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Drosophila von Hippel-Lindau Tumor Suppressor Complex Possesses E3 Ubiquitin Ligase Activity

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Mutations of the von Hippel-Lindau (VHL) tumor suppressor gene predispose individuals to a variety of human tumors, including renal cell carcinoma, hemangioblastoma of the central nervous system, and pheochromocytoma. Here we report on the identification and characterization of the *Drosophila* homolog of VHL. The predicted amino acid sequence of *Drosophila* VHL protein shows 29% identity and 44% similarity to that of human VHL protein. Biochemical studies have shown that *Drosophila* VHL protein binds to Elongins B and C directly, and via this Elongin BC complex, associates with Cul-2 and Rbx1. Like human VHL, *Drosophila* VHL complex containing Cul-2, Rbx1, Elongins B and C, exhibits E3 ubiquitin ligase activity. In addition, we provide evidence that hypoxia-inducible factor (HIF)-1α is the ubiquitination target of both human and *Drosophila* VHL complexes.

Key Words: **VHL; Elongin; cullin; ubiquitin ligase; E3; hypoxia-inducible factor.**

The VHL tumor suppressor gene is mutated in the majority of sporadic clear-cell renal carcinomas and in VHL disease, a hereditary cancer syndrome characterized by the development of various tumors including renal cell carcinoma, retinal angioma, hemangioblastoma of the central nervous system, and pheochromocytoma (1, 2). VHL protein is expressed in all tissues and appears to perform multiple functions, including regulation of transcription elongation (3, 4), repression of hypoxia-inducible genes (5–7), and regulation of the fibronectin matrix assembly (8).

VHL protein forms a complex with B and C subunits of the transcription elongation factor Elongin (referred to as the VBC complex) (3, 9–11). Recently, the VBC complex has been shown to interact with Cul-2 and Rbx1 to form a multiprotein complex, VBC · Cul-2 · Rbx1 (12–14). Interestingly, Elongin C and Cul-2 share sequence similarity with the Skp1 and Cdc53 components of the SCF (Skp1/Cdc53/F-box protein) E3 ubiquitin ligase (13), and Rbx1 is also an integral component of the SCF complex (14). The structural analogy between the VBC · Cul-2 · Rbx1 complex and the SCF complex suggests that the former functions as an E3 ubiquitin ligase. Indeed, both endogenous VHL complex purified from cell extracts and recombinant VBC · Cul-2 · Rbx1 complex purified from insect cells have been shown to possess E3 ubiquitin ligase activity, although the ubiquitination target was not identified (15, 16).

In this paper, we describe the identification of *Drosophila melanogaster* (dm) VHL and show that the multiprotein complex containing dm VHL possesses E3 ubiquitin ligase activity. Furthermore, we provide evidence that HIF-1α is the ubiquitination target of both human and dm VHL complexes.

MATERIALS AND METHODS

Materials. Sources of materials are as follows: ubiquitin (Ub)-aldehyde (MBL); bovine Ub, creatine kinase (Sigma); creatine phosphate (Calbiochem); mouse monoclonal anti-ubiquitin (Babco); goat polyclonal anti-HIF-1α (Santa Cruz); mouse monoclonal anti-FLAG (M2) (Sigma); mouse monoclonal anti-HA (12CA5), anti-MYC (9E10), and anti-HPC4 (Roche); mouse monoclonal anti-HSV (Novagen). Recombinant E1 enzyme was purified from Sf9 insect cells infected with baculovirus containing histidine-tagged E1 from *Arapidopsis thaliana* (provided by W. Krek). Recombinant E2 (UbcH5b) was expressed in *E. coli* using pRSET B vector (Invitrogen).

