A domain responsible for HIF-1α degradation by YC-1, a novel anticancer agent

HYE-LIM KIM[1], EUN-JIN YEO[1], YANG-SOOK CHUN[2] and JONG-WAN PARK[1]

[1]Department of Pharmacology, [2]Department of Physiology and Cancer Research Institute, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-799, Korea

Received January 20, 2006; Accepted March 14, 2006

Abstract. HIF-1α is believed to promote tumor growth and metastasis, and many efforts have been made to develop new anticancer agents based on HIF-1α inhibition. YC-1 is a widely used HIF-1α inhibitor both *in vitro* and *in vivo*, and is being developed as a novel class of anticancer drug. However, little is known about the mechanism by which YC-1 degrades HIF-1α. As the first step for understanding the mechanism of action of YC-1, we here identified the HIF-1α domain responsible for YC-1-induced protein degradation. YC-1 blocked the HIF-1α induction by hypoxia, iron chelation, and proteasomal inhibition and also degraded ectopically expressed HIF-1α. In deletion analyses, C-terminal HIF-1α was found to be sensitively degraded by YC-1. Using a GFP-fusion method, the YC-1-induced degradation domain was identified as the aa. 720-780 region of HIF-1α. We next tested the possible involvement of HDAC7 or OS-9 in YC-1-induced HIF-1α degradation. However, their binding to HIF-1α was not affected by YC-1, suggesting that they are not involved in the YC-1 action. It is also suggested that YC-1 targets a novel pathway regulating HIF-1α stability.

Correspondence to: Dr Jong-Wan Park, Department of Pharmacology, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-799, Korea
E-mail: parkjw@snu.ac.kr

Abbreviations: HIF, hypoxia-inducible factor; ARNT, aryl hydrocarbon receptor nuclear translocator; bHLH, basic helix-loop-helix; PAS, per-arnt-sim; ODDD, oxygen-dependent degradation domain; NDD, N-terminal degradation domain; CDD, C-terminal degradation domain; TAD, transactivation domain; ID, inhibitory domain; PHD, HIF-1 prolyl hydroxylase; VHL, von Hippel-Lindau; FIH, factor inhibiting HIF; GFP, green fluorescence protein; HA, hemagglutinin; DFO, desferrioxamine; HDAC, histone deacetylase; IP, immunoprecipitation

Key words: HIF-1α, YC-1, degradation domain, cancer chemotherapy

Introduction

Hypoxia develops within solid tumors because the tumor growth outpaces the vessel formation and because the aberrant tumor vessels are easily obstructed or ruptured. Tumor cells have developed numerous adaptive mechanisms to enable them to survive under hypoxic conditions. Tumor adaptation to hypoxia includes angiogenesis, anaerobic glycolysis, and expression of stress proteins related to cell survival or death (1). The regulation of most proteins required for hypoxic adaptation occurs at the gene level, which involves transcriptional induction by hypoxia-inducible factor-1 (HIF-1). To date, ≥ 60 genes have been found to be up-regulated by HIF-1 (2). HIF-1 is composed of HIF-1α and HIF-1ß/aryl hydrocarbon nuclear receptor translocator (ARNT). Of these, HIF-1α is the key protein that determines the presence and the transcriptional activity of HIF-1. HIF-1α levels have been reported to be elevated in tumor specimens from cancer patients, and its expression was found to be positively related to tumor aggressiveness and a poor prognosis (3,4). Moreover, animal xenograft studies have shown that the overexpression of HIF-1α enhances tumor growth and angiogenesis (5,6). Therefore, HIF-1α is believed to promote tumor growth and metastasis, and many efforts have been made to develop new anticancer agents based on HIF-1α inhibitors (7).

HIF-1α is composed of 826 amino acids (8). Its N-terminal half contains the basic domain (aa. 17-30), a helix-loop-helix domain (aa. 31-71), and a PAS domain (aa. 85-298), which are required for dimerization with ARNT and binding to the HRE DNA core recognition sequence (5'-RCGTG-3'). The C-terminal half of HIF-1α is required for transactivation. The transactivation domains (TADs) are localized to aa. 531-575 (N-terminal TAD) and aa. 786-826 (C-terminal TAD), which are separated by an inhibitory domain (ID). Nuclear localization signals (NLSs) are located at N-terminal (aa. 17-74) and C-terminal (aa. 718-721) of HIF-1α. In addition, the central portion of HIF-1α (aa. 401-603) contains the oxygen-dependent degradation domain (ODDD), which determines the stability of HIF-1α protein (9). Of ODDD, located in the N-terminal end (aa. 390-417) and C-terminal end (aa. 549-582) are the specific degradation sites targeted by von Hippel-Lindau (VHL) protein (10-12). In aerobic conditions, HIF-1-prolyl hydroxylases (PHD1-3) modify two proline residues (P402 and P564) in

these degradation domains (13,14). Then, von Hippel-Lindau protein (pVHL), a part of the E3 ubiquitin ligase protein complex, binds to the modified HIF-1α, which results in the ubiquitination and proteasomal degradation of HIF-1α (15). Since the enzymatic reaction of prolyl hydroxylation requires molecular oxygen and iron, hypoxia or iron deficiency limit this hydroxylation, thereby precluding the binding of pVHL and thus leading to the stabilization of HIF-1α (16).

