmids and the establishment of a transfected cell line

Haemagglutinin (HA)-tagged *HIF-1α* and *ARNT* expression plasmids (pcDNA3) were generous gifts from Eric Huang and Jie Gu. HA-tagged *HIF-1α* variant expression plasmid was made using a PCR-based mutagenesis kit (Stratagene). Mutagenesis with specific oligonucleotides (sense, 5-AACTACAGTTCCTGAGGAAGA-3; antisense, 5-CTGAGTAGAAAATGGGTTCTT-3) was employed to delete the nucleotides derived from the 12th exon. The fidelity of the PCR and identity of the construct were confirmed by sequencing the insert.

HEK 293 cells were stably transfected with the pcDNA3 or pcDNA3-*HA/HIF-1α* variant using the calcium phosphate method. Transfected cells were incubated for 36 hours in supplemented Dulbecco's modified Eagle's medium and then 0.45 mg/ml of G418 was added to select the transfected cells. Stable transfectants from three different transfections were pooled after 30 days to avoid bias in gene expression due to variable sites of chromosomal integration.

Reporter assays

The EPO and VEGF enhancer-driven luciferase reporter genes were constructed as previously described (Chun et al, 2000a). The synthetic DNAs coding the HIF-1-binding enhancer regions (EPO, 5-GGT-ACCGGCCCTACGTGCTGTCTCACACAGCCTGTCTGACCTCT-CGACCTACCGGCCAGATCT-3; VEGF, 5-GTACCCACAGTGC-ATACGTGGGCTCCAACAGGTCCTCTTGATC-3) were inserted into the pGL3 promoter plasmid (Promega). To assay the *HIF-1* activity, HEK 293 cells were cotransfected with the luciferase reporter genes and 10 μg of 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 hours. After stabilizing, the cells were incubated for 16 hours at 20% or 1% O_2. They were then lysed and assayed for luciferase activity using a Biocounter M1500 luminometer (Lumac). β-gal assays were performed for normalization of transfection efficiency.

RT-PCR for *HIF-1α* and β-actin

Total RNAs were isolated from HEK 293 cells by TRIZOL (GIBCO/BRL) and qualified on a denaturing agarose gel. RT-PCR was performed using a PCR-Access kit (Promega) as previously described (Chun et al., 2000b). The RT-PCR conditions used were: one cycle of reverse transcription at 48°C for 1 hour and 25 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and elongation at 68°C for 1 minute. The resulting PCR fragments (5 μl) were electrophoresed on a 2% agarose gel at 100 V in 0.5×TAE, and the gels were then stained with ethidium bromide. The nucleotide sequences of the primers are summarized in Table 1.

Semiquantitative RT-PCR for the hypoxia-inducible genes

The expression of the hypoxia-inducible genes was measured by semi-quantitative RT-PCR, as previously described (Chun et al., 2000b). One μg of total RNA was added to a 50 μl RT-PCR reaction mixture containing 5 μCi [α-^{32}P]CTP and 250 nM primers. RT-PCR was conducted by one cycle of reverse transcription at 48°C for 1 hour and 18 cycles of denaturation at 94°C for 30 seconds, annealing at 53°C for 30 seconds, and elongation at 68°C for 1 minute. The resulting PCR fragments (5 μl) were electrophoresed on 4% polyacrylamide gels at 100 V in a 0.3 × TBE buffer at 4°C, and the dried gels were autoradiographed. The nucleotide sequences of the primers are summarized in Table 1.

Extractions of nuclear and cytosolic proteins

Nuclear proteins were extracted as previously described (Chun et al., 2000b). Cells were quickly cooled by placing the plates on ice and then washing twice in ice-cold PBS. Scraped cells were quickly cooled by placing the plates on ice and washing twice in ice-cold phosphate-buffered saline before the cells were removed by scraping. Cells were centrifuged at 1000 ***g*** for 5 minutes at 4°C and then washed twice with ice-cold phosphate-buffered saline. Cells were then

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

Primer name	Strand	Nucleotide sequence
HIF-1α	Sense	CCCCAGATTCAGGATCAGACA
	Antisense	CCATCATGTTCCATTTTTCGC
VEGF	Sense	AACTTTCTGCTGTCTTGG
	Antisense	TTTGGTCTGCATTCACAT
Enolase 1	Sense	AAGAAACTGAACGTCACAGA
	Antisense	GATCTTCGATAGACACCACT
Aldolase A	Sense	GTCATCCTCTTCCATGAGAC
	Antisense	AGGTAGATGTGGTGGTCACT
LDH A	Sense	CTTTTCCTTAGAACACCAAA
	Antisense	TTGAGTTTGATCACCTCATA
β-Actin	Sense	AAGAGAGGCATCCTCACCCT
	Antisense	ATCTCTTGCTCGAAGTCCAG

