Interaction of Hydroxylated Collagen IV with the von Hippel-Lindau Tumor Suppressor*

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The von Hippel-Lindau tumor suppressor (pVHL) targets hydroxylated α-subunits of hypoxia-inducible factor (HIF) for ubiquitin-mediated proteasomal destruction through direct interaction with the hydroxyproline binding pocket in its β-domain. Although disruption of this process may contribute to VHL-associated tumor predisposition by up-regulation of HIF target genes, genetic and biochemical analyses support the existence of additional functions, including a role in the assembly of extracellular matrix. In an attempt to delineate these pathways, we searched for novel pVHL-binding proteins. Here we report a direct, hydroxylation-dependent interaction with α-chains of collagen IV. Interaction with pVHL was also observed with fibrillar collagen chains, but not the folded collagen triple helix. The interaction was suppressed by a wide range of tumor-associated mutations, including those that do not disturb the regulation of HIF, supporting a role in HIF-independent tumor suppressor functions.

Germ line mutations in the von Hippel-Lindau (VHL) tumor suppressor gene predispose affected individuals to a variety of highly vascularized benign and malignant tumors, including central nervous system hemangioblastoma, pheochromocytoma, and clear-cell renal carcinomas (CCRC) (1). In addition, most sporadic CCRC manifest biallelic somatic inactivation of VHL. Exactly how pVHL functions as a tumor suppressor remains unclear but, in part, involves its action in an E3 ligase complex that targets prolyl-hydroxylated α-subunits of hypoxia-inducible factor (HIF) for degradation (2–4). In VHL-defective cells, HIF-α subunits are stabilized, leading to constitutive up-regulation of the HIF system (2). Although the precise mode of tumor promotion is unclear, HIF target gene products play a central role in a wide range of functions in tumor biology (5), and genetic studies have demonstrated a clear positive role for the HIF-2α isoform at least in the later stages of tumor development (6–8).

Nevertheless, the mutational spectrum associated with VHL disease exhibits genotype/phenotype correlations that suggest more than one tumor suppressor action (1). Specific patterns of mutations are associated with type 1 disease (hemangioblastoma, CCRC), type 2A (hemangioblastoma, pheochromocytoma), type 2B (hemangioblastoma, pheochromocytoma, CCRC), and type 2C (pheochromocytoma only). Mutational dysregulation of HIF does not concord precisely with tumor predisposition (9, 10), and a substantial body of evidence points to HIF-independent VHL functions (1, 11). Importantly a number of these are disrupted by tumor-associated mutations, suggesting a causal role in tumor suppressor function. One well studied but as yet poorly understood effect is the defective assembly of extracellular matrix (ECM) that is displayed by VHL-deficient CCRC cells (10, 12–14). Several lines of investigation have suggested that this defect reflects a distinct HIF-independent function, and a link to the interaction of pVHL with an intracellular fraction of the ECM protein fibronectin has been proposed (12). First, mutations associated with type 2C VHL disease are competent for HIF regulation, but not ECM assembly (9, 10). Second, comparative gene expression studies in Caenorhabditis elegans mutants that are defective for the single HIF-α homologue hif-1, or doubly defective for hif-1 and the VHL homologue vhl-1, have provided genetic evidence for a HIF-1-independent VHL-1 pathway that also appears connected with an ECM function (15). Third, studies of human CCRC cells expressing a non-degradable form of HIF-2α support the HIF independence of the VHL-associated ECM defect, also linking this pathway (as opposed to HIF activation) to enhanced angiogenesis (14).

To better understand this HIF-independent function, we have continued to screen for pVHL-associated proteins using co-immunoprecipitation and analysis by tandem mass spectrometry (MS/MS). The structure of pVHL bound to hydroxylated HIF-α peptides reveals a discrete hydroxyproline binding pocket in the β-domain of pVHL that forms specific hydrogen bonds with hydroxylated, but not non-hydroxylated, HIF-α (16, 17). These studies predict that other proteins directly binding pVHL might contain sites of prolyl hydroxylation. Interestingly, the transcriptionally active hyperphosphorylated large subunit of RNA polymerase II (Rbp1) has been identified as a second hydroxylation-dependent binding partner of pVHL (18). However, it seems unlikely that this interaction is responsible for the observed effects on matrix assembly, suggesting that there might be...
other hydroxylated species that account for this function. Here we report the identification of collagen IV chains as pVHL-binding proteins and the characterization of a specific, direct, hydroxylation-dependent interaction between pVHL and unfolded collagen chains that is suppressed by tumor-associated mutations in a manner that is concordant with effects on ECM assembly.

