VHL-mediated Hypoxia Regulation of Cyclin D1 in Renal Carcinoma Cells

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Abstract

Renal cell carcinoma is associated with mutation of the von Hippel-Lindau (VHL) tumor suppressor gene. Cell lines derived from these tumors cannot exit the cell cycle when deprived of growth factors, and the ability to exit the cell cycle can be restored by the reintroduction of wild-type protein VHL (pVHL). Here, we report that cyclin D1 is overexpressed and remains inappropriately high during contact inhibition in pVHL-deficient cell lines. In addition, hypoxia increased the expression of cyclin D1 specifically in pVHL-negative cell lines into which pVHL expression was restored. Hypoxic induction of cyclin D1 was not observed in other pVHL-positive cell lines. This suggests a model whereby in some kidney cell types, pVHL may regulate a proliferative response to hypoxia, whereas the loss of pVHL leads to constitutively elevated cyclin D1 and abnormal proliferation under normal growth conditions.

Introduction

The VHL\(^2\) tumor suppressor gene is frequently mutated in a high percentage of RCCs (1). Over the last several years, the function of the pVHL has been elucidated (2). The pVHL acts as the substrate recognition module of an E3 ubiquitin ligase that targets proteins for degradation. The only known targets for pVHL are the hypoxia-inducible transcription factors HIF-1\(\alpha\) and HIF-2\(\alpha\). These results explain the apparent misregulation of hypoxia-inducible genes under normal oxygen levels in cell lines not expressing pVHL. The HIF targets that are overexpressed in VHL-deficient cell lines include the GLUT-1, vascular endothelial growth factor, and several metabolic enzymes (3). Presumably, the overexpression of vascular endothelial growth factor leads to the highly vascular nature of these tumors. Current knowledge about cancer suggests that all of the cancer cells exhibit relaxed control over entrance into and/or progression through the cell cycle (4). It is now generally believed that some component of the Rb pathway is altered in a large percentage of human tumors (5). The Rb pathway regulates cell cycle progression from G\(_1\) to S phase. Loss of either Rb or p16 expression releases cell cycle control via loss of p16 inhibition of the cyclin D1/CDK4 complex or loss of Rb inhibition of the E2F transcription factor. Alternatively, the Rb pathway can be disrupted by overexpression of cyclin D1, as is commonly observed in breast cancer. RCC cell lines that arise because of inactivation of the VHL gene have been shown to be resistant to cell cycle arrest when deprived of exogenous growth factors (6). Furthermore, this in vitro phenotype is reversed with the reintroduction of WT pVHL. Studies of Rb in RCC have demonstrated that loss of Rb expression is rarely seen in these cancers (7). On the other hand, p16 expression is lost in only \(\sim 30\%\) of the RCCs, with p16 loss occurring by a variety of mechanisms including gene mutation, deletion, or silencing via hypermethylation (7, 8). We decided to undertake a survey of cell cycle proteins in RCC cell lines to determine the source of their failure to remain quiescent on growth factor withdrawal. Here we demonstrate that the absence of pVHL expression is responsible for enhanced expression of cyclin D1 and that reintroduction of WT pVHL represses cyclin D1 overexpression. In addition, our work reveals that pVHL mediates the hypoxia-inducible, regulated expression of this critical cell cycle regulator. This unexpected regulation is observed only after reintroduction of pVHL into cancer cell lines that emerged as the result of loss of functional pVHL.

