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Biochemistry

Characterization of the VHL tumor suppressor gene product: Localization, complex formation, and the effect of natural inactivating mutations

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ABSTRACT **The human *VHL* tumor suppressor gene has been implicated in the inherited disorder von Hippel–Lindau disease and in sporadic renal carcinoma. The homologous rat gene encodes a 185-amino acid protein that is 88% sequence identical to the aligned 213-amino acid human VHL gene product. When expressed in COS-7 cells, both the human and the rat VHL proteins showed predominant nuclear, nuclear and cytosolic, or predominant cytosolic VHL staining by immunofluorescence. A complicated pattern of cellular proteins was seen that could be specifically coimmunoprecipitated with the introduced VHL protein. A complex containing VHL and proteins of apparent molecular masses 16 and 9 kDa was the most consistently observed. Certain naturally occurring VHL missense mutations demonstrated either complete or partial loss of the p16–p9 complex. Thus, the VHL tumor suppressor gene product is a nuclear protein, perhaps capable of specifically translocating between the nucleus and the cytosol. It is likely that VHL executes its functions via formation of specific multiprotein complexes. Identification of these VHL-associated proteins will likely clarify the physiology of this tumor suppressor gene.**

The genetic dissection of both hereditary and sporadic cancers has provided insights into both oncogenesis and the most fundamental cellular processes of growth stimulation, growth control, cell cycle progression and regulation, response to injury, DNA damage and loss of genomic integrity, and cell death. Ten or more tumor suppressor genes have been identified to date and their number is likely to grow rapidly (see refs. 1 and 2 and references therein). The vast majority of mechanistic information has come from biochemical and genetic studies of the products of two of these—pRb, encoded by the retinoblastoma gene, and p53. The *VHL* gene found on human chromosome 3p25.5 was reported in 1993 (3) as the likely tumor suppressor gene whose germ-line mutation is associated with the rare inherited disorder von Hippel–Lindau disease (3–5). This disorder is characterized by development of multiple benign and malignant tumors in a number of organs including kidney, retina, central nervous system, pancreas, and adrenal gland (see ref. 6 and references therein). Subsequent analysis revealed that mutations (7, 8) and/or transcriptional inactivation (9) of the *VHL* gene are associated with the majority of cases of sporadic clear cell renal carcinoma, a disease that is newly diagnosed in >20,000 Americans each year.

In contrast to the extensive knowledge of p53 or retinoblastoma functions, little is known about the biology or biochemistry of the VHL gene product. Sequence of the cloned human cDNA predicted a 24.2-kDa protein with no significant homology to any known protein. In this study, we report the cloning of the rat homolog of the human *VHL* gene.¶ Alignment of the two highly homologous sequences supports the assignment of the initiating methionines for the two genes. We also examine the expression, localization, and coimmunoprecipitation with other cellular proteins of both human and rat VHL proteins.

MATERIALS AND METHODS

cDNA Library Screening and Sequence Analyses. DNA probes for cDNA library screening were generated by PCRs using the human VHL g7 cDNA as a template. Exon 1 probe spans nt 229–506 and exon 2 probe spans nt 577–760 of the g7 cDNA (3). A rat liver cDNA library (10) (a total of 3×10^5 plaques) was screened according to established procedures (11). Bacteriophage clones that hybridized with both exon 1 and exon 2 probes were purified. The cDNAs were subcloned into Bluescript vector pKS+ (Stratagene) and sequenced.

Antibody Production. Anti-VHL polyclonal antisera 4939 and 4940 were raised in New Zealand rabbits against a truncated human VHL protein (amino acids 54–213) expressed in baculovirus/Sf9 cells.

Expression Vectors and Transfection of COS-7 Cells. The VHL open reading frame (ORF) sequences from the rat and human cDNAs were generated by PCR, subcloned into pSX, a modified version of the mammalian expression vector pCDL-SRα (12), and named pSX-R8 and pSX-G7 for rat and human VHL, respectively. pSX-R8F and pSX-G7F DNA constructs had an 8-amino acid epitope tag, FLAG (DYKDDDDK), added to the C termini of the VHL ORFs. Mutations of human VHL FLAG DNA were constructed by PCR as described (13).