EXPERIMENTAL PROCEDURES

**Cells, Plasmids, Transfections, and 35S Labeling**—HA-tagged wild type or mutant pVHL plasmids, empty vector (pcDNA3), and the stably transfected RCC4 sublines have been described (9, 19). Human embryonic kidney 293T cells were grown under standard conditions and transfected using FuGENE 6 (Roche Applied Science). MG132 (BIOMOL), desferrioxamine (DFO; Sigma), and dimethyloxalylglycine (DMOG; Frontier Scientific) were used at final concentrations of 25 μM, 100 μM, and 1 mM, respectively. Radioisotopic labeling of cells was for 12 h as described (19). Collagen Iα1 plasmid (pcMV6-COL1A1) was from ORIGENE. pcDNAcol4α3V5 was a gift from M. Green and pcBFTcol4α3NC1FLAG a gift from R. Kalluri.

**Antibodies, Purified Proteins, and Peptides**—Anti-β tubulin (clone 2-28-33), anti-HA (clone HA7)-agarose conjugate, and anti-FLAG M2 peroxidase conjugate were from Sigma. Anti-HIF-1α (clone 54) and anti-pVHL (Ig32) antibodies were from BD Biosciences. Anti-collagen IV (ab6586) and anti-collagen I (ab34710) antibodies were from Abcam. Anti-fibronectin polyclonal antibody was from Dako Corp. Anti-HA (3F10) peroxidase conjugate antibody was from Roche Applied Science and anti-V5 peroxidase conjugate from Serotec. Purified collagens were from Abcam and purified fibronectin from Chemicon. HA peptide was from Roche Applied Science, and biotinylated HIF-1α peptides (residues 556–574) were a gift from K. S. Hewitson.

**Extract Preparation, Immunoprecipitation, and Immunoblotting**—Cell extracts were prepared in Igepal lysis buffer (10 mM Tris, pH 7.5, 0.25 M NaCl, 0.5% v/v Igepal CA630 with “Complete” Protease inhibitor (Roche)) and 25 μM MG132. For immunoblotting of “Input” 8 μg of cell extract was used, representing 5 or 10% (as indicated) of the total protein used for immunoprecipitation. For immunoprecipitation cell extract was precleared for 30 min at 4 °C with Protein A-agarose beads (Upstate) followed by 16 h of incubation (preparative scale) or 2 h (standard) with the appropriate antibody and affinity resin. Immunoprecipitates were washed five times with Igepal buffer and either analyzed directly or subjected to glycine elution and trichloroacetic acid precipitation (for anti-pVHL and preparative scale anti-HA immunoprecipitates) prior to addition of Laemmli sample buffer, heat denaturation (5 min at 95 °C), and SDS-PAGE. For HIF-1α, V5 (PK tag), collagen I, and collagen IV immunoblotting, samples were first resolved by 7.5% SDS-PAGE. For VHL, HA, FLAG, and β tubulin immunoblotting 15% SDS-PAGE was used. Immunoblotting of “Input” 8 μg of cell extract was used, representing 5 or 10% (as indicated) of the total protein used for immunoprecipitation. For immunoprecipitation cell extract was precleared for 30 min at 4 °C with Protein A-agarose beads (Upstate) followed by 16 h of incubation (preparative scale) or 2 h (standard) with the appropriate antibody and affinity resin. Immunoprecipitates were washed five times with Igepal buffer and either analyzed directly or subjected to glycine elution and trichloroacetic acid precipitation (for anti-pVHL and preparative scale anti-HA immunoprecipitates) prior to addition of Laemmli sample buffer, heat denaturation (5 min at 95 °C), and SDS-PAGE. For HIF-1α, V5 (PK tag), collagen I, and collagen IV immunoblotting, samples were first resolved by 7.5% SDS-PAGE. For VHL, HA, FLAG, and β tubulin immunoblotting 15% SDS-PAGE was used. Immunoblotting signals were visualized with Lumigen™ PS-3 detection reagent (Amersham Biosciences). For denatured, non-reduced conditions, samples were prepared in Laemmli

