Mammalian Tumor Suppressor Int6 Specifically Targets Hypoxia Inducible Factor 2α for Degradation by Hypoxia- and pVHL-independent Regulation*

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The hypoxia-inducible factors HIF-1α and HIF-2α are structurally similar as regards their DNA-binding and dimerization domains, but differ in their transactivation domains and, as is shown by experiments using hif-1α−/− and hif-2α−/− mice, in their functions. This implies that HIF-1α and HIF-2α may have unique target genes. To address this discrepancy and identify HIF-2α-specific target genes, we performed yeast two-hybrid analysis and identified the tumor suppressor Int6/eIF3e/p48 as a novel target gene product involved in HIF-2α regulation. The int6 gene was first identified from a screen in which the mouse mammary tumor virus was employed as an insertional mutagen to identify genes whose functions are critical for breast tumor formation. Here, by using two-hybrid analysis, immunoprecipitation in mammalian cells, and HRE-reporter assays, we report the specific interaction of HIF-2α (but not HIF-1α or HIF-3α) with Int6. The results indicate that the direct interaction of Int6 induces proteasome inhibitor-sensitive HIF-2α degradation. This degradation was clearly observed in renal cell carcinoma 786-O cells, and was found to be both hypoxia- and pVHL-independent. Furthermore, Int6 protein knockdown by int6-siRNA vectors or the dominant-negative mutant Int6-ΔC increased endogenous HIF-2α expression, even under normoxia, and induced sets of critical angiogenic factors comprising vascular endoplasmic growth factor, angiopoietin, and basic fibroblast growth factor mRNA. These results indicate that Int6 is a novel and critical determinant of HIF-2α-dependent angiogenesis as well as cancer formation, and that int6-siRNA transfer may be an effective therapeutic strategy in pathological conditions such as heart and brain ischemia, hepatic cirrhosis, and obstructive vessel diseases.

Hypoxia-responsive genes are involved in glucose transport, glycolysis, erythropoiesis, angiogenesis, vasodilation, and respiratory rate. However, they are also involved in the pathogenesis of many cardiovascular diseases and cancer (1, 2). Central to many molecular and physiological responses to hypoxia in most mammals are the hypoxia-inducible factors HIF-1α and HIF-2α (HIFs),2 heterodimers with HIF-1β (also referred to as aryl hydrocarbon receptor nuclear translocator, ARNT) (3–5). Both HIF-1α and ARNT belong to the basic helix loop helix Per-Arnt-Sim (PAS) family of transcription factors, which share several conserved structural domains (6).

Under normoxia, HIF-1α is ubiquitinated via interaction with the von Hippel-Lindau tumor suppressor protein (pVHL) and is subsequently degraded by the 26 S proteasome (7–12). pVHL, the recognition component of an E3 ubiquitin ligase complex, binds HIF-1α when it is hydroxylated at proline residues 402 and 577 (13–15). The proline hydroxylation of HIF-1α is catalyzed by prolyl hydroxylase domain-containing proteins, which are members of the 2-oxoglutarate-dependent dioxygenase superfamily whose activity requires O2 as a cofactor (16, 17). During hypoxia, the activity of prolyl hydroxylase domain-containing proteins is suppressed, resulting in a reduction in both the proline hydroxylation of HIF-1α and its association with pVHL. The stabilized HIF-1α proteins migrate into the nucleus and heterodimerize with the obligate ARNT. The heterodimer then binds the hypoxic response elements (HREs) that are present within the regulatory regions of a variety of target genes to up-regulate their expression. Typically, these genes function both to increase O2 supply to tissues and to facilitate metabolic adaptation to hypoxia (18–20).

Like HIF-1α, the HIF-2α protein is also stabilized during hypoxia, forms a heterodimer with ARNT, and transactivates the vascular endothelial growth factor (VEGF) and erythropoietin promoter (21, 22). Structurally, HIF-2α and HIF-1α share 48% amino acid sequence similarity. In particular, there is 83% identity in their basic helix-loop-helix domains and ~70% homology between their PAS regions. Furthermore, the oxygen-dependent degradation domains of the two HIF-α subunits, including the two critical proline residues, also exhibit a high

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2 The abbreviations used are: HIF, hypoxia inducible factor; MMTV, mouse mammary tumor virus; ARNT, aryl hydrocarbon receptor nuclear translocator; PAS, Per-Arnt-Sim; pVHL, von Hippel-Lindau gene product; HRE, hypoxia responsive element; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; TAD, transactivation domain; Tie-2, tuutina intima endothelial kinase-2; eIF3e, eukaryotic translational initiation factor 3e; CSN, the COP9 signalosome; PINT, Proteasome/Int6/Nip-1/TRIP-15; siRNA, small interfering RNA; RT, real time; siRNA, small interfering RNA; HA, hemagglutinin.
degree of homology (Fig. 1A). Thus, as described above, the stability of both proteins appears to be subject to similar regulation by O₂ (23–26). Nevertheless, HIF-2α was found to be stable and localized in the nucleus of bovine arterial endothelial cells, even under normoxia (27, 28), and the re-expression of HIF-2α could regulate the expression of tunica intima endothelial kinase 2 (Tie-2), thus rescuing vascular development (29).

