Histone Deacetylase 7 Associates with Hypoxia-inducible Factor 1α and Increases Transcriptional Activity*

Received for publication, June 7, 2004, and in revised form, July 13, 2004
Published, JBC Papers in Press, July 26, 2004, DOI 10.1074/jbc.M406320200

Hiroyuki Kato§, Shiori Tamamizu-Kato†, and Futoshi Shibasaki

From the Department of Molecular Cell Physiology, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan

Hypoxia-inducible factor (HIF)-1α is a transcription factor that controls expression of genes responsive to low oxygen tension, including vascular endothelial growth factor (VEGF), erythropoietin, and glycolytic enzymes. The activity of HIF-1α is regulated by binding to the transcriptional co-activator cAMP-response element-binding protein-binding protein (CBP)/p300. Using the yeast two-hybrid screening system, we found that the inhibitory domain of HIF-1α strongly interacted with the C-terminal domain of histone deacetylase (HDAC) 7. The o-nitrophenyl β-O-galactopyranoside assay revealed that regions containing amino acids 735-785 of HIF-1α and amino acids 689-952 of HDAC7 were minimum contact sites of the interaction. The binding of HDAC7 with HIF-1α was reproduced in HEK293 cells grown under normoxic and hypoxic conditions (2% O2). HDAC7 bound solely to HIF-1α among other HIF-1α family members, including HIF-2α and HIF-3α, whereas HIF-1α only interacted with HDAC7 in the class II HDAC family. Although HDAC7 was localized dominantly in the cytoplasm at normal oxygen concentrations, HDAC7 co-translocated to the nucleus with HIF-1α under hypoxic conditions. In the nucleus, HDAC7 increased transcriptional activity of HIF-1α through the formation of a complex with HIF-1α, HDAC7, and p300. Taken together, these results indicate that HDAC7 is a novel transcriptional activator of HIF-1α.

The abbreviations used are: HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor; HDAC, histone deacetylase; ONPG, o-nitrophenyl β-O-galactopyranoside; TAD, transactivation domain; NLS, nuclear localization signal sequence; CBP, cAMP-response element-binding protein-binding protein; ID, inhibitory domain; E3, ubiquitin-protein isopeptide ligase; HA, hemagglutinin; aa, amino acid(s); DBD, DNA-binding domain; AD, activation domain.

* This work was supported by Grant-in-aid for Scientific Research C 15590287 (to H. K.) and Grant-in-aid for Scientific Research A 12090404 (to F. S.) from the Ministry of Education, Science, Sports and Culture of Japan; the Human Science Foundation (F. S.); and a research grant from the Tokyo Metropolitan Medical Science Organization (to H. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

† Both authors contributed equally to this work.

§ To whom correspondence should be addressed. Tel.: 81-3-3823-2105, ext. 5511; Fax: 81-3-3823-6065; E-mail: h-kato@rinshoken.or.jp.

© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.
a role in repression of gene transcription by associating with HDAC5 in vivo via recruitment of co-repressors N-CoR and SMRT in the nucleus. HDAC7, like other HDAC family members HDAC4 and HDAC5, requires HDAC3 for enzymatic activity (25–28). HDAC4, HDAC5, and HDAC7 contain a high homologous conserved domain (HDAC domain) containing catalytic domain in the C-terminal region. The N-terminal region and tail of HDAC7 is less homologous to the corresponding regions of HDAC4 and HDAC5. HDAC4, HDAC5, and HDAC7 also contain N-terminal nuclear localization signal sequences and C-terminal signal-responsive nuclear export sequences (29, 30). In addition, HDAC4, HDAC5, and HDAC7 are known to shuttle between the cytoplasm and the nucleus in a process regulated by calcium/calmodulin-dependent protein kinase (31, 32).

In this study, we show that HDAC7 forms a complex with HIF-1α and p300 in the nucleus under hypoxic conditions, resulting in increased levels of HIF-1α target genes (VEGF and Glut-1). Conversely, HDAC4 and HDAC5 did not bind HIF-1α. Moreover, HDAC7 translocates from the cytoplasm to the nucleus under hypoxic conditions. We therefore propose a novel role for HDAC7 in regulating the transcriptional activity of HIF-1α.

MATERIALS AND METHODS

Plasmids and Clones—The N-terminal Myc-, HA-, and FLAG-tagged pcDNA3.1 (+) vectors (Invitrogen) were from Dr. Toshiaki Suzuki, and human HIF-2α and rat HIF-3α full-length cDNAs were kindly provided by Dr. Thomas Kietzman. Human HIF-1α cDNA was purchased from Novus Biologics. The full-length HDAC4 and HDAC5 cDNAs were cloned from a human brain library (Takara) and mouse heart, respectively. All mutants were generated by PCR-based site-directed mutagenesis, carried out by mismatch amplification using two sequential PCRs (33) by the Expand High Fidelity PCR System (Roche Applied Science). All constructs were sequenced to confirm no undesired mutations.

