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Binding and regulation of HIF-1α by a subunit of the proteasome complex, PSMA7

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Abstract The hypoxia-inducible factor-1α (HIF-1α) is an important transcription factor for cellular responses to oxygen tension. It is rapidly degraded under normoxic conditions by the ubiquitin-dependent proteasome pathway. Here we report a critical role of the 20S proteasome subunit PSMA7 in HIF-1α regulation. PSMA7 was found to interact specifically with two subdomains of HIF-1α. PSMA7 inhibited the transactivation function of HIF-1α under both normoxic and hypoxia-mimicking conditions. In addition, we show that the PSMA7-mediated regulation of HIF-1α activity is associated with the proteasome pathway. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Hypoxia; Hypoxia-inducible factor-1α; Proteasome; PSMA7

1. Introduction

Oxygen tension is an important factor for the regulation of mammalian genes that are involved in angiogenesis, vasculogenesis, glucose metabolism, and apoptosis [1]. The induction of these oxygen-sensitive genes by low oxygen tension (hypoxia) is mainly mediated through hypoxia-inducible factor-1 (HIF-1), a basic helix–loop–helix transcription factor [2,3]. HIF-1 is a dimeric transcription factor that is composed of HIF-1α and HIF-1β, and binds to a consensus sequence (5′-RCGTG-3′) [4]. Under hypoxic conditions, it up-regulates messenger RNAs encoding vascular endothelial growth factor (VEGF), erythropoietin, and glycolytic enzymes. This stimulatory effect is due to the stabilization of oxygen-sensitive HIF-1α. At normoxia, HIF-1α is rapidly degraded, while HIF-1β is stably expressed regardless of oxygen tension [5,6]. HIF-1α has two transactivation domains localized to amino acid residues 531–575 and 786–826. In addition, amino acid sequences between the two domains were found to inhibit transactivation function, and thus this region is referred to as an inhibitory domain [7].

Recent studies have shown that HIF-1α is targeted by the von Hippel–Lindau (VHL) tumor suppressor protein for poly-ubiquitination [8]. The VHL protein forms a complex with elongins B and C, and cullin-2 through the elongin C binding domain [9,10], and acts as the recognition component of an E3 ubiquitin ligase complex for poly-ubiquitination [10,11]. The poly-ubiquitinated HIF-1α protein is degraded by the ubiquitin-dependent 26S proteasomes [12,13]. The 26S proteasome complex is a ubiquitous multicatalytic protease complex composed of two large complexes: the 20S catalytic core complex and the 19S regulatory complex [14]. The 19S complex is required for the recognition of poly-ubiquitinated protein substrates that are degraded inside of the 20S core complex. The barrel-shaped 20S particle is made up of four rings, each of which contains seven different subunits. The two inner rings contain β-type subunits and the outer rings comprise α-type subunits. The proteasome is involved in many different cellular processes, ranging from the cell cycle process to antigen processing [15]. In general, the half-lives of the short-lived protein targets of the proteasome complex span from 30 min to several hours. In contrast, the half-life of HIF-1α was reported to be less than 5 min under normoxic conditions [6]. It is still not clear how HIF-1α is degraded so quickly, as compared to other substrates of the ubiquitin-dependent 26S proteasome complex.

Here we show that PSMA7, an α-type subunit of the 20S proteasome core complex, physically interacts with HIF-1α and inhibits the transactivation activity of HIF-1α by causing the proteasome-dependent degradation of HIF-1α.

2. Materials and methods

2.1. Cell culture

293T and BOSC 23 [16] cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) with 0.375% sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS) in a 5% CO_2 incubator at 37°C.