Currently, YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl-indazole is used as an inhibitor of HIF-1α. We previously found that YC-1 reduced the protein level of HIF-1α and inhibited the expression of hypoxia-inducible genes in cultured hepatoma cells (17). YC-1 was also found to halt the growth of five xenografted human tumors without inducing apparent toxicity. Moreover, tumors from YC-1-treated mice showed fewer blood vessels, reduced HIF-1α levels, and lower levels of HIF-1-regulated gene transcription (18). Thus, YC-1 is regarded as a good lead compound for the development of HIF-1-targeting anticancer agents (19). However, little is known about the mechanism by which YC-1 degrades HIF-1α. As the first step for understanding the mechanism of action of YC-1, we here identified the HIF-1α domain responsible for YC-1-induced protein degradation. YC-1 blocked the HIF-1α induction by hypoxia, iron chelation, and proteasomal inhibition. Moreover, it also degraded ectopically expressed HIF-1α. In deletion analyses, the C-terminal HIF-1α was found to be sensitively degraded by YC-1. Using a GFP-fusion method, the YC-1-induced degradation domain was identified as the aa. 720-780 region of HIF-1α.

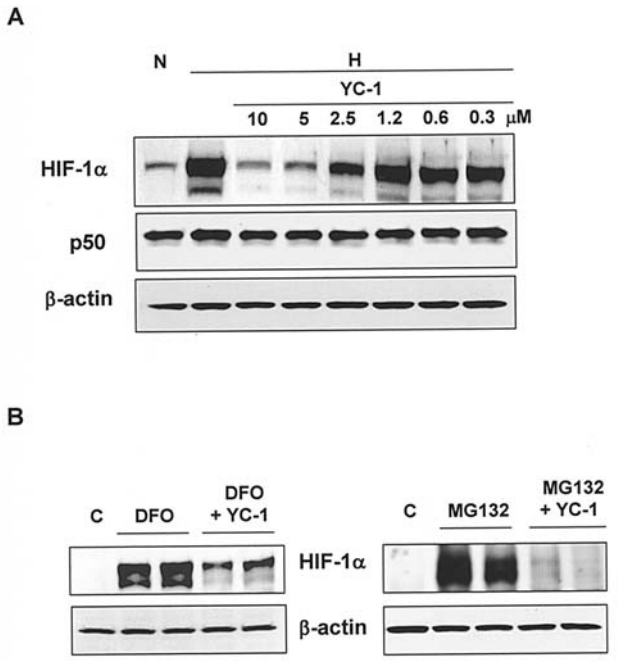

Figure 1. YC-1 suppresses HIF-1α expression in Hep3B cells. (A) After 8 h normoxic (N) or hypoxic (H) incubation, HIF-1α, NF-κB p50, ß-actin proteins were isolated from Hep3B cells, and analyzed by Western blotting. Various concentrations of YC-1 were added to the culture media 5 min prior to hypoxic incubation. (B) After 8-h incubation with 130 μM desferrioxamine (DFO) or 10 μM MG132, HIF-1α and ß-actin proteins were analyzed by Western blotting. YC-1 (2 μM) was added to the culture media 5 min prior to the drug treatment. The data shown are representative of 3 separate experiments.

Materials and methods

Reagents and antibodies. YC-1 was purchased from A.G. Scientific Inc. (San Diego, CA), dissolved in dimethyl sulfoxide (DMSO) to 100 mM stock solution. Desferioxamine (DFO), MG132 and other chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Culture media and fetal calf serum were purchased from Gibco/BRL (Grand Island, NY). Anti-HIF-1α antiserum was generated in rabbits against a bacterially expressed fragment encompassing amino acids 418-698 of human HIF-1α, as previously described (20). Antibodies, mouse anti-ß-actin, rabbit anti-NF-κB p50, mouse anti-green fluorescent protein (GFP) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rat anti-HA and mouse anti-Flag antibodies were obtained from Roche Ltd. (Basel, Switzerland) and Sigma-Aldrich Corp., respectively.

