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Role of the von Hippel-Lindau Tumor Suppressor Gene in the Formation of β1-Integrin Fibrillar Adhesions[1]

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ABSTRACT

The von Hippel-Lindau tumor suppressor gene (*VHL*) is absent or inactivated in the VHL cancer syndrome and in most sporadic renal cancers. VHL is required for the assembly of a proper extracellular fibronectin matrix, although the exact mechanism remains unknown. In this report, we demonstrate that 786-O renal cancer cells are unable to organize an adequate matrix even in the presence of an excess of exogenous fibronectin. Because the formation of integrin fibrillar adhesions plays a pivotal role in the organization of extracellular fibronectin, we next examined the expression and subcellular distribution of integrins in VHL(−) cells and their wild-type VHL stably transfected counterparts. The levels of β1 and αv integrins were increased in VHL(−) cells when compared with VHL(+) transfectants. Early after plating, both VHL(+) and VHL(−) cells were capable of assembling classic "patch-like" αv focal contacts. As the culture advanced and cells became confluent, αv integrins partly relocated to the intercellular junctions in VHL(+) transfectants, which then developed large β1 fibrillar-type adhesions and anchored firmly to the substrate. In contrast, confluent VHL(−) cells were unable to assemble β1 fibrillar adhesions, and αv focal contacts remained unchanged at all stages of the culture. Exogenous activation of β1 integrins with either divalent cations or activating antibodies partly restored the capability of VHL(−) cells to assemble β1 fibrillar adhesions and fibronectin fibers. Finally, pulse-chase studies of metabolically labeled 786-O cells revealed that the maturation of the common β1-integrin chain was delayed in VHL(−) cells when compared with VHL(+) cells. Our results show that VHL is an important regulator of integrins and is essential for the formation of β1 fibrillar adhesions. These findings help to explain the abnormal extracellular matrix organization and increased motility of VHL(−) renal cancer cells.

INTRODUCTION

Germline mutations of the *VHL*[3] tumor suppressor gene cause the hereditary VHL disease, which is characterized by the development of multiple tumors, mainly hemangioblastomas of the central nervous system and renal cancers. VHL is also inactivated in up to 80% of sporadic renal cancers (1). VHL does not closely resemble other known proteins. It binds, however, to elongin B, elongin C, and CUL2, forming the so-called VCB ternary complex (2). On the basis of the functions of yeast homologues of these VHL-interacting proteins, a role for VHL in the proteolysis of target substrates has emerged. Indeed, recent studies have shown that VHL binds to the transcription factor hypoxia-inducible factor and promotes its ubiquitination and degradation by the proteasome (3–6). Although most recent research on VHL has focused on the regulation of hypoxia-inducible factor, VHL has been involved in the control of cell shape and motility. In this regard, VHL has been functionally associated with modulation of cell migration across transwell filters, with control of differentiation and morphogenesis (7, 8), and with regulation of matrix metalloproteinases and their inhibitors (9). In addition, VHL physically interacts with fibronectin, and its inactivation leads to impaired extracellular fibronectin organization in VHL(−) renal cancer cells and VHL −/− mouse embryo fibroblasts (10). Because fibronectin has profound effects on many aspects of cell behavior (11), these observations indicate that altered matrix organization might contribute to the aggressive behavior of VHL(−) tumors. However, the mechanisms by which VHL controls the assembly of this ECM protein have not been established.

Integrins are a family of heterodimeric transmembrane proteins, composed of different α and β chains, that provide a link between cells and the surrounding matrix through the formation of specialized structures called cell-matrix adhesions (12, 13). The organization of cell-matrix adhesions is a strongly regulated process and requires the association of multiple proteins to the integrin-ligand complex. These proteins include vinculin, talin, and particularly, F-actin, which is essential for the stability of the whole complex (14, 15). There are two major types of cell-matrix adhesions, namely classic FCs and fibrillar adhesions (16–19), which not only differ in their morphology but also in their constituents. Classic FCs are arrowhead shaped, contain αv or β1 integrins, and are rich in talin and vinculin. Conversely, fibrillar adhesions are elongated, contain exclusively β1 integrins, and possess low levels of vinculin or talin. The formation of fibrillar adhesions involves cytoskeletal rearrangement of the previously formed β1 classic FCs and is an absolute requirement for the organization of extracellular fibronectin into fibrillar arrays (20–22).

