Hypoxia Inducible Factor-α Binding and Ubiquitylation by the von Hippel-Lindau Tumor Suppressor Protein*

The von Hippel-Lindau tumor suppressor protein (pVHL) has emerged as a key factor in cellular responses to oxygen availability, being required for the oxygen-dependent proteolysis of α subunits of hypoxia inducible factor-1 (HIF). Mutations in VHL cause a hereditary cancer syndrome associated with dysregulated angiogenesis, and up-regulation of hypoxia inducible genes. Here we investigate the mechanisms underlying these processes and show that extracts from VHL-deficient renal carcinoma cells have a defect in HIF-α ubiquitylation activity which is complemented by exogenous pVHL. This defect was specific for HIF-α among a range of substrates tested. Furthermore, HIF-α subunits were the only pVHL-associated proteasomal substrates identified by comparison of metabolically labeled anti-pVHL immunoprecipitates from proteosomally inhibited cells and normal cells. Analysis of pVHL/HIF-α interactions defined short sequences of conserved residues within the internal transactivation domains of HIF-α molecules sufficient for recognition by pVHL. In contrast, while full-length pVHL and the p19 variant interact with HIF-α, the association was abrogated by further N-terminal and C-terminal truncations. The interaction was also disrupted by tumor-associated mutations in the β-domain of pVHL and loss of interaction was associated with defective HIF-α ubiquitylation and regulation, defining a mechanism by which these mutations generate a constitutively hypoxic pattern of gene expression promoting angiogenesis. The findings indicate that pVHL regulates HIF-α proteolysis by acting as the recognition component of a ubiquitin ligase complex, and support a model in which its β domain interacts with short recognition sequences in HIF-α subunits.

Matthew E. Cockman‡§, Norma Masson‡§, David R. Mole‡, Panu Jaakkola‡, Gin-Wen Chang‡, Steven C. Clifford‡, Eamonn R. Maher‡, Christopher W. Pugh‡, Peter J. Ratcliffe‡§, and Patrick H. Maxwell‡

From the ‡Wellcome Trust Centre for Human Genetics, Oxford OX3 7BN, United Kingdom and the §Section of Medical and Molecular Genetics, Department of Paediatrics and Child Health, University of Birmingham, Birmingham B15 2TT, United Kingdom

The von Hippel-Lindau (VHL) tumor suppressor gene contributes to the development of inherited and sporadic cancer in a pattern which conforms to the predictions of Knudson’s “two hit” hypothesis. In the inherited syndrome, affected individuals bearing a germ-line mutation are strongly predisposed to highly angiogenic tumors of the retina and central nervous system, phaeochromocytoma, and renal cell carcinoma, whereas in sporadic renal cell carcinoma an important pathogenetic role is indicated by the high frequency of somatic mutation or inactivation of both VHl alleles (for review, see Ref. 1). A function in oxygen-regulated gene expression was first suggested by the observation that the hypoxia inducible mRNAs encoding vascular endothelial growth factor and glucose transporter-1 (GLUT1) were up-regulated in VHL defective renal cell carcinoma cell lines (2, 3). Important mechanistic insights into this phenomenon have recently been gained through studies of the action of pVHL on the transcriptional complex hypoxia inducible factor-1 (HIF-1) (4). HIF-1 is a heterodimer of basic helix-loop-helix PAS proteins, HIF-α and HIF-β (5), which plays a critical role in a broad range of responses to hypoxia. These include the regulation of genes involved in angiogenesis, erythropoiesis, energy metabolism, iron metabolism, vasomotor control, inflammation, tissue matrix metabolism, and cell survival decisions (6). A central mode of regulation of HIF-1 is through oxygen-regulated proteolysis of HIF-α subunits (HIF-1α and HIF-2α) involving the ubiquitin-proteasome pathway (7–9). Studies in VHL defective renal cell carcinoma lines indicated that pVHL has a critical function in this process, since in VHL-defective cells HIF-α subunits were found to be constitutively stabilized, and re-expression of pVHL restored oxygen-dependent proteolysis. Immunoprecipitation and electrophoretic mobility supershift assays demonstrated that pVHL and HIF-α subunits were physically associated in a range of cell types, consistent with a general role for pVHL in the regulation of HIF-α proteolysis (4). However, the precise mechanism of HIF-α targeting by pVHL was not defined.