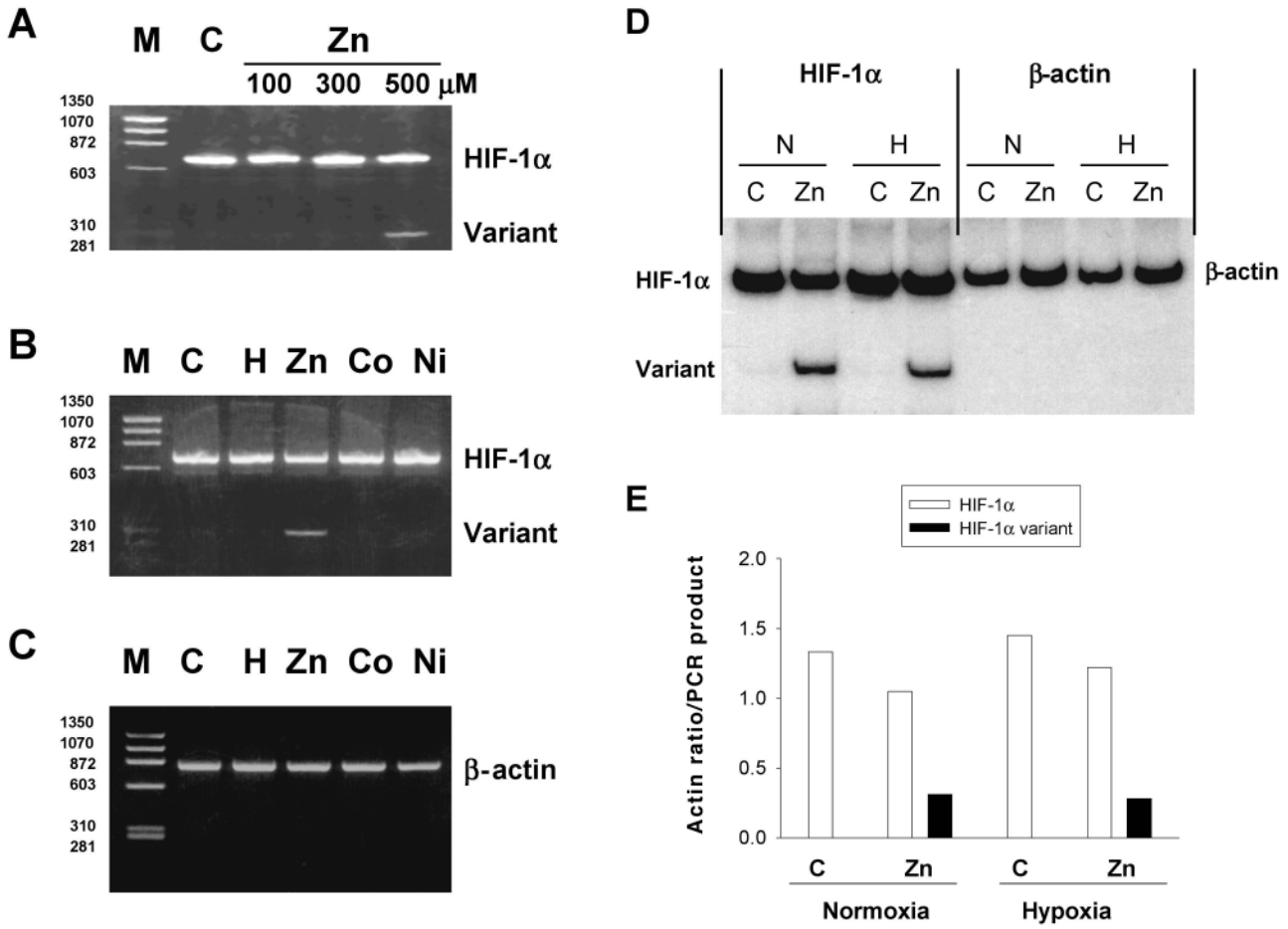

Fig. 1. Expression of a human *HIF-1α* mRNA isoform induced by the zinc ion. HEK 293 cells were cultured for 4 hours in the absence (C) and presence of $ZnCl_2$ (100, 300 and 500 μM) (Zn), $CoCl_2$ (100 μM) (Co), or $NiCl_2$ (300 μM) (Ni), or subjected to 4 hours of hypoxia (H). *HIF-1α*, its isoform, and β-actin mRNAs were amplified using RT-PCR, and analyzed by electrophoresis and ethidium bromide staining, as described in Materials and Methods (A, B, C). The molecular size of PCR product was assessed by comparing its mobility with those of DNA markers (M). These mRNA levels were analyzed by semiquantitative RT-PCR using [α-^{32}P]CTP (D). The relative amounts of the mRNAs were expressed as the ratios of the cpm value of HIF-1α or its variant fragments to β-actin under each set of conditions (E). The results presented in each panel represent three separate experiments.

resuspended in three packed cell volumes of lysis buffer consisting of 10 mM Tris, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, and 0.2% Nonidet P-40, 0.5 mM dithiothreitol, 1 mM Na_3VO_4, and 0.4 mM phenylmethylsulfonyl fluoride. Cells were then vortexed at medium speed for 10 seconds and incubated on ice for 5 minutes. Nuclei were then pelleted at 1000 ***g*** for 5 minutes at 4°C, and the supernatant was collected for the cytosolic fraction. One packed volume of extract buffer, consisting of 20 mM Tris, pH 7.8, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 20% glycerol, 0.5 mM dithiothreitol, 1 mM Na_3VO_4, and 0.4 mM phenylmethylsulfonyl fluoride, was added to the nuclei and the whole vortexed at medium speed for 5 seconds per minute for 10 minutes. The nuclear extracts were then centrifuged at 20,000 ***g*** at 4°C for 5 minutes, aliquoted into chilled tubes, frozen quickly in liquid nitrogen and stored at −70°C. The cytosolic fraction was centrifuged at 80,000 ***g*** at 4°C for 1 hour and the resulting supernatant was stored at −70°C. Protein concentration was measured using BCA (bicinchoninic acid) method (Bio-Rad) with bovine serum albumin as a standard.

Electrophoretic Mobility Gel Shift (EMSA) assay

The oligonucleotide probe used in the gel shift assay for HIF-1 consisted of the sequence 5-ACCGGCCCTACGTGCTGTCTCAC-3. The ^{32}P-labelled double-stranded probe was prepared and EMSA assay was performed as previously described (Chun et al., 2000b). DNA-protein binding reactions were carried out for 20 minutes at 4°C and run on a 5% non-denaturing polyacrylamide gel. For supershift analysis, 1 μl of rat HIF-1α antiserum was added to the completed EMSA reaction mixture and incubated for 2 hours at 4°C prior to loading.

Immunoblotting and immunoprecipitation

For the immunoblotting of HIF-1α and its variant protein, 20 μg of cell extract was separated on a 6% and 10% SDS/polyacrylamide gel, respectively, and transferred to an Immobilon-P membrane (Millipore). Immobilized proteins were incubated overnight at 4°C with rabbit anti-HIF-1α antibody, diluted 1:5000 in 5% nonfat milk in TBS/0.1% Tween-20 (TTBS). Horseradish peroxidase-conjugated anti-rabbit antiserum (Amersham Pharmacia Biotech) was used as a secondary antibody (1:5000 dilution in 5% nonfat milk in TTBS). After extensive washing with TTBS, the complexes were visualized by enhanced chemiluminescence plus (Amersham Pharmacia Biotech). HIF-1 antiserum was generated in rabbits against a bacterially expressed fragment encompassing amino acids 418-698 of human HIF-1α, as previously described (Chun et al., 2000a). For immunoprecipitation, HEK 293 cells were cotransfected with the pHA/HIF-1α variant and pARNT. Forty-two hours after transfection,

the cells were solubilized, and the cell lysates incubated with rabbit anti-HA (SantaCruz Biotechnology) or goat anti-ARNT antibodies (SantaCruz Biotechnology), followed by incubation with protein A-Sepharose beads (Amersham Pharmacia Biotech). After washing, the immunocomplexes were eluted by boiling for 3 minutes in the SDS sample buffer containing 10 mM DTT and subjected to SDS-PAGE and immunoblotting.

Immunofluorescence microscopy

Transfected HEK 293 cells were subjected to normoxia or hypoxia (4 hours), and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) at room temperature for 15 minutes. The cells were washed three times with 100 mM glycine in PBS, permeabilized with 0.1% Triton X-100 in PBS for 1 minute and washed three times with PBS. After blocking nonspecific binding with 5% normal goat serum in PBS for 1 hour, the cells were incubated overnight at 4ºC with rabbit anti-HIF-1α antibody (1:1000), rabbit anti-HA antibody (1:250) purchased from SantaCruz Biotechnology, or the mouse monoclonal anti-ARNT 2B10 antibody (1:500) purchased from Affinity BioReagents. The primary antibodies were stained by incubation with FITC-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories), FITC-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories), or FITC-conjugated goat anti-mouse antibody (Zymed Laboratories) at room temperature for 1 hour, respectively. After washing in PBS, the cells were analyzed by confocal laser scanning microscopy (BioRad MRC1024).

RESULTS

Identification of alternatively spliced variant of human HIF-1α induced by $ZnCl_2$

A pair of PCR primers were designed to identify zinc-induced splicing of *HIF-1α* mRNA. Total RNAs from HEK 293 cells, which had been treated with 500 μM $ZnCl_2$ for 4 hours, were reverse-transcribed and amplified with specific primers. Two distinct DNA bands of different sizes were reproducibly observed by gel electrophoresis (at approximately 700 and 300 bp; Fig. 1A). The RT-PCR of the RNAs extracted from the Hep3B cells treated with 300 μM $ZnCl_2$ also revealed two DNA bands of the same size (data not shown). The lower DNA band was smaller than the PCR product expected from the structure of wild-type *HIF-1α*, whereas the size of the upper band matched the expected DNA size (704 bp). Other metal ions such as cobalt chloride and nickel chloride did not produce the splicing variant of *HIF-1α* mRNA (Fig. 1B). The mRNA of β-actin, which was used as a negative control, was not altered by either the zinc ion or the other metal ions (Fig. 1C). These results indicated that the zinc ion specifically alters the splicing of *HIF-1α* mRNA.