Isolation of Drosophila VHL gene. A homology search of the GenBank database using human VHL protein sequence as the query revealed that nucleotides 92853–93386 of *Drosophila melanogaster* P1 clones DS00724 and DS00284 contained a predicted open reading frame (ORF) encoding a protein highly homologous to portions of the VHL protein. A DNA fragment of 534 bp encoding the full-length

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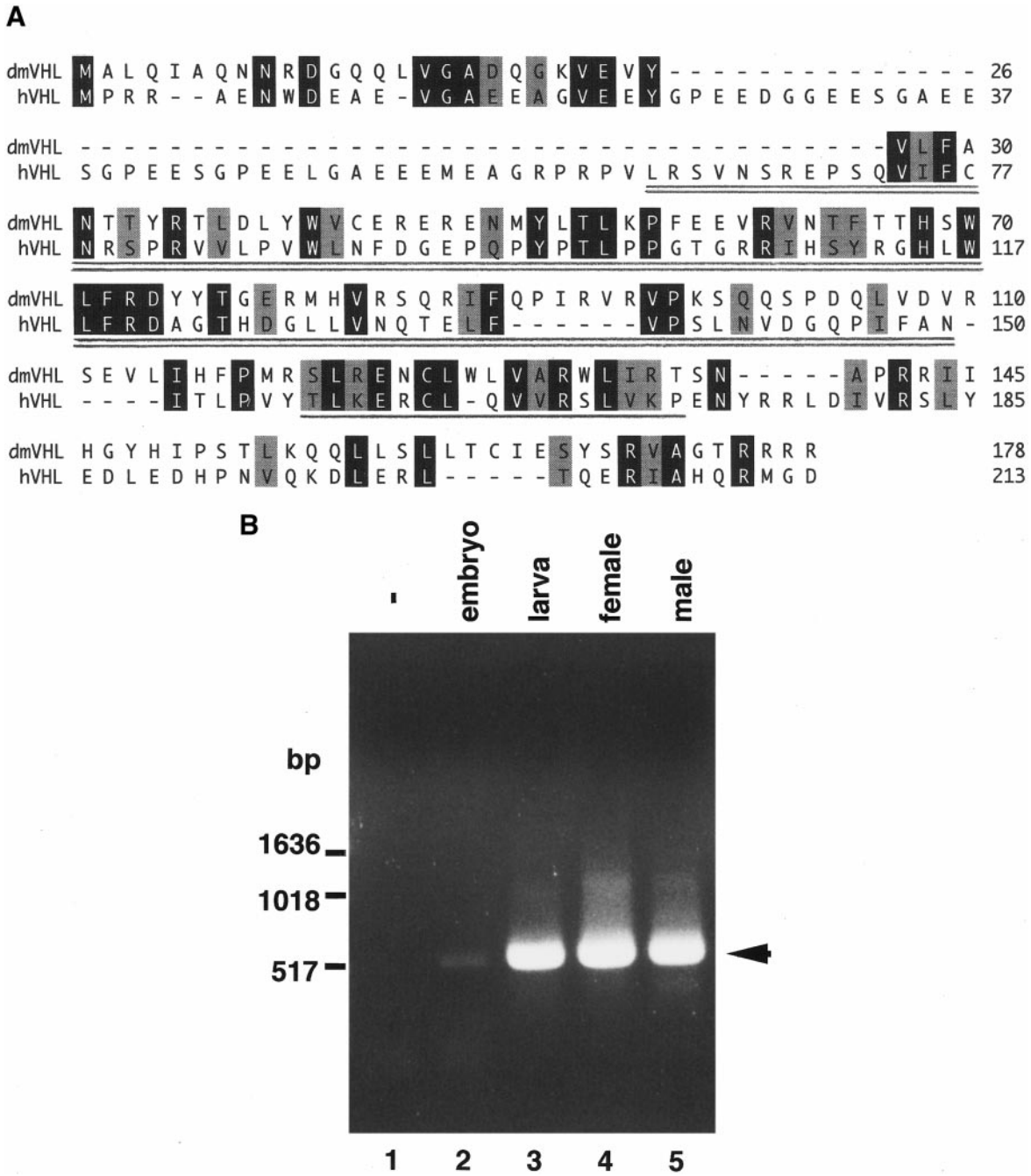