Materials and Methods

Cells and Transfections. 786–0 cell lines (truncated pVHL and amino acids 1–115) expressing hemagglutinin-tagged pVHL were a gift from W. G. Kaelin (Dana Farber Cancer Institute, Boston, MA; Ref. 9). The VHL-deficient RCC lines (111, 121, and T20) and VHL-positive RCC lines (112, 171, and 181) were obtained from the laboratory of W. M. Linehan, National Cancer Institute, Bethesda, MD (10). The cell lines 111, 121, T20, 171, and 181 were all from tumors classified histologically as renal clear cell, whereas cell line 112 was from a histologically papillary tumor. In addition, cell line 121 contains a hypermethylated VHL gene. Only T20 was derived from a VHL patient; the others were from sporadic kidney cancer. HEK293 and MCF7 were obtained from the American Type Culture Collection, and other cell lines used for the hypoxia analyses were obtained from J. Bonifacino and T. Rouault (National Institute of Child and Health Development, Bethesda, MD). Cell lines were grown in complete DMEM with 10% FCS (BioFluids, Inc.) at 37°C with 5% CO\(_2\). To contact inhibit the cell lines, log-phase cells were grown to confluence, after which the medium was changed. The medium was replaced 3–4 days later, and the cells allowed to grow for an additional 3–4 days. FACS analysis (described below) indicated \(<1.0\%\) of the cells were in S phase at this point in the culture.

Retrovirial Infection. Recombinant retroviruses were obtained by transfection of the relevant retroviral construct into Phoenix cells. Medium from these cells was collected 48 h after transfection and was used to infect exponentially growing 111, 121, and T20 RCC cells. Puromycin resistance suggested an \(<75\%\) infection efficiency.

Cell Cycle Analysis. Cells were trypsinized, washed with 1× PBS, and treated with RNase A, followed by incubation in PI. Stained cells were analyzed on a Becton Dickinson FACScan flow cytometer. For BrdUrd analysis, cells were cultured in medium (containing 10% or 0% serum depending on the experimental conditions) containing 10 \(\mu\)M BrdUrd for 1 h. Cells were then trypsinized, washed with 1× PBS, and fixed in methanol for 1 h at \(-20^\circ\text{C}\), followed by staining with FITC-conjugated anti-BrdUrd antibodies and PI in accordance with the manufacturer’s specifications. BrdUrd/PI-stained cells were also analyzed on a Becton Dickinson FACScan flow cytometer.

Antibodies. The following antibodies were used as probes for Western blotting: cyclin D1 (DCS-6; PharMingen and Santa Cruz), p27\(^{kip}\) (57; Transduction), cyclin A (BP683; PharMingen), CDK4 and CDK6 (PharMingen and Calbiochem), GLUT-1 (Alpha Diagnostic), HIF-1\(\alpha\) (54; Transduction), cyclin D2 (34B1–3; Oncogene), and cyclin D3

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(G107–565; PharMingen). The following antibodies were used specifically for immunoprecipitation: cyclin D1 (Neomarkers) and VHL (Ig32; PharMingen).

**Cell Lysis, Western Blotting, and Immunoprecipitation.** For protein analysis, cells were scraped and lysed on ice in IP-B buffer [20 mM Tris-HCl (pH 7.4), 1% NP40, 1 mM sodium orthovanadate, and “complete-mini” protease inhibitor tablet (Boehringer)] for 45 min on ice. After centrifugation and protein concentration determination (D/C Protein Assay; Bio-Rad), 100-μg aliquots of lysates were resolved by SDS-PAGE analysis. Proteins were transferred to Immobilon-P membrane (Millipore), blocked for 1 h in 5% nonfat dry milk (in 1× PBS and 0.1% Tween 20, 1× PBS-T). Blots were probed with the indicated antibodies for either 1 h at room temperature or overnight at 4°C. Protein bands were visualized with horseradish peroxidase-conjugated antimouse or antirabbit IgG and enhanced chemiluminescence detection system (Amersham). For immunoprecipitation, 1–3 μg of protein lysates were precleared with 50 μl of packed Gamma-Bind Sepharose beads (Pharmacia) for 2–3 h at 4°C. Lysates were then mixed with 100-μl packed beads and 10 μg of the indicated antibody, and then rocked overnight at 4°C. Beads were washed three to five times in 1× PBS-T, eluted with SDS sample buffer, and resolved by SDS-PAGE analysis followed by Western blotting.

