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von Hippel-Lindau Tumor Suppressor Protein Represses Platelet-Derived Growth Factor B-Chain Gene Expression Via the Sp1 Binding Element in the Proximal PDGF-B Promoter

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Abstract VHL is the causative gene for von Hippel-Lindau disease and sporadic clear cell renal cancer. It has been shown that pVHL can suppress the expression of certain genes that are overexpressed in renal carcinomas. One such gene is that encoding the potent mitogen and chemoattractant, platelet-derived growth factor B-chain (PDGF-B). The regulatory mechanisms underlying pVHL suppression of PDGF-B expression, however, are completely unknown. This understanding would shed vital light on the control of growth factor gene expression by this tumor suppressor. Here we report that pVHL can repress both endogenous steady-state PDGF-B mRNA expression and PDGF-B promoter-dependent transcription in WKY12-22 cells. Transient transfection analysis utilizing PDGF-B promoter-chloramphenicol acetyl transferase (CAT) reporter constructs revealed that pVHL inhibition of PDGF-B expression is mediated via the Sp1-binding element in the proximal region of the PDGF-B promoter. Recent studies have demonstrated a physical interaction between pVHL and Sp1, which activates the PDGF-B promoter. We show that Sp1 can rescue PDGF-B promoter activity and endogenous PDGF-B mRNA expression from pVHL repression. These findings thus demonstrate a pivotal role for Sp1 in pVHL inhibition of PDGF-B transcription. J. Cell. Biochem. 85: 490–495, 2002.

Key words: platelet-derived growth factor B-chain; von Hippel-Lindau protein; transcription; gene expression

The von Hippel-Lindau tumor suppressor gene, VHL, was identified in 1993 as the causative gene for von Hippel-Lindau disease, an inherited cancer family syndrome comprising several types of well-vascularized neoplasms, including clear-cell renal cancers [Cohen et al., 1999]. The VHL gene, mapped to chromosome 3p25–p26 [Latif et al., 1993] and spans approximately 20 kb [Latif et al., 1993], is mutated in nearly all cases of sporadic clear-cell renal cancer [Crossey et al., 1994; Foster et al., 1994]. Crystallographic analysis demonstrates that VHL contains two major domains, the elongin C-binding domain (residues 157–188) comprising alpha-helical regions, and a beta-domain (residues 63–154) comprising an alpha-helix at 193–204 [Stebbins et al., 1999].

Several reports have found that pVHL can shuttle back and forth between the nucleus and cytoplasm [Duan et al., 1995; Lee et al., 1996, 1999; reviewed in Ivan and Kealin, 2001]. This suggests that it, like other tumor suppressors (such as pRb and p53) may influence cellular phenotype at the level of transcription [Duan et al., 1995]. Recent reports indicating pVHL can regulate transcription and promoter activity of certain genes have supported this concept. For example, reintroduction of VHL into cell lines in vitro downregulates the expression of several hypoxia-inducible genes, such as vascular endothelial growth factor (VEGF) [Iliopoulos et al., 1996; Siemeister et al., 1996; Mukhopadhyay et al., 1997], glut-1 [Iliopoulos et al., 1996], and PDGF-B chain [Iliopoulos et al.,

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1996], which are all overexpressed in renal cancer. The transcriptional mechanisms mediating pVHL repression of VEGF have been partially delineated. pVHL directly binds the transcriptional activator Sp1 and inhibits Sp1 activity [Mukhopadhyay et al., 1997; Pal et al., 1998]. Recently, Cohen et al. [1999] described the domains involved in this interaction. VHL interacts directly with the Sp1 zinc finger region and self associates via the VHL 96-122 AA domain. Whether Sp1 is involved in pVHL repression of PDGF-B expression is unknown.