FIG. 1. (A) Comparison of the deduced amino acid sequences between *Drosophila* (dm) and human (h) VHL proteins. Identical amino acids are shown in white letters on a black background and chemically similar amino acids are shown in black letters on a gray background. The region required for binding to the Elongin BC complex and the β-sheet domain of VHL protein are indicated by single and double underlines, respectively. Numbers indicate amino acid position in each protein. (B) Analysis of expression of the VHL gene during *Drosophila* development by RT-PCR. Arrowhead indicates position of the PCR product stained with ethidium bromide. The size of the DNA standards is indicated on the left.

Drosophila homolog of the VHL protein was amplified by PCR using oligonucleotide primers 5′-ATGGCGCTCCAAATAGCGCAGAAC-3′ and 5′-ACGCCGACGTCGCGTGCCCGC-3′.

RT-PCR. Poly(A)$^+$ RNA was isolated from embryos, third instar larvae, and adult flies using the QuickPrep Micro mRNA Purification kit (Amersham Pharmacia Biotech) as described previously (17). mRNA was reverse-transcribed using the First-strand cDNA synthesis kit (Amersham Pharmacia Biotech) with a NotI site-flanked oligo-(dT) primer (5′-AACTGGAAGAATTCGCGGCCGCAGGAAT-TTTTTTTTTTTTTTTTT-3′) (Amersham Pharmacia Biotech) and then PCR reaction was performed using sense primer 5′-ATGGCGCTCCAAATAGCGCAGAAC-3′ and antisense primer 5′-

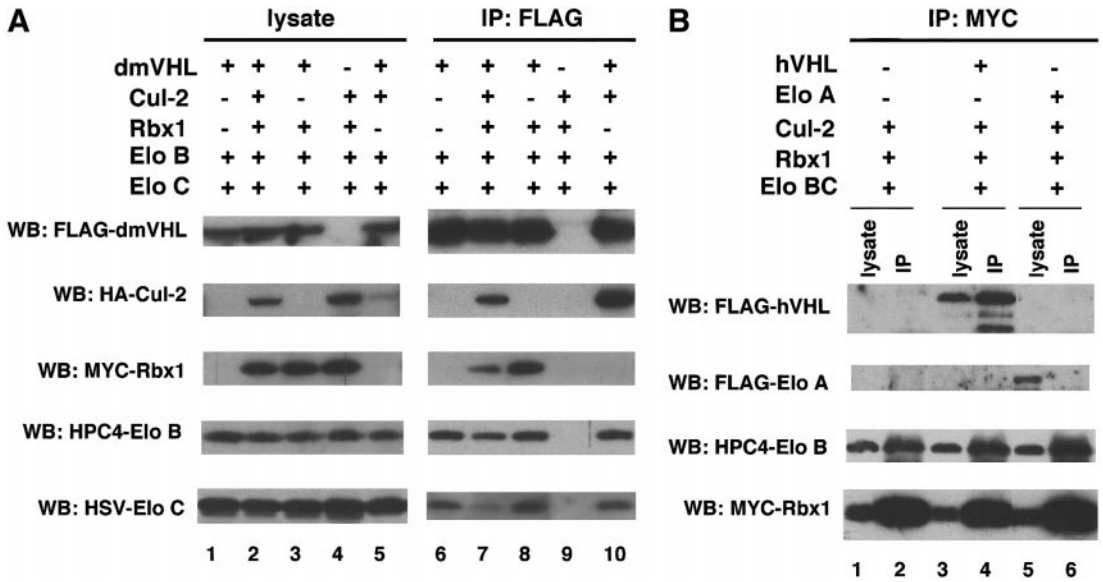

FIG. 2. *Drosophila* VHL protein, but not Elongin A, associates with Cul-2 and Rbx1 in the presence of Elongins B and C. Lysates from Sf9 cells expressing the indicated baculoviruses were immunoprecipitated with anti-FLAG (A) or anti-MYC (B), respectively. Immunoprecipitated proteins were detected by Western blotting using the indicated antibodies.