Yeast Two-hybrid Screening and β-Galactosidase Assays (ONPG Assay)—Yeast two-hybrid screenings were carried out using MATCHMAKER GALA two-hybrid system 3 (Clontech), and all methods were performed according to the manufacturer’s protocols. For the bait construct, the cDNA encoding the inhibitory domain of human HIF-1α (HIF-1α-ID) comprising amino acid (aa) residues 601–785 was subcloned into pGBKTK7, a GAL4 DNA-binding domain (DBD) vector (pGBKTK7-HIF-1α-ID). The yeast host strain, AH109 (MATa strain), was transformed with pGBKTK7-HIF-1α-ID to confirm that pGBKTK7-HIF-1α-ID did not autonomously activate reporter genes. Briefly, the bait strain was mated with MATCHMAKER Pretransformed library (human brain) (Clontech). A total of 1.2 × 107 library clones were screened. Positive clones were selected by two steps, complementation by auxotrophy (growth on media lacking tryptophan, leucine, histidine, and adenine and supplemented with 2.5 mM 3-amino-1,2,4-triazole) and β-galactosidase filter assays.

cDNA fragments of HIF-1α, HIF-2α, and HIF-3α were amplified by PCR, subcloned into pGBKTK7, and transformed into strain AH109. Amplified by PCR, cDNA fragments of HDAC4, HDAC5, and HDAC7 were subcloned into pGADT7, a GAL4 activation domain (AD) vector and transformed into strain Y187 (MATα strain). After mating of these transformed strains, protein–protein interaction was quantified by β-galactosidase activities using ONPG as a substrate.

Cloning of Full-length HDAC7 cDNA—A full-length cDNA of HDAC7 was cloned using 5’-rapid amplification of cDNA ends system, version 2 (Invitrogen) and Expand High Fidelity PCR System (Roche Applied Science). 5′-CCCTTTCGGACGATGGATTC-3′ primer was used for the first step and 5′-GGGGATCTGGCGCGCAAGG-3′ primer was used for the second step. The amplified cDNA fragments were subcloned into Myc- and HA-tagged pcDNA3.1 (+) as described above.

Yeast Three-hybrid Assay—Yeast three-hybrid assays were performed using pGADT7 and the pBridge vector (Clontech). In this assay, the effects of a third protein on specific protein interactions can be measured. The pBridge vector allows constitutive expression of a cloned protein (DBD fusion protein) through the alcohol dehydrogenase promoter. In addition, a third protein is conditionally expressed from the MET25 promoter in pBridge in response to changes in methionine concentrations in the media. In the presence of 1 mM methionine, protein expression is repressed, and in the absence of methionine, the expression of protein is induced. cDNAs of HIF-1α comprising amino acids 601–826 and 735–785 were subcloned into pGADT7, and the resulting vector constructs were transformed into strain Y187. The cDNAs of HDAC7 (aa 669–952) and the N-terminal domain of p300 (aa 1–437) or CBP (aa 1–452) were subcloned into the two multiple cloning sites in the pBridge vector and transformed into Y187. After mating of these colonies, diploid yeast were grown on media with or without methionine. The effects of the third protein (HDAC7, p300, or CBP) on the protein-protein interaction between HIF-1α and HDAC7 or p300 or CBP were measured by β-galactosidase filter assay.

Cell Culture and Transfection—HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (Sigma) containing 10% heat-inactivated fetal bovine serum (Hyclone), 50 units/ml penicillin G, and 50 µg/streptomycin (Invitrogen) in a 5% CO2 atmosphere. Cells were transiently transfected using Lipofectamine 2000 (Invitrogen) with 10 µg plasmid DNA/well (6-well tissue culture dishes) or the Ca/phosphate method with 2 µg plasmid/18-mm micro-cover glass (Matsunami).

Western Blot Analysis—Whole cell lysates were prepared using a Nuclear Extraction Kit (Active Motif). Protein was boiled at 95 °C in SDS sample buffer for 5 min, run on 7.5% polyacrylamide gels, and transferred to polyvinylidine difluoride membranes. Membranes were incubated overnight at 4 °C with blocking buffer, followed by exposure to primary antibodies for 1 h. Anti-HIF-1α antibody was obtained from Transduction Laboratories. Anti-Myc antibody and anti-HA antibody were purified from 9E10 and 12CA5 hybridomas, respectively (MBL). Monoclonal anti-FLAG antibody (M2-FLAG) and polyclonal anti-p300 antibody were from Sigma and Santa Cruz Biotechnology. The membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG (Chemicon) or horseradish peroxidase-conjugated anti-rabbit IgG (Chemicon) and washed six times with phosphate-buffered saline containing 0.1% Tween 20. Chemiluminescence was detected with ECL Western Blotting System (Amersham).