COS-7 cells ($4–8 \times 10^6$) grown in Dulbecco's modified Eagle's medium (DMEM) H21 with 10% fetal calf serum (FCS) were electroporated with 40 μg of DNA (four pulses of 0.5 kV × 99 μsec; BTX Electro square porator model T820; BTX, San Diego) and incubated in DMEM/FCS for 48 h before lysis.

Cell Lysis and Western Blotting. COS-7 cells (5×10^6) were washed with ice-cold DMEM H21 and lysed in 1 ml of Triton X-100 lysis buffer [20 mM Tris·HCl, pH 8.0/137 mM NaCl/1% Triton X-100/10% (vol/vol) glycerol/1 mM phenylmethylsulfonyl fluoride (PMSF)/0.5 μg of leupeptin per ml/1 μg of aprotinin per ml/1 mM NaF/1 mM sodium orthovanadate]. Lysates were immunoprecipitated with antibody (Ab)4940 (1:500 dilution) or a monoclonal anti-FLAG Ab (M2 mAb; Kodak–IBI; 1 μg/ml). Immunoprecipitates were analyzed on

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Abbreviations: ORF, open reading frame; Ab, antibody; mAb, monoclonal antibody.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. U14746).

SDS/14% polyacrylamide gel and Western blots were probed with Ab4939 at 1:2000 dilution.

Metabolic Labeling Experiments. Thirty-six hours after transfection, COS-7 cells were incubated with labeling medium [DMEM H21 without methionine and cysteine, 2% FCS, and 0.1 mCi of Trans^{35}S-label (ICN) per ml (1 Ci = 37 GBq)] for 16 h at 37°C. Cells were lysed as described and lysates were immunoprecipitated with M2 mAb. The immunoprecipitates were analyzed on a split percentage SDS/polyacrylamide gel (10% on top and 15% on bottom).

Subcellular Fractionation. Pellets of 1×10^7 transfected COS-7 cells were suspended in 2 ml of homogenization buffer (0.25 M sucrose/10 mM Tris·HCl, pH 7.5/10 mM KCl/1.5 mM $MgCl_2$/1 mM PMSF/0.5 μg of leupeptin per ml/1 μg of aprotinin per ml/1 mM NaF/1 mM sodium orthovanadate) for 20 min on ice and broken by 30 strokes in a B-type Dounce homogenizer. The cell homogenates were centrifuged at 600 × *g* for 10 min at 4°C. The pellets were washed and named nuclear fractions, and the supernatants were centrifuged at 100,000 × *g* for 1 h at 4°C. The 100,000 × *g* pellets and supernatants were named membrane fractions and cytosolic fractions, respectively.

Sucrose Gradients. A 5–20% sucrose gradient was made by mixing 6.2 ml of solution A (10 mM Tris·HCl, pH 7.5/150 mM NaCl/0.1% Triton X-100/0.2 mM NaN_3/5% sucrose) and 6.2 ml of solution B (10 mM Tris·HCl, pH 7.5/150 mM NaCl/0.1% Triton X-100/0.2 mM NaN_3/20% sucrose) using a gradient maker (Gradient Master; Biocomp, New Brunswick, Canada). Lysates of 2×10^7 cells were loaded on the top of the gradient solutions and centrifuged at 200,000 × *g* (average) for 16 h at 4°C. The gradients were fractionated and each fraction was immunoprecipitated with M2 mAb.

Immunofluorescence. COS-7, HeLa, or NRK cells were fixed in 1% formaldehyde at room temperature for 20 min, stained with M2 mAb (1 μg/ml), mounted, and photographed by immunofluorescence and phase-contrast microscopy as described (13).

RESULTS

Comparison of Amino Acid and Nucleotide Sequences of Rat and Human VHL cDNAs. Six independent rat cDNA clones were obtained that hybridized with DNA probes spanning exon 1 and exon 2 of the human VHL g7 cDNA. The nucleotide sequence of the longest cDNA, BRL-8, was analyzed. BRL-8 cDNA is 2.81 kb long and hybridized to a single mRNA species of ≈2.9 kb. At the 5′ end of the BRL-8 cDNA, an ORF predicting the homolog of the human VHL protein is found following a 5′ untranslated region of 119 nt. There is an in-frame translation stop codon 60 nt upstream from the first ATG codon. At the 3′ end, a candidate polyadenylylation signal, AATAAA, was found 16 nt upstream from a stretch of six A residues. These results suggest that clone BRL-8 is a near full-length rat VHL cDNA and that it contains the full-length ORF for the rat VHL protein.