In an independent line of investigation into pVHL function, protein association experiments defined a series of molecules that interact with pVHL, including elongins B and C and a member of the cullin family (CUL2) (10–13). The recognition that another cullin (Cdc53) played a key role in ubiquitin ligase complexes, which are necessary for proteolytic destruction at cell cycle transitions in yeast, suggested a role for pVHL in proteolysis (12), and led to the proposal of a model based on homology with the SCF (Skp-1-Cdc53/Cullin-F-box) class of ubiquitin ligases (13). Combinatorial arrangements of Skp-1, Cullin, and F-box proteins in SCF complexes provide different E3 target specificities, which are known or predicted to depend on association between substrate and an interaction domain on the F-box component (for review, see Ref. 14). It was proposed that the VHL-elongin B/C/CUL2 complex might form a new class of ubiquitin ligases with pVHL playing a role analogous to...
the F-box protein, although the substrate(s) were undefined. This model has recently been supported by further similarities to SCF complexes noted in structural studies of the VHL-elongin B/C complex (15), and by recognition that another protein, Rbx1, is a common component of SCF and VHL-elongin B/C/UC2 complexes (16).

Taken together these observations suggested a specific hypothesis, that pVHL might act as the recognition component of a ubiquitin ligase complex that targets HIF-α subunits for ubiquitin-dependent proteolysis. If correct, this hypothesis has clear implications for understanding the role of pVHL in cellular oxygen sensing, and the mechanism of tumor suppressor function. Furthermore, it raises important questions concerning the range of pVHL substrates, the domains responsible for substrate capture, and the relationship to sites of oncogenic mutation.

In this work we provide direct evidence in support of this hypothesis and address the associated questions. We demonstrate a critical role for pVHL in HIF-α ubiquitylation using in vitro ubiquitylation assays. Using a display of metabolically labeled proteins which co-precipitated with pVHL in untreated and proteasomally blocked cells we show that the range of pVHL-associated proteolytic targets is limited and confined in and proteasomally blocked cells we show that the range of labeled proteins which co-precipitated with pVHL in untreated more, we show that the HIF-α transactivation domains that interact with pVHL. Furthermore, we show that the HIF-α interactions are disrupted by tumor-associated mutations in the β-domain of pVHL and that this is associated with defective HIF-α ubiquitylation in vitro, defective HIF-α regulation in vivo, and up-regulation of HIF target gene expression.

EXPERIMENTAL PROCEDURES

Cell Culture—RC4 cells (4), originally a gift from C. H. M. Cu Bay, and COS7 cells were maintained in Dulbecco's modified Eagles medium with 10% fetal calf serum. RC4/VHL is a subline re-expressing wild type pVHL following stable transfection with pcDNA3-VHL (4). Further series of constructs were generated from RC4/VHL with wild type or mutant pVHL expression plasmids (see below), or empty vector, using Fugene6 (Roche Molecular Biochemicals), followed by selection in G418 (1 mg/ml). Clones were picked as individual colonies and maintained in G418. Expression of stably transfected VHL genes was checked by immunoblotting with anti-pVHL or anti-HA antibody. To determine if two independent clones were studied for each expression plasmid. Twenty-four hours before the experimental exposures, cells were subdivided onto 75-m² dishes in medium lacking G418. Parallel exposures to control and test conditions were generally for 4 h. Hypoxic incubation was in an atmosphere of 1% oxygen, 5% CO2, balance nitrogen. 1 ml of 100 m M NaCl, 0.5% Igepal CA630 (Sigma), 20 m M Tris-HCl (pH 7.5), 1.5 m M MgCl2, 1 m M sodium orthovanadate, 5 m M leupeptin, with 1 m M benzoxyl-L-leucinyl-L-leucinyl-L-norvalinal and 100 m M calpain inhibitor I (Sigma). In Vitro Translation—[35S]Methionine-labeled proteins were prepared by coupled transcription and translation reactions of expression plasmids in rabbit reticulocyte lysate (TNT, Promega).