There has been a continuing interest in distinguishing the roles of HIF-1α and HIF-2α. HIF-1α−/− mice exhibit midgestation lethality and severe blood vessel defects (30, 31). In contrast, hif-2α-deficient mice are reported to manifest defective vascular remodeling during embryonic development (32), as well as during catecholamine homeostasis (33), fetal lung maturation (34), and hematopoietic cell production (35). Thus, HIF-1α and HIF-2α are thought to play critical roles during the angiogenic hypoxic response. Unlike HIF-1α, HIF-2α is mainly expressed in vascular endothelial cells. Therefore, HIF-2α is thought to regulate endothelial-specific genes, and it is considered to function differently from HIF-1α. Alternatively, the nonoverlapping expression patterns of HIF-1α and HIF-2α may contribute to mutant lethality, even if they regulate similar genes, thus revealing the limitation of gene targeting in the analysis of HIF-α function. Despite this information, however, little is known about the mechanisms underlying these functional differences.

Recently, HIF-2α (but not HIF-1α) has been shown to promote tumor growth in a renal carcinoma xenograft model (36, 37), suggesting that it plays an important and unique role in tumorigenesis. Thus, it is important to analyze the target genes of HIF-1α and HIF-2α, and to understand their individual functions. Indeed, HIF-2α was reported to transactivate endothelial-specific genes such as Tie-2 and fetal liver kinase-1 (38–40). DNA microarray analysis using renal cell carcinoma 786-O cells that exclusively express HIF-2α (but not HIF-1α) exhibited a number of hypoxia-inducible genes, including some novel hypoxia-responsive genes such as that coding for adipose differentiation-related protein (41). However, the expression profiles of genes induced by HIF-2α and the functions of HIF-2α in vascular endothelial cells have not been elucidated.

VEGF is the most important angiogenic factor and its function is essential in both embryonic vasculogenesis as well as adult angiogenesis (42, 43). However, VEGF alone is not sufficient for the assembly of a physiologically functional vasculature. Results obtained from therapeutic angiogenesis studies using VEGF demonstrated that the new blood vessels whose formation is induced by VEGF are leaky (44) and do not persist for long (45). Thus, in addition to VEGF, some other molecules and their combinations are expected to contribute to the production of functional and persistent mature blood vessels.

In this study, we attempted to identify new factors that bind HIF-2α by using a two-hybrid system screen method. Of the 28 clones selected under high-stringency conditions, 3 contained an identical 372-bp fragment encoding the N-terminal domain of Int6 (amino acids 4–128).

The int6 gene was first identified from a screen in that the mouse mammary tumor virus (MMTV) was used as an insertional mutagen to identify genes whose functions are critical for breast tumor formation (46). The MMTV insertion in mouse Int6 appears to create a C-terminal truncated protein (Int6-ΔC, shown in Fig. 1A) that is dominant negative. The overexpression of these truncated proteins can transform cells in culture, and injection of these transformed cells into nude mice can induce tumor formation (47, 48). Furthermore, human Int6 may also indirectly influence proteolysis via at least three proteins that have been identified as Int6-binding proteins, Tax (49), Ret finger protein (50), and p56 (51). Tax, a transactivator of the human T-cell leukemia virus type I binds to Int6 and stimulates the activity of the proteasome (49, 52). Ret finger protein contains a RING finger, which is widely present among ubiquitin ligases (53), and localizes to promyelocytic leukemia gene product nuclear bodies, which are proteasome-rich (49). p56 was first identified as one of the molecules whose expression is induced by interferons; it contains two ubiquitin-like domains, suggesting that it can also bind the proteasome (51). The Int6 protein has been independently identified as a subunit (eIF3e) of the eukaryotic translation initiation factor eIF3. In addition, this protein can interact with two other multisubunit complexes: the COP9 signalosome (CSN) and the proteasome (54). Nonetheless, the role of Int6 in tumorigenesis is currently unclear.