Immunofluorescence Staining—HEK293 cells were plated on 18-mm micro-cover glasses (Matsunami) and transfected with 2 µg of the appropriate plasmid using the Ca/phosphate method. After 5 h, cells were washed with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and incubated at normal or hypoxic conditions (1% O2) for 12 h. Cells were fixed in 3.7% paraformaldehyde in phosphate-buffered saline and washed with phosphate-buffered saline containing 0.1% Nonidet P-40. For immunostaining, fixed cells were incubated with antibodies for 1 h, washed, and incubated with Cy3-conjugated secondary antibody for 30 min. Cells were washed and mounted with Vectashield (Vector Laboratories) mounting medium for fluorescence with 4′,6-diamidino-2-phenylindole. Images were visualized with an Olympus 1x70 inverted system microscope equipped with charge-coupled device. The resulting images were stored using Meta-morph computer software.

Immunoprecipitation—After 24 h of transfection, HEK293 cells were washed with phosphate-buffered saline and lysed with lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 0.5 mM dithiothreitol, and protease inhibitor mixture (Roche Applied Science). The cell lysate was centrifuged at 14,000 × g for 30 min, and the supernatant was removed. Anti-Myc-Sepharose (Santa Cruz Biotechnology) or anti-FLAG M2-agarose (Sigma) was added to the supernatant and incubated for 2 h. After washing with the lysis buffer five times, SDS sample buffer was added and boiled for Western blot analysis.

Real-time PCR—Total RNA was isolated from HEK293 cells (plated on 6-well dishes) using Isogen (Nippon Gene). First-strand DNA was synthesized by SuperScript First-Strand Synthesis System for reverse transcription-PCR (Invitrogen). Real-time quantitative PCR was performed on a TaqMan PCR system (ABI/Prism 7700 Sequence Detection System, Applied Biosystems). A standard curve for serial dilutions of 18S rRNA was generated. The relative standard curve method (Applied Biosystems) was used to calculate the amounts of VEGF and Glut-1 RNA.

RESULTS

HIF-1α Associates with HDAC7 in Yeast—HIF-1α has been shown to interact with several proteins such as CBP/p300 and factor inhibiting HIF/asparaginyl hydroxylase. Regulation of HIF-1α transcriptional activity, however, is not well understood. To clarify the transcriptional activation mechanisms of HIF-1α, we set out to identify proteins that interact with HIF-1α using the yeast two-hybrid system. Because both the N-terminal transactivation domain (N-TAD) and C-terminal transactivation domain (C-TAD) of HIF-1α showed high-back
Yeast two-hybrid assays were performed to measure interaction between HIF-α family members and HDACs. The relative strength of the interactions of the proteins was measured by a quantitative β-galactosidase assay (ONPG assay). AD, a GAL4 activation domain; DBD, a GAL4 DNA-binding domain. A, analysis of the HDAC7-interacting domain in HIF-1α. The top box represents full-length HIF-1α cDNA with specific domains. Amino acid residues 601–735 in HIF-1α were used as bait. Two of 13 positive clones were identified as HDAC7 (aa 669–952) after screening 1.2 x 10⁶ clones from a human brain library. Numbers indicate the position of amino acids in HIF-1α. Various fragments of HIF-1α were tested for their ability to interact with HDAC7 (aa 669–952). PAS, PER-ARNT-SIM domain. B,
HDAC7 Increases HIF-1α Activity

Fig. 2. Interaction of HIF-1α and class II HDACs in HEK293 cells. Subcellular localization of HIF-1α and class II HDACs (HDAC4, HDAC5, and HDAC7) under normoxic conditions (Norm. or Normoxia) and hypoxic conditions (Hypo. or Hypoxia). HEK293 cells were transfected with HA-tagged full-length HIF-1α, HDAC4, HDAC5, and HDAC7 and incubated under normoxic and hypoxic conditions (2% O₂ for 12 h). Immunofluorescence staining was performed to visualize each protein as described under “Materials and Methods” (top panels). 200 transfected cells were scored by four independent experiments. Histograms in the bottom panels show the percentage of cells containing HA-tagged proteins in the cytoplasm (C) and nucleus (N). None of the cells expressed the protein in both the cytoplasm and nucleus.

Ground in yeast two-hybrid screenings, we used the ID (aa 601–785) located between the N-TAD and C-TAD as bait. 1.2 × 10⁶ clones from a human brain library were screened. Two of 13 β-galactosidase-positive clones contained the same ~1.8-kb fragment encoding the C-terminal domain of HDAC7 (aa 669–952), a member of the class II family of mammalian HDACs. The cloned HDAC7 contained the C-terminal conserved domain (HDAC domain), the catalytic domain, and the C-terminal unique tail.

We further characterized the interaction between HDAC7 and HIF-1α to map the essential binding domains of HDAC7 and HIF-1α. Fig. 1A shows the relative strength of the interactions of the proteins in yeast determined by a quantitative β-galactosidase assay using ONPG. Full-length HIF-1α and constructs containing amino acids 401–600, 601–826, 601–785, 681–826, 735–826, 735–785, and 786–826 of HIF-1α retained β-galactosidase activities. Constructs with amino acids 1–481, 601–734, and 601–680 had no activity. Amino acids 401–600 and 786–826 of HIF-1α (the N-TAD and the C-TAD) alone showed relatively strong β-galactosidase activity without the presence of AD-HDAC7. These activities were due to the autonomous β-galactosidase activity as reported in the manufacturer’s protocol. From this assay, the region containing amino acids 735–785 (within the ID of HIF-1α) seemed to be the minimum region required for binding to HDAC7.