To compare the mRNA levels of *HIF-1α* and the *HIF-1α* variant, a semiquantitative RT-PCR using [α-^{32}P]CTP and autoradiography were performed as described in Materials and Methods. As shown in Fig. 1D, the zinc ion produced a smaller PCR fragment in normoxic and hypoxic cells. These PCR fragments were quantified by cutting out each radioactive band from the dried gel, and measuring the radioactivity of each gel band with a beta-counter. The relative amounts of *HIF-1α* and its variant fragment were assessed by dividing their cpm values by that of the corresponding β-actin. Fig. 1E shows that the short form of *HIF-1α* was present at about 30% of the full-length *HIF-1α* mRNA level. Although full-length *HIF-1α* was somehow reduced by the zinc treatment in both normoxic and hypoxic cells, the sum of *HIF-1α* and the *HIF-1α* variant mRNA equalled the mRNA level of the control cells. This result supports the possibility that the zinc ion may change the splicing process of *HIF-1α* pre-mRNA but that it does not affect the transcription of the *HIF-1α* gene.

Structure of *HIF-1α* cDNA isoform

The *HIF-1α* cDNA isoform was cloned and sequenced as described in Materials and Methods, and its structure is summarized in Fig. 2. The cloned cDNA was 2047 bp in size and its nucleotide sequence was identical to the 2481 bp *HIF-1α* cDNA, except for a 434 bp deletion in the middle. A comparison with the genomic organization of *HIF-1α* (Iyer et al., 1998) showed that the cloned cDNA was derived

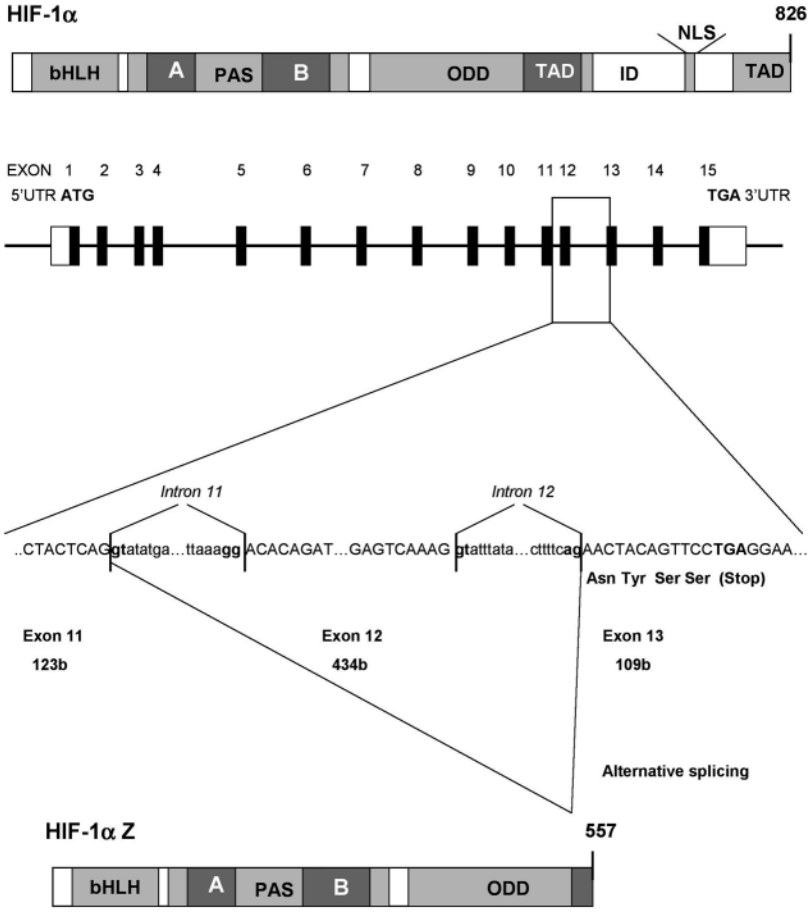

Fig. 2. Schematic representation of a newly cloned HIF-1α isoform, HIF-1αZ, compared with HIF-1α. The 11th and 13th exons were directly joined, which generated an immediate termination codon and a new frame in the 13th exon. This alternative splicing introduces four new amino acids following the Gln^{553} of HIF-1α. It translates into a 557 amino acid polypeptide. Compared with HIF-1α, it conserves both bHLH and PAS but loses a part of the ODD domain, TAD and the NLS motif.

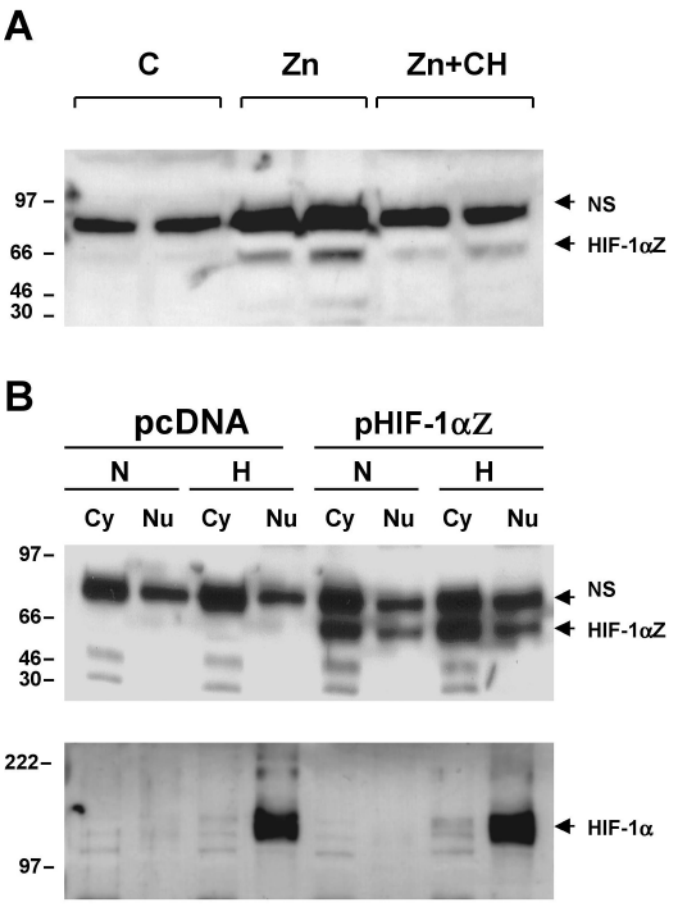

Fig. 3. Expression of HIF-1αZ protein. HIF-1αZ protein was identified using anti-HIF-1α antibody 8 hours after HEK 293 cells were treated with 500 μM $ZnCl_2$ in the absence and presence of cycloheximide (CH) (10 μg/ml). The HIF-1αZ protein band (HIF-1αZ) and nonspecific protein band (NS) are indicated. The molecular mass of HIF-1αZ was determined to be 62 kDa on SDS-polyacrylamide gel (A). C, Control. HEK 293 cells were transfected with pcDNA3 (pcDNA) or pcDNA3-HIF-1αZ (pHIF-1αZ), using the calcium phosphate method. The transfected cells were subjected to normoxia (N) or hypoxia (H) for 4 hours, and then homogenized to isolate the cytosolic (Cy) and nuclear proteins (Nu). Expressed HIF-1αZ and endogenous HIF-1α proteins were analyzed on 10% and 6% poylacrylamide gels (B).

from an alternatively spliced mRNA that lacked the 12th exon of the *HIF-1α* gene (Fig. 2). In this mRNA, the 11th exon and the 13th exon were directly joined, which generated an immediate termination codon and a new frame in the 13th exon. Consequently, this alternative splicing introduces four new amino acids, Asn-Tyr-Ser-Ser, as a new C-terminus following the Gln^{553} of the wild-type 826 amino acid HIF-1α protein. This novel form of *HIF-1α* mRNA translates into a 557 amino acid polypeptide, which we designated HIF-1αZ. HIF-1αZ conserves both the bHLH and PAS domains of HIF-1α, which are essential for binding with ARNT. However, it loses a part of the ODD domain, TAD and NLS motif, which suggests that it may be partly regulated by oxygen tension and may have no transactivation activity and an impaired ability to translocate into the nucleus.