PDGF is a potent mitogen and chemoattractant implicated in the pathogenesis of a diverse range of diseases. PDGF transcription is positively regulated by Sp1 in a diverse range of cell types, including large arterial endothelial cells [Khachigian et al., 1994], venous endothelial cells [Scarpati and DiCorleto, 1996], smooth muscle cells [Silverman et al., 1997], Jurkat T cells [Trejo et al., 1996], and U2-OS osteosarcoma cells [Liang et al., 1996]. Here we report that pVHL is a potent repressor of PDGF-B promoter activity and endogenous PDGF-B expression. We show that the integrity of the Sp1 binding site in the proximal region of the PDGF-B promoter is necessary for pVHL-repression of PDGF-B transcription. Moreover, we show that overexpression of Sp1 rescues the PDGF-B promoter and the endogenous gene from pVHL repression. These findings demonstrate the tight regulation of PDGF-B expression by Sp1 and pVHL at the level of transcription.

MATERIALS AND METHODS

Cell Culture

WKY12-22 rat aortic smooth muscle cells were cultured in Waymouth's MB752/1 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 30 μg/ml L-glutamine, 10 U/ml penicillin and 10 μg/ml streptomycin at 37°C, and 5% CO_2. Cultures were passaged every 4–5 days in 75-cm^2 flasks.

Transient Cell Transfection

WKY12-22 SMCs were seeded in 100-mm tissue culture plates for 48 h before transfection. When approximately 60–70% confluent, the cells were transfected with 8 μg of the indicated PDGF-B promoter-reporter plasmids [Khachigian et al., 1994], pCMV-WT-VHL and CMV-Sp1 as indicated, using FuGENE6 (Roche Molecular Biochemicals). A precipitate was formed using 3 μl of FuGENE6/ microgram of transfected DNA, and the transfection mix made up to 1 ml with serum-free Waymouths' medium. After incubation at 22°C for 10 min, the DNA/FuGENE6 mixture was added to cells containing 4 ml of complete Waymouth's medium. Two days post transfection, cell lysates were prepared for assessment of chloramphenicol acetyltransferase (CAT) activity [Khachigian et al., 1994]. The concentration of protein in the cell lysates were assessed using the BCA protein assay kit and used to correct CAT reporter activity.

RT-PCR

Total RNA was prepared using the TRIzol reagent (GIBCO-BRL, Life Technologies) in accordance with manufacturer's instructions. For the RT reaction, 15 μg of RNA, 300 ng random primer (Promega), and 150 U of M-MLV reverse transcriptase (Stratagene cloning systems) were combined in a total volume of 50 μl containing 50 mmol/L Tris-HCl, pH 8.3, 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 40 U Rnasin, and 0.5 mmol/L of each dNTP. Reaction tubes were incubated at 65°C for 5 min, and then tubes were cooled slowly to room temperature. PDGF-B amplification was performed as follows. One hundred picomoles of 5′ and 3′ PDGF-B primers were combined in a total volume of 100 μl containing 20 mmol/L $(NH_4)_2SO_4$, 75 mmol/L Tris-HCl, pH 9.0, 0.1% (wt/vol) Tween 20, 4.0 mmol/L $MgCl_2$ and 0.25 mmol/L of each dNTP and the reverse transcribed cDNA. Samples were heated to 91°C for 4 min before the addition of 1 U Taq Polymerase. The samples were run through 91°C for 1 min, 56°C for 1 min, and 72°C for 1 min for 28 cycles, followed by a further 3 min extension at 72°C to facilitate complete extension of the product. GAPDH amplification was performed to allow relative quantification of PCR products. GAPDH amplification was performed as follows. One hundred picomoles of 5′ and 3′ primers were combined in a total volume of 50 μl containing 20 mmol/L $(NH_4)_2SO_4$, 75 mmol/L Tris-HCl, pH 9.0, 0.1% (wt/vol) Tween 20, 2 mmol/L $MgCl_2$ and 0.25 mmol/L of each dNTP. The samples were cycled through 94°C for 1 min, 53°C for 1 min, followed by 72°C for 1 min. Thirty cycles were followed by a further 4 min extension at 72°C. The PCR reaction was loaded onto 1% agarose gels, electrophoresed, stained with ethidium bromide, and photographed under