![FIGURE 1. Co-immunoprecipitation of collagen IV with pVHL. A, Coomassie blue stain of preparative anti-HA immunoprecipitations from RCC4-pVHLHA cells displayed by 8–16% gradient SDS-PAGE. Cells were untreated or incubated with MG132 for 4 h. pVHL and known interacting proteins re-identified by MS are indicated; the ~220-kDa protein identified as α1- and α2-chains of collagen IV has an asterisk. Nonspecific bands are marked ns. B, tryptic peptides identified by MS/MS analysis of the ~220-kDa species. C, autoradiograph of 35S-labeled proteins resolved by 8–16% gradient SDS-PAGE. Lanes 1–6 display the indicated immunoprecipitates (IP) from RCC4-p19VHLHA cells (P19) or vector alone cells (VA). FN, anti-fibronectin; Col IV, anti-collagen IV. Cells were untreated or exposed to MG132 for 12 h. Lanes 7–12 display proteins re-immunoprecipitated by the indicated antibodies after HA peptide elution of anti-HA IP (lanes 4 and 5).](image-url)
sample buffer without dithiothreitol and heated for 5 min at 95 °C.

Far Western Analysis—Proteins were resolved by 10% SDS-PAGE, transferred to membrane, blocked using 4% (w/v) dried skimmed milk in phosphate-buffered saline/0.1% Tween 20 (PBS/T) for 30 min and then incubated for 1 h with recombinant pVHL-elongin C-elongin B complex (VCB) (16) at 100 nM in PBS/T with 4% milk. Membranes were washed four times with PBS/T and analyzed by anti-pVHL immunoblotting. VCB was omitted in mock far Western.

Mass Spectrometry—Proteins were resolved by SDS-PAGE, and excised gel fragments were digested with trypsin. Peptides were analyzed by liquid chromatography coupled to tandem mass spectrometry as described previously (20). MS/MS spectra were matched to the SwissProt data base using the Mascot algorithm (Matrix Science).

RESULTS

Collagen IV Binds pVHL—Following large-scale anti-HA immunoprecipitation of extract from a stably transfected CCRC cell line expressing VHLHA (RCC4-VHLHA), several co-immunoprecipitating species were identified by MS/MS. Most corresponded to known pVHL-binding proteins (Elongin B, Cullin 2, TRIC chaperonin components (TCP-1α, e, η), HIF-1α, HIF-2α), verifying the fidelity of the analysis. An additional species migrating with an apparent molecular mass of ~220 kDa was newly assigned as containing the α1- and α2-chains of collagen IV (Fig. 1, A and B).

A similar pVHL binding species has previously been visualized by 35S labeling in RCC4 and 786-O cells (9, 10, 19, 21) and, in 786-O cells, assigned as fibronectin (12). To clarify the relationship between this species and the newly defined pVHL-collagen IV interaction, we performed further HA immunoprecipitations from 35S-labeled RCC4-VHLHA cells3 and similar transfectants expressing an HA-tagged internally initiated pVHL (RCC4-p19VHLHA) (Fig. 1C). Comparison of anti-HA, anti-collagen IV, and anti-fibronectin immunoprecipitates demonstrated exact co-migration of the major high molecular mass p19VHLHA-collagen IV species with collagen IV, but not with fibronectin (Fig. 1C). Interestingly, treatment with the proteasomal inhibitor MG132 markedly increased capture of HIF-1α but had no effect on this species. To confirm the identity of the ~220-kDa species we eluted the anti-HA immunoprecipitates using HA peptide and re-immunoprecipitated the eluates with anti-collagen IV, anti-fibronectin, or anti-HIF-1α antibodies (Fig. 1C). Collagen IV was detected in the eluates in all conditions, whereas HIF-1α was only detected after MG132 treatment. These results confirm a specific association between collagen IV and pVHL that is independent of HIF.