From further analysis of the specific interaction between Int6 and HIF-2α, we observed that the N-terminal region of Int6 specifically binds to the C-terminal inhibitory domain of HIF-2α, but not to that of HIF-1α or HIF-3α. However, the Int6 binding induced instability of HIF-2α in MCF-7 and HeLa cells, although the dominant negative mutant Int6-ΔC, in which the C-terminal Proteasome/Int6/Nip-1/TRIP-15 (PINT) domain is deleted (54), induced stable HIF-2α expression, even under normoxia. Unlike the mechanism of HIF-1α degradation, HIF-2α degradation was shown to depend on the Int6/proteasome pathway, but not on pVHL, which is a ubiquitin ligase. A luciferase assay using the HRE promoter plasmid also demonstrated that Int6-ΔC expression increased the stability and transcriptional activity of HIF-2α. Moreover, by using small interfering RNA (siRNA) against HIF-2α, we found that the silencing of endogenous Int6 was sufficient to induce HIF-2α expression, even under normoxia, and it enhanced the expression of angiogenic factors such as angiopoietin, fetal liver kinase-1, Tie-2, and VEGF. Taken together, our present results suggest that the novel regulation of HIF-2α by Int6 plays a critical role in the regulation of both tumorigenesis and angiogenesis.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Assay and Plasmid Construction**—We used the MATCHMAKER two-hybrid system (Clontech) as described previously (55). Because human HIF-2α autoactivates the reporter gene when fused with the DNA-binding domain, we used an HIF-2α (55) fragment from amino acids 571 to 828 to reduce background. For the two-hybrid screen, the HIF-2α fragment (amino acids 571–828) was fused to the GAL4 DNA-binding domain of the pGBKT7 yeast expression vector; pGBKT7-HIF-2α was used as bait and was transformed into strain AH109. Pretransformed human heart MATCHMAKER cDNA library also was used as a prey (Clontech).
cDNA fragments of HIF-1α, HIF-2α, HIF-3α, and HIF-2α mutants were subcloned into the pGBKTK vector and transformed into the AH109 yeast strain. The Int6 N-terminal (4–150), Int6 full-length, and the HIF-2α (571–828) fragments were amplified by PCR, and subcloned into the pGADT7 vector and transfected into strain Y187 (MATα strain). After mating of these transformed strains, the protein-protein interaction was quantified by β-galactosidase activity using o-nitrophenyl β-D-galactopyranoside as a substrate.

The int6-wt gene was generated by PCR using a human heart cDNA library and subcloned into the pcDNA3-HA or pcDNA3-Myc vectors (Invitrogen). Int6-ΔC (amino acids 1–326), a C-terminal-truncated Int6, and other truncated mutants indicated in the figures were produced by PCR with pcDNA3-HA-Int6 as the template. For expression of HIF-2α, we subcloned the open reading frame cDNA into the pcDNA3-Myc vector.

Vector Constructs for Expressing int6-siRNA—The int6-siRNA constructs were prepared according to the manufacturer’s instructions. Briefly, four sequences were used: human int6-siRNA219, 5’-AAGAACCACAGTGGTTGCA-3’ (nucleotides 219–236); human int6-siRNA358, 5’-AAGCATGTGTTTTAGGCAAGG-3’ (nucleotides 358–376), and a green fluorescent protein sequence or non-coding siRNA as the controls. These siRNAs were individually expressed under the control of the U6 promoter in the pSilencer vector (Ambion).

Cell Culture, Transfection, and Reporter Assays—MCF-7 (ATCC) and HeLa cells (ATCC) were routinely grown at 37 °C and 5% CO₂ in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), 50 units/ml penicillin G, and 50 μg/ml streptomycin (Invitrogen). Transient transfection into 40–60% confluent MCF-7 and HeLa cells was performed by calcium/phosphate precipitation.

Immunofluorescence Staining—Monoclonal and polyclonal antibodies were generated using peptide sequences, and the whole cell lysate was assayed for the protein concentration by using the DC protein assay (Bio-Rad). Total protein (50 μg/sample) was separated on 4–20% SDS-PAGE gels, transferred to polyvinylidene fluoride membranes, and blocked overnight with 5% skim milk in phosphate-buffered saline. The primary antibodies were incubated at room temperature for 1 h. Horseradish peroxidase-conjugated anti-rabbit or -mouse IgG (Pierce) secondary antibodies were incubated at room temperature for 1 h, and then washed 6 times with phosphate-buffered saline containing 0.1% Tween 20.