To determine the region within HDAC7 essential for binding to HIF-1α, we constructed vectors containing the AD fused to HDAC7 fragments and introduced them into yeast along with a vector expressing the DBD fused to a fragment of HIF-1α (aa 735–785). Fig. 1B shows that the C-terminal fragment of HDAC7 (aa 669–952), which contains the catalytic domain, C-terminal conserved domain (HDAC domain), and C-terminal tail, bound to HIF-1α. Constructs containing other C-terminal fragments of HDAC7 (Fig. 1B) or the N-terminal fragment of HDAC7, were assessed for their ability to interact with HIF-1α.

Analysis of the HIF-1α-interacting domain in HDAC7. The top box represents full-length HDAC7 cDNA with specific domains. A fragment containing amino acids 735–785 of HIF-1α was used for the binding assay. Numbers indicate the position of amino acids in HDAC7. Various fragments of the C-terminal domain of HDAC7 were tested for their ability to interact with HIF-1α. C, comparison of the binding ability of HIF-1α, HIF-2α, and HIF-3α to HDAC7. The boxes (left) represent full-length HIF-1α, HIF-2α, and HIF-3α with their specific domains. Numbers indicate the position of amino acids in HIF-2α and HIF-3α. The C-terminal domains of HIF-2α (aa 571–870 and 571–927) and HIF-3α (aa 506–682 and 506–828) were tested for their ability to interact with HDAC7. D, comparison of the binding ability of HDAC7, HDAC4, and HDAC5 to HIF-1α. The boxes (left) represent full-length HDAC family HDAC4, HDAC5, and HDAC7 with their domains. Numbers indicate the position of amino acids in HDAC4 and HDAC5. The C-terminal domains of HDAC4 (aa 802–1084) and HDAC5 (aa 823–1113), corresponding to the C-terminal domain of HDAC7, were assessed for their ability to interact with HIF-1α.
HDAC7 (data not shown) showed no β-galactosidase activity. These data demonstrate that HIF-1α binds to HDAC7 in yeast and suggest that the minimum contact sites included amino acids 735–785 of HIF-1α and amino acids 669–852 of HDAC7.

HIF-1α and HDAC7 belong to their respective families, the HIF-α family and the class II HDAC family. To identify whether other members of each family also associated in yeast, we performed the ONPG assay using DBD fusions with the C-terminal domains of HIF-2α (aa 571–870 or 571–828) and HIF-3α (aa 506–662 or 506–635). Because other class II HDAC family members (HDAC4 and HDAC5) were structurally similar to HDAC7, we also constructed AD fusions with the C-terminal domains of HDAC4 (aa 802–1084) and HDAC5 (aa 823–1113), corresponding to the HIF-1α-binding domain of HDAC7 (aa 669–952).

As shown in Fig. 1C, HIF-1α had strong β-galactosidase activity, whereas HIF-2α and HIF-3α did not show any activity. Moreover, HDAC4 and HDAC7 retained β-galactosidase activities, although, quantitatively, the level for HDAC4 was approximately one-fourth weaker than that of HDAC7. HDAC5 did not have any β-galactosidase activity (Fig. 1D). These data suggest that, in yeast, HIF-1α specifically binds HDAC4 and HDAC7. HIF-1α also appears to show stronger binding to HDAC7 when compared with HDAC4.

HIF-1α and HDAC7 Co-localize to the Nucleus—To examine the subcellular localization of HDAC7, HEK293 cells were transfected with a plasmid expressing HDAC7 and visualized by immunofluorescence staining. As shown in Fig. 2, HDAC7 was mainly expressed in the cytoplasm under normal oxygen concentrations; expression of HDAC7 was observed in the cytoplasm of 65.5 ± 0.67% of the transfected cells. Other class II HDAC family members, HDAC4 and HDAC5, were also visualized, and the results are shown in Fig. 2. HDAC4 was localized to the cytoplasm, whereas HDAC5 was localized to the nucleus. HDAC7 changed expression pattern from the cytoplasm to the nucleus when transfected cells were incubated under hypoxic conditions (2% O2, 12 h); 94.4 ± 0.37% of the cells expressed HDAC7 in the nucleus. On the other hand, localization of HDAC4 and HDAC5 was not altered by hypoxic treatment. During hypoxia, HIF-1α was expressed solely in the nucleus, suggesting that HDAC7 and HIF-1α co-localize to the nucleus. Similar changes in HDAC7 localization induced by hypoxia were observed in HeLa cells (data not shown).