Collagen IV Binding Correlates with pVHL Tumor Suppressor Function—Previous studies of pVHL tumor suppressor action have examined 35S-labeled protein interaction profiles of disease-causing pVHL mutants (9, 10, 19) and shown that essentially all tumor-associated mutations reduce capture of the species that we now assign in RCC4 cells as collagen IV. We therefore wished to test whether capture of collagen IV is indeed reduced by tumor-associated VHL mutations. We first tested RCC4 stable transfectants expressing HA-tagged pVHL mutants associated with sporadic CCRC, type 2A VHL disease, or type 2B VHL disease in anti-HA co-immunoprecipitation assays and found that all these mutations ablate the interaction of pVHL with collagen IV (Fig. 2A). We next assayed further mutations associated with types 2B and 2C VHL disease in similar co-immunoprecipitation assays following transient transfection into 293T cells and found that they also impaired collagen IV binding (Fig. 2B). Although the 2C mutants V84L and L188V retained some interaction with collagen IV, capture was substantially less than with wild type pVHL. Thus, consistent with previous observations on capture of the ~220-kDa 35S-labeled pVHL-associated species (10), collagen IV capture was reduced by all types of tumor-associated VHL mutation. Because the interaction of wild type pVHL with collagen IV was clearly observed in 293T cells and was also demonstrated in 786-O and U2OS cells, these findings also indicate that the pVHL-collagen IV interaction is not restricted to RCC4 cells.

VCB Complex Binds Directly to Collagen Chains—To determine whether VCB and collagen IV interact directly, we performed Far Western assays using purified recombinant VCB complex. Collagen IV and HA immunoprecipitates from

3 N. Masson, unpublished data.

4 A. Grosfeld and I. Stolze, unpublished data.
RCC4-p19VHLHA cells were treated with MG132 for 4 h prior to harvest to enable comparison with HIF-

Hydroxylation-dependent Interaction between pVHL and Collagen IV—Given the dependence of pVHL-HIF-α interaction on prolyl hydroxylation and the extensive prolyl and lysyl hydroxylation of collagens, including collagen IV (for review see Ref. 22), we next tested whether the pVHL-collagen IV interaction was regulated by hydroxylation. RCC4-p19VHLHA cells were exposed to hydroxylase inhibitors and tested for effects on collagen IV capture in anti-HA immunoprecipitates (or on HA (p19VHLHA) capture in anti-collagen IV immunoprecipitates). To provide an internal positive control, effects were compared with those on capture of HIF-1α in MG132-exposed cells (Fig. 4A). Both the 2-oxoglutarate analogue DMOG and the iron chelator DFO reduced the association of pVHL with collagen IV as well as with HIF-1α. Note that these treatments also altered the mobility of collagen IV (both in the Input and collagen IV immunoprecipitates) consistent with inhibition of post-translational modification (Fig. 4A). In addition, the VCB Far Western reactivity of the collagen IV immunoprecipitates was altered by treatment of cells with DMOG and DFO (Fig. 4A), confirming the expected dependence on hydroxylation. Taken together, these findings suggest that hydroxylated collagen IV binds to VCB.

To determine whether binding is competitive with HIF-α, we tested the ability of synthetic HIF-1α peptides to block the interaction of VCB with collagen IV by Far Western. Results illustrated in Fig. 4B show that the VCB-collagen IV interaction was competed by hydroxylated (HyPHI19), but not unmodified (ProHI19) HIF-1α peptides, suggesting that the hydroxyproline binding pocket in the β-domain of pVHL is required for the interaction (16).

pVHL Interaction with Fibrillar Collagen Chains—Collagen IV is the principal (non-fibrillar) basement membrane collagen. In common with fibrillar collagens it contains a collagenuous domain of Gly-X-Y triplets, where X and Y can be any amino acid residue but are commonly proline and hydroxyproline, respectively (22, 23). Following hydroxylation this domain folds into the triple helical conformation that is found in mature collagens. We next sought to determine whether pVHL binding was restricted to collagen IV or whether it could bind other collagens, including the folded triple helix. To address this, 293T cells were co-transfected with plasmids expressing either complete or truncated collagen chains and VHLHA. Interactions were tested by anti-HA immunoprecipitation, revealing association with full-length α-chains of collagen I as well as collagen IV, but not with an isolated non-collagenous domain from collagen IV (Fig. 5A). To test for interaction with folded IV (left panel) and is thus assigned as collagen IV on the basis of both mobility and immunoactivity.
collagens, VCB Far Western analysis was used to screen purified preparations of ECM, including collagen IV and the fibrillar collagens type I, II, and V (Fig. 5B). Interaction with all three collagen types was detected, but only when the samples were denatured. Taken together, these findings suggest that a variety of collagen chains, but not the folded triple helix, bind pVHL directly.