The rat BRL-8 cDNA predicts a protein of 185 amino acids, while the human g7 cDNA predicts a protein of 213 amino acids. The size difference between the two VHL proteins results largely from a single region of disparity in the two sequences and an addition of 6 amino acids at the C terminus of the rat protein. Near the N terminus, a unique Gly-Xaa-Glu-Glu-Xaa acidic motif is repeated eight times in the human VHL protein, while it occurs only once in the rat protein (Fig. 1). Aside from the acidic repeats, the predicted rat VHL protein shares 88% identity with the corresponding sequence in the predicted human VHL protein. The first methionine in the predicted human VHL protein is conserved in the predicted rat VHL protein in a sequence context of MPRXA, suggesting that this methionine may be the translation start site in the rat as well as in the human transcript.

In the 5′ untranslated region, both the nucleotide and amino acid sequence identity between rat and human *VHL* genes drop sharply from that of the ORF region. Thus, the nucleotide sequence identity drops from 82% to 40%, and the predicted amino acid sequence identity of the ORF and sequence 5′ to the first methionine drops from 88% to 25%. This observation further suggests that the first methionine in both rat and human transcripts is the translation start site for the VHL gene products.

Expression of Human and Rat VHL Proteins in COS-7 Cells. The cDNAs encoding the human or rat VHL ORFs either alone or modified by the addition of a C-terminal epitope tag, FLAG (DYKDDDDK), were expressed in COS-7 cells. The untagged human and rat proteins migrated at 28 and 21 kDa, respectively (Fig. 2*A*). The human cDNA gave rise to three additional minor species. Fig. 2*A* shows two of these species, 26 and 24 kDa, from the untagged construct that were recognized by an anti-VHL Ab. Each of the epitope-tagged species migrated more slowly than its untagged counterpart at 31, 29, 27, and 18 kDa (Fig. 2*A*).

When the transiently expressing cells were labeled with ^{35}S-amino acids and cell lysates were immunoprecipitated with the anti-FLAG Ab, the same pattern of VHL proteins was observed as shown in Fig. 2*A*. In addition, both the human and rat proteins coimmunoprecipitated an identical series of other protein bands (Fig. 2*B*). These included two low molecular mass bands (16 and 9 kDa) and a group of bands migrating between 50 and 75 kDa. The 16-kDa-associated protein is well resolved from the 18-kDa epitope-tagged VHL species. These other bands were not directly recognized by anti-VHL Abs by Western blotting, were identically coimmunoprecipitated with two different anti-FLAG mAbs, were seen whether the epitope tag was placed at the C or the N terminus of the VHL ORF, and were not seen in immunoprecipitates from control transfectants or from transfectants expressing an irrelevant FLAG-tagged protein, ARF6 (Fig. 2*B*). The detection of the lower-molecular mass bands (p16–p9) was the most reproducible, regardless of the level of expression of the VHL protein. In contrast, we had the impression that the higher molecular mass bands were predominantly seen in populations expressing high levels of VHL protein. The low molecular mass-associated bands are shown on a higher percentage gel in Fig. 2*C*, where one can appreciate that the 16-kDa "band" is actually a triplet. These coprecipitated bands can be selectively eluted from the specific immunoprecipitates by addition of increasing concentrations of sarcosyl (Fig. 2*D*), consistent with the hypothesis that these proteins may interact with the VHL protein.

Subcellular Localization of VHL in Transiently Transfected Cells. The epitope-tagged human and rat proteins were introduced into several different cell lines and examined by immu-

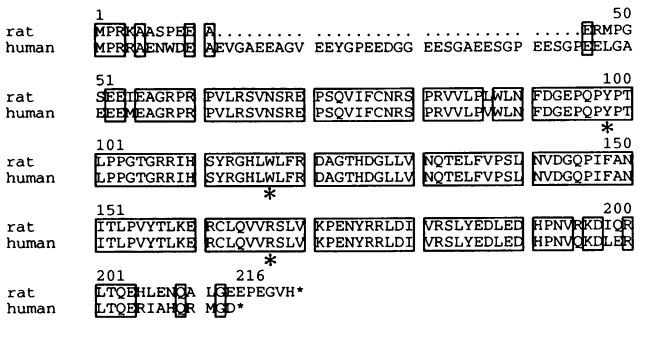

FIG. 1. Amino acid sequence comparison between predicted rat and human VHL proteins. Predicted sequences of the rat VHL protein (185 amino acids) and the human VHL protein (213 amino acids) are shown. Amino acids identical between rat and human VHL proteins are boxed. Positions of mutations introduced into the VHL protein for biochemical studies are marked by asterisks—namely, Y98, W117, and R167.