Our results show that the presence of VHL is strictly necessary for the formation of β1-integrin fibrillar adhesions in renal cancer cells and may help to explain the altered ECM assembly and increased motility of VHL(−) cells.

MATERIALS AND METHODS

Cells and Cell Cultures. Stable transfectants of the 786-O renal cancer cell line (pRC-HAVHL, clones WT8 and WT10; pRC alone, clones PRC3 and PRC9; and pRC-HAVHLMUT 1-115), parental RCC10 renal cancer cells, and the two VHL stably transfected clones WT63 and WT53 were grown in RPMI 1640 containing 10% heat-inactivated fetal bovine serum and supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin (BioWhittaker Europe, Verniers, Belgium). Except for parental RCC10, cells were also maintained with G418, which was removed before each experiment.

Antibodies. mAbs TS2/16 (23), LIA1/2 (23), HUTS21 (24), TS2/7 (anti-α1 integrin), Tea 1/41 (anti-α2 integrin), and VJ1/18 (anti-α3 integrin) have been described previously. mAb ABA-6D1 (anti-αv) and mAb P1D6 (anti-α5β1) were provided by Dr. C. Martínez-Alonzo (Centro Nacional de Biotecnología, Madrid, Spain) and Dr. E. A. Wayner (Fred Hutchinson Cancer Research Center, Seattle, WA), respectively. Antifibronectin polyclonal antibody was purchased from Calbiochem, preimmune polyclonal antirabbit serum from Dako, and anti-α-tubulin and antivinculin mAbs from Sigma. The P3X63 myeloma protein was used as a control.

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[3] The abbreviations used are: VHL, von Hippel-Lindau; ECM, extracellular matrix; FC, focal contact; mAb, monoclonal antibody; TBS, Tris-buffered saline.

Flow Cytometry Analysis. 786-O cells (4.8×10^5) and RCC10 cells (4×10^5) were grown for 3 days and 36 h, respectively, in 35-mm culture dishes, trypsinized, washed, and resuspended in PBS. Cells were incubated with primary antibodies at 4°C for 30 min, washed, and incubated with FITC-conjugated antimouse immunoglobulins (DAKO-PATTS, Copenhagen, Denmark) for 30 min on ice. After being washed, cells were resuspended in PBS and analyzed in a FACScan flow cytometer (Becton Dickinson, Lincoln Park, NJ). To analyze the β1-integrin activation state, cells were detached, treated with freshly prepared $MnCl_2$ (1 mM), incubated with the primary antibody HUTS21 for 30 min at 37°C (24), and analyzed as described above.

Immunofluorescence Microscopy. Cells were grown on glass coverslips in 24-well culture dishes, fixed at the indicated times with 3% paraformaldehyde in PBS for 15 min at room temperature, and washed with TBS (0.1 M Tris-HCl, 0.15 M NaCl). Cells were then permeabilized with 0.5% Triton X-100 for 10 min, and nonspecific binding sites were blocked by incubation with TNB (0.1 M Tris-HCl, 0.15 M NaCl, 0.5% blocking reagent; Boehringer Mannheim GmbH) for 30 min. Cells were incubated with the appropriate primary antibody for 45 min at room temperature and washed with TBS. Secondary antibodies, Alexa 488 goat antimouse IgG or Alexa 488 goat antirabbit IgG (Molecular Probes, Inc. Eugene, OR) were then added, and cells were incubated for 30 min. Finally, cells were washed with TBS, washed briefly with distilled water, and mounted with Mowiol. For double-label immunofluorescence studies, mouse serum was also used to prevent antibody cross-reactions. For F-actin double immunostaining, Texas red-phalloidin (Molecular Probes) was added to the appropriate secondary antibody. Samples were examined with a Nikon Labophot-2 photomicroscope, and images were acquired with a COHU high-performance CCD camera (Cohu, Tokyo, Japan) connected to a LEICA Q550 CW workstation (Leica Imaging Systems, Ltd. Cambridge, United Kingdom) using LEICA QFISH V1.01 Software (Leica).