ACGCCGACGTCGCGTGCCCGC-3′. The PCR product was size-fractionated by electrophoresis in 2% agarose gel.

Plasmid construction and expression of recombinant proteins in Sf9 cells. *Drosophila* VHL containing amino-terminal 6-histidine and FLAG tags, and human HIF-1α containing amino-terminal 6-histidine and HA tags were subcloned into pBacPAK8, and recombinant baculoviruses were generated using the BacPAK baculovirus expression system (Clontech). The baculovirus vectors encoding FLAG-tagged human VHL (14), HA-tagged human Cul-2 (14), Myc-tagged mouse Rbx1 (14), HPC4-tagged human Elongin B (14), HSV-tagged human Elongin C (14), and FLAG-tagged rat Elongin A (18) were described previously. Sf9 cells were cultured in Grace's insect medium (GIBCO) supplemented with 10% fetal bovine serum at 27°C and infected with the indicated recombinant baculoviruses. Seventy hours after infection, the cells were collected and lysed as described previously (18). The cell lysates were then centrifuged at 10,000*g* for 20 min and the supernatants were used for immunoprecipitation and protein purification. Human HIF-1α with 6-histidine and HA tags was purified consecutively using Ni^{2+}-agarose (Invitrogen) and anti-HA conjugated agarose beads (Roche).

Immunoprecipitation and Western blotting. Baculovirus-infected cell lysates were incubated with an appropriate antibody for 1 h at 4°C and then with protein A-Sepharose CL-4B for 1 h, or incubated with anti-FLAG M2 conjugated agarose beads (Sigma) for 2 h at 4°C. The beads were washed four times with buffer containing 40 mM Hepes-NaOH (pH 7.9), 150 mM NaCl, 1 mM DTT, 0.5% Triton X-100, and 10% glycerol. Immunoprecipitated proteins were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to a polyvinylidene difluoride membrane (Millipore), and visualized by Western blotting using a chemiluminescence reagent (NEN).

In vitro ubiquitination assay. Lysates from Sf9 cells (1×10^6) infected with the indicated baculoviruses were immunoprecipitated with either 3 μg of anti-FLAG antibody and 10 μl of protein A-Sepharose or 10 μl of anti-FLAG conjugated agarose beads. The beads were mixed with 100 ng of E1, 200 ng of UbcH5b, 10 μg of ubiquitin, and 0.5 μg of ubiquitin aldehyde in a 15 μl reaction containing 20 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 2 mM DTT, 2 mM ATP, and an ATP-regenerating system (10 mM creatine phosphate, 10 μg of creatine phosphokinase). For ubiquitination of HIF-1α, approximately 50 ng of purified HIF-1α was added to each reaction. Reaction mixtures were incubated for 60 min at 37°C. Fifteen microliters of sample buffer was added to stop the reaction. Samples were boiled for 10 min, separated by SDS–PAGE, and processed for Western blotting with the indicated antibody.

RESULTS AND DISCUSSION

Isolation of Drosophila VHL gene. In an effort to identify model systems with which to study the function of VHL protein *in vivo,* we are identifying and characterizing homologs of VHL in genetically tractable organisms. A TBLASTN search of the GenBank database using human VHL as the query sequence identified a predicted *Drosophila melanogaster* ORF encoding a potential VHL homolog. A genomic DNA fragment containing dm VHL cDNA sequence was obtained by PCR. The dm VHL cDNA contained an ORF encoding a protein of 178 amino acids with a calculated molecular mass of 21,241 Da (Fig. 1A). Comparison of the predicted amino acid sequences between human VHL and dm VHL homolog revealed that the two proteins are 29% identical and 44% similar over a 158 amino acid overlap. Notably, the dm VHL homolog exhibited the greatest similarity to human VHL residues 157–174, which include the region critical for binding to the Elongin BC complex, and are frequently mutated in VHL kindreds and sporadic renal cell carcinomas (1, 9, 19). The β-sheet domain of human VHL also shares significant sequence similarity to that of dm VHL protein. This domain is believed to be responsible for the recognition of as yet unidentified proteins (19).