Detection of HIF-1αZ protein in Zn-treated cells and its expression

To demonstrate the presence of *HIF-1αZ* mRNA translation products, an immunoblotting experiment was carried out using anti-HIF-1α antibody to recognize the remaining ODD domain in HIF-1αZ. After cells were incubated with zinc ion for 8 hours, the cells were harvested and total proteins extracted with a denaturing sample buffer for SDS-PAGE. An immunoreactive protein was detected in the lysate, whereas the protein was not expressed in the control cells (Fig. 3A). When the zinc-treated cells were incubated with cycloheximide (10 μg/ml), a protein synthesis inhibitor, the protein expression diminished, indicating that the protein was newly synthesized after zinc treatment. On SDS-polyacrylamide gel, the molecular mass of HIF-1αZ was determined to be 62 kDa and matched the theoretical molecular weight (63.2 kDa) calculated using the compute pI/Mw program in ExPASy Proteomics tools, which suggests that the protein is translated from *HIF-1αZ*. To characterize the HIF-1αZ protein, we constructed a HA-tagged *HIF-1αZ* expression plasmid and transfected HEK 293 cells. The expressed HIF-1αZ protein showed the same migration pattern as the zinc-induced protein (Fig. 3B), which confirmed that the zinc-induced protein is the product of *HIF-1αZ* mRNA. The majority of the expressed HIF-1αZ protein was present in the cytosolic fraction; a small amount was observed in the nuclear fraction. HIF-1αZ protein was induced slightly by hypoxia, whereas wild-type HIF-1α was dramatically induced only in the nuclear fraction. To re-examine the subcellular localization and oxygen-independent expression of HIF-1αZ, we stained HIF-1αZ under normoxic or hypoxic conditions using immunofluorescence and confocal laser scanning microscopy. Expressed HIF-1αZ was not localized to the nuclei of HEK 293 cells under either normoxic or hypoxic conditions (Fig. 4, first and second rows). By contrast, HIF-1α was detected only in hypoxic cells and localized to the nuclei. The immunofluorescence images observed in a higher magnification showed that HIF-1αZ was localized to the cytoplasm, whereas HIF-1α was to the nuclei (Fig. 4, third row). These properties of HIF-1αZ represent the loss of the ODD domain and the NLS motif as expected from its protein structure, which is shown in Fig. 2.

Inhibitory effect of HIF-1αZ on hypoxic responses

Recently, Maemura et al. (Maemura et al., 1999) reported that a truncated endothelial PAS domain protein 1 (EPAS1), lacking the transactivation domain at the C-terminus, functions as a dominant-negative mutant of endogenous EPAS1. Because HIF-1αZ also loses the transactivation domains, we tested the possibility that HIF-1αZ functions as a dominant-negative isoform of endogenous HIF-1α. For this purpose, stable transfectant cell lines of *pHA/HIF-1αZ* and pcDNA were subjected to normoxia or hypoxia for 16 hours. The mRNA levels of the hypoxia-inducible genes such as VEGF, aldolase A, enolase 1 and LDH A were measured by semiquantitative RT-PCR. The mRNA of β-actin was determined as a control. In pcDNA-transfected cells exposed to hypoxia, all mRNAs of the hypoxia-inducible genes were markedly increased, compared with the normoxic control. In *pHIF-1αZ*-transfected cells exposed to hypoxia, however, this hypoxic induction was not as great as was observed in pcDNA-transfected cells (Fig. 5). This result indicates that the

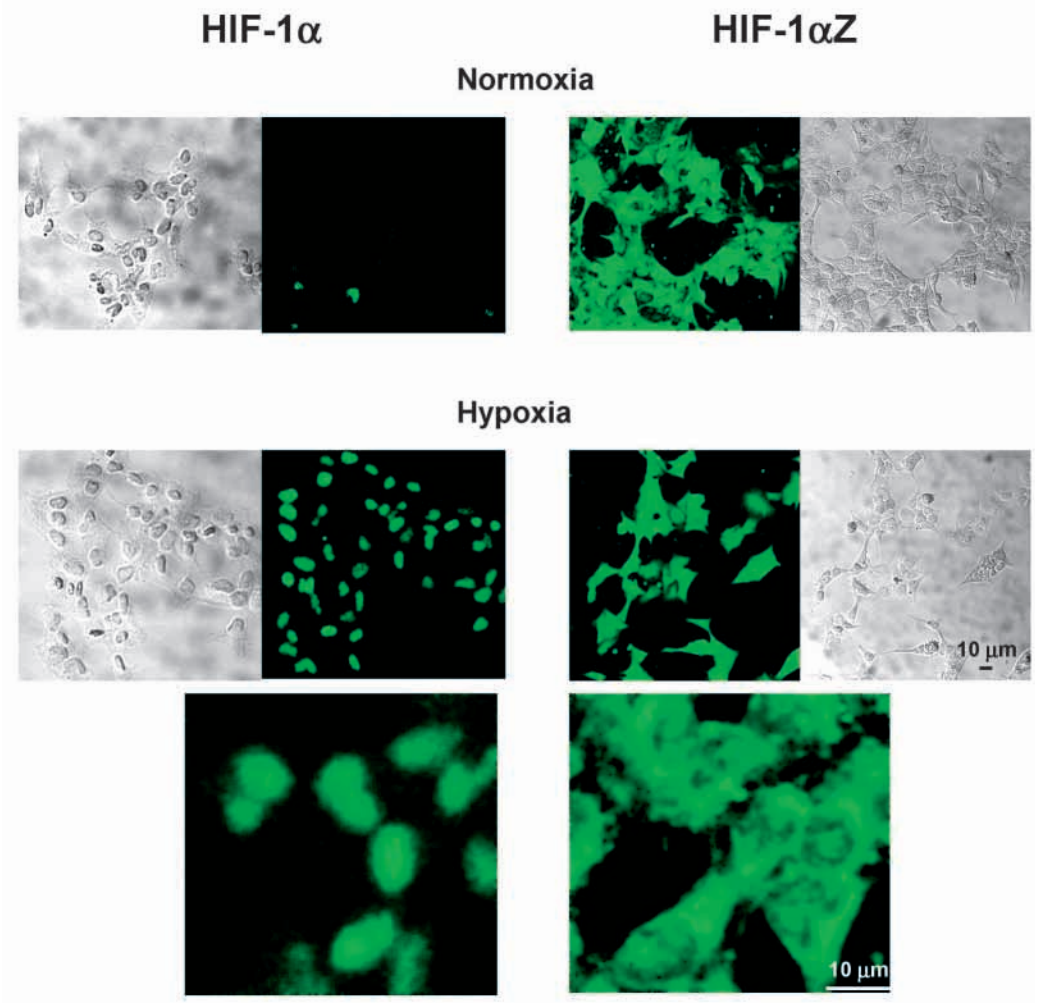

Fig. 4. Subcellular localization of HIF-1α and HIF-1αZ. HEK 293 cells (left panel) and pHA-HIF-1αZ transfectant cells (right panel) were subjected to normoxia or 4 hours of hypoxia, and then incubated with rabbit anti-HIF-1α antibody and rabbit anti-HA antibody, respectively, followed by a FITC-conjugated goat anti-rabbit antibody. The corresponding transmission images are shown in the outer columns.

mRNA expressions of hypoxia-inducible genes are suppressed by HIF-1αZ.