Immunoprecipitation assays were performed using a modified protocol from Sigma. Transfected or nontransfected HeLa cells, MCF-7 cells, or 786-O cells were grown in 21% O₂ and lysed with RIPA buffer. Following sonication, the whole cell lysate was centrifuged at 15,000 × g for 45 min. The supernatant was incubated overnight at 4 °C with anti-HA-conjugated agarose (Sigma), or anti-Int6 monoclonal antibody or anti-Bax monoclonal antibody (2D2; Sigma) with protein G-Sepharose beads (Amersham Biosciences), on an orbital shaker, and then washed four times with RIPA buffer. Immunoprecipitates were boiled in 2 × SDS sample buffer for 10 min. Coimmunoprecipitated HIF-2α was analyzed by Western blotting with anti-Myc polyclonal and anti-HIF-2α monoclonal antibody, and then visualized with the SuperSignal West Pico chemiluminescence detection system (Pierce).

Real Time-PCR—Real time-PCR was performed as described previously (55). To measure the angiogenic factors, HeLa cells were transfected with pSilencer 2.1-U6-int6-siRNA219 and pSilencer 2.1-U6 negative control under normoxia or hypoxia. Total RNA was isolated from transfected cells by using IsoGen.
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The first-strand DNA was synthesized using SuperScript First-strand Synthesis (Invitrogen). The primers and probes were selected from the TaqMan gene expression assay inventories (Applied Biosystems). Quantitative real-time PCR was performed by using the ABI PRISM7000 Sequence Detection System (Applied Biosystems). The DNAs were heated for 2 min at 50 °C and 10 min at 95 °C before the PCR that consisted of 40 cycles at 95 °C for 15 s and 65 °C for 1 min. A standard curve for VEGF, and bFGF was calculated in relation to a master reference by using standard curves.

RESULTS

Identification of Int6 (eIF3e/p48) as an HIF-2α-specific Interacting Protein— The activities of both HIF-1α and HIF-2α are controlled by the hydroxylation of key proline and asparagine residues present in the conserved oxygen-dependent degradation sequence and transactivation domain (TAD) that regulate protein stability and transactivation potential, respectively (57) (Fig. 1A). To investigate differential HIF-α regulation, yeast two-hybrid analysis was performed using the C-terminal region of HIF-2α (amino acids 571–828) as bait; this region represents the region of greatest sequence divergence between the HIF-α proteins and is devoid of autoactivity (55).

From a total of 1.2 × 10⁶ cDNA clones in the initial screen of the human heart cDNA library, 326 were positive clones when assayed for β-galactosidase expression for the first screen. An N-terminal Int6 fragment (amino acids 4–128, accession number U94175) was identified in 3 of the 28 clones in a high-stringency screening. Int6 has been previously identified as eIF3e/p48, which is one of the translational initiation factors (54). The dominant negative mutant, Int6-ΔC, lacked the C-terminal PINT domain (Fig. 1A) that has been reported to be generated by MMTV integration. Overexpression of this truncated protein transforms cells in culture, and the injection of these cells into nude mice induces tumor formation (47, 48).

To determine the binding specificity of Int6 to HIF-α subtypes or regions, we performed a β-galactosidase assay by using DNA-binding domain fusions with the C-terminal domains of HIF-1α (amino acids 601–826), HIF-2α (amino acids 571–828), and HIF-3α (amino acids 506–662). The Int6 fragment bound to HIF-2α, but neither to HIF-1α nor HIF-3α; this indicated subtype specificity (Fig. 1B).

We further characterized the interaction between Int6 and HIF-2α to determine the essential binding domains of both proteins. Fig. 1C shows the relative strength of the interactions between the N-terminal of Int6 and truncated HIF-2α in yeast that were analyzed by a quantitative β-galactosidase assay. The N-terminal of Int6 (Int6-N; amino acids 4–128) retained its β-galactosidase activity in HIF-2α constructs that contained amino acids 571–828, 641–828, and 571–700. Constructs with amino acids 701–828 exhibited no activity. From these results, the region containing amino acids 571–700 of HIF-2α appeared to be the minimum region required for Int6 binding.

Finally, to determine the region within Int6 that is essential for HIF-2α binding, we constructed a vector containing the activated domain fused to the HIF-2α fragment (amino acids 571–828) (indicated in Fig. 1D) and introduced this into yeast along with a vector expressing the DNA-binding domain fused to Int6 mutant fragments. The C-terminal HIF-2α fragment also binds the N-terminal region (amino acids 4–128) of Int6 (Fig. 1D). The N-terminal region of Int6 contains a possible nuclear export signal sequence, suggesting that the specific binding modulates the cellular localization or HIF-2α transcriptional activity. These data demonstrate that in yeast, Int6 binds specifically to HIF-2α, and the minimum contact sites include amino acids 4–128 of Int6 and amino acids 571–700 of HIF-2α.