To confirm the interaction between HIF-1α and HDAC7 in HEK293 cells, immunoprecipitation experiments were conducted. Cells were co-transfected with Myc-HIF-1α and HA-HDACs (Fig. 3A) or Myc-HDAC7 and HA-HIF-α family members (Fig. 3B). Cell lysates were prepared and immunoprecipitated with anti-Myc antibodies conjugated to Sepharose. Co-immunoprecipitated HDACs or HIF-α family members were detected by anti-HA antibody. In support of our results in yeast, only HDAC7 was co-precipitated by HIF-1α, and HIF-1α was co-precipitated by HDAC7 under normoxic and hypoxic conditions. These results indicated specific interaction between HIF-1α and HDAC7 because other HIF-α family members and class II HDACs did not interact with each other.

HIF-1α Brings HDAC7 to the Nucleus—HIF-1α and HDAC7 contain nuclear localization signal sequences at amino acids 718–721 and 157–192, respectively. To determine whether the signal sequence of each protein was important for localization to the nucleus, we constructed nuclear localization signal sequence (NLS) deletion mutants of HIF-1α and HDAC7 (Myc-HIF-1α-ΔNLS and HA-HDAC7-ΔNLS). The wild-type or each mutant construct was transfected into HEK293 cells and incubated under normoxic or hypoxic conditions. The transfected cells were subjected to immunofluorescence staining. As shown in Fig. 4, A and B, overexpressed Myc-HIF-1α-ΔNLS and HA-HDAC7-ΔNLS localized to the cytoplasm in cells incubated under normoxic or hypoxic conditions, whereas wild-type Myc-HIF-1α (Myc-HIF-1α-WT) and HA-HDAC7 (HA-HDAC7-WT) localized to the nucleus under hypoxic conditions. We conducted co-transfection experiments using different combinations of the full-length and ΔNLS mutant expression vectors under hypoxic conditions. When expressed together, Myc-HIF-1α-WT and HA-HDAC7-WT were co-localized to the nucleus. Interestingly, when Myc-HIF-1α-WT and HA-HDAC7-WT were co-expressed in the cells, HA-HDAC7-ΔNLS was localized with Myc-HIF-1α-WT in the nucleus (Fig. 4C). Conversely, when Myc-HIF-1α-ΔNLS and HA-HDAC7-WT were co-expressed, both proteins were localized to the cytoplasm (Fig. 4C). The binding of ΔNLS mutants and the wild-type constructs of Myc-HIF-1α and HA-HDAC7 were confirmed by immunoprecipitation (data not shown). These results suggest that translocation of HDAC7 from the cytoplasm to the nucleus under hypoxic conditions requires association of HDAC7 with HIF-1α and that NLS of HIF-1α plays an important role in this translocation of HDAC7.

HIF-1α Increases Transcriptional Activity of HIF-1α—We next investigated the role of HDAC7 association with HIF-1α in the nucleus under hypoxic conditions. To determine whether HDAC7 could affect transcriptional activity of HIF-1α, we used real-time PCR to quantify expression of two HIF-1α target genes, VEGF and Glut-1. HEK293 cells were transfected with HIF-1α and HDAC7 and incubated under normoxic or hypoxic conditions. 18S rRNA was used as an internal control because glyceraldehyde-3-phosphate dehydrogenase, the most commonly used for assay control, was one of HIF-1α target genes. The relative amount of VEGF and Glut-1 RNAs was quantified. Fig. 5 shows that overexpression of HIF-1α causes an increase...
HDAC7 Increases HIF-1α Activity

Fig. 4. Co-localization of HIF-1α and HDAC7 in transfected HEK293 cells. The vectors encoding full-length and mutant Myc-HIF-1α and HA-HDAC7 were transiently transfected into HEK293 cells. Localization of Myc-HIF-1α and HA-HDAC7 was detected by immunofluorescence staining using anti-Myc antibody and anti-HA antibody, respectively. A, localization of the full-length (WT) and nuclear localization signal sequence-deleted (ΔNLS) mutants of HIF-1α (HIF-1α-WT and HIF-1α-ΔNLS). The NLS (aa 718–721) in HIF-1α was deleted. Localization of Myc-HIF-1α-WT and Myc-HIF-1α-ΔNLS was detected under normoxic and hypoxic conditions (2% O₂, 12 h). B, localization of the full-length (WT) and nuclear localization signal sequence-deleted (ΔNLS) mutants of HDAC7 (HA-HDAC7-WT and HA-HDAC7-ΔNLS). The NLS (aa 157–192) in HDAC7 was deleted. Localization of HA-HDAC7-WT and HA-HDAC7-ΔNLS was detected under normoxic and hypoxic conditions (2% O₂, 12 h). C, vectors encoding full-length and mutant Myc-HIF-1α and HA-HDAC7 were transiently co-transfected HEK293 cells. Histograms to the right show the percentage of the cells containing wild-type and NLS mutants of HIF-1α or HDAC7 in the cytoplasm (C) and nucleus (N). 200 transfected cells were scored by four independent experiments. None of the cells expressed the protein in both the cytoplasm and nucleus.