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

Hypoxia induces transcriptions of the hypoxia-inducible genes through the activation of an enhancer, which contains a HIF-1 consensus (Semenza, 2000b). To investigate whether the inhibitory effects of HIF-1αZ occur at the transcriptional level, the effects of HIF-1αZ on the expressions of reporter genes, which are under the control of the hypoxic enhancer, were examined. Transfected cells were further transfected with reporter plasmids, which contained EPO or VEGF enhancer element fused to the luciferase gene. In hypoxic pcDNA-transfected cells, luciferase activities of EPO enhancer reporter and VEGF enhancer reporter increased by 9.5-fold and 2.1-fold, respectively, compared with those of normoxic cells. However, in *pHIF-1αZ*-tranfected cells, luciferase activities were not increased by hypoxia (Fig. 6A,B). These findings suggest that HIF-1αZ suppresses the hypoxic response of the hypoxic enhancers, resulting in reduced mRNA levels of the hypoxia-inducible genes.

Inhibitory effect of HIF-1αZ on HIF-1 binding to the hypoxia response element

HIF-1 DNA binding activity to the 24 bp hypoxia response element of the EPO gene was examined to determine whether HIF-1αZ affects HIF-1 binding to the enhancer; this was found to be the case, as HIF-1 binding was greatly increased by hypoxia and HIF-1αZ inhibited this binding activity (Fig. 6C). No significant HIF-1 DNA binding was observed even when 10 μg of nuclear protein extracted from HIF-1αZ-transfectant was loaded on the gel. The lower bands (letter NS) represent the constitutive binding and nonspecific binding. Super-shift (letter SS) by anti-HIF-1α antibody indicates that the upper band represented the HIF-1/DNA complex. Recently, Maxwell et al. (Maxwell et al., 1999) found a high and low mobility species of DNA-binding HIF-1 in EMSA, the latter of which contains the von Hippel-Lindau (VHL) gene product pVHL. In hypoxic HIF-1αZ-transfected cells, both high-mobility and low-mobility species were suppressed. These results suggest that HIF-1αZ inhibits the formation of the HIF-1 complex in the nucleus.

Recovery effect of ARNT on the HIF-1 inhibition by HIF-1αZ

How does HIF-1αZ inhibit the HIF-1 formation? We thought of two possible answers for this question: first, it substitutes for wild-type HIF-1α in the HIF-1 complex, and second, it inhibits the accumulation of HIF-1α or ARNT in the nucleus. Because HIF-1αZ conserves intact basic regions for DNA-binding and bHLH/PAS domains for ARNT-binding, HIF-1αZ might retain the ability to DNA-bind and dimerize with ARNT.

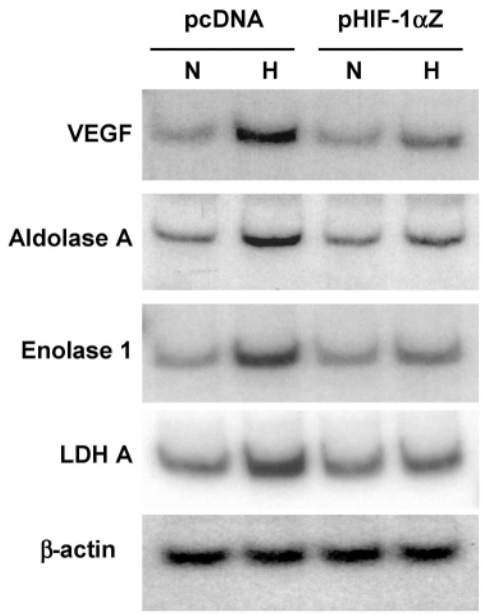

Fig. 5. mRNA expressions of the hypoxia-inducible genes. The mRNAs were isolated from transfected HEK 293 cells subjected to normoxia (N) or 16 hours of hypoxia (H), and analyzed by semi-quantitative RT-PCR. The results presented in each panel are representative of three separate experiments.

Therefore, HIF-1αZ can substitute for HIF-1α in the HIF-1 complex in the nucleus, and a new band for the HIF-1αZ-ARNT complex might be searched for by EMSA. However, no new band was found in the EMSA of the pHIF-1αZ-transfected cell line (Fig. 6C). This finding suggests that HIF-1αZ does not compete with endogenous HIF-1α in the formation of HIF-1 in the nucleus. To examine further whether HIF-1αZ competes with HIF-1α, increasing amounts of pHIF-1α were cotransfected into a pHIF-1αZ-tranfected cell line with EPO-enhancer reporter plasmid. Although the HIF-1 activity suppressed by HIF-1αZ was somehow increased by HIF-1α, it did not fully recover (Fig. 7A). Surprisingly, however, ARNT fully restored the HIF-1 activity in hypoxic pHIF-1αZ-transfected cells (Fig. 7B). This result suggests that HIF-1αZ inhibits ARNT, but not HIF-1α. Therefore, we propose that HIF-1αZ, by sequestering ARNT, works against HIF-1 as a dominant-negative isoform.

Sequestering of ARNT by HIF-1αZ

To test whether HIF-1αZ sequesters ARNT in the cytosol, the nuclear translocation of ARNT was examined. As shown in Fig. 8, ARNT protein was increased in the nuclear extract of hypoxic pcDNA-transfected cells, but not in normoxic cells. The hypoxic induction of the nuclear translocation of ARNT was markedly diminished in the *pHIF-1αZ*-transfected cells. By contrast, in total lysates, ARNT levels were unaffected by hypoxia and no differences were found between the two cell lines (Fig. 8). Because ARNT is prone to leak from the nuclei during isolation of the nuclei in the absence of its partner proteins including HIF-1α, we examined the subellular localization of ARNT by immunofluorescence microscopy. In HIF-1αZ(–) cells cultured under normoxic conditions, strong immunofluorescence was detected in the nuclei, whereas the immunofluorescence in the cytoplasm was very weak (Fig. 9, first row on the left column). The immunofluorescence in the nuclei became stronger in hypoxic cells than that in normoxic cells (Fig. 9, second row on the left column). These findings support the previous report (Chilov et al., 1999) that ARNT is a constitutive nuclear protein and easily leaked from the nuclear comparment during biochemical separation of the nuclei. In HIF-1αZ(+) cells, however, strong immunofluorescence was not localized to the nuclei under either normoxic or hypoxic conditions (Fig. 9, first and second