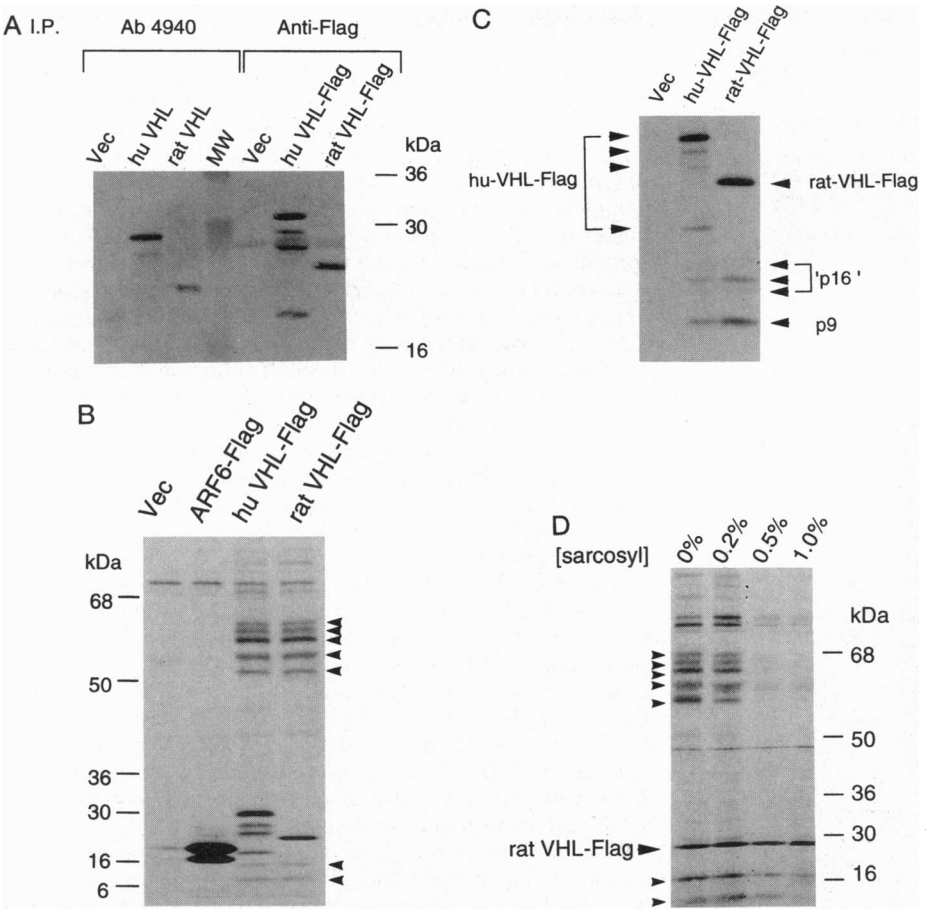

FIG. 2. (*A*) Expression of VHL protein in COS-7 cells. VHL proteins were immunoprecipitated with Ab4940 from lysates of COS-7 cells transfected with pSX vector (Vec), pSX-G7 (hu VHL), or pSX-R8 (rat VHL), or by an anti-FLAG Ab from lysates of COS-7 cells transfected with pSX-G7F (hu VHL-Flag) and pSX-R8F (rat VHL-Flag). Western blot was probed with Ab4939. Lane MW, molecular size markers. (*B*) Coimmunoprecipitation of VHL protein with multiple cellular proteins. COS-7 cells were transfected with pSX vector (Vec), pSX-ARF6-FLAG (ARF6-Flag), pSX-G7F (hu VHL-Flag), or pSX-R8F (rat VHL-Flag) and metabolically labeled. Lysates were immunoprecipitated with M2 mAb and analyzed by SDS/PAGE and autoradiography. Arrowheads point to a quintuplet of proteins at 55–65 kDa, a 16-kDa protein, and a 9-kDa protein that coimmunoprecipitated with the VHL protein. (*C*) Low molecular mass VHL-associated proteins. Arrowheads point to human and rat VHL proteins and apparent triplet of the 16-kDa protein (p16) and the 9-kDa protein (p9). (*D*) Disruption of coimmunoprecipitation of VHL with cellular proteins by sarcosyl. COS-7 cells transfected with pSX-R8F (rat VHL-Flag) and metabolically labeled were lysed in Triton X-100 lysis buffer plus various concentrations of sarcosyl. Lysates were immunoprecipitated with M2 mAb and analyzed by SDS/PAGE and autoradiography. Arrowheads show cellular proteins that coimmunoprecipitated with VHL.