2.2. Plasmid constructions

A series of glutathione-*S*-transferase (GST) fused HIF-1α deletion mutant plasmids (pEBG/HIF-1α) for expression in mammalian cells was constructed by polymerase chain reaction, followed by cloning into the pEBG plasmid. All deletion mutants were confirmed by DNA sequencing analysis. The N-terminal hemagglutinin (HA)-tagged HIF-1α expression plasmids were constructed by transferring the HIF-1α

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Abbreviations: HIF-1α, hypoxia-inducible factor-1α; VHL, von Hippel–Lindau protein; GST, glutathione-*S*-transferase; HA, hemagglutinin; ODD, oxygen-dependent degradation

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deletion mutants from pEBG/HIF-1α plasmids into the pcDNA3/HA plasmid. The luciferase reporter plasmid (pFR-Luc) containing the promoter with five tandem repeats of GAL4 binding sites was purchased from Stratagene. The full-length HIF-1α cDNA was inserted into *Bam*HI and *Xba*I sites of pFA-CMV containing the GAL4 DNA binding domain to construct pFA-CMV-HIF-1α. pFLAG/PSMA7 was made by inserting the full-length PSMA7 cDNA into pFLAG-CMV2.

2.3. In vitro binding assays

GST fusion proteins were expressed in *Escherichia coli* and were purified as described [17]. The GST fusion proteins were immobilized on glutathione-Sepharose 4B beads (Amersham Pharmacia), and then were incubated with in vitro-translated [^{35}S]methionine-labeled HIF-1α for 5 h at 4°C in Nonidet P-40 lysis buffer (0.137 M NaCl, 0.02 M Tris–HCl pH 7.5, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 1 mM PMSF, 1 μg/ml each of leupeptin, aprotinin, and pepstatin). The beads were washed five times with the same buffer. The bound proteins were eluted with SDS–PAGE sample buffer and were separated by SDS–PAGE, followed by autoradiography.

2.4. In vivo binding assays and immunoblotting

Transfected BOSC 23 cells were treated with 125 μM $CoCl_2$ to mimic hypoxic conditions. In each set of transfections described in this report, the total DNA concentrations were equalized with empty plasmids. Unless noted, all transfected cells were subjected to $CoCl_2$ exposure for 16 h. After 48 h of incubation, the cells were washed with phosphate-buffered saline buffer and were lysed with the Nonidet P-40 lysis buffer for 20 min at 4°C, followed by centrifugation at $10\,000\times g$ for 10 min. The supernatant was incubated with glutathione-Sepharose 4B beads or an anti-FLAG M2 affinity gel (Sigma) for at least 5 h at 4°C with rocking. The beads were washed five times in the Nonidet P-40 lysis buffer. The bound proteins were eluted with SDS–PAGE sample buffer, separated by SDS–PAGE, and transferred to a nitrocellulose membrane. Immunoblotting assays were performed using an anti-FLAG M2 antibody (Sigma), an anti-GST antibody (Sigma), or an anti-HA antibody (Santa Cruz Biotechnology, Inc.), followed by an incubation with an appropriate secondary antibody conjugated to horseradish peroxidase. The protein bands were visualized by the ECL system.

2.5. Luciferase assay

293T cells were grown to 50–80% confluence in 6 well plates, and were transfected by lipofectamine (Gibco BRL) with 1 μg each of pFR-Luc and pFA-CMV-HIF-1α, and 0.5 μg of pCMV/β-gal with or without 1 μg of pFLAG/PSMA7. Total amounts of DNA were equalized with empty plasmids. After 5 h of transfection in 1 ml of serum-free medium, an equal volume of medium containing 10% FBS was added, and the cells were incubated for an additional 24 h. The medium was then replaced with DMEM with 2% FBS in the absence or presence of 125 μM $CoCl_2$ for 24 h before harvesting. The luciferase activity was determined using an assay system (Promega) with a luminometer. The relative fold induction of luciferase activity was determined and normalized to β-galactosidase activity. All transfections were done in duplicate and were repeated at least three times.

3. Results and discussion

3.1. Interaction of HIF-1α with the proteasome complex subunit PSMA7

In order to clarify the regulation mechanisms of the HIF-1α inhibitory domain (residues 576–785), we carried out yeast two hybrid screening with a Hela cDNA library, using the inhibitory domain as the bait [18]. One of the strongly interacting groups contained several independent clones of the proteasome subunit PSMA7 [19], all of which included a common amino acid sequence (residues 163–248) (data not shown). To further confirm the interaction between the HIF-1α and PSMA7 proteins, we performed in vitro binding assays with ^{35}S-labeled HIF-1α and GST–PSMA7 (residues 139–248) (Fig. 1A). GST and GST–VHL fusion proteins were used as negative and positive controls, respectively.