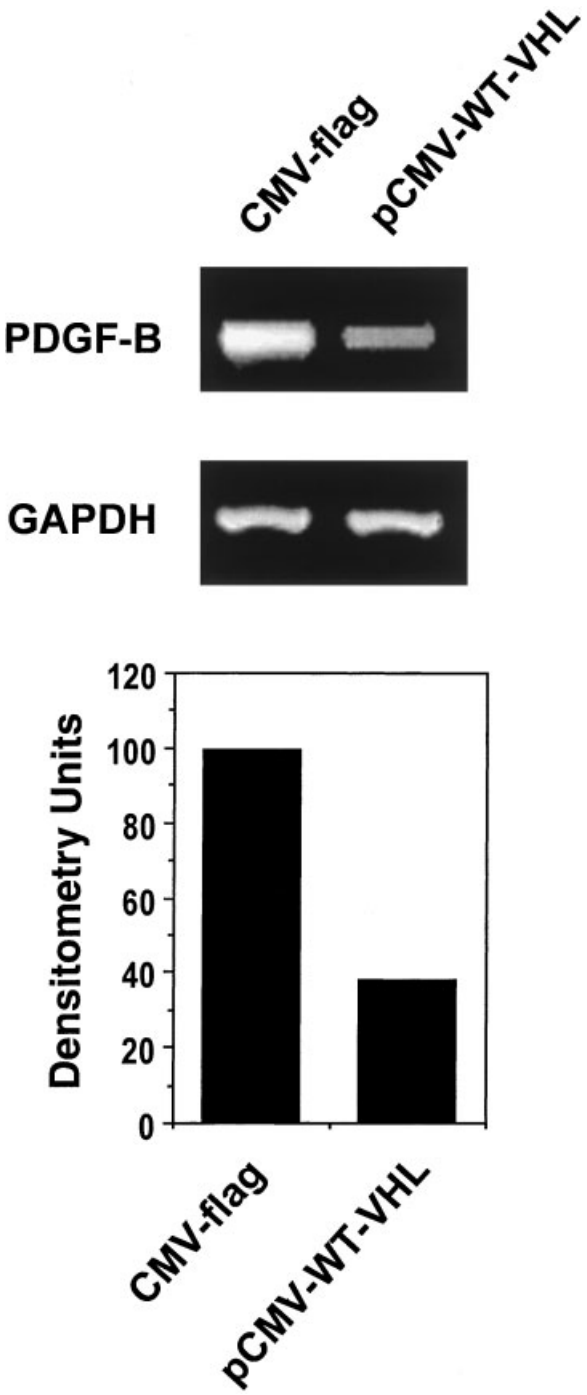

Fig. 1. pVHL downregulates PDGF-B mRNA expression. WKY12–22 cells were transiently transfected with 20 μg of pCMV-WT-VHL or the control backbone CMV-flag for 24 h before extraction of total RNA and semi-quantitative RT-PCR analysis for PDGF-B or GAPDH mRNA as described under Materials and Methods. The data is representative of two independent determinations.

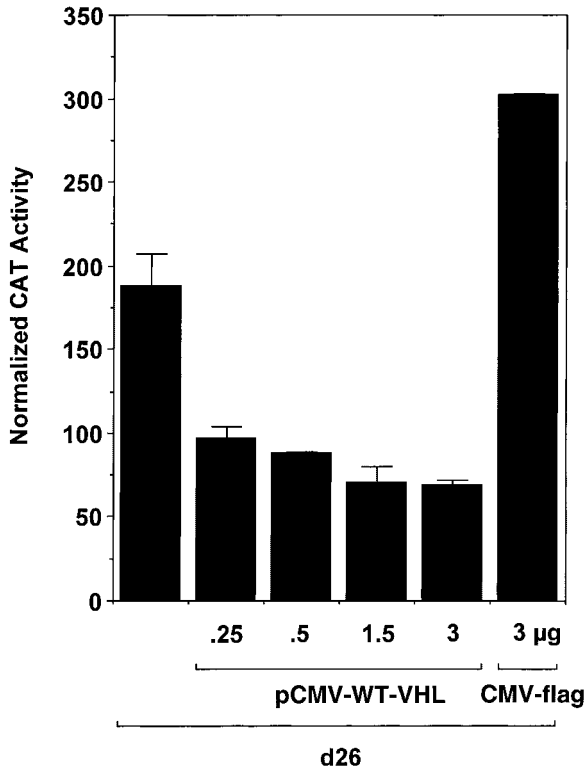

Fig. 2. pVHL represses PDGF-B transcription. WKY12-22 cells were transiently transfected with 8 μg of the PDGF-B promoter-CAT construct d26 and the indicated amounts of pCMV-WT-VHL, or control backbone CMV-flag. The total amount of transfected DNA was normalized to 3 μg with CMV-flag. Twenty-four hours post transfection cells were harvested, CAT activity determined and normalized to the concentration of protein in the cell lysates. The data is representative of two independent determinations. Error bars represent SE of the mean.