To assess the expression pattern of the dm VHL gene, RT-PCR was carried out using Poly(A)$^+$ RNA

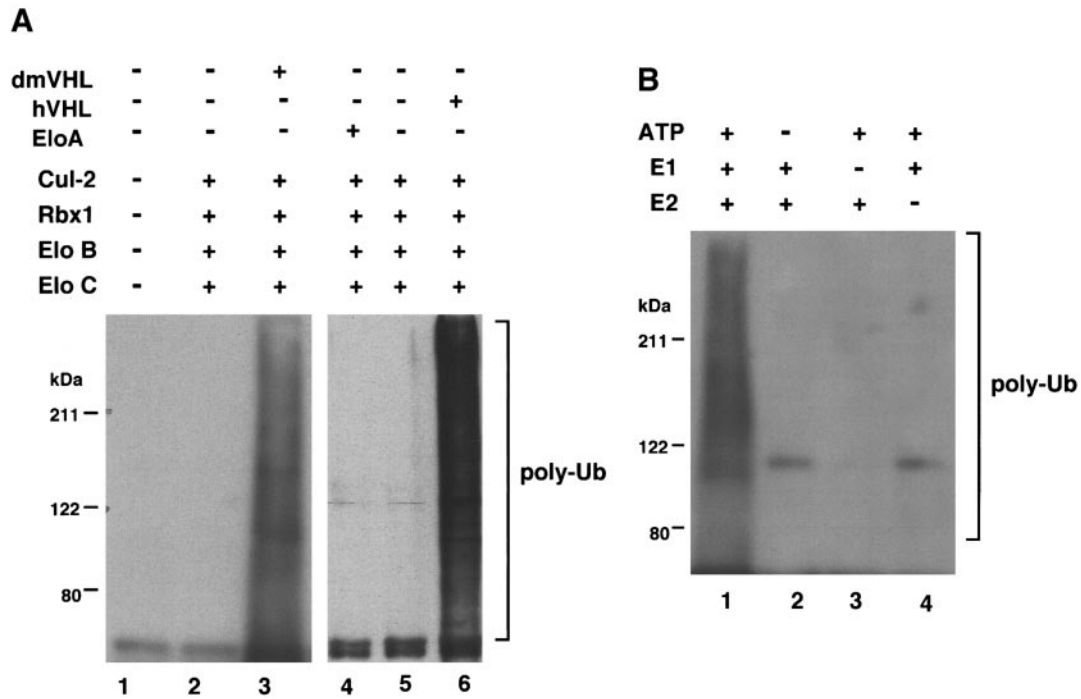

FIG. 3. The multiprotein complex containing *Drosophila* VHL protein, but not Elongin A, possesses E3 ubiquitin ligase activity. (A) Lysates from Sf9 cells expressing the indicated baculoviruses were immunoprecipitated with anti-FLAG antibody and were subjected to *in vitro* ubiquitination assay as described under Materials and Methods. Samples were analyzed on 8% SDS–PAGE and processed for Western blotting with anti-ubiquitin antibody. (B) Lysates containing *Drosophila* VHL, Cul-2, Rbx1, and Elongins B and C were immunoprecipitated with anti-FLAG antibody and were subjected to ubiquitination assay as described in Materials and Methods, omitting ATP (lane 2), E1 (lane 3), or E2 (lane 4) from reaction mixtures. Samples were analyzed on 8% SDS–PAGE and processed for Western blotting with anti-ubiquitin antibody.

prepared from embryo, larva, and adult fly. PCR products of the expected size (~540 base pairs) were detected at a low level in embryo and at high levels in larva and adult fly, suggesting that the dm VHL gene is expressed throughout the development (Fig. 1B).