Cell culture. Hep3B hepatoma and HEK293 embryonal kidney cell-lines were obtained from ATCC (Manassas, VA). Hep3B and HEK293 cells were cultured in α-modified Eagle's medium and in Dulbecco's modified Eagle's medium, respectively. Both culture media were supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere containing 5% CO_2 at 37˚C. Oxygen tensions in the incubator (Vision Sci Co., Korea) were either 140 mm Hg (20% O_2, v/v, normoxia) or 7 mm Hg (1% O_2, v/v, hypoxia). All culture media and sera were purchased from Gibco/BRL.

Expression plasmids and transfection. The *hemagglutinin-tagged HIF-1α (HA/HIF-1α)* expression plasmid was a generous gift from Dr Eric Huang (NCI, Bethesda, MD). The plasmid used to stably express HA/HIF-1α mutant (HA/sHIF-1α) was made by deleting three degradation domains (aa. 397-405, 513-553, and 554-595) using a PCR-based mutagenesis kit (Stratagene, Cedar Creek, TX). The plasmids for truncated HA/HIF-1α mutants were reconstructed from the *HA/HIF-1α* plasmid by deleting aa. 517-826, 1-490, or 1-576 of HIF-1α using the mutagenesis kit. The *GFP-HIF-1α* plasmids were constructed by inserting full-length or truncated *HIF-1α* cDNA (cloned by PCR) into pcDNA-GFP. The *Flag-HDAC7* and *Flag-OS-9* plasmids were constructed by inserting full-length *HDAC7* and *OS-9* cDNA (cloned by RT-PCR) into *pcDNA-Flag*. The nucleotide sequences of the primers used for RT-PCR were 5'-CATGGACCTGCGGGTGGGCC-3' and 5'-CA AGGGCATGGTGGGCGGGC-3' for *HDAC7* or 5'-GATG GCGGCGGAAACGCT-3' and 5'-TCAGAAGTCAAATTC GTCC-3' for *OS-9*. All constructs were verified by DNA sequencing. For transient expression, approximately 40% of the confluent HEK293 cells in 60-mm cell culture dishes were transfected with 0.5-8 μg of plasmids using the calcium phosphate method. Transfected cells were incubated for 48 h in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and then used for experiments.

Immunoblotting and immunoprecipitation. For immunoblotting, total proteins were separated on 8-15% SDS/polyacrylamide gels, and transferred to an Immobilon-P membrane

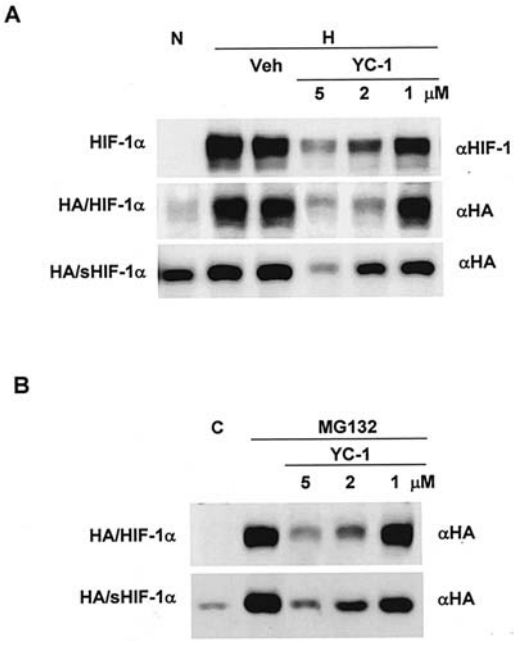

Figure 2. YC-1 suppresses ectopically expressed HIF-1α or its mutant in HEK293 cells. (A) HEK293 cells were transfected with 4 μg of *pcDNA*, the plasmid *HA-tagged HIF-1α* (HA/HIF-1α) or the plasmid HA-tagged stable HIF-1α (HA/sHIF-1α), and then incubated under normoxic (N) or hypoxic (H) conditions for 8 h. Endogenous HIF-1α in *pcDNA*-transfected cells and expressed HA/HIF-1α proteins were detected using anti-HIF-1α (αHIF-1) and anti-HA (αHA) antibodies, respectively. DMSO (Veh) or 2 μM YC-1 was added to the culture media 5 min prior to hypoxic incubation. (B) The transfected HEK293 cells were treated with 10 μM MG132 for 8 h, and prepared for Western blotting. YC-1 (2 μM) was added to the culture media 5 min prior to MG132 treatment. The data shown are representative of 3 separate experiments.