ultraviolet illumination. Expected size products were GAPDH of 287 bp and PDGF-B of 317 bp.

RESULTS

pVHL Downregulates Endogenous PDGF-B mRNA Expression

We assessed the effect of overexpressing pVHL on steady-state levels of PDGF-B mRNA. WKY12-22 cells were transfected with 20 μg of pCMV-WT-VHL, or the control backbone CMV-flag, and left undisturbed for 24 h prior to preparation of total RNA and semi-quantitative RT-PCR analysis, which were performed in the linear amplification range based on pilot experiments. We observed significant inhibition of PDGF-B mRNA expression upon overexpression of pVHL compared to the backbone control, CMV-flag (Fig. 1). In contrast, GAPDH expression was unaltered by pVHL (Fig. 1), indicating

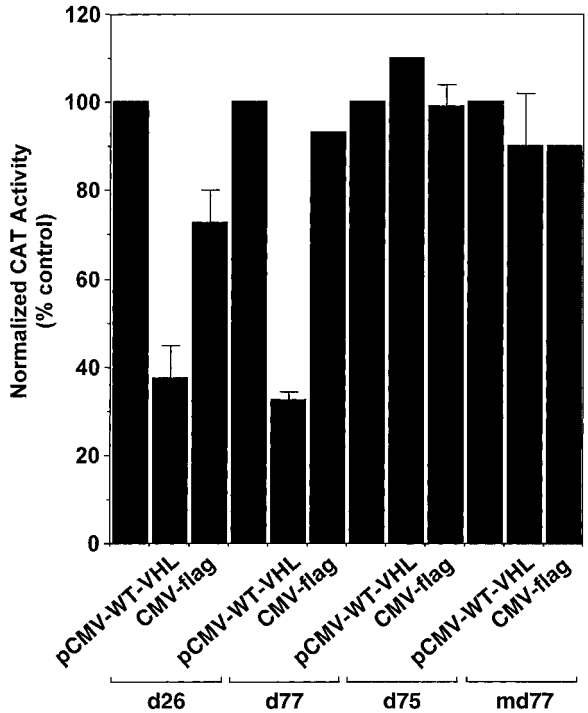

Fig. 3. pVHL repression of the PDGF-B promoter is mediated via the Sp1 site in the proximal promoter. WKY12-22 cells were transfected with 8 μg of the indicated PDGF-B promoter-CAT constructs and 5 μg of either VHL or CMV-flag. Twenty-four hours post transfection CAT activity was normalized to the concentration of protein in the cell lysates. The data, representative of two independent determinations, is expressed as a percentage of CAT activity generated in cells transfected with the reporter vector alone. Error bars represent SE of the mean.