Fibronectin-coated coverslips were prepared by incubation for at least 3 h at 37°C with a solution containing 10 μg/ml fibronectin (Sigma).

Measurement of the Strength of Cell Adherence. 786-O cells (125×10^3) were grown in 24-well dishes. After 10–20 h, supernatants (250 μl) from P3X63, TS2/16, or LIA1/2 were added to the culture medium. After 3–4 days, the medium was removed, and a solution containing 1% Triton X-100 in PBS, which extracts noncytoskeletal-associated material, was added to intact monolayers. The time at which VHL(+) and VHL(−) cell monolayers were detached was measured. Cell detachment could be easily observed macroscopically, but was also verified with a phase-contrast microscope.

Western Blots. 786-O cells (1.4×10^6) were grown in 60-mm culture dishes for 3–4 days. To analyze total fibronectin, after one wash with PBS, cell monolayers were lysed for 15 min in RIPA buffer [150 mM NaCl, 10 mM Tris (pH 7.4), 1% NP40, 0.5% deoxycholate, and 0.1% SDS] supplemented with protease and phosphatase inhibitors. To quantify intracellular fibronectin, cells were mildly trypsinized, the reaction was stopped with fibronectin-free serum, and cells were washed three times with excess RPMI before lysis in RIPA buffer. Cell lysates were harvested and centrifuged for 20 min at 14,000 rpm and 4°C. Protein concentrations were determined using the BIO-RAD D_c Protein Assay Kit, and equal amounts of lysates were subjected to SDS-PAGE electrophoresis, transferred to a nitrocellulose membrane (Bio-Rad), and finally blotted with the indicated antibody. Immunodetection was performed using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Pulse-Chase Experiments. Pulse-chase labeling of cells with [^{35}S]methionine was carried out as described in *Current Protocols in Immunology* (25) and as described by others (26–28). 786-O cells (clones WT8 and MUT1-115) were plated in 150-mm dishes 1 day before the experiment. Monolayers at 70–90% confluence were then washed with HBSS (BioWhittaker Europe, Verniers, Belgium). Labeling medium [RPMI without methionine (Sigma), 5% dialyzed FCS, and 2 mM L-glutamine] was added to the dishes and incubated for 2 h at 37°C to deplete intracellular pools of methionine. After this medium was removed, 6 ml of labeling medium and 1.0 mCi of [^{35}S]methionine were added to each dish, and cells were incubated at 37°C for 30 min to allow the incorporation of radioactive methionine to biosynthetic precursors. The monolayers were washed with chase medium [RPMI, 10% FCS, and 15 mg/liter nonradioactive L-methionine (Sigma)]. Maturation of labeled precursors was followed by lysis at different chase times. At each chase time, monolayers were scraped off and centrifuged for 5 min at 14,000 rpm and 4°C. After being washed with PBS, pellets were lysed in EBC buffer [50 mM Tris (pH 8.0), 120 mM NaCl, 0.5% NP40] supplemented with protease and phosphatase inhibitors and clarified by centrifugation at 14,000 rpm for 15 min. Supernatants were then precleared with protein G-Sepharose, and equal amounts of radioactive lysates were incubated with the appropriate antibody coupled to protein G-Sepharose at 4°C under continuous mixing. Immunoprecipitates were washed with EBC buffer and PBS and boiled for 5 min in Laemmli buffer under nonreducing conditions; samples were run in 7% SDS-PAGE. Gels were fixed and treated with Amplify (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom), dried under vacuum at 60°C for 2 h, and exposed to an X-ray film at −80°C.