Int6 Associates with HIF-2α, and the N-terminal Activation Domain of Int6 Increases HIF-2α Activity Independent of Hypoxia—The localization of Int6 in mammalian cells varies among cell types and tissues (50). HIF-α family members are expressed in the cytosol and translocated into the nucleus in...
**FIGURE 2.** Subtype-specific interaction of Int6 with HIF-2α, and the dominant negative mutant Int6-DΔC promotes HRE-driven transcriptional activation even under normoxia. A, colocalization of HA-Int6-wt with Myc-HIF-2α-wt was detected in MCF-7 cells by immunofluorescence analysis under normoxia as described previously (55). Cytoplasmic localization of HA-Int6-wt changed to nuclear following coexpression with Myc-HIF-2α; both proteins were expressed in the nucleus. B, coimmunoprecipitation of Int6 mutants and HIF-2α. Lysates from cells cotransfected with pCDNA3-Myc-HIF-2α and pCDNA3-HA-Int6-wt (lane 1), pCDNA3-HA-Int6-DΔC (lane 2, amino acids 1–326), pCDNA3-HA-Int6-N (lane 3, amino acids 1–150), or pCDNA3-HA-Bax (lane 4) were immunoprecipitated (IP) with anti-HA-conjugated agarose (middle panel, input), and detected by anti-HA antibody on Western blots. Coimmunoprecipitated (Co-IP) Myc-HIF-2α was detected by Western blotting with anti-Myc polyclonal antibody (top panel). Bottom panel shows expression of Myc-HIF-2α (input) in whole cell lysate (whole) of cotransfected cells. C, coimmunoprecipitation of endogenous Int6 and HIF-2α in HeLa (lane 1), MCF7 (lane 2), and 786-O cell lines (lane 3). Whole lysates were immunoprecipitated with Int6-monoclonal antibody (second panel, lanes 1–3), and Bax-monoclonal antibody (2D2, third panel, lane 4), and coimmunoprecipitated HIF-2α with Int6 or Bax was analyzed by Western blotting with anti-HIF-2α polyclonal antibody (top panel). Bottom panel shows expression of endogenous HIF-2α (Endo-HIF-2α) of whole cell lysates (whole). D, HRE-driven luciferase activity was measured in HeLa cell lysates in which Myc-HIF-1α (lanes 1–3), Myc-HIF-2α (lanes 4–6), or Myc-HIF-3α (lanes 7–9) were cotransfected under normoxic (open columns) or hypoxic conditions (closed columns) for 14 h with the control vector (lanes 1, 4, and 7), HA-Int6-wt (lanes 2, 5, and 8) or HA-Int6-DΔC (lanes 3, 6, and 9). The asterisks (*) indicate specificity (p < 0.001, t test) with error bars.

A hypoxia-dependent manner (58). In contrast to the expression of HIF-1α, that of HIF-2α is not regulated strictly by O2 concentration (26, 59). To confirm the Int6/HIF-2α interaction in mammalian cells, MCF-7 cells were transfected with a plasmid expressing Myc-HIF-2α and/or HA-Int6 and visualized by immunofluorescence staining. As shown in Fig. 2A, our observations of the cultured cells indicated the nuclear localization of endogenous and overexpressed HIF-2α even under normoxia (upper left). Int6 was expressed in both the cytoplasm and the nucleus under normoxia (Fig. 2A, upper right). In coexpression, both proteins were clearly colocalized in the nucleus even under normoxia (Fig. 2A, lower panels). Thus, the Int6/HIF-2α interaction depends on HIF-2α expression, but not hypoxia, and Int6 may function in the nucleus after its translocation with HIF-2α from the cytoplasm.

Cells were cotransfected with Myc-HIF-2α and HA-Int6-wt (Fig. 2B, lane 1), HA-Int6-truncated mutants (amino acids 1–326: Fig. 2B, lane 2, amino acids 1–150, and lane 3), and that it induced HIF-2α degradation or reduced the viability of the transfected cells by chromosome instability or aberrant mitosis (60).