**Metabolic Labeling.** Cells growing in tissue culture dishes were incubated at 37°C for 1 h in DMEM (-methylionine, -cysteine) with 10% diazyed FCS. The cells were pulse-labeled for 30 min in complete DMEM with 0.1 mM/ml [35S]methylionine/cysteine (ICN) and chased for indicated times. Cyclin D1 was immunoprecipitated as described earlier and quantitated by a phosphorimager.

**Northern Blotting.** Total RNA was prepared using Trizol (Life Technologies, Inc.), and 2.5–5 μg was denatured with glyoxal and then run on a 1.25% agarose gel in 4-morpholinopropanesulfonic acid buffer. The RNA was transferred to Nytran (S & S), heated to remove the glyoxal modification, and hybridized in Hybrisol I (Intergen). DNA probes, filter hybridization, and washing were carried out using standard protocols.

**Hypoxia Assays.** Cells growing in 15-cm dishes were incubated for 12–15 h in hypoxia chambers (Billups-Rothenberg) containing 0.5% oxygen, 5% carbon dioxide, and 95% nitrogen at 37°C. Parallel normoxic plates were run under standard conditions.

**Results**

We wanted to examine the molecular mechanism responsible for the altered cell cycle response of RCC cells on serum deprivation through an analysis of G1-phase regulatory proteins. We studied the 786-0 RCC cell line (VHL-deficient) stably transfected with the WT VHL gene or vector alone (pRc; Ref. 9). These cell lines were synchronized by contact inhibition (S phase was 65%). By 140 h after release, the pRc cells underwent massive apoptosis (as measured by annexin V FACS analysis; data not shown), whereas the arrested WT cells remained adherent and viable for the remainder of the time course.

The majority of cell cycle-related proteins examined during the time course correlated well with the observed patterns of proliferation (Ref. 5; Fig. 1B). As both lines synchronously progressed through a single cell cycle, cyclin A expression and hyperphosphorylated ppRb (hyperphosphorylated retinoblastoma protein) increased and then decreased as would be expected. Additionally, p27kip, a CDK inhibitor, was down-regulated during proliferation and up-regulated during cell cycle arrest. As pRc cells re-entered the cell cycle, cyclin A and ppRb expression were induced, whereas p27kip expression was attenuated. However, cyclin D1 remained high throughout the time course in the pRc cells. In contrast, cyclin D1 remained low in WT cells increasing slightly only during the initial proliferation phase at 26 h. In pRc cells, cyclin D1 overexpression was observed at the zero time point (contact inhibition in 10% serum), suggesting that overexpression of cyclin D1 was independent of growth. Cyclin D3 did not show any significant differences during this time course, whereas cyclin D2 was not detectable in either cell line. We examined the effect of reintroducing WT pVHL into three additional VHL-deficient RCC cell lines. Reintroduced pVHL into these cell lines resulted in attenuation of cyclin D1 overexpression (Fig. 1C). Given these data, cyclin D1 appeared to be specifically and constitutively overexpressed in VHL mutant RCC cells, and reintroduction of WT pVHL into these cells resulted in a dramatic decrease in the cyclin D1 level.

Cyclin D1 is regulated by many post-transcriptional mechanisms.
including the ubiquitin-mediated proteolysis pathway (11). Recent evidence has demonstrated that pVHL can function as an E3-ubiquitin ligase (12), so we investigated whether pVHL regulated cyclin D1 protein half-life. The stability of cyclin D1 protein in VHL mutant and WT cells was assessed by [35S]methionine/cysteine pulse-chase analysis. As shown in Fig. 2A, the kinetics of cyclin D1 degradation were similar in 786–0 WT and pRc cell lines at log phase and at contact inhibition (t1/2 = 15–30 min). However, the pRc cells synthesized 3-fold more cyclin D1 protein than did the WT cells during log phase increasing to 6-fold more at contact inhibition. This difference in synthetic rate was also seen in the three “VHL-rescued” RCC lines presented earlier (data not shown). Using four independent WT and pRc clones, we examined the expression of cyclin D1 mRNA at contact inhibition by Northern blotting (Fig. 2B). All four of the pRc cell lines manifested significantly higher levels of cyclin D1 mRNA in comparison to the four WT cell lines studied (a 5–10-fold difference). There was also a close correlation between cyclin D1 mRNA and protein levels in each of the eight cell lines. These results support the increased expression of cyclin D1 in these cell lines is because of increased mRNA expression and not because of a post-translational mechanism.