**DISCUSSION**

Our data reveal a HIF-independent interaction between pVHL and collagen IV α-chains that is affected by a wide range of tumor-associated mutations. Although type 2C mutants retain some collagen IV binding, capture was substantially less than with wild type pVHL. These findings fit well with literature describing abnormal extracellular matrix assembly in VHL-defective cells (12, 14), and concordance between the mutational disruption of collagen IV binding and the ECM defect strongly suggests a causal link. Nevertheless, the precise mechanism remains unclear. pVHL does not contain a secretory leader sequence, and we found no extracellular immunoreactivity. Furthermore, pVHL does not appear to interact with native extracellular (triple helical) collagen but with unassembled intracellular collagen IV chains. Several different intracellular routes of degradation for unassembled collagen chains have been described, including proteasomal degradation (24, 25). However, despite the established function in degradation of HIF-1α, we have so far been unable to demonstrate similar pVHL-dependent processing of collagen IV chains.5 Nevertheless, our findings should provide a new lead in understanding tumor suppressor mechanisms. They also suggest that hydroxylated α-chains of collagen IV and HIF may compete for pVHL binding, potentially providing a mechanism by which cellular stresses that might enhance production of unassembled collagen chains could induce normoxic expression of HIF.

5 N. Masson and A. Grosfeld, unpublished data.
Previous studies of 786-O cells have identified an interaction between pVHL and fibronectin (12), and it has been assumed that 35S-labeled pVHL-associated species in CCRC cells migrating at a similar (~220 kDa) position are fibronectin. Our data indicate that, at least in RCC4 cells, the major pVHL-associated species in this region are in fact collagen IVα-chains. Interestingly, we found that 786-O cells express more fibronectin and less collagen IV than RCC4, perhaps explaining why fibronectin was identified as a pVHL-interacting protein in earlier studies of 786-O, but not in our current MS/MS analysis in RCC4 cells. Whether fibronectin interacts directly or indirectly with pVHL has been controversial (10, 21). However, using purified fibronectin we were able to demonstrate an interaction with VCB by Far Western specifically under non-reducing conditions (Fig. 4B). The structural and physiological implications of this finding and whether it explains previous discrepancies will require further investigation.

In the MS analysis we identified peptides representing the α1- and α2-chains of collagen IV, but not other collagens. However, in further experiments using either denatured purified fibrillar collagens (I, II, and V) in VCB Far Western analysis or co-immunoprecipitation of overexpressed α-chains of collagen I with VHLHA, we found interaction, suggesting that pVHL binding might not be restricted to collagen IV. Because we were unable to demonstrate interaction with the non-collagenous domain of a collagen IV α-chain and both co-immunoprecipitation and Far Western interactions were reduced by two different procollagen hydroxylase inhibitors, our results suggest that the interaction most likely occurs through the heavily hydroxylated collagenous domain. Though competition with prolyl-hydroxylated, but not unmodified, HIF peptide would be consistent with the interaction involving sites of prolyl hydroxylation within collagen, this is not proven, as both DFO and DMOG affect lysyl as well as prolyl hydroxylation. Modeling the structure of the triple helical folded collagenous domain onto the hydroxyproline binding site in the β-domain of pVHL (16, 17) reveals a major steric clash that would be predicted to prevent binding. In keeping with this, recombinant VCB complex did not bind native folded collagen of either fibrillar or non-fibrillar forms. In future studies it will be interesting to map the binding determinants more precisely. Notably, unlike the hydroxylated HIF-1α 19-mer peptides, a denatured (Gly-Pro-Hyp)10 peptide did not compete the interaction with collagen IV,6 suggesting that the interaction determinants are more complex than the simple presence of hydroxyproline in the triplet repeat.

In the wider context of tumor biology many lines of investigation have implicated collagen IV proteolysis both in tumor growth and angiogenic regulation (26, 27). Our findings now reveal one of the most direct molecular links to date between collagen and a major tumor suppressor.

6 A. Grosfeld, unpublished data.

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