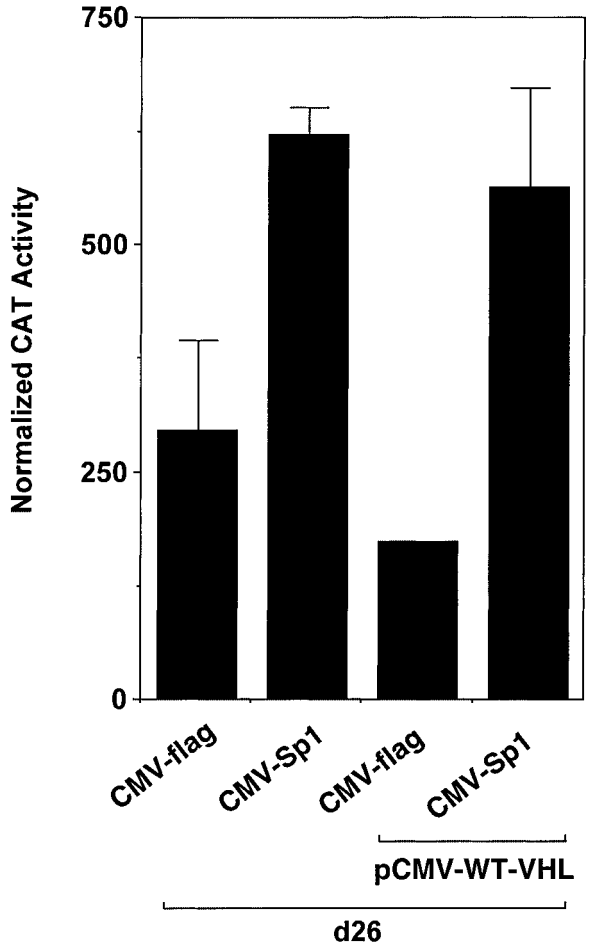

Fig. 4. Sp1 overexpression rescues repression of PDGF-B promoter activity by pVHL. WKY12-22 cells were transfected with 8 μg of the PDGF-B promoter-CAT reporter construct, d26 and 5 μg of either CMV or CMV-Sp1. After 24 h, CAT activity in the lysates was normalized to the concentration of protein as described under Materials and Methods. The data is representative of two independent determinations. Error bars represent SE of the mean.

that pVHL inhibition was selective and not the consequence of non-specific squelching.

pVHL Represses PDGF-B Transcription

To begin dissecting the mechanism underlying pVHL downregulation of PDGF-B expression, we transfected the WKY12-22 cells with construct d26, a CAT reporter construct bearing 153 bp of the PDGF-B promoter relative to the TATA box, together with varying amounts of pCMV-WT-VHL, or its backbone counterpart. Reporter activity was readily detectable in cells transfected with d26 and CMV-flag (Fig. 2). In contrast, we observed repression of PDGF-B promoter activity by pVHL over a range of concentrations in a dose-dependent manner (Fig. 2).

pVHL Repression Is Mediated Via the Sp1 Site in the Proximal PDGF-B Promoter

The preceding findings showed that both endogenous PDGF-B mRNA and PDGF-B promoter-dependent gene expression are inhibited by pVHL. To identify the region in the PDGF-B promoter mediating this pVHL repression, we transfected the cells with a series of CAT reporter constructs driven by various sized fragments of the PDGF-B promoter [Khachigian et al., 1994]. Consistent with observations in Figure 2, pVHL suppressed transcription driven by construct d26 (− 153) (Fig. 3). pVHL also repressed CAT activity generated in cells transfected with construct d77, containing 82 bp of PDGF-B promoter sequence (Fig. 3). However, reporter activity driven by construct d75, lacking the Sp1 binding element at position − 34/− 18, was