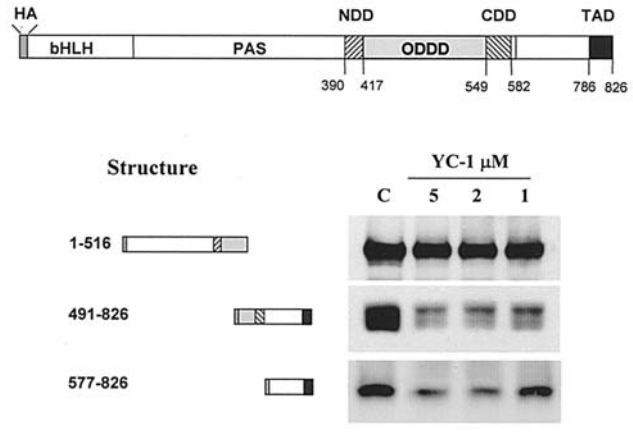

Figure 3. The C-terminal portion of HIF-1α is degraded by YC-1. The upper panel illustrates the structure of HIF-α protein and the positions of the important domains. HA, hemaglutinin; bHLH, basic helix-loop-helix; PAS, per-ant-sim; ODDD, oxygen-dependent degradation domain; NDD, N-terminal degradation domain; CDD, C-terminal degradation domain; TAD, transactivation domain. The left panel in the lower panel illustrates the structures of mutated HIF-α HEK293 cells were transfected with 4 μg of *HA-tagged truncated HIF-1α* plasmids, and then incubated in the presence of 10 μM MG132 for 8 h. Expressed HA/HIF-1α proteins were detected using anti-HA antibody. Various concentrations of YC-1 were added to the culture media 5 min prior to MG132 treatment. The data shown are representative of 3 separate experiments.

(Millipore, Bedford, MA). Membranes were then blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TTBS) at room temperature for 1 h and incubated overnight at 4˚C with the primary antibodies, diluted 1:1000 in 5% non-fat milk in TTBS. Horseradish peroxidase-conjugated anti-rabbit, anti-mouse or anti-rat antiserum was used as a secondary antibody (1:5000 dilution in 5% non-fat milk in TTBS, 1-h incubation) and the antigen-antibody complexes were visualized using an Enhanced Chemiluminescence Plus kit (Amersham Biosciences Corp., Piscataway, NJ). For immunoprecipitation, HEK293 cells were co-transfected with 4 μg of *pHA/HIF-1α* and 2 μg of *pFlag/HDAC7* or *pFlag/OS-9*. Forty-two hours after transfection, the cells were solubilized, and the cell lysates were incubated with anti-HA antiserum and then with protein A/G-Sepharose beads (Amersham Pharmacia Biotech). After washing, the immunocomplexes were eluted in SDS sample buffer containing 10 mM DTT, and then subjected to SDS-PAGE and immunoblotting using anti-Flag antibody.

Results

YC-1 degrades HIF-1α irrelevantly of the oxygen and proteasome-dependent pathway. In aerobic conditions, HIF-1α is hydroxylated and finally degraded by the 26S proteasome. We first examined whether YC-1 degraded HIF-1α via this pathway. YC-1 reduced the HIF-1α levels in a dose-dependent manner in hypoxic Hep3B cells, whereas it did not affect the levels of other proteins such as p50 and ß-actin (Fig. 1A). The effective concentration 50% (EC_{50}) of YC-1 is approximately 2 μM. YC-1 was also found to inhibit HIF-1α expression induced by an iron chelator DFO (Fig. 1B, left panel). However, YC-1 strongly inhibited the HIF-1α expression induced by proteasome inhibitor MG132 (Fig. 1B, right panel). This suggests that YC-1 degrades HIF-1α independently of the proteasomal pathway. To examine the oxygen-independent degradation of HIF-1α, we expressed HA-tagged HIF-1α (HA/HIF-1α) and its stable mutant (HA/sHIF-1α) which is stable regardless of the oxygen tension. YC-1 suppressed the hypoxic induction of HA/HIF-1α as effectively as that of endogenous HIF-1α. Surprisingly, YC-1 also suppressed the expression of HA/sHIF-1α (Fig. 2A). Moreover, YC-1 suppressed MG132-induced expression of both HA/HIF-1α and HA/sHIF-1α (Fig. 2B). These results further support the oxygen and proteasome-independent HIF-1α degradation by YC-1, and suggest the possibility that YC-1 degrades HIF-1α by targeting domain(s) other than the two responsible for oxygen-dependent degradation.