Immunoprecipitation of cyclin D1 was used to examine its biochemical state in a contact-inhibited VHL-mutant cell (13). Using the 786–0 WT and pRc cell lines, it was found that the immunoprecipitated cyclin D1 in pRc cells was complexed with CDK4 (Fig. 3A). In addition, cyclin D1 coprecipitated p27Kip. The total amount of CDK4 protein but not CDK6 protein was elevated in the pRc cells (Fig. 3B), whereas CDK4 mRNA levels were similar in the two cell lines (data not shown), and this difference was most significant in contact-inhibited cells. This suggests a post-transcriptional mechanism to account for the elevated CDK4 protein level in the pRc cells. As shown in Fig. 3C, Western blot analysis of cycloheximide-treated cells indicated that the CDK4 protein was extrapolated to be significantly more stable in pRc cells (t1/2 = 5.7 h) than in WT cells (t1/2 = 2.2 h).

Fig. 2. Analysis of the regulation cyclin D1 expression. A, pulse-chase analysis of log-phase and contact-inhibited 786–0 WT (gray) and pRc (black) cells. Equal amounts of radiolabeled protein extract were immunoprecipitated, as determined by TCA precipitation of protein from lysates and quantitation of radioactivity in each lysate. B, correlation of cyclin D1 protein and mRNA expression in contact-inhibited cells. Multiple 786–0 WT and pRc clones (Clone ID 6 and 7) were analyzed by Western (WB) and Northern blotting (NB). 786–0 par is the original parental cell line. The mRNA levels were normalized to transketolase (TK) as a housekeeping gene. All values were then normalized to 786–0 parental, which was expressing the highest ratio of cyclin D1:transketolase.

Fig. 3. Interaction of cyclin D1 with CDK4 and p27KIP1. A, cyclin D1 was immunoprecipitated from multiple (Clone ID), contact-inhibited 786–0 WT and pRc cell lines. The immune complexes were analyzed by Western blotting for known binding partners of cyclin D1. B, Western blot analysis of CDK4 and CDK6 in log-phase and contact-inhibited 786–0 WT and pRc cell lines. As a loading control, blots were stripped and probed for cyclin D3, which has been shown not to vary between WT and pRc cell lines at any point in the cell cycle (Fig. 1B; data not shown). C, stability of CDK4 in contact-inhibited 786–0 WT and pRc cell lines. Cycloheximide was added at the zero time, extracts were prepared at the indicated times, and CDK4 levels were determined by Western blot analysis. Note that data beyond 4 h is not available because of cycloheximide toxicity in RCC cell lines.

pVHL has been shown to be responsible for the regulated expression of a number of hypoxia-controlled genes (3, 14). This regulation is through the ability of the pVHL ubiquitin-ligase complex to recognize HIF-1α and HIF-2α, targeting them for degradation under normoxic but not hypoxic conditions (2, 14, 15). It is pVHL, acting as an F-box protein, which recognizes the HIF transcription factors. With the loss of pVHL, numerous hypoxia-inducible genes are constitutionally expressed because the HIFs are stabilized. The elevated expression of the cyclin D1 mRNA in the absence of pVHL led us to examine whether cyclin D1 was also a hypoxia-induced gene.