in the amount of VEGF and Glut-1 RNAs (157 ± 5.1% and 202.8 ± 5.1%, compared with hypoxia-treated mock cells, respectively), whereas HDAC7 did not change the amounts of both RNAs. Interestingly, co-transfection of HIF-1α with HDAC7 further enhanced the amount of VEGF and Glut-1 RNAs (200.0 ± 4.5% and 188.9 ± 6.7%, compared with cells transfected with HIF-1α alone). This enhancement of the transcriptional activity of HIF-1α by HDAC7 was not observed under normal O₂ conditions. In addition, when the HDAC7-binding domain (aa 735–785) was deleted from HIF-1α (HIF-1α-Δ), the enhancement of HIF-1α transcriptional activity by HDAC7 was diminished. These results demonstrate that association of HDAC7 with HIF-1α enhances transcriptional activity of HIF-1α in hypoxia-stressed cells (2% O₂).

HIF-1α, HDAC7, and p300 Form a Complex—The C-TAD of HIF-1α has been shown to bind with transcriptional co-activators via the CH1 domain in the N-terminal domain of CBP and p300 (6, 12). To determine whether HDAC7, HIF-1α, and p300/CBP form a complex or whether there is competition for binding between these proteins, yeast three-hybrid experiments were performed. We utilized an AD vector and the pBridge vector, a three-hybrid vector that expresses two proteins, a DBD fusion protein and an additional protein (the third protein). The third protein, under control of the Met25 promoter, was expressed in methionine-free media and repressed in the presence of 1 mM methionine. We constructed pBridge vectors expressing HDAC7, CBP, or p300 as the DBD fusion proteins or as the methionine-regulated third proteins. We tested the effects of the third protein on binding between the DBD-HIF-1α fragment (aa 601–826) containing the CH1 domain and the AD-N-terminal domain of p300 (aa 1–437) or the AD-N-terminal domain of CBP (aa 1–452). The N-terminal domains of CBP and p300 containing the CH1 domain bind the C-TAD of HIF-1α (aa 786–826). Therefore, HIF-1α fragments (aa 601–826) containing the binding sites of HDAC7 and CBP/p300 were used. As summarized in Table I, the β-galactosidase filter assays show that HIF-1α fragment (aa 601–826) binds to HDAC7, CBP, or p300. CBP and p300 do not inhibit the binding between HIF-1α and HDAC7, whereas HDAC7 does not inhibit the binding between HIF-1α and CBP or HIF-1α and p300. On the other hand, the minimal binding fragment of HIF-1α (aa 735–785) binds only to HDAC7. CBP and p300 do not inhibit the binding between this HIF-1α fragment and HDAC7. These data indicate that CBP and p300 do not appear to interfere with the interaction between HDAC7 and HIF-1α. In addition, HDAC7 does not replace CBP and p300 on HIF-1α.
HDAC7 Increases HIF-1α Activity

The cDNAs of HIF-1α comprising amino acids 601–826 and 735–785 were subcloned into the AD-vector and transformed into yeast strain Y187. The cDNAs of HDAC7 (aa 669–952) or the N-terminal domain of p300 (aa 1–437) or CBP (aa 1–826) were subcloned into two multiple cloning sites (MCS-I and MCS-II) in pBridge vector, a three-hybrid vector. pBridge constitutively expresses an AD-protein (MCS-I) and the additional third protein (MCS-II) under control of the MET25 promoter. The AD-vector-HIF-1α fragments and pBridge vectors harboring HDAC7 or p300 and CBP were transformed into strain AH109. After mating of these colonies, the diploid cells were grown on media with methionine (not inducing the third protein) or without methionine (inducing the third protein). The effects of the third protein (HDAC7 or p300 or CBP) on protein-protein interaction between HIF-1α and HDAC7 or p300 or CBP were measured by β-galactosidase filter assay.

**Table I. Effects of HDAC7, CBP and p300 on binding to HIF-1α in yeast using yeast three-hybrid system**

| AD protein | DBD protein | The third protein | β-Gal* activity |
|------------|-------------|------------------|----------------|
| No         | No          | No               | –              |
| No         | HDAC7       | No               | –              |
| No         | CBP         | No               | –              |
| No         | p300        | No               | –              |
| HIF1-α (aa 601–826) | No | HDAC7 | – |
| No         | CBP         | No               | –              |
| No         | p300        | No               | –              |
| HIF1-α (aa 735–785) | No | HDAC7 | + |
| No         | CBP         | No               | –              |
| p300       | HDAC7       | No               | –              |
| No         | CBP         | No               | –              |
| No         | p300        | No               | –              |
| HIF1-α (aa 735–785) | No | HDAC7 | + |
| No         | CBP         | No               | –              |
| p300       | HDAC7       | No               | –              |

*β-Gal, β-galactosidase; –, negative clones; +, positive clones.