Drosophila VHL forms a complex with Cul-2, Rbx1, and Elongins B and C. We have previously reported that human VHL protein associates with Elongins B and C to form a stable complex (3). Subsequently, Cul-2 (human Cdc53 homologue) and Rbx1 were also found to be the components of the human VHL complex (12–14). We therefore tested whether dm VHL protein is able to form a multiprotein complex containing Cul-2, Rbx1, and Elongins B and C. Sf9 insect cells were coinfected with various combinations of baculoviruses encoding FLAG-dm VHL, HA-Cul-2, MYC-Rbx1, HPC4-Elongin B, and HSV-Elongin C, and complexes were immunoprecipitated from cell lysates using anti-FLAG antibody. dm VHL protein associated with Elongins B and C directly (Fig. 2A, lane 6) and assembled into a complex with Cul-2 and Rbx1 in the presence of Elongins B and C (lane 7). In addition, dm VHL protein assembled with Rbx1 and Elongins B and C in the absence of Cul-2 (lane 8), and with Cul-2 and Elongins B and C in the absence of Rbx1 (lane 10). These findings are consistent with the results previously obtained for human VHL protein (14), suggesting that the isolated *Drosophila* sequence is the true homolog of human VHL.

Elongin A, which contains the F-box sequence at its C-terminus, is also able to form a stable complex with Elongins B and C (10, 11). Thus, we next tested whether Elongin A is able to associate with Cul-2 and Rbx1 as in the case of VHL. Sf9 insect cells were coinfected with baculoviruses encoding HA-Cul-2, MYC-Rbx1, HPC4-Elongin B, and HSV-Elongin C, in the presence or absence of either FLAG-Elongin A or FLAG-human VHL, and complexes were immunoprecipitated from cell lysates using anti-MYC antibody. Unlike human VHL protein, Elongin A did not assemble into a complex with Cul-2 and Rbx1 (Fig. 2B, lanes 3–6), suggesting that Elongin A does not function as a F-box protein albeit its sequence homology.

Drosophila VHL multiprotein complex possesses E3 ubiquitin ligase activity. Human VHL complex was recently shown to possess E3 ubiquitin ligase activity (15, 16). We therefore tested whether the isolated dm VHL protein, together with Cul-2, Rbx1, and Elongins B and C is able to reconstitute this activity.