In Vitro Interaction Assay—[35S]Methionine-labeled proteins were produced in reticulocyte lysates programmed with plasmids encoding HA epitope-tagged pVHL and HIF-α sequences. 1 μl of the indicated lysates was mixed in 100 μl of NETN buffer (150 m M NaCl, 0.5 m M EDTA, 10 m M Tris-HCl, 1 m M MgCl2, 0.5% Igepal CA630 (Sigma), 10 m M dithiothreitol). After removal of buffer, cells were disrupted in a Dounce homogenizer. Following lysis, crude extract was centrifuged at 10,000 × g for 10 min at 4 °C to remove cell debris and nuclei, and stored in aliquots at −70 °C. Ubiquitylation assays were carried out at 30 °C in a total volume of 40 μl, containing 2 μl of programmed reticulocyte lysate, 27 μl of cell extract, 4 μl of 10 × ATP-regenerating system (20 m M Tris, pH 7.5, 10 m M ATP, 10 m M magnesium acetate, 300 m M creatine phosphate, 0.5 m M creatine phosphokinase), 4 μl of 5 mg/ml ubiquitin (Sigma) or methylated ubiquitin (AFFINITI Research Products), 0.83 μl of 150 μg/ml ubiquitin aldehyde (AFFINITI Research Products). For pVHL reconstitution experiments, [35S]methionine-labeled wild type or mutant pVHL (4 μl of programmed reticulocyte lysate) was preincubated with the reaction mixture at room temperature for 5 min prior to addition of substrate. Aliquots were removed at indicated times, mixed with SDS sample buffer, and analyzed by SDS-PAGE and autoradiography. Gels were quantitated using a Storm 840 PhosphorImager (Molecular Dynamics).

RESULTS

To test the hypothesis that pVHL is a necessary component of a ubiquitin ligase complex recognizing HIF-α we developed an in vitro ubiquitylation assay for HIF-1α. In this assay, cell extracts (initially from COS7) were incubated with [35S]methionine-labeled HIF-1α prepared in vitro in reticulocyte lysates. Incubation with extract alone converted the HIF-1α substrate to a slower migrating form, an effect which was enhanced by an


ATP-regenerating system (Fig. 1) and was prevented by addition of ubiquitin, which prevents the formation of multiubiquitin chains and acts as a chain terminator (20), consistent with the inherent stability of this subunit (21). Myc is a highly unstable transcription factor, which is destroyed by ubiquitin-mediated proteolysis (22). Myc substrate was ubiquitylated, but in contrast with HIF-1α, Myc ubiquitylation was similar between VHL defective and pVHL re-expressing RCC4 sublines. Although HIF-1α ubiquitylation was clearly more efficient in VHL-deficient cells, a low level was apparent in the defective cells.

We next tested the specificity of pVHL-dependent ubiquitylation using a range of other transcription factors. No ubiquitylation of HIF-1β could be demonstrated, irrespective of VHL status (Fig. 3, second panel), consistent with the inherent stability of this subunit (21). Myc is a highly unstable transcription factor, which is destroyed by ubiquitin-mediated proteolysis (22). Myc substrate was ubiquitylated, but in contrast with HIF-1α, Myc ubiquitylation was similar between VHL defective and VHL competent extracts (Fig. 3, third panel). Iron regulatory protein-2 (IRP-2) which, like HIF-1α, is destroyed in an oxygen-dependent manner by the ubiquitin-proteasome pathway (23) also showed no evidence of pVHL-dependent ubiquitylation (Fig. 3, fourth panel). Thus pVHL-dependent ubiquitylation was specific for HIF-α among a range of substrates tested in this assay. To provide a further unprejudiced insight into the range of proteolytic substrates targeted by pVHL we sought to display metabolically labeled substrates captured in vitro in pVHL immunoprecipitates. We performed a comparative display of anti-pVHL immunoprecipitates from normal cells and cells treated with proteasomal inhibitors, arguing that this should reveal differentially expressed species corresponding to HIF-α subunits, and should also provide a display of other pVHL target proteins which are degraded in a similar manner.