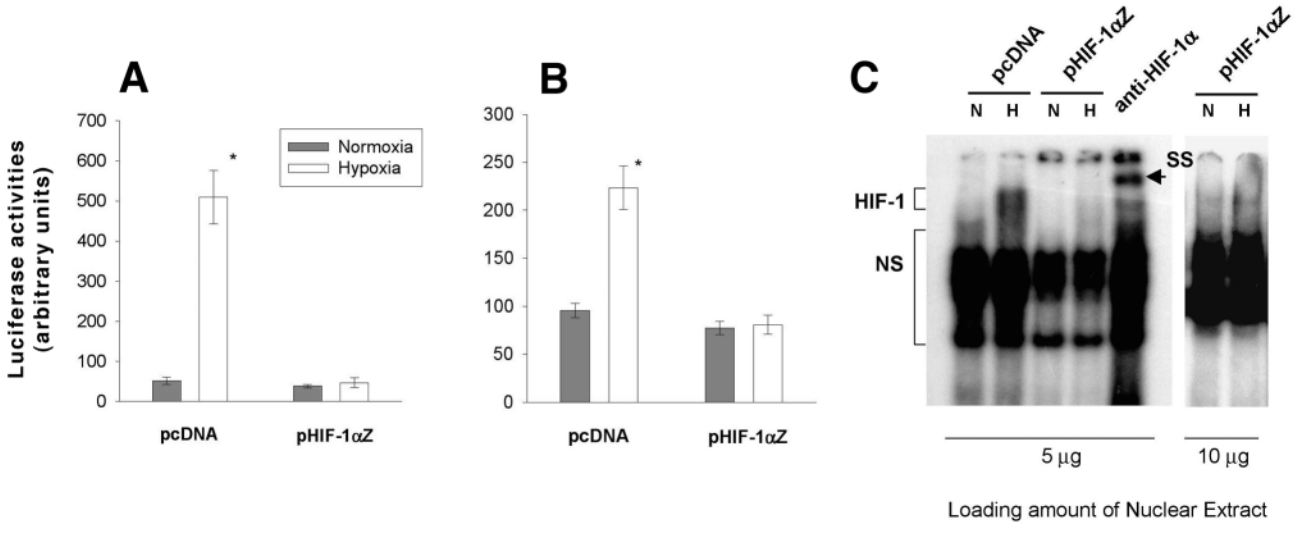

Fig. 6. Effect of HIF-1αZ on HIF-1 activity. Luciferase reporter plasmids containing the Epo enhancer (A) and the VEGF enhancer (B) were transfected into HEK 293 cells transfected with pcDNA and pHIF-1αZ. After 16 hours of hypoxia, the luciferase activity of the reporter gene was measured. Each bar, in arbitrary units, represents the mean±s.d. of six experiments. HIF-1 was extracted from the nuclei of the transfected cells subjected to normoxia (N) or 4 hours of hypoxia (H), and analyzed by EMSA (C). For supershift analysis, each 1 μl of rabbit HIF-1α anti-serum, used in Fig. 3B, was added to the completed EMSA reaction mixture. HIF-1 binding (HIF-1), nonspecific binding (NS), and supershifted bands (SS) are indicated. The loading amount of nuclear extract is indicated below the corresponding result. **P*<0.05 vs the normoxic control by using unpaired Student's *t*-test.

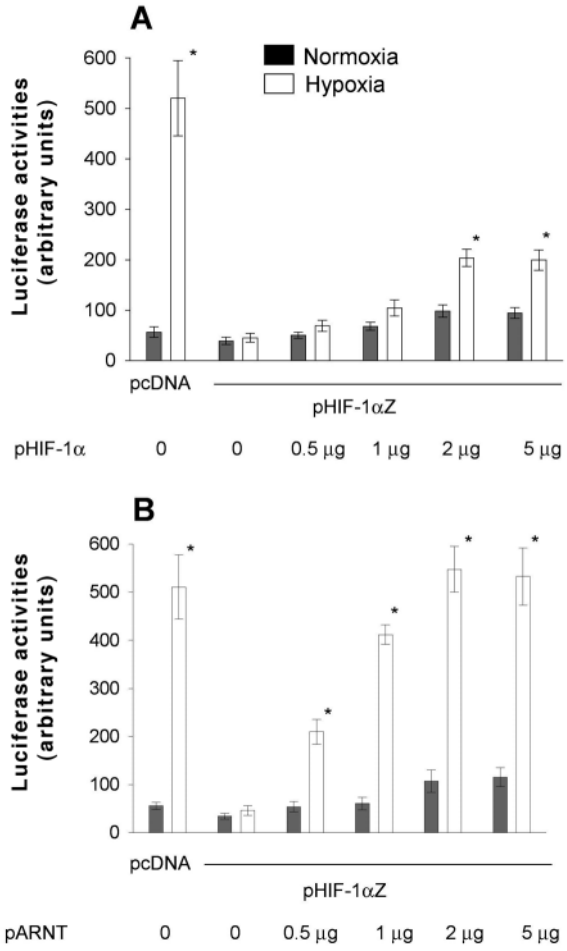

Fig. 7. The effect of competition by HIF-1α or ARNT with HIF-1αZ upon HIF-1 activity. *pHIF-1α* (A) and *pARNT* (B) plasmids were cotransfected with the Epo enhancer reporter vector into the stable transfectant cell line of *pHIF-1αZ*. After 16 hours of hypoxia, the luciferase activities of the reporter gene were measured. Each bar, in arbitrary units, represents the mean±s.d. of six experiments, and the loading amount of the plasmid is indicated below the corresponding result. **P*<0.05 vs the normoxic control in each condition by using unpaired Student's *t*-test.

rows on the right column). When the HIF-1αZ(+) cells were examined at a higher magnification, the immunofluorescence of ARNT was detected to the cytoplasm, whereas the immunofluorescence was detected to the nuclei in the HIF-1αZ(–) cells. These immunoblotting and immunocytochemistry experiments demonstrated that ARNT is no longer translocated to the nucleus when the cells overexpress HIF-1αZ, thus suggesting that the HIF-1αZ inhibits the nuclear translocation of ARNT.

To test whether HIF-1αZ captures ARNT in the cytosol, co-immunoprecipitation of HIF-1αZ with ARNT was examined in the cytosolic fraction of hypoxic HEK 293 cells cotransfected with HA-tagged HIF-1αZ and ARNT. HA-tagged HIF-1αZ and ARNT proteins were identified using anti-HA antibody and anti-ARNT antibody, respectively. Accordingly, HIF-1αZ protein was found in an immune complex precipitated by the anti-ARNT antibody. Likewise, ARNT protein was also found in the immunoprecipitates of an

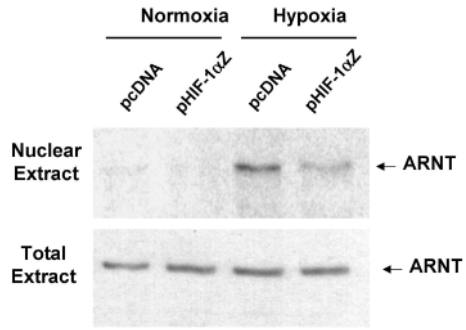

Fig. 8. Nuclear translocation of ARNT. ARNT protein was extracted from the nuclei and the cytosol of the stable transfectant pHIF-1αZ cell line. The cells were subjected to normoxia or hypoxia (4 hours), and 5 μg of protein from nuclear extracts or 40 μg of protein from total cellular lysates were separated by SDS-PAGE followed by western blotting with goat anti-ARNT antibody.

anti-HA antibody (Fig. 10, lane 2), which indicated an association between HIF-1αZ and ARNT in the cytosol. These proteins were not immunoprecipitated in pcDNA-transfected cells (Fig. 10, lane 1).