Identification of the YC-1-degraded domain in HIF-1α. To examine the involvement of two oxygen-sensitive degradation domains (NDD and CDD) in YC-1-induced HIF-1α degradation, we expressed two truncated HIF-1α mutants, each of which contains one of two degradation domains. Of the two mutants, C-terminal HIF-1α (aa. 491-826) was sensitively degraded by YC-1 but N-terminal HIF-1α (aa. 1-516) was not (Fig. 3). We further deleted CDD from the C-terminal HIF-1α to make aa. 577-826. This mutant was still found to be degraded by YC-1 (Fig. 3), which suggests that both oxygen-sensitive degradation domains are not required

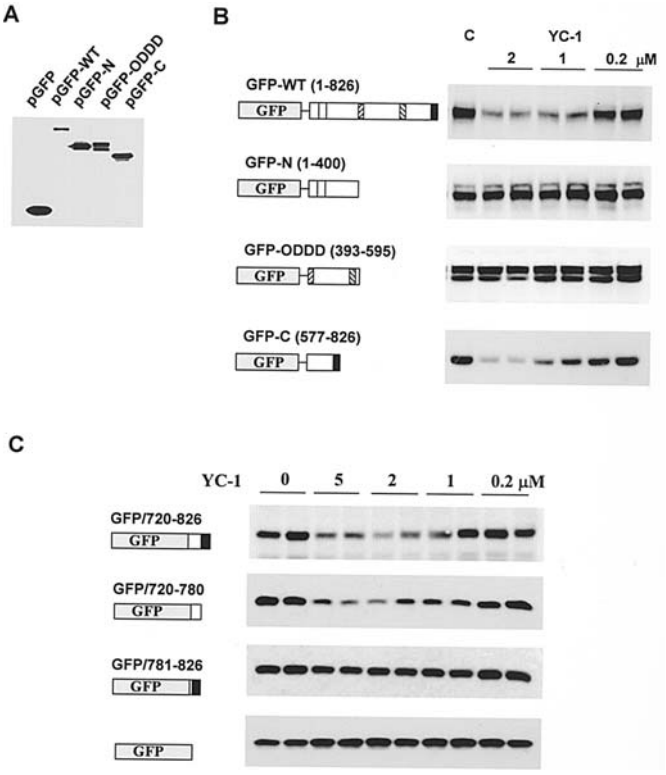

Figure 4. YC-1-degraded domain is aa. 720-780 of HIF-17. The upper panel illustrates the structure of HIF-α protein and the positions of the important domains. (A) HEK293 cells were transfected with 4 μg of *GFP-fused HIF-1α* plasmids, and then incubated in the presence of 10 μM MG132 for 8 h. Expressed proteins were electrophoresed on a 12% SDS/PAGE gel and detected using anti-GFP antibody. (B) The left panel illustrates the structures of *GFP-fused HIF-α* proteins. HEK293 cells were transfected with 4 μg of *GFP-fused HIF-1α* plasmids, and then incubated in the presence of 10 μM MG132 for 8 h. Various concentrations of YC-1 were added to the culture media 5 min prior to MG132 treatment. Expressed proteins were electrophoresed on 10% SDS/PAGE gels and detected using anti-GFP antibody. (C) HEK293 cells were transfected with 4 μg of *GFP-fused HIF-1α* plasmids, and then incubated in the presence of 10 μM MG132 for 8 h. Various concentrations of YC-1 were added to the culture media 5 min prior to MG132 treatment. Expressed proteins were electrophoresed on 12% SDS/PAGE gels and detected using anti-GFP antibody. The data shown are representative of 3 separate experiments.

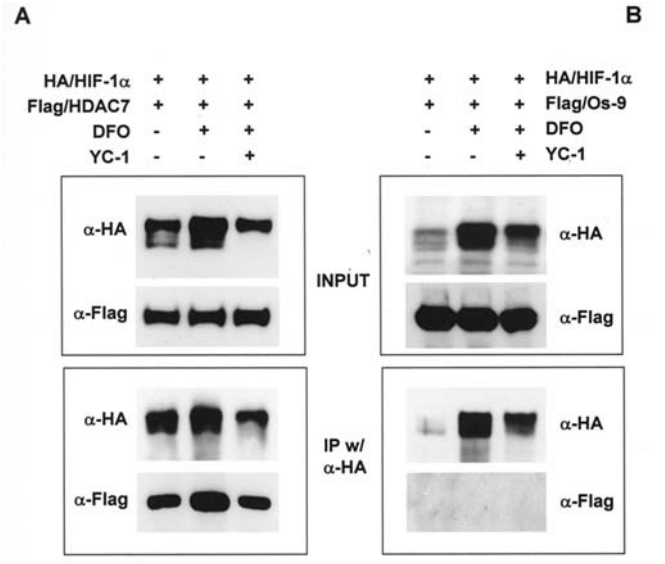

Figure 5. HDAC7 or OS-9 is not related with YC-1-induced HIF-1α degradation. HEK293 cells were co-transfected with 4 μg of *HA/HIF-1α* plasmid and 2 μg of *Flag/HDAC7* plasmid (A) or 2 μg of *Flag/Os-9* plasmid (B), and incubated with 130 μM DFO or 2 μM YC-1 for 4 h. Lysates were prepared and immunoprecipitations were performed using anti-HA antibody. The co-immunoprecipitation of HA/HIF-1α and Flag/HDAC7 was identified by Western blotting using anti-HA and anti-Flag antibodies (lower panel, IP w/α-HA). Protein quantities in the samples were verified by Western blotting (upper panel, INPUT). The data shown are representative of 3 separate experiments.