Contact-inhibited 786–0 WT and pRc cells were incubated in hypoxia for 14 h, and the expression patterns of GLUT-1 and cyclin D1 were examined. As shown in Fig. 4A, GLUT-1 expression was significantly up-regulated in hypoxia compared with normoxia in the WT cells, whereas it was constitutively elevated in the pRc cells. In pRc cells, cyclin D1 expression was attenuated on exposure to hy-
poxia although still significantly higher than in normoxic WT cells. Interestingly, the expression of cyclin D1 in WT cells was induced ~3-fold by hypoxia. Northern blot analysis indicated that the induction of cyclin D1 in WT cells was the result of higher cyclin D1 mRNA. Western blots were repeated several times with two different pairs of WT and pRc clones to obtain a hypoxia:normoxia ratio of cyclin D1 and GLUT-1 protein expression. In hypoxia, cyclin D1 protein was induced by 5.5-fold in the WT cells, whereas it was slightly reduced by 0.7-fold in pRc. GLUT-1 protein was induced in hypoxia by 12.1-fold in WT, whereas it remained relatively constant in pRc (1.2-fold).

To test whether the pVHL-dependent hypoxia effect on cyclin D1 was dependent on contact inhibition, log-phase and serum-starved cells were also examined. Similar results were observed under both conditions in the 786–0 WT and pRc cells (Fig. 4B). The ratio of cyclin D1 protein induction by hypoxia was greatest in serum-starved cells (~10-fold) in the WT cells. This effect was not unique to 786–0 cells but was also observed in the independent VHL-negative lines 121 and T20. We next examined whether the pVHL-dependent hypoxia induction of cyclin D1 was observed in VHL-positive kidney cell lines. Western blot analysis of HIF-1α and cyclin D1 in several VHL-positive kidney and nonkidney cancer cell lines after exposure to hypoxia. In addition, the 171 cells were retrovirally transfected to express additional pVHL (171 + pVHL). All cell lines were in log-phase.

One question is whether in VHL-negative RCC cells the dysregulation of cyclin D1 expression is the locus of the Rb pathway inactivation. Rb protein was detected by Western blot analysis in all of the RCC cell lines used in this study (data not shown). However, p16 loss could also inactivate the Rb pathway, and this has been found in ~30% of RCC tumors (8). Therefore, we examined the RCC cell lines to determine their p16 status by genomic PCR, RT-PCR, and Western blot analysis using HEK293 and MCF7 cell lines as controls that express normal levels of pVHL (data not shown). The HEK293 cell line was found to retain the p16 gene, exhibited a strong RT-PCR product, whereas two of the other three (the VHL-positive 112 cell line) gave weak p16 RT-PCR product, whereas two of the other three (the VHL-positive 171 and VHL-hypermethylated 121 cell lines) gave weak p16 RT-PCR product.
 PCR products. The 121 cell line did not have detectable p16 protein by Western blot analysis. In summary, there was no consistent pattern to the presence or absence of the p16 gene or the p16 protein in relation to the VHL status of the cell lines.

Discussion

Cancer cells typically show misregulation in their control of the cell cycle. While this can occur through a variety of mechanisms and at a number of points in the cell cycle, virtually all cancer cells appear to have defects in the cell cycle controlling Rb pathway (4). Previous work established a defect in RCC-derived, VHL-negative cell lines in their ability to exit the cell cycle (6). Interestingly, whereas serum withdrawal (growth factor deprivation) from VHL-negative cells failed to persistently arrest the cycling of these cells, contact-inhibited withdrawal (growth factor deprivation) from VHL-negative cells were first synchronized by contact inhibition into G0. The cells were then released from G0 by plating at low density in serum-free medium, and a time course was carried out examining a wide variety of cell cycle proteins. Of the proteins examined, cyclin D1 was up-regulated in the VHL-negative cell lines during normal log-phase growth as well as during the G0 release into serum-free medium.