**Fig. 5. Effect of HDAC7 on the expression levels of VEGF and Glut-1 RNA.** Vectors encoding HIF-1α or HIF-1α deletion mutant (lacking aa 735–785; indicated as HIF-1α-Δ) and HDAC7 were transiently transfected into HEK293 cells, and the cells were incubated under normoxic and hypoxic conditions (2% O₂ for 4 or 12 h). The relative expression levels of VEGF (12 h) (A) and Glut-1 (4 h) (B) RNA were assayed by quantitative PCR analysis. Results from three independent quantitative PCR experiments were averaged. Data (the mean ± S.E.) represent the percentage of control (RNA contents in mock-transfected cells).

To further confirm these results, immunoprecipitation was performed using HEK293 cells. Myc-HIF-1α, FLAG-HDAC7, and HA-p300 were co-transfected into the cells and incubated under normoxic and hypoxic conditions. Cell lysates were immunoprecipitated with anti-Myc-Sepharose or anti-FLAG M2-agarose. As shown in Fig. 6A, HDAC7 was immunoprecipitated with HIF-1α under normoxic or hypoxic conditions, whereas p300 was immunoprecipitated with HIF-1α only in cells incubated under hypoxic conditions. The expression levels of HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At normal O₂ levels, endogenous factor inhibiting HDAC7 and p300 were the same under normoxic and hypoxic conditions. At norma...
the O2 tension surrounding the cells. This shuttling of HDAC7 was more clearly observed when HDAC7 and HIF-1α were precipitated with anti-FLAG antibody and anti-p300 antibody. The amounts of p300 and HDAC7 expressed in HEK293 cells were equal in cells incubated under normoxic and hypoxic conditions. B, p300 and HDAC7 were immunoprecipitated with anti-FLAG M2-agarose (FLAG-HDAC7). Co-immunoprecipitated p300 (arrows) and HDAC7 were analyzed by Western blot (WB) with anti-p300 antibody and anti-HIF-1α antibody.

Our study also showed that HDAC4 localized to the cytoplasm and HDAC5 localized to the nucleus under normoxic conditions. These proteins did not translocate to the nucleus or cytoplasm under hypoxia. One feasible explanation why HDAC4 did not associate with HIF-1α in mammalian cells is that the two proteins are localized to different areas of the cell.

HDAC4, HDAC5, and HDAC7 possess the NLS and the nuclear export sequence in their N-terminal domain and C-terminal domain, respectively. The subcellular localizations of the HDACs, however, are reported to be regulated by a mechanism involving calcium/calmodulin-dependent protein kinase and an intracellular chaperone protein, 14-3-3. 14-3-3 binds to calcium/calmodulin-dependent protein kinase-phosphorylated serine residues in the N-terminal domain of the HDACs, resulting in the export of HDACs with a cellular export factor, CRM1 (30, 31, 41). Our data show that HDAC7 localizes to the cytoplasm under normoxic conditions and translocates to the nucleus under hypoxia. Co-transfection experiments using HIF-1αΔNLS and HDAC7ΔNLS mutants demonstrated that the NLS in HIF-1α is involved in translocation of the HIF-1α-HDAC7 complex into the nucleus. Deletion of the HDAC7 NLS, however, did not affect subcellular localization of HIF-1α. Therefore, it is less likely that phosphorylation of HDAC7 plays a role in translocation of HDAC7 under hypoxia. Because hypoxia causes an increase in intracellular calcium concentration in epithelial cells (42), hypoxia-induced phosphorylation of HDAC7 might be involved in stabilization of HIF-1α-HDAC7 complex or export of HIF-1α from the nucleus to the cytoplasm.