Association with Int6, but Not Int6-DΔC, Increases HIF-2α Instability—Cells tolerated the overexpression of Int6-DΔC and/or HIF-2α well; however, Int6-wt coexpression suppressed HIF-2α expression and reduced cell viability. Therefore, it was important to determine whether the binding affects the stability of HIF-2α. In HeLa cells (as well as in MCF-7 cells; data not shown), the coexpression of Int6-wt reduced HIF-2α expression (Fig. 3A, lanes 3 and 4), whereas the dominant negative mutant Int6-DΔC increased HIF-2α expression by 2- and 5-fold under normoxia (N) and hypoxia (H), respectively (Fig. 3A, lanes 5 and 6). To confirm the interaction between the Int6/Int6-DΔC and HIF-2α on the endogenous protein level of HeLa cells, we transfected the Int6 and Int6-DΔC plasmids into cells and harvested the whole cell lysate for Western blotting. We found that Int6-wt reduced the expression of endogenous HIF-2α not only under normoxia (Fig. 3B, lane 2), but also
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FIGURE 3. Int6-wt, but not Int6-ΔC, promotes hypoxia-independent HIF-2α degradation. A, to evaluate HIF-2α stability by Western blotting, the control vector (lanes 1 and 2), Int6-wt (lanes 3 and 4), and the Int6-ΔC mutant (lanes 5 and 6) were cotransfected with HIF-2α into HeLa cells under normoxic (N: 21% O2) and hypoxic conditions (H: 2% O2) for 16 h. The expressed HIF-2α, Int6, and α-tubulin (endogenous marker) are shown in the upper, middle, and lower panels, respectively. B, for the evaluation of endogenous HIF-2α (Endo-HIF-2α) expression by Western blotting, whole cell lysates were transfected with control vector (lanes 1 and 4), Int6 (lanes 2 and 5), and the Int6-ΔC mutant (lanes 3 and 6) into HeLa cells under normoxic (21% O2) and hypoxic conditions (2% O2) for 16 h. The expressed Endo-HIF-2α, HA-Int6, HA-Int6-ΔC, and α-tubulin (endogenous marker) are shown in the upper, middle, and lower panels, respectively. C, Myc-HIF-2α was transfected into HeLa cells with the control vector (lanes 1 and 2) or the indicated amounts (μg) of the HA-Int6-wt plasmid (lanes 3–8) under normoxic or hypoxic conditions. The expressed Myc-HIF-2α and control tubulin are shown in the upper and lower panels, respectively.

FIGURE 4. HIF-2α degradation is sensitive to proteasome activity in 789-O cells that lack endogenous pVHL expression. A, HeLa cells were cotransfected with the Myc-HIF-2α and HA-Int6-wt plasmids and incubated for 6 h with the proteasome inhibitors, MG132 (10 μM, lane 3) or MG101 (100 μM, lane 4), or the buffer alone (lane 2). Lane 1 shows control Myc-HIF-2α expression. B, cotransfection of Myc-HIF-2α (upper panel) and HA-Int6-wt in dose-dependent concentrations (middle panel) into renal carcinoma cells (786-O) that lack endogenous pVHL expression. Overexpressed Myc-HIF-2α was detected. C, transfection with HA-Int6-wt in dose-dependent concentrations (middle panel) into 786-O cell lines. Endogenous HIF-2α (Endo-HIF-2α) was detected (upper panel).

under hypoxic conditions (Fig. 3B, lane 5), whereas Int6-ΔC in particular enhanced the expression of endogenous HIF-2α more under hypoxia than under normoxia (Fig. 3B, lanes 3 and 6).

To further examine the dose-dependent effect of Int6, we transfected 1 μg of vectors expressing HIF-2α with Int6-wt at the indicated amount (adjusted to 2 μg by the addition of control vector) into cells. We observed no significant decrease in HIF-2α expression when the cells were transfected with a small amount of Int6 plasmid (0.25 μg) (Fig. 3C, lanes 3 and 4). Higher Int6 expression clearly suppressed HIF-2α expression (0.75 μg; lanes 5 and 6), and cotransfection with Int6 and HIF-2α at a 1:1 ratio completely suppressed HIF-2α expression under both normoxia and hypoxia (1.0 μg; lanes 7 and 8, respectively). These results demonstrated that the suppression of HIF-2α expression depends on Int6 expression, but not on O2 concentration.