nofluorescence microscopy. In any population of cells, a variety of patterns were observed (Fig. 3). Many cells had a predominantly nuclear localization. Even this was varied, with some cells demonstrating a fine punctate nuclear pattern and others, generally with higher total staining, having a more diffuse nuclear pattern with exclusion of nucleoli. In other cells, there was both nuclear and cytoplasmic staining and, finally, there were cells with only cytoplasmic staining. It was not obvious whether the localization depended on the total level of fluorescence intensity. These three patterns were about equally represented in the population. The same pattern was seen in transiently transfected COS and HeLa cells and in stably transformed NRK cells using either human or rat cDNAs.

Subcellular fractionation of transiently transfected populations expressing either human or rat proteins supported the varied immunofluorescence patterns (Fig. 4*A*). While the most enriched fraction was the nucleus, significant portions of the total protein were found in the cytosolic fraction. In addition, a significant fraction of the total VHL protein was found in the 100,000 × *g* pellet of the cytosolic fraction. Finally, VHL protein could be recovered from the Triton-insoluble nuclear fraction and 100,000 × *g* pellet. Fractionation of metabolically labeled cells followed by immunoprecipitation of either human or rat proteins revealed a distinct pattern of coprecipitated proteins from the different fractions (Fig. 4*B*). All of the coprecipitated bands were seen in the nuclear-derived material. In contrast, the amounts of the higher molecular mass proteins were dramatically reduced in precipitations of the cytosol. Conversely, the 100,000 × *g* pellet contained none of the lower molecular mass bands, but the full pattern of larger proteins was present. This suggested that the expressed VHL protein may exist as part of more than one distinct protein complex. To further assess this, transfected, metabolically labeled cells were lysed in Triton X-100 and separated on a continuous 5–20% sucrose gradient before being subjected to immunoblotting or immunoprecipitation (Fig. 5*A*). The dis-

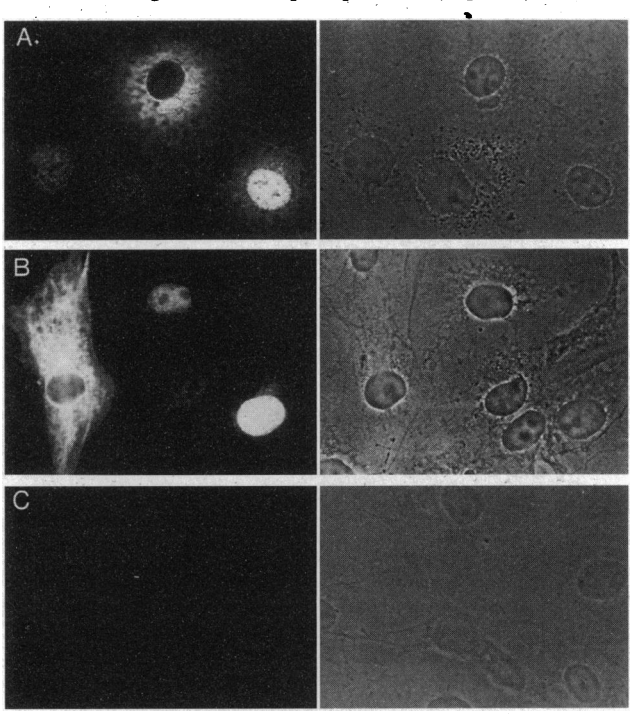

FIG. 3. Immunolocalization of epitope-tagged rat and human VHL in transfected COS-7 cells. COS-7 cells transfected with plasmid encoding rat (*A*) or human (*B*) FLAG-tagged VHL or with plasmid alone (*C*) were stained with M2 mAb, followed by rhodamine-conjugated donkey antibodies to mouse IgG. Rhodamine channel images (*Left*) are shown next to the phase-contrast image (*Right*) of the same field.