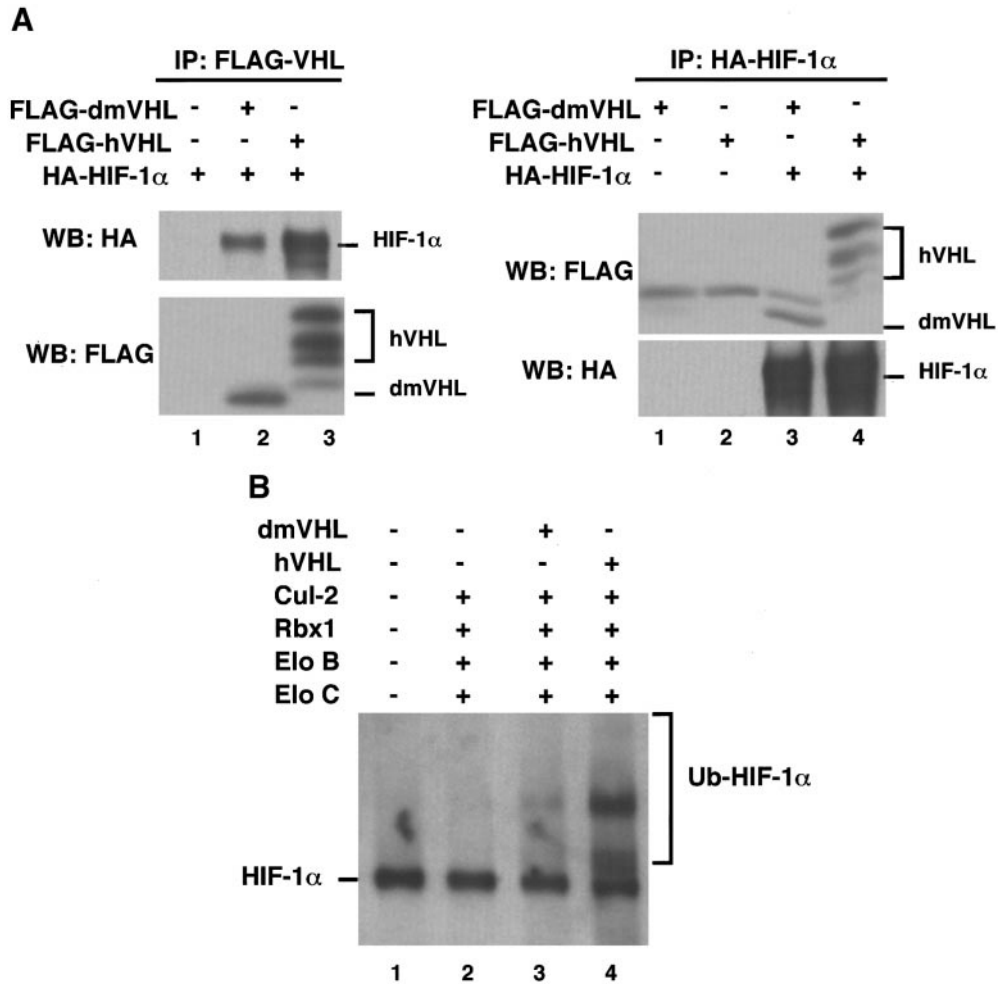

FIG. 4. Both *Drosophila* and human VHL proteins promote the ubiquitination of HIF-1α *in vitro.* (A) Interaction of *Drosophila* and human VHL proteins with HIF-1α. Lysates from Sf9 cells expressing the indicated baculoviruses were immunoprecipitated with anti-FLAG (left panel) or anti-HA (right panel). Immunoprecipitated proteins were detected by Western blotting using the indicated antibodies. (B) Lysates from Sf9 cells expressing the indicated baculoviruses were immunoprecipitated with anti-FLAG M2 beads and were subjected to *in vitro* ubiquitination assay as described under Materials and Methods. Samples were analyzed on 8% SDS–PAGE and processed for Western blotting with anti-HIF-1α antibody.

Sf9 insect cells were coinfected with various combinations of baculoviruses encoding FLAG-tagged human or dm VHL, and Cul-2, Rbx1, and Elongins B and C, and complexes were immunoprecipitated with anti-FLAG antibody. After washing, purified complexes were supplemented with purified E1, E2, ubiquitin, and ATP. After an incubation period of 60 min at 37°C, the reaction mixture was separated by SDS–PAGE and analyzed by anti-ubiquitin immunoblotting to detect ubiquitin conjugates. Almost no ubiquitin conjugates were detected in reactions containing either uninfected Sf9 extracts or extracts expressing only Cul-2, Rbx1, and Elongins B and C (Fig. 3A, lanes 1, 2 and 5). In contrast, in the presence of dm VHL protein, a high-molecular-mass smear characteristic of ubiquitin conjugates was produced, although the amounts of smear formed were smaller compared to the reaction containing human VHL protein (Fig. 3A, lanes 3 and 6). To confirm if the formation of this high-molecular-mass ubiquitin conjugate by the dm VHL complex is based on E3 ubiquitin ligase activity, the essential components for ubiquitin conjugation reactions, which are E1, E2, and ATP, were individually omitted from the reaction mixture containing the dm VHL complex. As shown in Fig. 3B, the formation of ubiquitin conjugates was strictly dependent on the presence of ATP (lane 2), E1 (lane 3), and E2 (lane 4). These results indicate that dm VHL multiprotein complex containing Cul-2, Rbx1, and Elongins B and C exhibits E3 ubiquitin ligase activity.