for the YC-1 action and that the YC-1-degraded domain is present between aa. 577 and 826. To confirm the YC-1-degraded domain, HIF-1α and truncated mutants were fused with GFP and the expression of the fusion proteins was verified using anti-GFP antibody, as shown in Fig. 4A. As expected, full-length HIF-1α fused with GFP (GFP-WT) was sensitively degraded by YC-1, which indicates that the GFP fusion does not interfere with the action of YC-1. Of the three truncated HIF-1α fused with GFP, only C-terminal HIF-1α (GFP-C) was degraded by YC-1, whereas both the N-terminal (GFP-N) and the ODDD (GFP-ODDD) were not (Fig. 4B). This confirms that the YC-1-degraded domain is present within the C-terminal portion of HIF-1α. To identify the YC-1-degraded domain more specifically, we further deleted the N-terminal portion of GFP-C to remain as aa. 720-826. This mutant still showed YC-1-induced degradation (Fig. 4C, GFP/720-826). Since this domain contains TAD (aa. 786-826), we divided this region into two; GFP/720-780 and GFP/781-826 (TAD). Of these fusion proteins, only GFP/720-780 was degraded by YC-1 (Fig. 4C). These results suggest that the aa. 720-780 region is responsible for HIF-1α degradation by YC-1.

YC-1 does not affect the binding of HDAC7 or OS-9 to HIF-1α. The YC-1-degraded domain has been known as the inhibitory domain (ID) (21). Recently, yeast two hybrid studies demonstrated that HDAC7 and OS-9 were bound to ID and enhanced HIF-1 activity or stabilized HIF-1α (22,23). Since YC-1 degraded the domain interacting with HDAC7 and OS-9, we proposed that YC-1 destabilizes HIF-1α by interfering with HDAC7 or OS-9. To test this possibility, we co-expressed HA/HIF-1α with Flag/HDAC7 or with Flag/OS-9 and co-immunoprecipitated these proteins using anti-HA antibody. YC-1 treatment for 4 h reduced HA/HIF-1α levels, but did not affect the expression of Flag/HDAC7 or Flag/OS-9 (Fig. 5, upper panel). The efficiency of HA/HIF-1α immunoprecipitation (IP) was verified by measuring HA/HIF-1α levels in the IP samples. Of the two candidates, Flag/HDAC7 was co-immunoprecipitated with HA/HIF-1α, as shown in Fig. 5A. Although the amount of Flag/HDAC7 in IP samples was reduced by YC-1, this is due to the reduction of HA/HIF-1α, because there was no significant difference in the ratio of Flag/HDAC7 to HA/HIF-1α in IP samples (Fig. 5A, lower panel). This suggests that the HDAC7-HIF-1α interaction is not affected by YC-1. We also examined the interaction between Flag/OS-9 and HA/HIF-1α, as shown in Fig. 5B. Although both proteins were highly expressed, Flag/OS-9 was not co-precipitated with HA/HIF-1α. This suggests that OS-9 is not associated with HIF-1α and that OS-9 does not participate in HIF-1α degradation in our experimental settings. Collectively, these results suggest that YC-1-induced HIF-1α

degradation is unlikely to be associated with either HDAC7 or OS-9. The detailed mechanism underlying YC-1-induced HIF-1α degradation remains to be investigated.

Discussion

YC-1 is a widely used HIF-1α inhibitor both *in vitro* and *in vivo*, and is being developed as a novel class of anticancer drug (7). In this study, we show that: i) YC-1 degrades HIF-1α irrelevantly of the oxygen and proteasome-dependent pathway; ii) previously known HIF-1α-regulation domains (ODDD and C-terminal TAD) do not participate in YC-1-induced HIF-1α degradation; and iii) the domain (aa. 720-780) within the inhibitory domain is responsible for YC-1-induced degradation. These results suggest that YC-1 targets a novel pathway regulating HIF-1α stability.