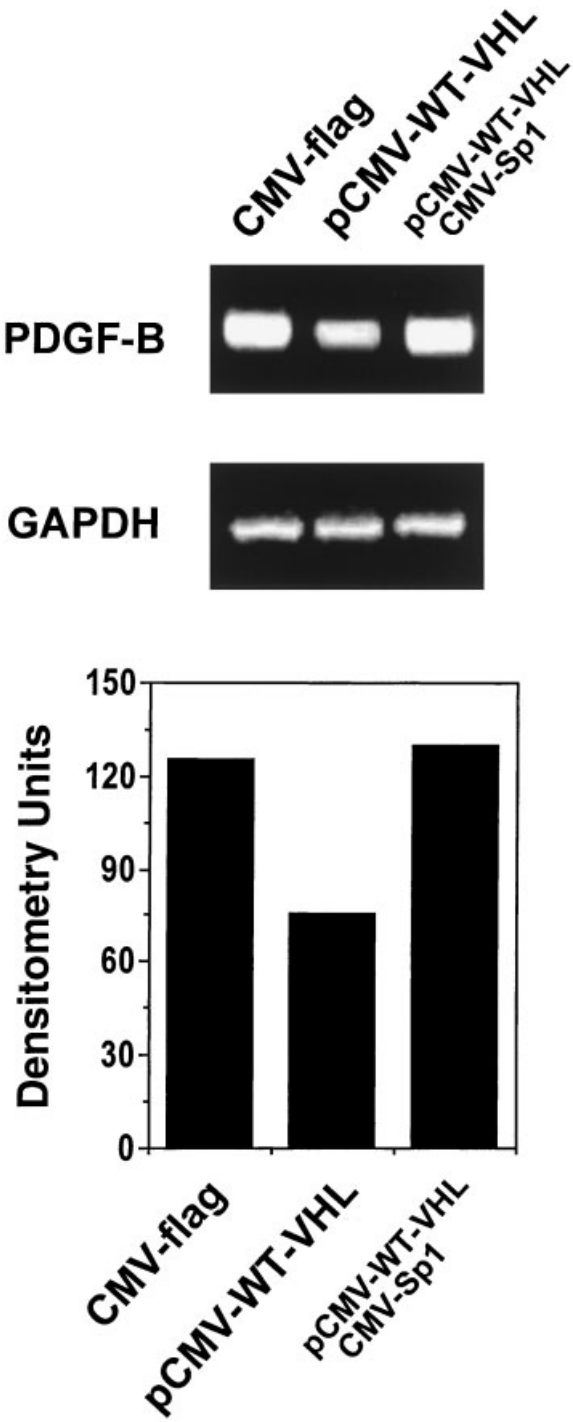

Fig. 5. Sp1 overexpression rescues repression of PDGF-β mRNA by pVHL. WKY12-22 cells were transfected with 3 μg of pCMV-WT-VHL alone or in combination with 5 μg of CMV-Sp1. Total amounts of transfected DNA were normalized to 8 μg with the control backbone CMV-flag. Twenty-four hours post transfection total RNA was extracted using TRIzol as described under Materials and Methods. Semi-quantitative RT-PCR analysis for PDGF-B or GAPDH mRNA was performed as described under Materials and Methods. The data is representative of two independent determinations.

unaffected by pVHL (Fig. 3). We next evaluated the effect of pCMV-WT-VHL in cells transfected with construct md77, which bears a mutation in the Sp1 binding element. This Sp1 mutant also failed to support pVHL repression of PDGF-B promoter activity (Fig. 3), demonstrating the requirement of an intact Sp1 binding site in the proximal region of the PDGF-B promoter for pVHL repression.

Sp1 Overexpression Rescues pVHL Repression of the PDGF-B Promoter and Endogenous PDGF-B Expression

Given the preceding data, we hypothesized that overexpression of Sp1 would reverse inhibition of PDGF-B promoter activity by pVHL. CAT activity generated by construct d26 was, as expected, suppressed by pVHL (Fig. 4). However, overexpression of CMV-Sp1 rescued the PDGF-B promoter from pVHL repression (Fig. 4). Semi-quantitative RT-PCR supported these findings, demonstrating the capacity of Sp1 to rescue endogenous PDGF-B mRNA expression from pVHL inhibition (Fig. 5). In contrast, levels of GAPDH mRNA was unaltered (Fig. 5).

DISCUSSION

This study demonstrates for the first time that the proximal region of the PDGF-B promoter is under the negative transcriptional control of the von Hippel-Lindau tumor suppressor protein. pVHL repressed PDGF-B promoter-dependent expression and endogenous PDGF-B mRNA. This is mediated by the Sp1 binding element located at position − 34/− 18. Mutation of the Sp1 binding site in the PDGF-B promoter abrogated inhibition by pVHL. Conversely, overexpression of Sp1, thereby altering the ratio of negative to positive regulators, rescued the PDGF-B promoter from pVHL repression. Since Sp1 exerts a positive influence on PDGF-B transcription [Khachigian et al., 1994; Liang et al., 1996; Scarpati and DiCorleto, 1996; Trejo et al., 1996; Silverman et al., 1997], and pVHL interacts with Sp1 [Mukhopadhyay et al., 1997], proteins that interfere with Sp1 function may inhibit the expression of potent growth factor. Indeed, the present study defines one such factor as pVHL, demonstrating for the first time the inhibitory influence of a tumor suppressor protein on PDGF-B at the promoter level.

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