We also tested whether Elongin A possesses ubiquitination activity. Sf9 insect cells were coinfected with baculoviruses encoding FLAG-Elongin A, Cul-2,

Rbx1, and Elongins B and C. Then, the complexes were purified with anti-FLAG antibody, and subjected to *in vitro* ubiquitination reaction. As predicted from the above finding that Elongin A does not associate with Cul-2 and Rbx1, Elongin A displayed no detectable E3 activity (Fig. 3A, lane 4).

HIF-1α is a substrate for VHL multiprotein complex. Mutations in the VHL gene predispose individuals to highly vascularized tumors (1, 2). Consistent with this finding, hypoxia-inducible mRNAs such as vascular endothelial growth factor (VEGF) and glucose transporter-1 (GLUT-1) are upregulated in VHL-defective renal carcinoma cells (5–7). Moreover, it was reported recently that HIF-1α, an upstream transcription factor that positively regulates the expression of VEGF and GLUT-1 mRNAs, is constitutively stabilized in VHL defective cells (20). From these findings, we speculated that HIF-1α is the ubiquitination target of the VHL complex and inappropriate accumulation of HIF-1α by VHL inactivation causes the development of vascularized tumors. We therefore first tested for the interaction between VHL protein and HIF-1α. Sf9 insect cells were coinfected with baculoviruses encoding FLAG-tagged human or dm VHL protein, and HA-tagged HIF-1α, and complexes were immunoprecipitated using antibodies to epitope tags. As shown in Fig. 4A, both human and dm VHL proteins were coprecipitated with HIF-1α by anti-FLAG (left panel) and by anti-HA (right panel) antibody. These results indicate that both human and dm VHL proteins bind to HIF-1α. We then tested whether VHL complex is able to ubiquitinate HIF-1α using *in vitro* ubiquitination assay. Sf9 insect cells were coinfected with baculoviruses encoding Cul-2, Rbx1, and Elongins B and C, in the presence or absence of FLAG-tagged human or dm VHL, and complexes were immunoprecipitated with anti-FLAG antibody. After washing, purified complexes were supplemented with purified HIF-1α, E1, E2, ubiquitin, and ATP. After an incubation period of 60 min at 37°C, the reaction mixture was separated by SDS-PAGE and analyzed by anti-HIF-1α immunoblotting to detect ubiquitin conjugated HIF-1α. Almost no ubiquitinated form of HIF-1α was detected in reactions containing either uninfected extracts or extracts expressing only Cul-2, Rbx1, and Elongins B and C (Fig. 4B, lanes 1 and 2). In contrast, in the presence of either dm VHL or human VHL complex, increased amounts of polyubiquitinated HIF-1α were detected, although significantly low amounts of the ubiquitinated form of HIF-1α were produced by dm VHL complex compared with that by human VHL complex (Fig. 4B, lanes 3 and 4). These results suggest that [1] HIF-1α is, indeed, the ubiquitination target of VHL ubiquitin ligase complex; [2] VHL protein functions as the substrate recognition component, similar to F-box protein of the SCF complex; and [3] these five components of the VHL complex, including VHL, Cul-2, Rbx1, and Elongins B and C, are sufficient for the reconstitution of E3 ubiquitin ligase activity against HIF-1α.

Finally, what is the *in vivo* ubiquitination target of dm VHL complex? Since, hypoxia-responsive systems are conserved between human and *Drosophila*, some of the HIF-1α-related proteins identified in *Drosophila* might be the targets (21). Determination of the true substrate of dm VHL complex would provide an insight into the role of VHL *in vivo* in the growth and development of multicellular organisms.

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Note added in proof. While the manuscript was in preparation, Ohh *et al.* (22) reported that cell extracts containing wild-type, but not mutant, VHL possess an activity to ubiquitinate both HIF-1α and HIF-2α.

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