Previously, YC-1 was used to produce cGMP by activating soluble guanylyl cyclase (reviewed in ref. 24). It increases the catalytic rate of soluble guanylyl cyclase and enhances the enzyme response to nitric oxide or carbon monoxide (25). In terms of its pharmacological action, YC-1 prevents thrombus formation by inhibiting platelet aggregation (26), reduces the blood pressure by relaxing vascular smooth muscle (27), and helps penile erection by relaxing corpus cavernosal smooth muscle (28). In addition to cGMP elevation, we for the first time found that YC-1 had a novel effect on HIF-1 activity (17). YC-1 was found to diminish the hypoxic activation of the *erythropoietin* and *VEGF* genes, and to suppress HRE-binding by HIF-1 and HIF-1α protein expression. However, sGC inhibitors failed to block these effects of YC-1 on HIF-1α, and further treatment with 8-bromo-cGMP did not inhibit the hypoxic induction of HIF-1α. Thus, the HIF-1α inhibition may not result from cGMP elevation (17). Since HIF-1α plays a crucial role in tumor promotion and angiogenesis, YC-1, as a novel HIF-1α inhibitor, could be further developed as a novel anticancer agent targeting HIF-1 and tumor angiogenesis. Indeed, we have demonstrated that YC-1 effectively inhibited tumor growth in immunodeficient mice grafted with five types of human tumor cells. These tumors showed reduced HIF-1α expression and poor vascularization. Moreover, YC-1 suppressed the expression of the HIF-1-regulated genes, i.e. VEGF and glycolytic enzymes, in grafted tumor tissue (18). Thus, YC-1 is regarded as a good candidate molecule for the development of novel anti-angiogenic, anticancer agents. However, the target molecule of YC-1 has not yet been found. Considering the fact that YC-1 has the property of protein binding (e.g. soluble guanylyl cyclase), YC-1 may bind to some protein(s) related with HIF-1α stability.

In the present study, we identified the HIF-1α domain responsible for YC-1-induced protein degradation. The YC-1-degraded domain is located at the C-terminal portion of HIF-1α. In addition to protein degradation via the ODDD, the modification of C-terminal HIF-1α is another regulatory mechanism of hypoxia-inducible gene expression. The TAD at the C-terminal end of HIF-1α requires recruitment of transcriptional co-activators such as p300 or CBP to activate hypoxia-inducible genes. Protein structural analyses of HIF-1 and p300/CBP revealed that the cysteine/histidine-rich 1 (CH1) domain of p300/CBP is associated with the TAD (29,30). Asn803 in the TAD is hydroxylated oxygen-dependently by factor inhibiting HIF-1 (FIH), which in turn inhibits the binding of p300/CBP to HIF-1α and thereby inhibits HIF-1-dependent transcription (31). Since the enzymatic reaction of FIH requires oxygen and iron, hypoxia or iron deficiency limits Asn803 hydroxylation, thereby recruiting p300/CBP and activating gene transcription. We considered the possible involvement of p300/CBP or FIH in YC-1-induced HIF-1α degradation. However, the domain (aa. 780-826) interacting with p300/CBP and FIH was stable in the presence of YC-1, which suggests that these proteins do not participate in the YC-1 effect. We next tested the possibility that the inhibitory domain (ID) is related with the YC-1 effect. The inhibitory domain (ID, aa. 576-785) is located between the ODDD and the TAD. Since the deletion of the ID enhances the transcriptional activity of HIF-1α, the ID is considered to inhibit the activity of the TAD. However, the detailed mechanism of action of the ID has not been clarified. We found that the ID was responsible for YC-1-induced HIF-1α degradation. However, the entire structure of the ID was not required for the YC-1 effect because the short domain containing aa. 720-780 was enough for it to be degraded by YC-1. We next tried to identify the protein that is related with the degradation of this domain by YC-1. We considered HDAC7 and OS-9 as candidates because they were reported to interact with the ID. However, their binding to HIF-1α was not affected by YC-1, which suggests that HDAC7 and OS-9 are not involved in the YC-1-induced HIF-1α degradation. Therefore, it is still unknown as to what controls the stability of the YC-1-degraded domain.

Acknowledgements

This study was supported by a Korea Research Foundation grant (2003-015-E00090).

References

1. Hockel M and Vaupel P: Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 93: 266-276, 2001.
2. Yeo EJ, Chun YS and Park JW: New anticancer strategies targeting HIF-1. Biochem Pharmacol 68: 1061-1069, 2004.
3. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL and Simons JW: Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 59: 5830-5835, 1999.
4. Birner P, Schindl M, Obermair A, Plank C, Breitenecker G and Oberhuber G: Overexpression of hypoxia-inducible factor 1alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. Cancer Res 60: 4693-4696, 2000.
5. Maxwell PH, Dachs GU, Gleadle JM, Nicholls LG, Harris AL, Stratford IJ, Hankinson O, Pugh CW and Ratcliffe PJ: Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc Natl Acad Sci USA 94: 8104-8109, 1997.
6. Ryan HE, Poloni M, McNulty W, Elson D, Gassmann M, Arbeit JM and Johnson RS: Hypoxia-inducible factor-1alpha is a positive factor in solid tumor growth. Cancer Res 60: 4010-4015, 2000.
7. Garber K: New drugs target hypoxia response in tumors. J Natl Cancer Inst 97: 1112-1114, 2005.
8. Wang GL and Semenza GL: Purification and characterization of hypoxia-inducible factor 1. J Biol Chem 270: 1230-1237, 1995.
9. Huang LE, Gu J, Schau M and Bunn HF: Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci USA 95: 7987-7992, 1998.

10. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS and Kaelin WG Jr: HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 292: 464-468, 2001.
11. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW and Ratcliffe PJ: Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science 292: 468-472, 2001.
12 Masson N, Willam C, Maxwell PH, Pugh CW and Ratcliffe PJ: Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. EMBO J 20: 5197-5206, 2001.
13. Bruick RK and McKnight SL: A conserved family of prolyl-4-hydroxylases that modify HIF. Science 294: 1337-1340, 2001.
14. Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ and Ratcliffe PJ: *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 107: 43-54, 2001.
15. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER and Ratcliffe PJ: The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 399: 271-275, 1999.
16. McNeill LA, Hewitson KS, Gleadle JM, Horsfall LE, Oldham NJ, Maxwell PH, Pugh CW, Ratcliffe PJ and Schofield CJ: The use of dioxygen by HIF prolyl hydroxylase (PHD1). Bioorg Med Chem Lett 12: 1547-1550, 2002.
17. Chun YS, Yeo EJ, Choi E, Teng CM, Bae JM, Kim MS and Park JW: Inhibitory effect of YC-1 on the hypoxic induction of erythropoietin and vascular endothelial growth factor in Hep3B cells. Biochem Pharmacol 61: 947-954, 2001.
18. Yeo EJ, Chun YS, Cho YS, Kim J, Lee JC, Kim MS and Park JW: YC-1: a potential anticancer drug targeting hypoxia-inducible factor 1. J Natl Cancer Inst 95: 516-525, 2003.
19. Giaccia A, Siim BG and Johnson RS: HIF-1 as a target for drug development. Nat Rev Drug Discov 2: 803-811, 2003.
20. Chun YS, Choi E, Kim TY, Kim MS and Park JW: A dominant-negative isoform lacking exons 11 and 12 of the human hypoxia-inducible factor-1alpha gene. Biochem J 362: 71-79, 2002.
21. Jiang BH, Zheng JZ, Leung SW, Roe R and Semenza GL: Transactivation and inhibitory domains of hypoxia-inducible factor 1alpha. Modulation of transcriptional activity by oxygen tension. J Biol Chem 272: 19253-19260, 1997.
22. Kato H, Tamamizu-Kato S and Shibasaki F: Histone deacetylase 7 associates with hypoxia-inducible factor 1alpha and increases transcriptional activity. J Biol Chem 279: 41966-41974, 2004.
23. Baek JH, Mahon PC, Oh J, Kelly B, Krishnamachary B, Pearson M, Chan DA, Giaccia AJ and Semenza GL: OS-9 interacts with hypoxia-inducible factor 1alpha and prolyl hydroxylases to promote oxygen-dependent degradation of HIF-1alpha. Mol Cell 17: 503-512, 2005.
24. Chun YS, Yeo EJ and Park JW: Versatile pharmacological actions of YC-1: anti-platelet to anticancer. Cancer Lett 207: 1-7, 2004.
25. Friebe A, Mullershausen F, Smolenski A, Walter U, Schultz G and Koesling D: YC-1 potentiates nitric oxide- and carbon monoxide-induced cyclic GMP effects in human platelets. Mol Pharmacol 54: 962-967, 1998.
26. Ko FN, Wu CC, Kuo SC, Lee FY and Teng CM: YC-1, a novel activator of platelet guanylate cyclase. Blood 84: 4226-4233, 1994.
27. Mulsch A, Bauersachs J, Schafer A, Stasch JP, Kast R and Busse R: Effect of YC-1, an NO-independent, superoxide-sensitive stimulator of soluble guanylyl cyclase, on smooth muscle responsiveness to nitrovasodilators. Br J Pharmacol 120: 681-689, 1997.
28. Mizusawa H, Hedlund P, Brioni JD, Sullivan JP and Andersson KE: Nitric oxide independent activation of guanylate cyclase by YC-1 causes erectile responses in the rat. J Urol 167: 2276-2281, 2002.
29. Dames SA, Martinez-Yamout M, De Guzman RN, Dyson HJ and Wright PE: Structural basis for Hif-1 alpha/CBP recognition in the cellular hypoxic response. Proc Natl Acad Sci USA 99: 5271-5276, 2002.
30. Freedman SJ, Sun ZY, Poy F, Kung AL, Livingston DM, Wagner G and Eck MJ: Structural basis for recruitment of CBP/p300 by hypoxia-inducible factor-1 alpha. Proc Natl Acad Sci USA 99: 5367-5372, 2002.
31. Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML and Bruick R: FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev 16: 1466-1471, 2002.