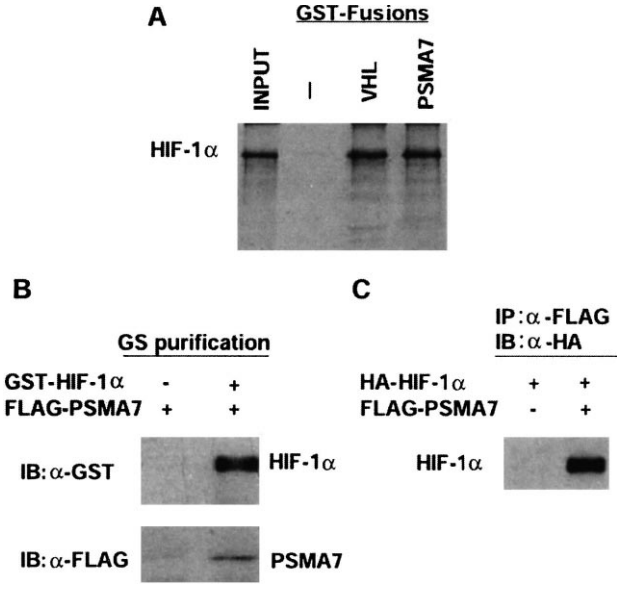

Fig. 1. HIF-1α associates with PSMA7 in vitro and in vivo. (A) The GST fusion proteins were immobilized on glutathione-Sepharose 4B, and were incubated with in vitro-translated [^{35}S]methionine-labeled HIF-1α for 5 h at 4°C. The bound proteins were eluted and were separated by 12% SDS–PAGE, followed by autoradiography. (B) BOSC 23 cells were co-transfected with 1 μg of pFLAG/PSMA7, and either the pEBG or pEBG/HIF-1α plasmid. After 48 h of transfection, lysates were prepared and were incubated with glutathione-Sepharose 4B beads. Bound proteins were eluted and were subjected to SDS–PAGE. Immunoblotting was performed with either an anti-GST or anti-FLAG M2 antibody. (C) The in vivo binding of HIF-1α to PSMA7 was confirmed by co-immunoprecipitation. BOSC 23 cells were co-transfected with pcDNA3/HA-HIF-1α, and either pFLAG/PSMA7 or empty pFLAG-CMV2. Transfected cell lysates were prepared and were immunoprecipitated with an anti-FLAG M2 affinity gel. Immunoblotting analysis was performed using an anti-HA antibody. IP, immunoprecipitation; IB, immunoblot.

GST fusion proteins were incubated with ^{35}S-labeled HIF-1α. The bound HIF-1α was eluted and subjected to SDS–PAGE, followed by autoradiography. HIF-1α was bound to GST–PSMA7 and GST–VHL but not to GST alone, suggesting that the interaction between HIF-1α and PSMA7 is specific.

To verify the intracellular interaction between HIF-1α and PSMA7, we transiently transfected the pEBG/HIF-1α full-length plasmid into BOSC 23 cells along with pFLAG/PSMA7. The intracellular interaction was confirmed by GST pull-down assays, followed by immunoblotting (Fig. 1B). While the GST alone did not pull down PSMA7, GST-fused HIF-1α pulled down PSMA7. We also performed co-immunoprecipitation assays with transiently expressed FLAG-PSMA7 and HA-HIF-1α to test if PSMA7 could bring down HIF-1α in a reciprocal way (Fig. 1C). The HIF-1α protein was detected in the pulled down PSMA7 complex. Taken together, these results indicate that HIF-1α interacts with PSMA7 in cells.