^{125}I Labeling of Cell Surface Proteins. To analyze cell surface integrins, cell monolayers (one 150-mm dish at 70–90% confluence, plated 1 day before the experiment) were harvested and washed in PBS by centrifugation for 5 min at 1200 rpm. Cells were then iodinated for 25 min with 1 mCi of sodium [^{125}I]iodine (Amersham Pharmacia Biotech) using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (IODO-GEN; Pierce). This compound selectively catalyzes the addition of radioiodine to the tyrosine residues exclusively on the outside of the cell membrane (29). The iodination reaction was stopped by incubating cells with PBS supplemented with 0.4% L-tyrosine (Sigma). After being washed twice with PBS, cells were lysed with EBC buffer, and equal amounts of radioactive lysates were subjected to immunoprecipitation as above.

RESULTS

Exogenous Fibronectin Does Not Restore Matrix Assembly in VHL(−) Renal Cancer Cells. VHL(−) 786-O renal cancer cells assemble very few fibronectin fibrils, whereas an extensive matrix can be observed when VHL is stably transfected (10). On the basis of the role of VHL in the proteolysis of target proteins, Ohh *et al.* (10) postulated that the absence of matrix organization could be attributable to a defect in the degradation of endogenous misfolded and/or misprocessed fibronectin. Accumulated intracellular aberrant fibronectin species would then, if secreted, interfere with the assembly of normal fibronectin.

To test this hypothesis, we cultured 786-O cells on coverslips in the presence or absence of an excess of normal fibronectin. Although the addition of normal fibronectin to the culture dish increased matrix assembly in VHL(+) cells, we did not detect any enhancement in VHL(−) cells (Fig. 1*A*). Likewise, culturing VHL(−) cells on fibronectin-coated coverslips did not significantly induce the assembly of fibronectin fibers. These results suggest that the defect in matrix formation might not be caused by the secretion of an altered form of fibronectin; otherwise, competition by exogenous fibronectin should have reversed the defect. Additionally, to rule out the possibility that fibronectin is abnormally accumulated inside VHL(−) cells, we quantified intracellular and total (extracellular and intracellular) fibronectin. To measure total fibronectin, we lysed VHL(+) and VHL(−) cells in their own culture dishes and then analyzed them by Western blotting. This method does not discriminate between assembled and surface adhered but nonassembled fibronectin. As shown in Fig. 1*B*, there were minimal differences in total fibronectin levels between VHL(+) and VHL(−) cells. Parallel mild trypsinization and washing of cell monolayers, a procedure that removes the ECM (30), was used to quantify exclusively intracellular fibronectin. Notably, the amount of intracellular fibronectin did not significantly differ between VHL(+) and VHL(−) cells (Fig. 1*B*).

Changes in Integrin Cell Surface Expression in VHL(−) Cells. Because integrins are essential for ECM assembly, diminished expression of integrins on the cell surface (31) could be a simple explanation for the defective fibronectin organization in 786-O VHL(−) cancer cells. To test this, we analyzed two different VHL(−) renal cancer cells lines (786-O and RCC10) and their respective VHL(+) stable transfectants for integrin expression by flow cytometry. Integrins αv, α3, and β1 were clearly up-regulated in VHL(−) cells compared with VHL(+) cells (Fig. 2). The levels of α2 and α5 integrins were also augmented in VHL(−) cells, but their expression