For these experiments we used the VHL defective renal carcinoma line RCC4, and stably transfected derivatives expressing wild type pVHL (RCC4/VHL) or C-terminal HA
epitope-tagged pVHL (RCC4/VHL.HA). Lysates were prepared from cells labeled with [35S]methionine/cysteine (4 h) under the indicated conditions. A, control (anti-SV40T) or anti-pVHL immunoprecipitates from wild type VHL stable transfectant (RCC4/VHL) cells (lanes 1–4), or untransfected VHL defective RCC4 cells (lanes 5 and 6). Several non-specifically precipitated species were retrieved (lanes 1, 5, and 6). pVHL was retrieved as three species of approximately 19, 24, and 27 kDa (lanes 2–4), present in RCC4/VHL but not RCC4 cells. Under standard conditions (lane 2) specific interacting proteins were identified with mobilities consistent with previous assignments as fibronectin (FN, ~200 kDa), CUL2 (~70 kDa), elongin B (~18 kDa), and elongin C (~15 kDa). In RCC4/VHL cells exposed to proteasomal inhibitors (PI; lanes 3 and 4) two additional species were precipitated migrating at ~135 kDa and ~120 kDa. Comparison with anti-HIF-1α and anti-HIF-2α immunoprecipitates in other gels (see part C and Figs. 5A and 6A) showed co-migration of these species with HIF-1α and HIF-2α, respectively. B, anti-HA immunoprecipitates of RCC4/VHL.HA cells stably transfected with HA epitope-tagged pVHL. The species identified were similar to those in A (in this experiment, elongin B and C were run off the gel). Capture of HIF-α subunits was observed in cells exposed to proteasomal inhibitors (lanes 2 and 4), and at a lower intensity in cells exposed to hypoxia without inhibitors (lane 3, HIF-1α was also visible on longer exposures). C, immunoprecipitates of RCC4 cells stably transfected with empty vector (lane 1), or pcDNA3.VHL(54–213).HA expressing epitope-tagged p19 pVHL (lanes 2–5). The p19 form of pVHL captured similar species to those captured by pVHL expressed from pcDNA3.VHL.HA (compare lane 2 with B, lane 4). Lanes 3 and 4 show anti-HIF-1α and anti-HIF-2α immunoprecipitates. Labeled species co-migrated with the species revealed by proteasomal blockade in the anti-HA precipitates (lane 2). Note, however, that labeled pVHL is not efficiently displayed in the labeled anti-HIF-α immunoprecipitates, although it can be detected by Western analysis of anti-HIF-α immunoprecipitates (4). This may in part reflect reduced labeling efficiency of pVHL compared with HIF-α, but also that co-immunoprecipitation of HIF-α associated species is inefficient with these antibodies.
and C), further truncation to codon 72 almost completely abolished the capture (Fig. 5A). Analysis of C-terminal truncations demonstrated that truncation to codon 187 greatly reduced capture of HIF-2α, and abolished capture of HIF-1α, while truncation to codon 156 abolished capture of both species (Fig. 5B). Effects on HIF-α capture correlated well with functional effects on the regulation of these molecules. Thus expression of the p19 form of pVHL suppressed the normoxic level of HIF-α subunits (data not shown) in keeping with its recognized ability to suppress expression of vascular endothelial growth factor and GLUT1 (24, 25), whereas failure of capture by the other truncations was associated with a constitutively high level of both HIF-α subunits in normoxia (Fig. 5C).

Many tumor-associated pVHL mutations are missense mutations which have been noted to cluster in two regions of the molecule (27). Previous studies have demonstrated reduced or absent elongin B/C capture by tumor-associated mutations in the core elongin-binding region of pVHL (residues 157–171) thus providing an explanation for defective pVHL function in this setting (10–13, 28). A second cluster of mutations lies nearer the N terminus, in the evolutionarily conserved region of exon 1 (residues 54 to 113); we hypothesized that these mutations might alter the ability to capture HIF-α. To investigate this we created a further series of stable RCC4 transfectants expressing epitope-tagged pVHL molecules with the following tumor-associated mutations: R82P, P86H, N90I, Q96P, Y98N, and Y112H. When compared with cells expressing wild type pVHL, HIF-α capture was greatly reduced or abolished by all the mutations except for Y112H, which did not capture HIF-1α and co-immunoprecipitated HIF-2α to a much reduced level (Fig. 6A). None of the mutant pVHLs co-precipitated the ~200 kDa species previously assigned as fibronectin (26). Y98N and Y112H captured elongin B and elongin C similarly to wild type pVHL, whereas the other mutants showed impaired capture. Functional analysis of HIF-α regulation showed dys-regulation of HIF-α levels for all mutants, with high normoxic levels compared with the wild type transfectant (Fig. 6B). The mutants R82P, P86H, N90I, and Q96P showed no regulation of HIF-α, whereas a low level of regulation was restored by Y98N and Y112H. Consistent with this, none of the mutants corrected constitutive up-regulation of GLUT1 in normoxic VHL defective cells as effectively as wild type, although partial correction was observed with Y98N and Y112H (Fig. 6C). Overall, these results indicate that the majority of the pVHL molecule beyond the second initiation site at codon 54 is required for effective HIF capture and regulation by oxygen. These properties are ablated by a series of tumor-associated mutations in the β-domain, some of which retain the ability to capture elongin B/C.