In summary, it appears that HIF-1αZ captures ARNT in the cytosol and then inhibits its nuclear translocation under hypoxic conditions, resulting in HIF-1 inactivation and the suppression of hypoxia-inducible gene expressions.

DISCUSSION

In this report, we have identified a new isoform of HIF-1α, which was generated by alternative splicing of the *HIF-1α* gene transcript in the presence of the zinc ion. As demonstrated by RT-PCR, *HIF-1αZ* mRNA was expressed in zinc-treated HEK 293 and Hep3B cells, but not in untreated cells. The difference between HIF-1αZ and the type HIF-1α (HIF-$1\alpha^{826}$) is that HIF-1αZ lacks the 12th exon, which introduces a frameshift resulting in a shorter form of HIF-1α (HIF-$1\alpha^{557}$). We have confirmed that HIF-$1\alpha^{557}$ was translated from *HIF-1αZ* mRNA by western blotting and that this was induced by the zinc ion. The HIF-1αZ expressed inhibited HIF-1 activity and reduced the mRNA expressions of hypoxia-inducible genes regulated by HIF-1. HIF-1αZ blocked the nuclear translocation of ARNT but not that of endogenous HIF-1α, and was also associated with ARNT in the cytosol. These results suggest that the inhibitory action of HIF-1αZ is due to the sequestering of ARNT in the cytosol. We summarized this hypothesis in Fig. 11. To our knowledge, this is the first report describing the zinc-induced alternative splicing of HIF-1α and a naturally produced HIF-1α variant functioning as a dominant-negative isoform. In addition, HIF-1αZ could be a useful tool in molecular biology for suppressing the cellular responses to hypoxia and dioxin, which require the nuclear import of ARNT.

These experiments demonstrated that ARNT is sequestered to the cytoplasm by expressed HIF-1αZ (Fig. 8; Fig. 9; Fig. 10). Likewise, an ARNT interacting protein (AINT) that sequesters ARNT in the cytoplasm has been found from a mouse embryo cDNA library using the yeast two-hybrid system (Sadek et al., 2000). The AINT protein interacts with

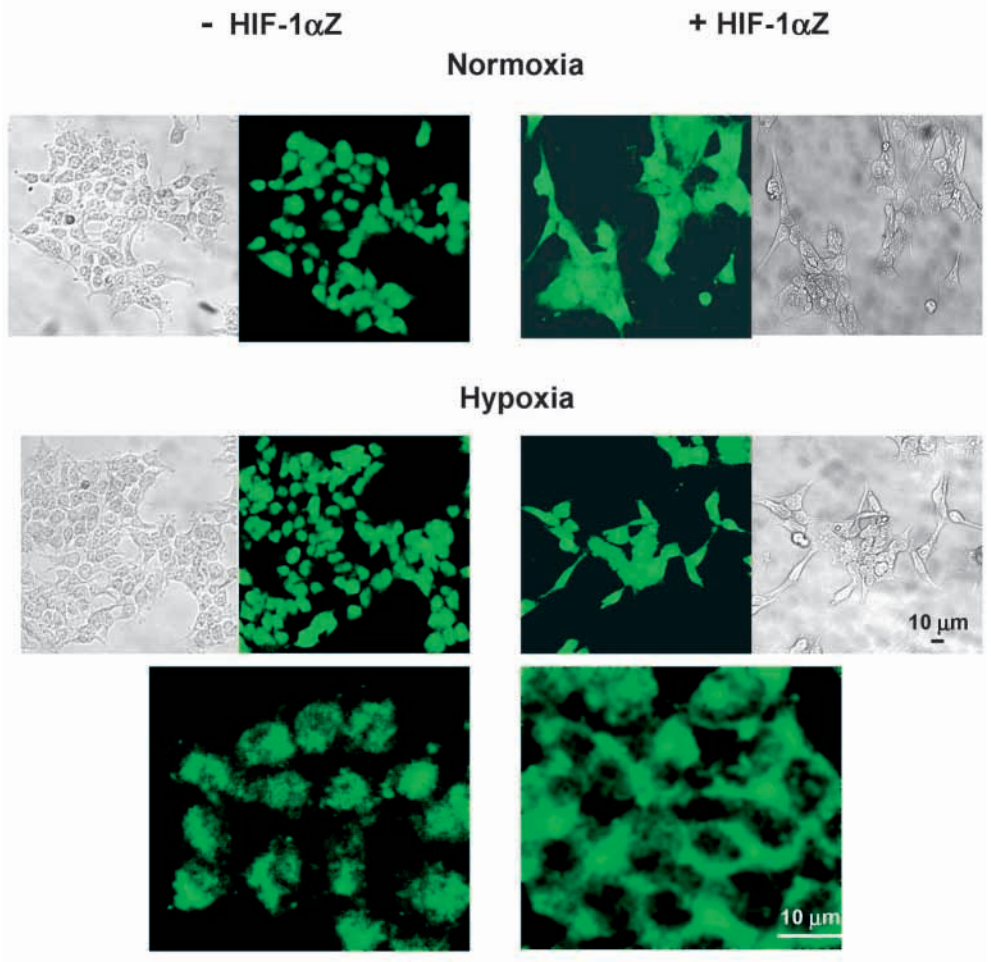

Fig. 9. Subcellular localization of ARNT. HEK 293 cells transfected with pcDNA (–HIF-1αZ) or pHA-HIF-1αZ (+HIF-1αZ) were subjected to normoxia or 4 hours of hypoxia, and then incubated with the monoclonal anti-ARNT antibody followed by an FITC-conjugated goat anti-mouse antibody. The corresponding transmission images are shown in the outer columns.

the PAS domain of ARNT and the protein is mainly localized to the cytoplasm in immunofluorescence microscopy. In the cells overexpressing AINT, immunofluorescence signal of endogenous ARNT was detected in the cytoplasm, but not in the nucleus. These properties of AINT are comparable to those of the HIF-1αZ in terms of ARNT binding, cytoplasmic localization and the inhibition of nuclear translocation of ARNT. Recently, the cytoplasmic anchoring of transcription factor is considered to be an important mechanism regulating gene expression. The best-studied example of this phenomenon is that of β-catenin. Its nuclear translocation is regulated by cytoplasmic anchoring to cadherins (Fagotto et al., 1996). Another example is given by β-amyloid precursor protein, which functions as a cytoplasmic anchoring site, inhibiting the nuclear translocation of Fe65, an adaptor protein that interacts with the transcription factorCP2/LSF/LBP1 (Minopoli et al., 2001). These examples suggest that a cytosolic interacting protein can redistribute a nuclear protein from the nucleus to the cytoplasm, thus supporting our hyothesis that the cytosolic HIF-1αZ interacting with ARNT inhibits the nuclear translocation of ARNT.