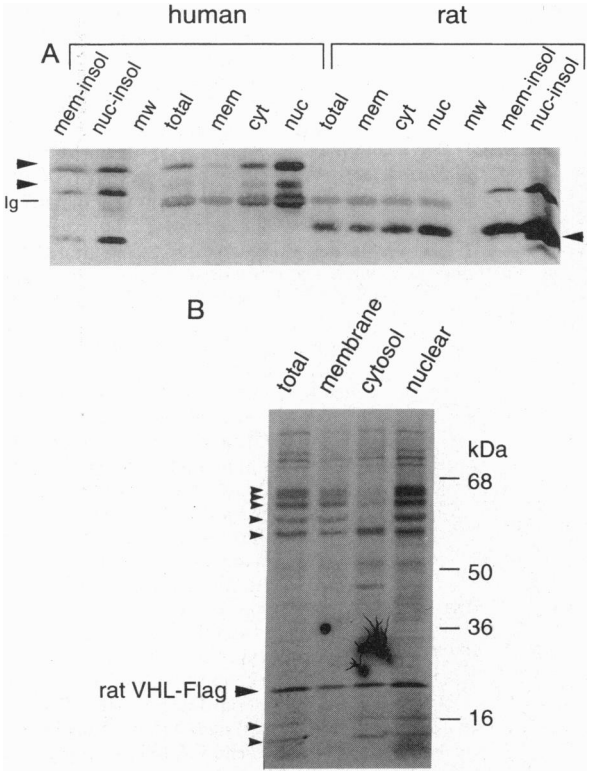

FIG. 4. (*A*) Subcellular fractionation of VHL protein. COS-7 cells transfected with either pSX-G7F (human) or pSX-R8F (rat) were fractionated into nuclear fraction (600 × *g* pellet), cytosolic fraction (100,000 × *g* supernatant), or membrane fraction (100,000 × *g* pellet). Total lysates (total) and subcellular fractions solubilized in Triton X-100 lysis buffer were immunoprecipitated with the M2 mAb. Triton-insoluble materials of the nuclear and membrane fractions were analyzed directly on SDS/polyacrylamide gel. Western blot was probed with Ab4940. Arrowheads point to VHL proteins. (*B*) Distinct subcellular localization of VHL-associated proteins. COS-7 cells transfected with pSX-R8F (rat VHL-Flag) and metabolically iabeled were fractionated as in *A*. Total lysate and Triton-soluble materials of each fraction were immunoprecipitated with the M2 mAb and analyzed by SDS/PAGE and autoradiography. Arrowheads point to the quintuplet of 55–65 kDa, p16, and p9.

tribution of total human VHL revealed two peaks, one in fractions 3 and 4 and the other in fractions 7 and 8. Immunoprecipitation revealed that all of the coprecipitated p16 and p9 proteins were found in fixed ratios at the lower densities comigrating with the major VHL protein peak (Fig. 5*B*), while at the higher density some of the 50- to 75-kDa coprecipitated proteins were seen. In no fraction was the full pattern of coprecipitated bands observed, suggesting that they were part of separate complexes, that there was a failure to recover some complexes, or that there was a selective loss of some of the higher molecular mass proteins on the sucrose gradient. Despite the uncertainties about the higher molecular mass bands, the separation of the regions containing the lower from the upper molecular mass-associated proteins strongly suggested that VHL can enter into several distinct multicomponent complexes, consistent with subcellular fractionation studies.