Cyclin D family of proteins (D1, D2, and D3) are important in the G1 to S phase transition (4). Cyclin D1 was the only member of the cyclin D family that showed differential expression in its level in a VHL-dependent manner. The cyclin D proteins are primarily involved in cell cycle progression through their binding to the CDKs CDK4 and CDK6. The cyclin D/CDK complex phosphorylates Rb, which then disassociates from the E2F transcription factor family of proteins. The E2F transcription factors are then able to reprogram the cell to enter the S phase. The CDK inhibitor p16 binds to the cyclin D/CDK complex and inhibits it from phosphorylating Rb causing the cell to arrest in G1. A similar model applies for arresting cells in G0, although this may be signalled through cell density (contact inhibition) as well as through growth factor levels via the RAS or MYC pathways (17).

Current literature strongly suggests that exposure to hypoxia generally inhibits cell proliferation, as well as global transcription and translation in multiple cell types (18). Specifically, it has been hypothesized that the cellular response to hypoxia involves reversible cell cycle arrest characterized by dephosphorylated Rb, loss of CDK activity, and decreased cyclin synthesis. However, relevant to our findings, hypoxia can actually induce certain cells in the kidney to proliferate (19, 20). Hypoxia induces the expression of erythropoietin in the kidney, which may act as a mitogenic growth factor for kidney cells (21). Alternatively, TGF-α is overexpressed in VHL-negative kidney cells in normoxia but is induced by hypoxia in a VHL-independent manner (22). TGF-α is also known to stimulate proximal renal tubule cells, which have been proposed but not proven to be the cell of origin of RCC (23). Perhaps in this manner, TGF-α is playing a significant role in stimulating cell proliferation of certain cell lineages in a VHL-dependent manner in the kidney.

The results reported here suggest that pVHL can mediate the hypoxia-regulated induction of cyclin D1 mRNA levels. This was only observed in VHL-negative tumor cell lines into which WT pVHL had been reintroduced. The overexpression of cyclin D1 that results from pVHL loss may be one mechanism by which the Rb pathway is deregulated in these tumors. No other cell line tested displayed hypoxia induction of cyclin D1. Perhaps these other cell lines have lost the normal hypoxia induction of cyclin D1 via other genetic changes.

An intriguing alternative is that only certain cell lineages are capable of pVHL-mediated hypoxia induction of cyclin D1. It would be only in these lineages that the loss of pVHL would induce cyclin D1, and thus only these lineages would give rise to cancer on VHL loss. While speculative, this idea provides a potential explanation for why the loss of VHL, a ubiquitously expressed tumor suppressor gene, gives rise to such a limited range of human cancers.

The link between VHL and the hypoxia-induced expression of cyclin D1 remains mysterious. First of all, we would like to know whether the apparent transcriptional effect is mediated by HIFs. The fact that VHL regulates HIFs and that cyclin D1 in these cells shows VHL-dependent hypoxia induction makes the argument connecting HIF with this effect more compelling. However, such a connection may be direct or indirect. The direct pathway would require the demonstration of functional HREs in the cyclin D1 promoter. Recent work using this approach showed that the immediate upstream promoter sequence (out to position −245) is needed for basal expression of cyclin D1 mRNA, especially, an important CRE element located between −52 and −45 (24). Careful examination of the cyclin D1 promoter region identified two consensus HREs with the canonical sequence 5′ RC GT C 3′ (25). These were located at positions −552 to −548 and −390 to −386 in a region not found to be important for basal expression of cyclin D1 mRNA. It will be interesting to determine whether these are in fact functional HREs and are responsible for the hypoxia regulation of cyclin D1 observed in these studies.

A more indirect pathway might involve the HIF induction of another pathway, such as TGF-β, which, in turn, regulates cyclin D1 transcription. Regardless of the precise mechanistic pathway, it is intriguing that hypoxia-induced expression of cyclin D1 appears to be restricted (to the extent thus far examined) to these VHL-negative RCC cells. Whether this reflects a property possessed by the lineage of cells that gives rise to these tumors or whether this is a property acquired during the development of VHL-negative RCC, remains unanswered by these observations. Whatever the explanation, these results demonstrate the profound context-specific regulation of even the most generalized biochemical pathways associated with cancer.

References


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