To further understand the functional effects of these mutations, we developed an in vitro reconstitution system for HIF-1α ubiquitylation using pVHL produced by in vitro transcription and translation. Addition of either full-length wild type pVHL, or p19 pVHL to RCC4 cell extract resulted in a large increase in the rate of HIF-1α ubiquitylation (Fig. 7A). The mutants R82P, P86H, N90I, Q96P, Y98N, and Y112H were all tested in this way. Since the pVHL molecules were labeled it was clear that a similar amount of each was added. Each of these mutants was markedly defective compared with wild type (Fig. 7, B and C), a result in keeping with the defective HIF-α regulation observed in vivo.

We next wished to define the subsequences of the HIF-α subunits that are recognized by pVHL. Since stable overexpression of transfected HIF-α subunits has presented substantial problems, we sought to define an in vitro system to enable further studies of the pVHL/HIF-α interaction. Fig. 8A shows that in a cell-free system using labeled proteins produced in rabbit reticulocyte lysates both HIF-1α and HIF-2α interact with pVHL. Testing of N-terminal pVHL truncations in this assay demonstrated that while truncation to the second initi-
We next tested the pVHL molecules bearing missense mutations in the β-domain of pVHL on co-immunoprecipitation of proteins and regulation of HIF-α subunits. A, immunoprecipitates from a series of RCC4 transfectants stably expressing HA epitope-tagged wild type pVHL (WT), the indicated mutant pVHL, or the vector alone. Cellular proteins were labeled with [35S]methionine/cysteine in the presence of hypoxia and proteasomal inhibitors (4 h). Despite retrieval of pVHL which is greater than that in the wild type transfectant used in this experiment, mutant pVHLS showed much reduced (Y112H) or absent co-precipitation of HIF-α subunits. The pVHL mutants Y98N and Y112H co-precipitate elongin B/C. B, immunoblot showing HIF-α subunit regulation by oxygen in the same series of transfectants. Regulation is restored by wild type but not mutant pVHLS, although Y98N and Y112H partially suppressed the normoxic level of HIF-α. C, immunoblot of glucose transporter GLUT1 in lysates of normoxic transfectants. Wild type pVHL transfectants but not the mutants R82P, P86H, N90I, and Q96P suppress normoxic GLUT1 expression. Mutants Y98N and Y112H show incomplete suppression. Experiments were performed on at least two independent clones for each mutation with similar results.

Fig. 6. Effect of tumor-associated missense mutations in the β-domain of pVHL on co-immunoprecipitation of proteins and regulation of HIF-α subunits. A, immunoprecipitates from a series of RCC4 transfectants stably expressing HA epitope-tagged wild type pVHL (WT), the indicated mutant pVHL, or the vector alone. Cellular proteins were labeled with [35S]methionine/cysteine in the presence of hypoxia and proteasomal inhibitors (4 h). Despite retrieval of pVHL which is greater than that in the wild type transfectant used in this experiment, mutant pVHLS showed much reduced (Y112H) or absent co-precipitation of HIF-α subunits. The pVHL mutants Y98N and Y112H co-precipitate elongin B/C. B, immunoblot showing HIF-α subunit regulation by oxygen in the same series of transfectants. Regulation is restored by wild type but not mutant pVHLS, although Y98N and Y112H partially suppressed the normoxic level of HIF-α. C, immunoblot of glucose transporter GLUT1 in lysates of normoxic transfectants. Wild type pVHL transfectants but not the mutants R82P, P86H, N90I, and Q96P suppress normoxic GLUT1 expression. Mutants Y98N and Y112H show incomplete suppression. Experiments were performed on at least two independent clones for each mutation with similar results.

Having established the fidelity of this in vitro pVHL interaction we proceeded to examine a series of fusion proteins containing full-length or truncated HIF-α subunits fused with either the glucocorticoid receptor or GAL4 DNA-binding domains. These fusion proteins have previously been used to characterize regulatory and transactivation domains in HIF-1α and HIF-2α (17, 18). In the first series of experiments we examined truncations of HIF-1α (Fig. 8C). Although there was some variation in the efficiency of capture among interacting molecules, we observed a marked decrease in HIF-α capture in vitro (results are illustrated for P86N and Y98N, Fig. 8A). Interestingly, the Y112H mutation showed some residual binding to HIF-2α in this assay (data not shown) similar to that seen in the cells stably transfected with this molecule. Overall, the results of this in vitro binding assay showed a striking correlation with the results of the in vivo experiments.