Int6-induced Degradation of HIF-2α Is Sensitive to Proteasome Activity, but Is Independent of pVHL—Int6 indirectly influences proteolysis via Ret finger protein (50), Tax (49), and p56 (51). In addition to the proteasome, Int6 also associates with the CSN and 26 S proteasome through the PINT domain (54, 61); this suggests that the Int6/HIF-2α interaction induces proteolysis via the ubiquitin/proteasome pathway. HIF-2α degradation was induced by the coexpression of Int6-wt (Fig. 4A, lane 2), and was partially inhibited by a 6-h treatment with proteasome inhibitors, MG132 (10 μM; lane 3) or MG101 (100 μM; lane 4) (Fig. 4A). pVHL is a key component of the complex that regulates HIF-1α turnover (11, 41). Furthermore, it mediates the ubiquitination of HIF-1α and HIF-2α, resulting in their rapid degradation via the ubiquitin-proteasome pathway under normoxia (16). To examine pVHL involvement in HIF-2α degradation by Int6, we used the renal
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The maximum silencing effect (>90%) was observed with the pSilencer vector that contained the Int6 target sequence (int6-siRNA219; nucleotides 219–236) in HeLa cells that overexpressed Int6-ΔC (Fig. 5A, upper panels). By using the same pSilencer vectors, the expression of cotransfected HIF-2α was stabilized by the silencing of endogenous Int6 even under normoxia (Fig. 5A, lower panels). Int6-ΔC expression was decreased by cotransfection of pSilencer int6-siRNA219 in an expression dependent manner (Fig. 5B) under both normoxia (N) and hypoxia (H). In determining how the pSilencer int6-siRNA219 effects endogenous HIF-2α expression under normoxic conditions, we found that endogenous HIF-2α expression was inversely correlated with that of endogenous Int6 (Fig. 5C), suggesting that the expression of HIF-2α is suppressed by Int6 independent of O2 concentration. Silencing Int6 eliminated its suppressive effect.

To confirm the effect of Int6 on the translation of HIF-2α, we performed an HRE-driven luciferase assay in HeLa cells that were cotransfected with a control vector (Fig. 5D, lanes 1 and 4), pSilencer int6-siRNA219 (lanes 2 and 5), or pSilencer int6-siRNA358 (lanes 3 and 6). The effect was further evaluated by cotransfecting HIF-2α with the previously mentioned vectors (lanes 4–6) under normoxia (white columns) and hypoxia (black columns). The results demonstrated that silencing of the Int6 by two different siRNAs increased HIF-2α activity even under normoxia (lane 2), and that the activity was considerably greater in cells that overexpressed HIF-2α (lanes 5 and 6). The activity under hypoxia was 2-fold higher than that under normoxia, clearly suggesting that HIF-2α expression and activity are regulated by pathways involving both Int6 and pVHL.

Silencing of Int6 Induces Angiogenic Factors through HIF-2α Activation—Although the transcription-activating properties of HIF-2α were very similar to those reported for HIF-1α, both are critical mediators for the inductive expression of VEGF, erythropoietin, and various glycolytic enzymes (21, 22). VEGF is the most important angiogenic factor, and its function is essential in embryonic vasculogenesis as well as adult angiogenesis (42, 43). Several research groups have generated HIF-2α knock-out mice and reported that HIF-2α knock-out resulted in the generation of different phenotypes. These results demonstrated that HIF-2α plays an essential role in vascular remodeling during embryonic development (32), catecholamine homeostasis (33), and fetal lung maturation (34).
Thus, HIF-1α and HIF-2α are thought to play critical roles during the angiogenic hypoxic response.

Our results revealed that the silencing of Int6 by pSilencer-siRNA219 induced selective expression and activation of HIF-2α, and that this effect may be useful for evaluating the differences between the roles played by HIF-1α and HIF-2α in tumor formation or angiogenesis. To evaluate the effect of HIF-2α/Int6 in angiogenesis, we transfected cells with pSilencer expressing Int6-siRNA219, which showed the strongest effect to suppress endogenous Int6 expression. We subsequently measured the mRNAs of several angiogenic factors, as well as HIFs, by quantitative RT-PCR using specific probes. We also detected the protein expressions of endogenous HIF-1α, HIF-2α, and Int6 by Western blotting.

First, we measured the mRNA of Int6, HIF-1α, and HIF-2α (Fig. 6A). Int6 was effectively silenced by both siRNA219, and this induced the expression of HIF-2α mRNA, but not HIF-1α mRNA. Under the same conditions, the expression of three major angiogenic factors, angiopoietin 1, VEGF, and FGF2 (bFGF), was induced with different patterns in cells overexpressing int6-siRNA219 under normoxia (white columns) or hypoxia (black columns) (Fig. 6B). Two angiogenic factors were induced under normoxia: angiopoietin 1 (2–2.5-fold higher than that under hypoxia) and bFGF (2–3-fold higher than that under hypoxia); however, VEGF induction was weak. On the other hand, VEGF was induced to a greater extent under hypoxia; this suggests that VEGF is regulated mainly by HIF-1α. However, VEGF alone is not sufficient to assemble physiologically functional vasculature. The results obtained from therapeutic angiogenesis studies using VEGF demonstrated that the new blood vessels whose formation is induced by VEGF are leaky (44).