3.2. Two domains of HIF-1α are involved in the interaction with PSMA7

We proceeded to map the domain of HIF-1α required for the interaction with PSMA7 in vivo. While the N-terminal region of HIF-1α is necessary for DNA binding and dimerization with HIF-1β, the C-terminal region has several regu-

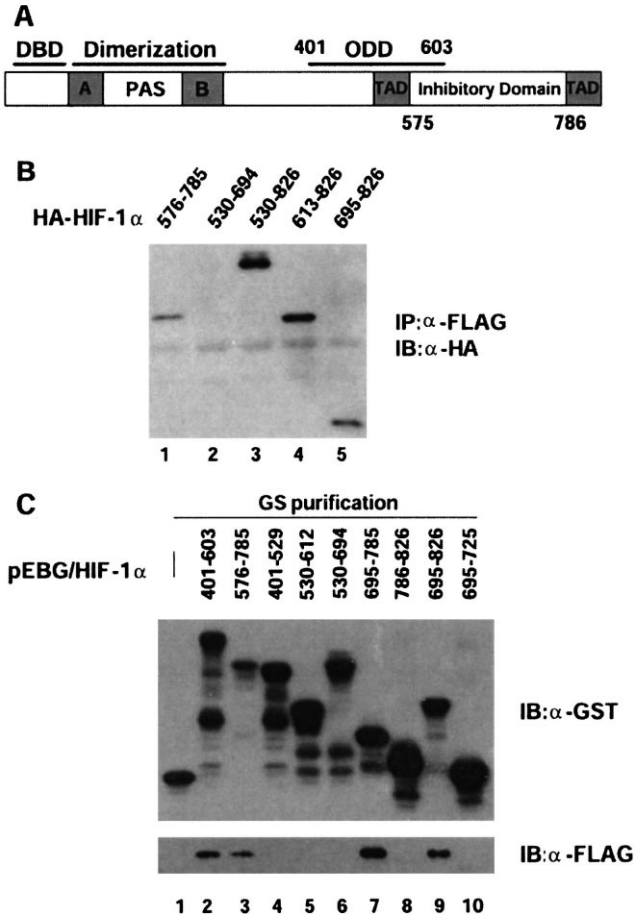

Fig. 2. Search for interaction domains of HIF-1α with PSMA7. (A) Schematic representation of full-length HIF-1α. DBD, DNA binding domain; PAS, period-ARNT-similar; TAD, transactivation domain; ODD, oxygen-dependent degradation. (B) BOSC 23 cells were co-transfected with pFLAG/PSMA7 and a series of pcDNA3/HA-HIF-1α, and were lysed. Co-immunoprecipitation assays were performed with an anti-FLAG M2 affinity gel, followed by immunoblotting with an anti-HA antibody. (C) A series of pEBG/HIF-1α deletion mutants was transfected with pFLAG/PSMA7 DNA. Binding assays were done as described in Fig. 1B. The pEBG plasmid was used for transfection in lane 1.

latory domains, including two transactivation domains, an oxygen-dependent degradation (ODD) domain, and an inhibitory domain (Fig. 2A). Since the C-terminal domains are involved in regulation, we performed binding assays to identify and localize the PSMA7 binding domains within the C-terminal region. We transfected a series of HA-HIF-1α deletion mutants together with pFLAG/PSMA7 into cells. From the lysates of these cells, PSMA7 was immunoprecipitated with anti-FLAG M2 beads, and the co-precipitated HIF-1α was probed by immunoblotting with an anti-HA antibody (Fig. 2B). PSMA7 co-immunoprecipitated the HIF-1α fragments of residues 576–785 (the inhibitory domain), 530–826, 613–826, and 695–826. The HIF-1α fragment of residues 530–694, however, failed to interact with PSMA7 (Fig. 2B, lane 2). These results show that the most critical domain for binding is within residues 695–785. We then constructed a series of pEBG/HIF-1α deletion mutants and performed binding assays, since the pEBG/HIF-1α constructs produced higher levels of recombinant proteins than the HA-HIF-1α constructs did (Fig. 2C). Consistent with the results shown in Fig. 2B, PSMA7 interacted with the whole inhibitory domain (residues 576–785) of HIF-1α. Further deletion analysis showed that the PSMA7 binding domain is localized within residues 726–785 of HIF-1α. Interestingly, the ODD domain comprising residues 401–603 also was found to interact with PSMA7 (Fig. 2C, lane 2). Based on these results, we conclude that the ODD domain and a portion of the inhibitory domain form complexes with PSMA7 independently. However, the separation of the ODD domain into two fragments resulted in a loss of the interaction with PSMA7 (Fig. 2C, lanes 4 and 5). Previously, the N-terminal transactivation domain (residues 532–585) [3,6,7] was shown to interact with VHL [20]. However, the fragment of residues 530–612, containing the VHL binding region, did not bind to PSMA7, suggesting that VHL and PSMA7 occupy different regions within the ODD domain of HIF-1α.