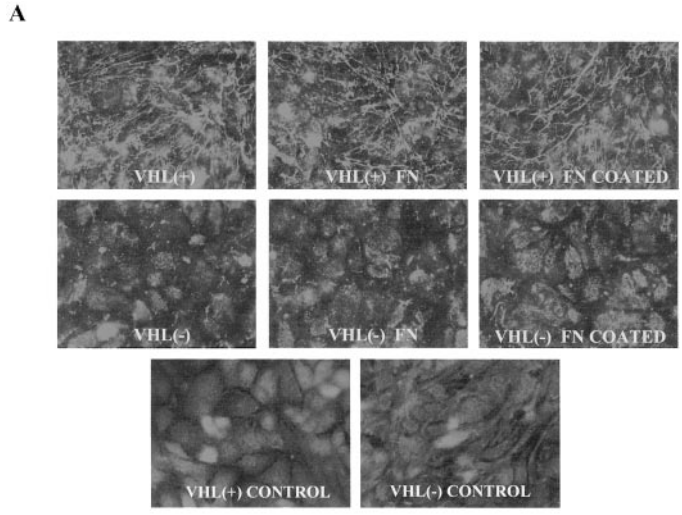

Fig. 1. Fibronectin assembly is impaired in VHL(−) cells. *A*, 786-O cells (125×10^3) were cultured on coverslips in the presence or absence of up to 50 μg/ml exogenous fibronectin (*FN*) or on fibronectin-coated coverslips (10 μg/ml; *FN COATED*). After 3–5 days of culture, cells were fixed, and fibronectin assembly was detected by indirect immunofluorescence. Preimmune polyclonal antirabbit serum (*CONTROL*) was used to show that most cytoplasmic staining obtained with the antifibronectin polyclonal antibody is nonspecific. *B*, Western blot analysis of intracellular and total fibronectin (*FN*) in 786-O cells. Tubulin (*α-TUB*) was used to show equal protein loading. One representative experiment is shown.

was low when compared with α3, particularly in RCC10 cells. Finally, no expression of α1, α4, and α6 integrins was detected in either renal cancer cell lines (data not shown).

In most cellular types, fibronectin assembly is mediated mainly by β1 integrins and specifically by $\alpha5\beta1$ (17, 32), although under certain circumstances other integrins have been involved. Our results indicate that the altered fibronectin assembly of VHL(−) renal cancer cells is not attributable to a decrease in β1 integrins on the cell surface. In addition, because we observed comparable increases in β1, α3, αv, and to a lesser extent, α2 and α5 integrins in both VHL(−) 786-O and RCC10 renal cancer cells, we propose that these changes may be a general consequence of VHL inactivation.

VHL Regulates Assembly of β1 Fibrillar Adhesions in Renal Cancer Cells. To explore different mechanisms by which VHL could modulate fibronectin assembly, we next analyzed the effects of VHL expression on the formation of cell-matrix adhesions in renal cancer cells. For this purpose, 786-O cells were cultured on coverslips and analyzed at different culture time points by immunofluorescence microscopy. To stain β1 integrins, we used the monoclonal antibody HUTS21, which selectively recognizes activated β1 integrins and is an excellent marker for β1 cell-matrix adhesions (24, 33).

Early after plating (10–15 h), both VHL(+) and VHL(−) cells adopted a fibroblastic-like morphology and assembled classic "patch-like" αv FCs (Fig. 3), which were coincident with the termini of actin stress fibers. Vinculin, which is an intracellular constituent of the classic FC complex, showed a similar pattern of arrowhead-shaped patches in both groups of cells. Under the conditions tested, β1 classic FCs were poor or absent at this early stage of culture and activated β1 integrins and fibronectin displayed a diffuse but coincident distribution in both VHL(+) and (−) cells (Fig. 3).

After 3–5 days, VHL(+) 786-O transfectants became confluent, and cells gradually acquired a rounded and epithelium-like phenotype (Fig. 4*A*, *panel a*). Correlating with this striking change in morphology (7), αv integrins partly relocated to the intercellular junctions (Fig. 4*A*, *panels c* and *e*). Parallel assessment of matrix assembly at this late stage of culture showed that VHL(+) cells had developed a well-defined pattern of extracellular fibronectin fibrillar arrays (Fig. 4*B*). Similarly, staining of activated β1 integrin with HUTS21 showed a clear-cut distribution in large fibril-type matrix adhesions, which were coincident with fibronectin fibers (Fig. 4*B*) and with the termini of actin cables (data not shown). Staining with $\alpha5\beta1$ showed a very similar pattern of fibrillar adhesions in VHL(+) cells (Fig. 4*B*) and displayed a coincident localization with fibronectin fibers (data not shown).