Effect of Naturally Occurring Point Mutations on VHL Complex Formation. A large number of hereditary and sporadic mutations in the VHL gene include missense mutations in the protein that are presumably partially or fully inactivating. We tested five of these point mutations, by transiently transfecting COS-7 cells, for their ability to form the associations described above. These mutations are shown in Fig. 1 and involve alterations of three different amino acids in the VHL sequence. Arg-167 is a hot spot for mutation in familial disease, and two observed substitutions to either a Trp or a Gln were examined (4, 5). Two mutations of Tyr-98, one of which is seen in VHL not associated with renal cell carcinoma (Tyr-98 to His) and one of which has been seen in sporadic renal cancer (Tyr-98 to Asn), were studied as well as a mutation at Trp-117, also seen in sporadic renal cancer (4, 5, 7, 8). Each of the overexpressed proteins coprecipitated the same pattern of higher molecular mass proteins from metabolically labeled cells (Fig. 6). However, three of the mutants appeared to assemble poorly, if at all, with the 16- and 9-kDa proteins. This was most apparent for the two Arg-167 mutants. The Trp-117 mutant had a greatly diminished but still detectable association with these proteins. In contrast, the two Tyr-98 mutants formed complexes that were indistinguishable from wild type.

DISCUSSION

The *VHL* tumor suppressor gene is a relatively recent addition to the rapidly growing list of tumor suppressor genes. The first step toward understanding how this gene functions in normal cells and how its loss of function predisposes to cancer is to provide the basic biochemical and cell biological characteriza-

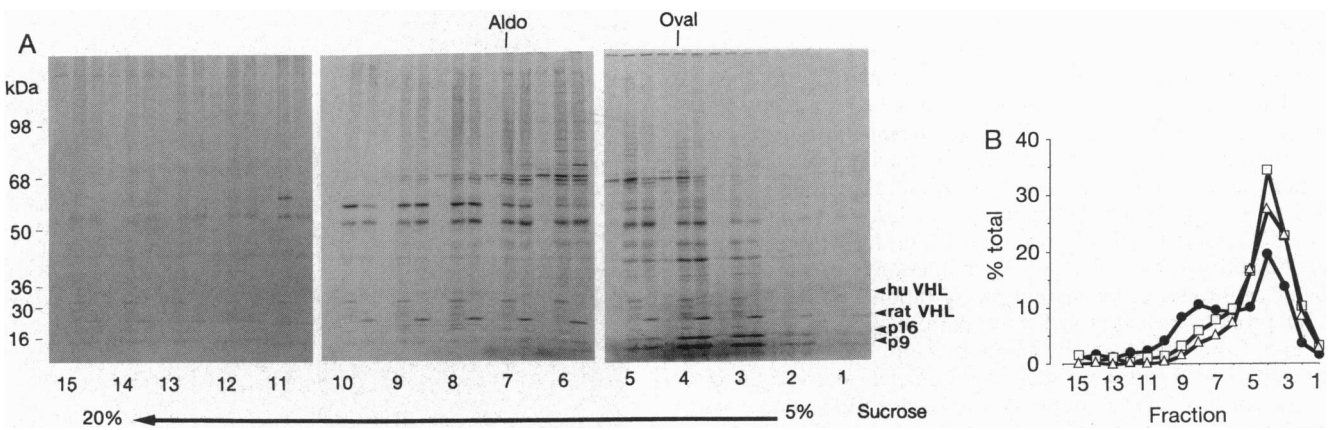

FIG. 5. (*A*) Migration of VHL protein and its associated proteins on a sucrose gradient. Lysates of COS-7 cells transfected with pSX, pSX-G7F, or pSX-R8F were analyzed on a 5–20% sucrose gradient. Equivalent fractions were immunoprecipitated with the M2 mAb and analyzed side by side in the order of vector control (pSX), human VHL (pSX-G7F), and rat VHL (pSX-R8F) (from left to right for each fraction) on SDS/polyacrylamide gel. Positions of soluble molecular mass standards aldolase (Aldo, 160 kDa) and ovalbumin (Oval, 43 kDa) are marked. Arrowheads point to human VHL protein (hu VHL), rat VHL protein, and VHL-associated p16 and p9. (*B*) Distribution of rat VHL protein, p16, and p9 on a sucrose gradient. Percentage of total VHL protein (●), p16 (△), and p9 (□) in each fraction of the 5–20% sucrose gradient (*A*) was assessed by scanning densitometry.