The endogenous expression of HIF-1α protein was markedly increased under hypoxia (Fig. 6C, lanes 3 and 4), but its up-regulation does not appear to be correlated with endogenous Int6 protein (Fig. 6C). However, we found that int6-siRNA219 enhanced the endogenous expression of HIF-2α under both normoxia (Nor) and hypoxia (Hyp) (Fig. 6C, lanes 2 and 4). These data suggest that int6-siRNA could enhance the protein and mRNA levels of HIF-2α irrespective of the oxygen supply. In respect to HIF-1α, int6-siRNA did not change its mRNA and protein levels, and the HIF-1α enhancement only took place after hypoxia. Hence, the increase of HIF-2α mRNA is a primary or secondary effect of HIF-2α induction caused by Int6 silencing, because the HIF-2α was capable of inducing its own expression via autoregulatory mechanisms (63). Further investigations are, however, necessary to elucidate the exact mechanisms responsible for these observations. These results suggest that the decrease in endogenous Int6 can induce several angiogenic factors, such as angiopoietin, Tie-2, and bFGF, and might result in angiogenesis and cancer formation.

**DISCUSSION**

In this study, we performed yeast two-hybrid and luciferase binding assays that demonstrated that Int6 differentially interacts with HIF-2α, but does not bind to HIF-1α and HIF-3α. The minimum contact sites include amino acids 4–128 of Int6 and amino acids 571–700 of HIF-2α. Furthermore, we demonstrated that this interaction has a functional consequence. First, the normoxic and hypoxic activities of HIF-2α decreased in a dose-dependent manner following Int6-wt overexpression; second, transfection of the dominant negative mutant Int6-ΔC stabilized HIF-2α protein expression and increased HIF-2α-dependent reporter gene activity, indicating that endogenous Int6 contributes to HIF-2α activity. This is consistent with a model in which the Int6-wt is able to bind HIF-2α and induce its instability through the ubiquitin/proteasome pathway. Proteasome inhibitors, MG132 and MG101, effectively protected HIF-2α from degradation by Int6. Furthermore, Int6-induced degrada-
tion of HIF-2α was observed in 786-O cells that lack endogen-
ous pVHL expression. The interaction of Int6 with HIF-2α may therefore contribute to a physiologically important nor-
moxic role, given that the silencing of endogenous Int6 by
siRNA expression specifically increases HIF-2α activity. Several
angiogenic factors such as VEGF, angiopoietin 1, and angiopi-
etin 2, are effectively induced by the silencing of endogenous
Int6. It is therefore necessary to gain an understanding of the
mechanisms by which the silencing of Int6 triggers the stabili-
zation of HIF-2α and transactivates the HIF-2α-dependent
induction of these factors.

The Int6 protein was independently identified as a subunit
e(lFl3ε) of the eukaryotic translational initiation factor elF3
(54). This protein is known to interact with two other multisub-
unit complexes: the CSN and the proteasome. However, the
role of Int6 in protein degradation is unclear. Our results indi-
cated a novel function of Int6, it directly binds to HIF-2α inde-
pendent of pVHL and causes its degradation via the ubiquitin-
proteasome pathway. Int6 has no clear homology with the
ubiquitin ligase family members, although the coexpression of
Int6 induced ubiquitination (data not shown). The mechanism
by which Int6 induces HIF-2α degradation probably involves
the binding of Int6 to Tax (49), Ret finger protein (50), and p56
(51), or an unknown ubiquitin ligase or the direct trapping of
the HIF-2α/Int6 complex into proteasomes; this is because the
C-terminal PINT domain of Int6 is reported to be a possible
adaptor for binding proteins such as Rpn5, a component of the
lid portion of the proteasome (46). These results reveal which
pathway (pVHL and/or Int6) is critical for the regulation of
HIF-2α stability. HIF-2α expression has been observed in many
cell lines and even normal tissues under normoxia. However, it
is considerably higher under hypoxia than under normoxia,
suggesting that HIF-2α degradation is regulated independently
by both proteins. Indeed, the yeast two-hybrid binding assays
demonstrated that HIF-2α had different binding sites for pVHL
and Int6, and that the binding was non-competitive. The asso-
ciation of pVHL with HIF-2α triggers ubiquitin-dependent
HIF-2α degradation in a hypoxia-dependent manner. However,
HIF-2α degradation by Int6 was independent of the O2 concen-
tration and was observed even in 786-O cells that lack endoge-
nous pVHL expression. These results clearly demonstrate that
Int6 association decides the fate of HIF-2α stability, independ-
ent of hypoxia and pVHL. These results are also confirmed by
several clinical reports. The Int6 expression in breast and lung
cancers is inversely correlated with angiogenesis and tumor-
genesis (64), suggesting that Int6 transcriptional regulation
plays a major role in the regulation of HIF-2α function.

The different roles of HIF-1α and HIF-2α in cancer forma-

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