Fig. 6. Interaction of HIF-1α, HDAC7, and p300 in HEK293 cells. Co-immunoprecipitation of Myc-HIF-1α, FLAG-HDAC7, and HA-p300. HEK293 cells were transfected with vectors encoding Myc-HIF-1α and FLAG-HDAC7 and HA-tagged full-length p300 (HA-p300). Cells were incubated under normoxic or hypoxic conditions (2% O2 for 12 h). A, after lysis of the cells, HDAC7 and p300 were immunoprecipitated with Myc-Sepharose (Myc-HIF-1α). Co-immunoprecipitated HDAC7 and p300 (arrows) were analyzed by Western blot (WB) with anti-FLAG antibody and anti-p300 antibody. The amounts of p300 and HDAC7 expressed in HEK293 cells were equal in cells incubated under normoxic and hypoxic conditions. B, p300 and HDAC7 were immunoprecipitated with anti-FLAG M2-agarose (FLAG-HDAC7). Co-immunoprecipitated p300 (arrows) and HDAC7 were analyzed by Western blot (WB) with anti-p300 antibody and anti-HIF-1α antibody.
14. Kallio, P., Okamoto, K., O'Brien, S., Carrero, P., Makino, Y., Tanaka, H., and Poellinger, L. (1998) EMBO J. 17, 6573–6586
15. Vo, N., and Goodman, R. H. (2001) J. Biol. Chem. 276, 13505–13508
16. Daines, S. A., Martinez-Yamout, M., De Guzman, R. N., Dyson, H. J., and Wright, P. E. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5271–5276
17. Freedman, S. J., Sun, Z.-Y. J., Poy, F., Kung, A. L., Livingston, D. M., Wagner, G., and Eck, M. J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5367–5372
18. Hewitson, K. S., McNeill, L. A., Riordan, M. V., Tian, Y.-M., Bullock, A. N., Welford, R. W., Elkins, J. M., Bhattacharya, S., Gleddie, J. M., Ratcliffe, P. J., Pugh, C. W., and Schofield, C. J. (2002) J. Biol. Chem. 277, 26351–26355
19. Lando, D., Peet, D. J., Gorman, J. J., Werlan, D. A., Whitelaw, M. L., and Bruck, R. K. (2002) Genes Dev. 16, 1466–1471
20. Lando, D., Peet, D. J., Werlan, D. A., Gorman, J. J., and Whitelaw, M. L. (2002) Science 295, 858–861
21. Mahon, P. C., Hirota, K., and Semenza, G. L. (2001) Genes Dev. 15, 2675–2686
22. Jiang, B.-H., Zheng, J. Z., Leung, S. W., Roe, R., and Semenza, G. L. (1997) J. Biol. Chem. 272, 19253–19260
23. Kao, H.-Y., Downes, M., Ordentlich, P., and Evans R. M. (2000) Genes Dev. 14, 55–66
24. de Ruijter, A. J. M., van Gennip, A. H., Caron, H. N., Kemp, S., and van Kuilenburg, A. B. P. (2003) Biochem. J. 370, 737–749
25. Fischle, W., Dequiedt, F., Pillon, M., Hendzel, M. J., Voelter, W., and Verdin, E. (2001) J. Biol. Chem. 276, 35826–35835
26. Fischle, W., Dequiedt, F., Hendzel, M. J., Guenther, M. G., Lazar, M. A., Voelter, W., and Verdin, E. (2002) Mol. Cell 9, 45–57
27. Guenther, M. G., Barak, O., and Lazar, M. A. (2001) Mol. Cell. Biol. 21, 6091–6101
28. Yang, W. M., Tsai, S. C., Wen, Y. D., Fejer, G., and Seto, E. (2002) J. Biol. Chem. 277, 9447–9454
29. Bertos, A. R., Wang, A. H., and Yang, X.-J. (2001) Biochem. Cell Biol. 79, 243–252
30. McKinsey, T. A., Zhang, C.-L., and Olson, E. N. (2001) Mol. Cell. Biol. 21, 6312–6321
31. Kao, H.-Y., Verdel, A., Tsai, C.-C., Simon, C., Juguilon, H., and Khochbin, S. (2001) J. Biol. Chem. 276, 47496–47507
32. McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 14400–14405
33. Horton, R. M., and Pease, L. R. (1991) in Indirect Mutagenesis (McPherson, M. J., ed), pp. 217–246, IRL Press, New York
34. Suhara, W., Yoneyama, M., Kitabayashi, I., and Fujita, T. (2002) J. Biol. Chem. 277, 22304–22313
35. Ema, M., Hirota, K., Mimura, J., Abe, H., Yoda, I., Sogawa, K., Poellinger, L., and Fujii-Kuriyama, Y. (1999) EMBO J. 18, 1905–1914
36. O'Rourke, J. F., Tian, Y.-M., Ratcliff, P. J., and Pugh, C. W. (1999) J. Biol. Chem. 274, 2060–2071
37. McKinsey, T. A., Zhang, C.-L., Lu, J., and Olson, E. N. (2000) Nature 408, 106–111
38. Zhou, X., Richon, V. M., Wang, A. H., Yang, X.-J., Rifkind, R. A., and Marks, P. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 14329–14333
39. Jiang, B.-H., Agani, F., Passaniti, A., and Semenza, G. L. (1997) Cancer Res. 57, 5328–5335
40. Zhong, H., Agani, F., Baccala, A. A., Laughner, E., Risso-Camacho, N., Issacs, W. B., Simon, J. W., and Semenza, G. L. (1998) Cancer Res. 58, 5280–5284
41. Grezinger, C. M., and Schreiber, S. L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7835–7840
42. Arnauld, T., Michiels, C., Alexandre, I., and Remacle, J. (1996) J. Cell. Physiol. 153, 215–221

41974

HDAC7 Increases HIF-1α Activity

by guest on July 28, 2018http://www.jbc.org/Downloaded from
Histone Deacetylase 7 Associates with Hypoxia-inducible Factor 1α and Increases Transcriptional Activity

Hiroyuki Kato, Shiori Tamamizu-Kato and Futoshi Shibasaki

J. Biol. Chem. 2004, 279:41966-41974. doi: 10.1074/jbc.M406320200 originally published online July 26, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406320200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 41 references, 30 of which can be accessed free at http://www.jbc.org/content/279/40/41966.full.html#ref-list-1