3.3. Effect of PSMA7 on the transactivation activity of HIF-1α

We performed luciferase reporter assays to test whether PSMA7 regulates the transactivation activity of HIF-1α. Transfected cells were either grown under normoxic conditions or treated with a hypoxia-mimicking reagent (125 μM $CoCl_2$). As shown in Fig. 3, transfection of GAL4/HIF-1α with the reporter plasmid resulted in an increase of the luciferase activity under both normoxic and $CoCl_2$-treated conditions. In addition to the global increase of luciferase activity by HIF-1α, when the cells were treated with $CoCl_2$, the luciferase activity was increased by 3-fold as compared to the activity under normoxic conditions. In contrast, co-transfection of pFLAG/PSMA7 decreased the transactivation activity of HIF-1α in a dose-dependent manner. Interestingly, under normoxic conditions, the relative reduction ratios of the HIF-1α activity were very similar to those under the hypoxia-mimicking conditions. Therefore, these results suggest that PSMA7 inhibits the HIF-1α transactivation activity and that its inhibitory function is not oxygen-dependent.

3.4. PSMA7 destabilizes HIF-1α

PSMA7 is a subunit of the 26S proteasome complex that degrades poly-ubiquitinated proteins. Since our results provided evidence that PSMA7 interacts with HIF-1α and inhibits the HIF-1α-mediated transactivation of the luciferase gene,

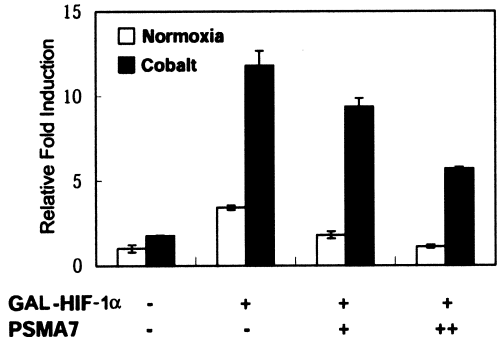

Fig. 3. PSMA7 inhibits the transactivation function of HIF-1α. 293T cells were transfected by lipofectamine with 1 μg each of the reporter plasmid pFR-Luc and GAL4/HIF-1α, and 0.5 μg of pCMV/β-gal together with 1 or 2 μg of the pFLAG/PSMA7 plasmid. Cells were treated with 125 μM $CoCl_2$ for 24 h. Luciferase activities were normalized to β-galactosidase activities. This is a representative of three independent experiments that were done in duplicate.