To further investigate the interaction we tested deletions and subsequences within this domain and sought to correlate pVHL binding in vitro with our previous in vivo functional analyses of oxygen regulated activity. For, HIF-1α amino acid sequences 530–634, 549–634, but not 572–634 could be captured by pVHL whereas for HIF-2α, amino acids 517–682, but not 534–682 could be captured by pVHL. In control experiments none of these interactions were observed using a mutant pVHL (P86H). The data show exact concordance between competence for pVHL binding and the previously reported ability of these fusion proteins to convey oxygen regulated responses in transiently transfected Hep3B cells (17, 18), and showed that HIF-1α residues 549–572 and HIF-2α residues 517–534 are critical for pVHL binding. Finally we tested previously defined minimal oxygen-regulated domains for pVHL interaction and found that HIF-1α sequences 549–582 (Fig. 8E), and the homologous HIF-2α sequences 517–552 (data not shown) were sufficient for interaction with pVHL.

DISCUSSION

Through the use of novel interaction and ubiquitylation assays this study provides direct evidence that pVHL regulates HIF-α subunits by acting as the recognition component of a ubiquitin ligase complex. It also addresses several questions raised by this finding. In particular, it indicates that the range of pVHL substrates appears limited, defines domains involved in HIF-α capture, and establishes that several oncogenic mutations in the β domain of pVHL prevent HIF-α capture.

The specificity of pVHL-dependent ubiquitylation for HIF-α demonstrated in vitro was reinforced by the differential display in vivo of pVHL-associated proteins in the presence and absence of proteasomal inhibitors. This readily demonstrated

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both HIF-1α and HIF-2α but did not reveal other proteosomal substrates targeted by pVHL. Although it is possible that HIF-1α and HIF-2α are unique targets, there are important caveats to this observation. Other species may associate less stably with pVHL, or may label poorly under the conditions used. Nevertheless we have obtained similar results using longer labeling times. Whether or not there are other substrates, these results support the importance of HIF-1α and HIF-2α as pVHL targets, and we wished to understand this interaction in greater detail.

Our findings establish first that the p19 form of pVHL could interact effectively with HIF-α, second that truncations at either end of this pVHL molecule prevent the interaction, third that a series of tumor-associated missense mutations in pVHL sited away from the core elongin-binding domain all disrupt the interaction, fourth that defective HIF-α capture could be observed in the presence of preserved elongin B/C capture, and finally that inability to capture HIF-α subunits correlated with inability to regulate their destruction.

During the course of this work the structure of the VBC complex was solved (15). The pVHL molecule has an α domain (residues 155 to 192) which forms the principal contacts with elongin C, and a larger β domain consisting of a seven-stranded β sandwich (residues 63 to 154) and an α helix (residues 193–204). Of the mutations tested, two (Y98N and Y112H) affected the HIF-α interaction without affecting elongin B/C capture. Both affect surface exposed residues at a predicted molecular interaction site formed from the β-domain. The other β-domain mutations appeared to have additional effects on elongin B/C capture in our study suggesting they may have effects on the overall molecular conformation. The effect of C-terminal truncation mutants on the interaction between pVHL and HIF-α may result from loss of the α helix which contributes to the β-domain. Overall the findings suggest the pVHL β-domain forms an interface which interacts directly or indirectly with HIF-α subunits, and which can be disturbed by mutations in many parts of the molecule. Previous studies of pVHL mutants have examined the effect of truncations and C-terminal missense mutations and concluded that capture of the elongin B/C–CUL2 complex by the C-terminal core elongin-binding domain was necessary but not sufficient for hypoxia inducible gene regulation (13). Taken together, the results suggest a model in which this regulation requires pVHL to interact with HIF-α through the β domain, and with the rest of the ubiquitin ligase complex through the core elongin C-binding domain.