The incorporation of transition metals other than iron, such as cobalt and nickel, in a putative oxygen-sensing ferroprotein is supposed to reduce oxygen binding to the ferroprotein, resulting in HIF-1α accumulation and EPO induction (Goldberg et al., 1988; Huang et al., 1999). Although zinc can also substitute for iron in haem moiety (Shibayama et al., 1986), it does not induce EPO under normoxic conditions (Goldberg et al., 1988). On the contrary, it has been reported that zinc suppresses EPO production in hypoxic cells (Gopfert et al., 1995; Dittmer and Bauer, 1992). Previously, we discussed this reciprocal behaviour of zinc on the oxygen-sensing pathway (Chun et al., 2000a). Zinc induced the accumulation and nuclear translocation of HIF-1α in the same manner as cobalt and nickel. However, it also blocked the nuclear translocation of ARNT under hypoxic conditions, which inhibited the formation of HIF-1 in the nucleus and suppressed EPO mRNA induction. We believe that the generation of HIF-1αZ seems to be responsible for the inhibitory effects of zinc ion on HIF-1-mediated hypoxic responses, because HIF-1αZ was expressed by the zinc ion and its recombinant protein showed the same effect as zinc on HIF-1 activity and ARNT translocation.

Recently, Gothie et al. (Gothie et al., 2000) reported two isoforms of human HIF-1α that result from alternative splicing of mRNA. One has an additional 3 bp at the junction of the 1st and the 2nd exons without a frameshift. The other loses the 14th exon resulting in a frameshift and an immediate termination of translation. The latter translates into a 736 amino acid polypeptide (HIF-1α^{736}). Because HIF-1α^{736} conserves most of the essential domains of HIF-1α, it is regulated by oxygen tension and transactivates VEGF promoter. They also demonstrated that HIF-1α^{736} can compete with endogenous HIF-1α, using a reporter assay, and that it

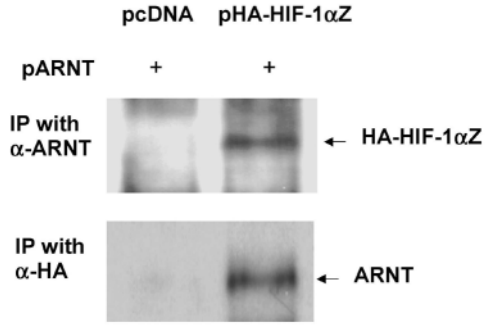

Fig. 10. Co-immunoprecipitation of HIF-1αZ and ARNT. pARNT plasmid was transfected into stable cell lines transfected with pcDNA and pHA-HIF-1αZ plasmids. After 4 hours of hypoxia, the cytosolic proteins were immunoprecipitated (IP) by rabbit anti-HA antibody (α-HA) or goat anti-ARNT antibody (α-ARNT). HA-HIF-1αZ protein and ARNT protein were identified by western blotting, and are indicated by arrows.

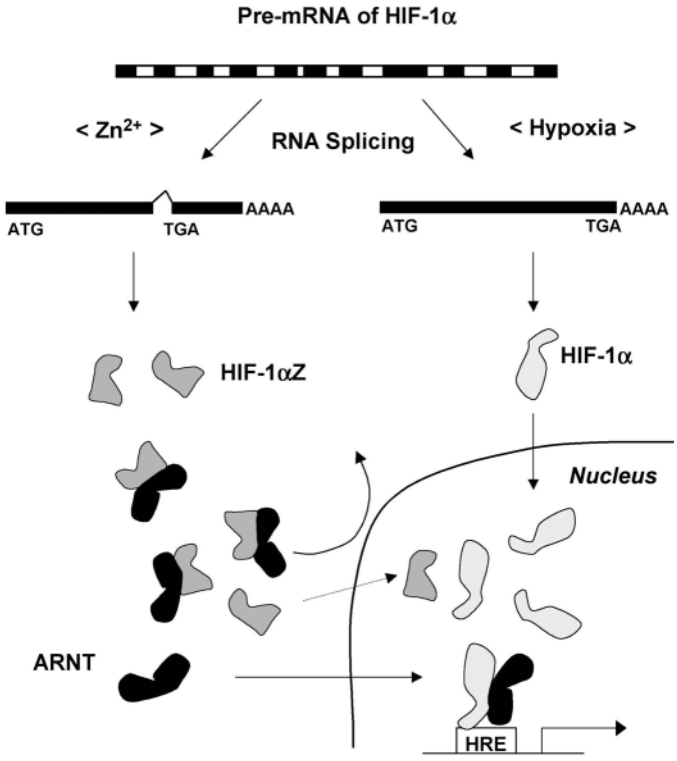

Fig. 11. Generation of HIF-1αZ and the mechanism of the HIF-1αZ-induced suppression of hypoxic responses.

inhibits the hypoxic induction of luciferase by up to 50%. This HIF-1α^{736} is comparable to HIF-1αZ in terms of mRNA and protein structure. Both proteins are generated by the deletion of one exon, producing shorter isoforms. However, there are big differences between them. HIF-1αZ lacked a part of ODD, both TADs, and NLS because of deletion of the 12th exon. HIF-1αZ was also more stable under normoxia and did not transactivate EPO and VEGF promoters, which suggests that HIF-1αZ is a better dominant-negative isoform at competing with HIF-1α. In fact, HIF-1αZ almost completely suppressed the tranactivation activities of HIF-1α as shown in Fig. 6A.

Endothelial PAS domain protein 1 (EPAS1) is homologous with HIF-1α and preferentially expressed in vascular endothelial cells. Like HIF-1α, it also dimerizes with ARNT and binds to the HIF-1-binding site. Maemura et al. (Maemura et al., 1999) found that a recombinant EPAS1^{485} lacking the TAD functions as a dominant-negative mutant of EPAS1^{870} and also competed with HIF-1α in the VEGF promoter assay. However, they did not investigate the association with ARNT and specifically its nuclear import. Although, as compared to HIF-1αZ, EPAS1^{485} is not a natural protein and has a different amino acid sequence, its protein structure is comparable with that of HIF-1αZ. It has intact bHLH and PAS domains like HIF-1αZ, and it might be able to bind with ARNT. Thus, we can speculate that it may share a common mechanism with HIF-1αZ, which is summarized in Fig. 11.

In zinc-treated cells, full-length *HIF-1α* mRNA was reduced and a new spliced variant emerged. The zinc ion probably changes the splicing process of the pre-mRNA, but does not affect the transcription of *HIF-1α* gene. To our knowledge, it has never been reported that the zinc ion can induce a newly spliced variant, even in other gene transcripts. How does the zinc ion change the splicing process of mRNA? The question remains to be answered. One possible explanation is that the zinc ion affects a putative zinc-dependent RNA-splicing process. The splicing of nuclear pre-mRNA occurs in a multicomponent complex containing nuclear ribonucleo-proteins and splicing factors. In higher eukaryotes, serine/arginine (SR) splicing factors are involved in the first steps of splice site recognition (Smith and Valcarcel, 2000). Human 9G8 SR factor contains a specific sequence in the median region, which is a zinc knuckle motif (CCHC motif) (Cavaloc et al., 1994). Moreover, splicing factors containing the zinc knuckle motif have also been found in an insect (Mazroui et al., 1999) and a plant (Lopato et al., 1999). Cavaloc et al. (Cavaloc et al., 1999) demonstrated that the zinc knuckle is involved in the RNA recognition specificity of 9G8. Therefore, because zinc is essential for the binding between RNA and the zinc knuckle motif, a large zinc concentration change may affect the RNA recognition specificity of splicing factor, and alter the splicing process. However, given that the zinc-induced alteration of RNA splicing occurs specifically at exon 12 of the *HIF-1α* gene, general RNA-splicing machinary like 9G8 SR factor may not be a key molecule to produce HIF-1αZ. Other putative molecule but 9G8 SR factor might be involved in the zinc effect and remains to be identified.

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