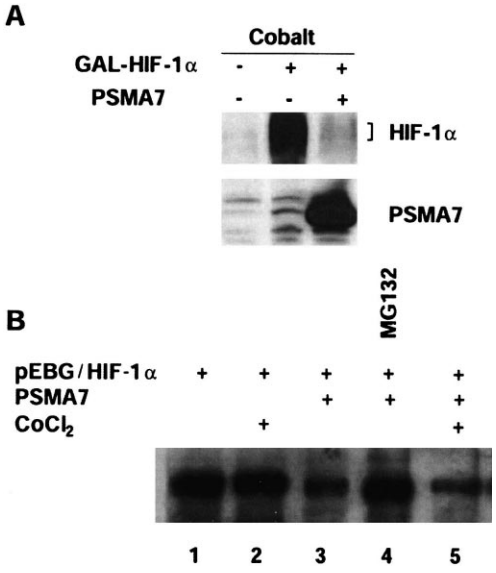

Fig. 4. HIF-1α is destabilized by PSMA7 in a proteasome-dependent manner. (A) GAL4/HIF-1α was co-transfected into 293T cells with or without pFLAG/PSMA7, as indicated. Total cell lysates from $CoCl_2$-treated cells were subjected to immunoblotting assays. The HIF-1α and PSMA7 proteins were detected with anti-HIF-1α and anti-FLAG M2 antibodies, respectively. (B) BOSC 23 cells were co-transfected with plasmids, as shown on top of the panel, and were treated with $CoCl_2$. For the sample used in lane 4, cells were exposed to 5 μM MG132, a specific proteasome inhibitor, for 4 h before harvest. GST–HIF-1α proteins were pulled down with glutathione-Sepharose beads. Immunoblotting analyses were performed as described in Fig. 1B.

we tested whether PSMA7 destabilizes the HIF-1α protein through the proteasome-dependent pathway. Cell lysates were prepared from transfected 293T cells grown under hypoxia-mimicking conditions and were subjected to immunoblotting with either an anti-HIF-1α antibody or anti-FLAG M2 antibody (Fig. 4A). Co-expression of PSMA7 decreased the HIF-1α protein level, suggesting that PSMA7 destabilizes HIF-1α.

To further study the effect of PSMA7 on HIF-1α, BOSC 23 cells were transfected with a large amount (2 μg) of the HIF-1α expression plasmid. This transfection condition produced a significant level of the HIF-1α protein and allowed us to analyze the HIF-1α expression levels clearly. Co-transfection of pFLAG/PSMA7 decreased the protein level of HIF-1α (Fig. 4B, lanes 1 and 3, and lanes 2 and 5). Treatment of co-transfected cells with MG132, a proteasome inhibitor, restored the HIF-1α protein level to that of cells transfected with the HIF-1α expression plasmid alone (Fig. 4B, lanes 1 and 4). This observation indicates that PSMA7 mediates the degradation of HIF-1α through the proteasome-dependent pathway. In addition, the $CoCl_2$ treatment of the PSMA7-transfected cells failed to stabilize the HIF-1α protein (Fig. 4B, lanes 3 and 5), suggesting that the PSMA7-mediated degradation of HIF-1α is not oxygen-dependent. These results coincide with the results of transient transfection assays, as described above (Fig. 3). The oxygen-independent regulation by PSMA7 may explain, at least in part, why HIF-1α is degraded relatively fast even when the cells were at hypoxia or under the hypoxia-mimicking conditions [6].

There have been several reports about cellular regulatory proteins and viral proteins that interact with subunits of the proteasome complex and participate in the proteasome-dependent regulation [21–25]. Since proteasome subunits can be exchanged with exogenous subunits in vivo and in vitro [23,26], the regulatory effect of the exogenous PSMA7 on HIF-1α may be due to the PSMA7-mediated recruitment of HIF-1α to the proteasome complex. Human hepatitis B virus X protein (HBX), another binding target of PSMA7, is also quickly degraded by the proteasome pathway [27], suggesting that PSMA7 may have a role in recruiting substrates to the proteasome complex.

Our finding of the interaction between HIF-1α and PSMA7 may be related to a recent report that HBX stimulates the transcription of VEGF, a target of HIF-1α, under both hypoxic and normoxic conditions [28]. Since HBX is known to interact with PSMA7 and to stimulate AP-1 activity by inhibiting AP-1 degradation [29,30], the binding of HBX to PSMA7 may also inhibit the degradation of HIF-1α.

The PSMA7 binding motif in the inhibitory domain of HIF-1α is closely related to the minimal repression domain (residues 757–785) [7], suggesting that the previously reported repressive effect of the inhibitory domain might be related to the interaction of HIF-1α with PSMA7 and the proteasome-dependent degradation. However, further analyses must be performed to test this hypothesis, since (i) multiple inhibitory sequences are present within the inhibitory domain; and (ii) the transactivation inhibitory function was more apparent at normoxia [7].

In conclusion, our results demonstrate that PSMA7 binds and regulates the HIF-1α activity by the oxygen-independent proteasome pathway.

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