At this late stage of culture, confluent VHL(−) cells were elongated and branched and displayed abundant αv classic FCs (Fig. 4*A*, *panels a* and *d*). Interestingly, they were unable to develop β1 fibrillar adhesions and instead displayed a punctate or diffuse but coincident distribution of β1 integrins and fibronectin (Fig. 4*B*). Random clusters of β1 integrins and fibronectin were also detected and may reflect

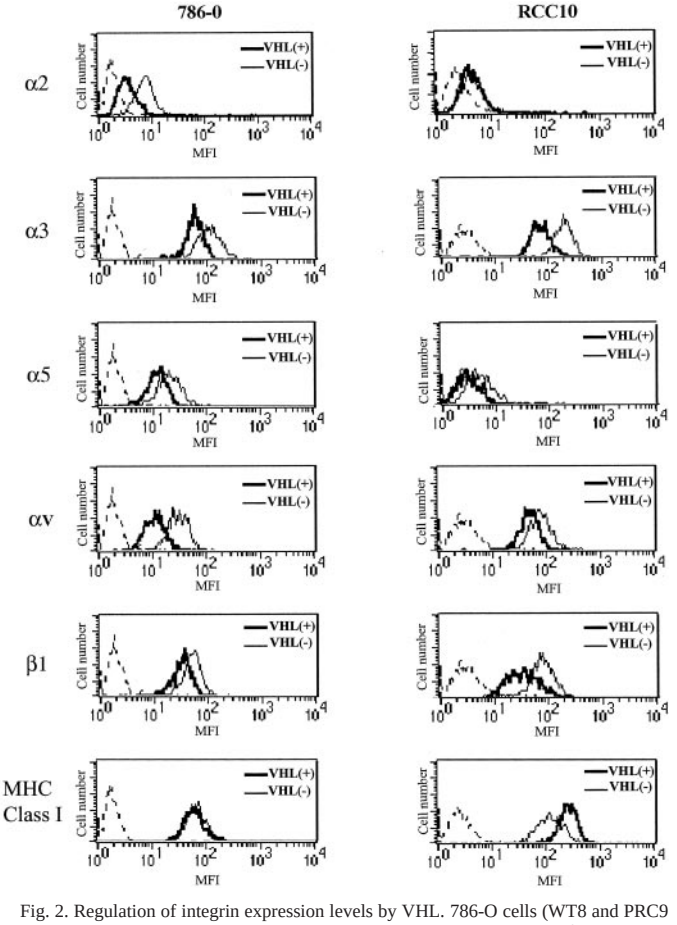

Fig. 2. Regulation of integrin expression levels by VHL. 786-O cells (WT8 and PRC9 clones) and RCC10 cells (parental and WT63 clone) were analyzed by fluorescence-activated cell sorting with antibodies against α2, α3, α5, αv, and β1. The myeloma protein P3X63 was used as a negative control (*dashed line*), and an antibody against MHC class I was used to demonstrate that there is no general up-regulation of cell surface markers when VHL is lost. One representative experiment is shown. Similar results were obtained with other clones of these cell lines (data not shown). *MFI*, mean fluorescence intensity.