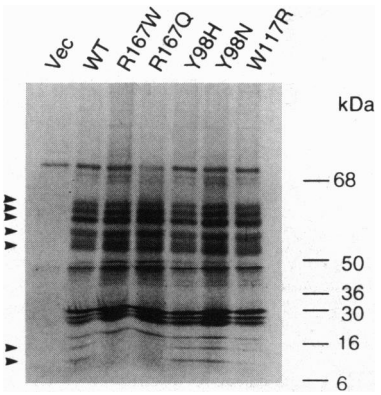

FIG. 6. Coimmunoprecipitation of wild-type and mutant VHL proteins with multiple cellular proteins. COS-7 cells were transfected with pSX vector (Vec), pSX-G7F (WT), and modified pSX-G7F encoding mutant forms of human VHL (R167W, R167Q, Y98H, Y98N, W117R) and metabolically labeled. Lysates were immunoprecipitated with M2 mAb and analyzed by SDS/PAGE and autoradiography. Arrowheads point to the quintuplet of 55–65 kDa, p16, and p9.

tions of the gene product. Because of difficulties in defining the 5′ end of the human VHL transcript, previous studies did not unambiguously define the extent of the protein product. Cloning of the homologous rat gene, we believe, has helped to clarify this. The two predicted protein sequences are extremely well aligned. In contrast to the human cDNAs, the most 5′ methionine in-frame with the conserved ORF is unambiguously identified in the rat because of an in-frame upstream stop codon. The sequence conservation between the two species begins at that first methionine, and the sudden drop in nucleotide conservation 5′ of this site strengthens the argument that sequences upstream of the first in-frame ATG in the human cDNA most likely represent 5′ untranslated region. During the preparation of this paper, a murine homologue of human VHL was reported (14). The murine protein is very similar to the rat, and the authors reported the comigration of *in vitro* translation product with an endogenous protein detected by an affinity-purified anti-VHL Ab. The original cloning of the human gene revealed a protein with no significant homology to any known proteins. The one exception was some similarity to a surface glycoprotein of trypanosomes due to the eight repeats of the sequence Gly-Xaa-Glu-Glu-Xaa (3). This intriguing repeat structure, however, is found in neither rat nor murine VHL homologs.

The variable subcellular localization of VHL is intriguing, albeit somewhat confusing. Some of this may arise from different levels of overexpression of the protein in these transient transfection systems. It is particularly striking that the protein is found exclusively in the nucleus of some cells and in the nucleus and cytosol of some cells, while in an equal number of cells the nucleus appears to be specifically excluded. Perhaps the VHL protein moves between the two compartments, but this remains to be tested. There are a variety of examples of proteins that can exist either in the cytosol or in the nucleus. For example, cyclin B translocates from the cytosol to the nucleus in a cell cycle-dependent manner (15). Despite this caveat, VHL is clearly capable of being a nuclear protein.

The finding that the VHL protein forms relatively tight complexes with specific sets of cellular proteins suggests that the identification of these associated proteins will yield clues about the function of VHL and may also lead to identification of other genes critical in growth and/or cell cycle control. Our data suggest that the introduced human and rat VHL proteins interact with the identical set of endogenous proteins. This finding informs us that the acidic repeat unique to the human protein is not required for these interactions. Both the subcellular fractionation and the sucrose density gradients suggest that the complexes containing p16 and the p9 complex may be distinct from those containing the higher molecular mass proteins. In addition, the ratio of p16 to p9 appears constant in all fractions in which they are seen in the sucrose gradient. One way of establishing the importance of the types of biochemical interactions observed in this study is to correlate the loss of function with the loss of specific interactions that results from mutations. A variety of documented missense mutations in the VHL protein seen either in the germ-line DNA of patients with VHL disease or in the DNA of human tumors point to these as likely loss of function mutations. Thus, the functional importance of the interaction of the VHL protein with p16 and p9 is suggested by the naturally occurring point mutations at Trp-117 and Arg-167 in the human protein. These mutations result in loss of binding to these two proteins observed with wild-type human or rat protein. In contrast, the Tyr-98 mutants demonstrate normal levels of association with these two proteins, suggesting that another effector function of the VHL is impaired in these mutants.

In summary, the human and rodent VHL proteins are highly conserved in sequence, in subcellular localization, and in their ability to form identical cross-species complexes with other cellular proteins. That VHL and its complexes are located to the nucleus suggests that it, like pRb and p53, may play a regulatory role in critical nuclear events related perhaps to progression through the cell cycle, gene transcription, and/or genomic integrity. The loss of specific complex formation in several naturally occurring inactivating mutants of VHL points to the functional importance of these complexes and promises that the identification of these VHL-associated proteins will likely lead to understanding the role of this tumor suppressor gene product in the life of the cell.

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