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

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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 it 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.

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-1β/aryl hydrocarbon receptor nuclear translocator (ARNT). Of these, HIF-1α is the key protein that determines the presence and 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-1α-prolyl hydroxylases (PHD1-3) modify two proline residues (P402 and P564) in

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

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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-benzylindazole 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.

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 and 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. Oxygen tensions in the incubator (Vision Sci Co., Korea) were either 140 mm Hg (20% O₂, v/v, normoxia) or 7 mm Hg (1% O₂, 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, 517-526, and 1-490) 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. 517-526, 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 PCR) into pcDNA-Flag. The nucleotide sequences of the primers used for RT-PCR were 5'-CATGGACCTGCGGGTGGGCC-3' and 5'-GAAGCCTATTGACGTCGGC-3' for HDAC7 or 5'-GATGGGCGCGAAACGCT-3' and 5'-TCAGAAGTCAAAATTCTGCC-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.
expressed HA/HIF-1α in separate experiments. 5 min prior to MG132 treatment. The data shown are representative of 3 prepared for Western blotting. YC-1 (2 μM) was added to the culture media transfected HEK293 cells were treated with 10 μM MG132 for 8 h, and was added to the culture media 5 min prior to hypoxic incubation. (B) The conditions for 8 h. Endogenous HIF-1α proteins were detected using anti-HIF-1α and anti-HA 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 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 (oHIF-1α) and anti-HA (oHA) 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.

(YC-1 degrades HIF-1α irreversibly 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 β-actin (Fig. 1A). The effective concentration 50% (EC50) 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 more sensitive 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 for YC-1 to degrade HIF-1α.

Results

YC-1 degrades HIF-1α irreversibly 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 hypoxic Hep3B cells, whereas it did not affect the levels of other proteins such as p50 and β-actin (Fig. 1A). The effective concentration 50% (EC50) 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.

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HIF-1α does not interfere with the action of YC-1. Of the three truncated domains, which indicate that the GFP fusion does not participate in HIF-1α degradation in our experimental settings. Although both proteins were highly expressed, Flag/OS-9 was not co-precipitated 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 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. The co-immunoprecipitation of HA/HIF-1α with 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.

YC-1 does not affect the binding of HDAC7 or OS-9 to HIF-1α. The YC-1-degraded domain has been 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.

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.
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-1α and HIF-1β 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-1α 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-1α 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-1α (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.

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References