Interestingly, the mutations we studied also affected the interaction with a species previously assigned as fibronectin (26). Whether HIF-α and fibronectin interactions represent connected or unconnected functions of pVHL is not yet known, but it could be of interest to examine the role of fibronectin regulation in HIF regulation or vice versa. In contrast with the analysis of pVHL in which truncations beyond the p19 variant all prevented HIF-α capture, we were able to define short subsequences within HIF-α which were sufficient for recognition by pVHL. Thus for HIF-1α we showed that sequences 549–582 were sufficient for pVHL interaction, and that HIF-1α residues 549–572, or HIF-2α residues 517–534 were necessary for this interaction. Interestingly, these sequences correspond to a region of striking conservation in the internal degradation domains of HIF-1α and HIF-2α which are otherwise surprisingly poorly conserved. It is also of interest that in both molecules these regions are contained within an internal transactivation domain, reflecting an emerging pattern of overlap between activation and destruction elements in unstable tran-

**Fig. 7. Complementation of defective HIF-1α ubiquitylation in RCC4 cell extracts by exogenous pVHL.** 35S-Labeled HIF-1α prepared in reticulocyte lysate was incubated with RCC4 extract, an ATP-regenerating system, ubiquitin, and ubiquitin aldehyde. The effect of adding 35S-labeled wild type or mutant pVHL prepared in separate reticulocyte lysates was tested. A, addition of either pVHL.HA or pVHL(54–213).HA in vitro to RCC4 cell extract greatly enhanced the ubiquitylation of HIF-1α. B, the addition of the mutant pVHL Y98N was much less effective than wild type pVHL.HA (WT) in enhancing HIF-1α ubiquitylation. C, pVHL molecules bearing the six missense mutations were tested for effects on ubiquitylation of HIF-1α in vitro by RCC4 cell extract over 30 min without ubiquitin aldehyde. No enhancement was seen over the control (vector programmed lysate), in contrast to the effects of wild type pVHL or pVHL(54–213). In other experiments (not shown) these mutant pVHL molecules showed no enhancement of ubiquitylation compared with controls both at longer time points and in the presence of ubiquitin aldehyde. For illustration the lane displaying unreacted HIF-1α substrate has been reproduced at the left-hand side of each panel in A and B.
scription factors (29). The definition of these residues as critical for an interaction with pVHL should help direct further studies of the function of the degradation domains. Previous studies have indicated that much larger domains including the non-conserved portions of both HIF-α subunits are required for the most efficient transfer of the oxygen-dependent degradation property (8, 18). Whether these sequences contain other pVHL interaction sites, or sequences that can transmit degradation triggering signals separate from the interaction with pVHL, is not clear. In extracts of stimulated mammalian cells HIF-1α is heavily phosphorylated (19). For many SCF complexes, target phosphorylation has been shown to be necessary for substrate recognition (14). Phosphorylation of HIF-1α in reticulocyte lysate is incomplete (Ref. 19 and data not shown), so the interaction of HIF-1α produced in reticulocyte lysate with pVHL suggests that full phosphorylation of HIF-1α is not necessary for binding.

In the in vitro studies of ubiquitylation, pVHL-dependent ubiquitylation was specific for HIF-α among a group of substrates tested. Important among these was IRP-2, an iron-regulated RNA-binding protein which is targeted for ubiquitylation-dependent proteolysis by a mechanism which is postulated to involve oxidative marking of a degradation domain (23). Like HIF-α, IRP-2 proteolysis is dependent on oxygen and iron, and is inhibited by cobaltous ions (30). Nevertheless we found that IRP-2 is not a pVHL substrate. This indicates that similar regulatory characteristics apparently affect two different ubiquitylation systems and raises an inter-
esting question as to how general the action of oxygen and iron might be in such processes.

At longer incubation times we observed some ubiquitylation of HIF-α by RCC4 extracts consistent with the possibility that there may be other, pVHL independent, ubiquitylation mechanisms (31). However, the enhancement of HIF-α ubiquitylation by pVHL was striking, and provided clear evidence for the function of pVHL in this process. Two reports published during the course of this work complement the current study (32, 33). These demonstrated that multiprotein complexes containing pVHL precipitated from cell extracts have E3 ubiquitin ligase activity and contain ubiquitylated species without identifying these proteins precisely. In one study two predominant ubiquitylated species were described with approximate mobilities of 100 and 220 kDa and similar mobility species bound recombinant VBC complex in a “Far Western” experiment (33). Although the faster mobility species might correspond to one or other HIF-α subunit, the approach taken is likely to be insensitive in the detection of these proteins because rapid degradation in the presence of oxygen would result in low levels in the normoxic cell extracts used. Conversely, the approach we have used might not efficiently label or capture certain pVHL-bind-

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