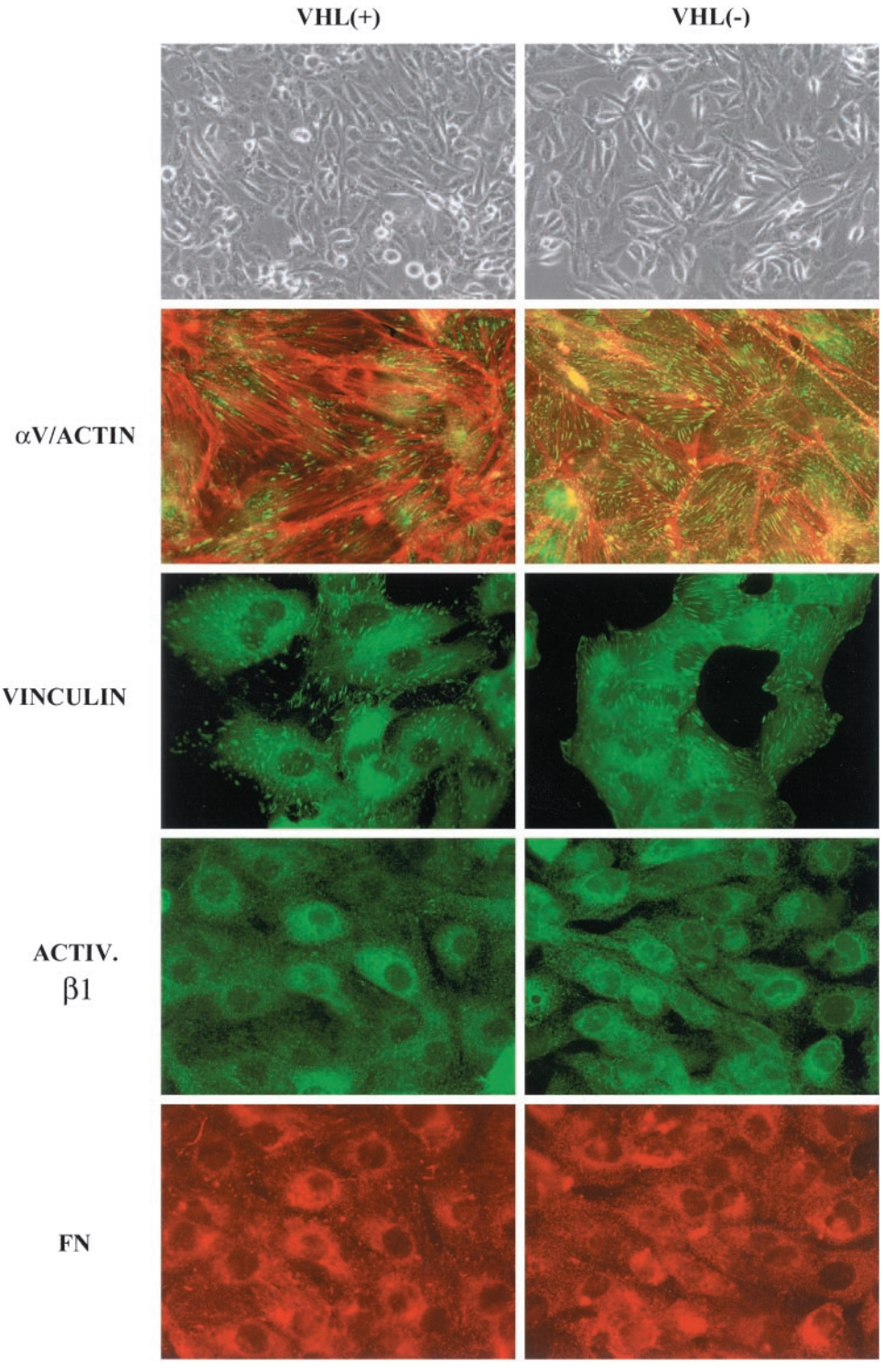

Fig. 3. Effect of VHL expression on cell morphology and the distribution of vinculin, αv, fibronectin, and β1 integrins at early stages of culture. 786-O cells (125×10^3) were grown on coverslips for 10–15 h and fixed. Cells were photographed with a phase-contrast microscope and then double stained with anti-αv and Texas red-phalloidin (to visualize actin), double stained with antiactivated β1 (HUTS21) and antifibronectin, or stained with antivinculin.

inadequate translocation of β1 integrins to the mature fibrillar adhesion. As expected, α5β1 integrins were diffusely distributed in VHL(−) cells.

To determine whether the formation of β1-integrin fibrillar adhesions in VHL(+) cells results in firmer cell adhesion to the substrate, 786-O cells were cultured for several days in the presence of either β1-activating (TS2/16) or β1-blocking (LIA1/2) antibodies. The medium was then removed, and intact monolayers were treated with a solution containing 1% Triton X-100, which rapidly extracts noncytoskeletal-associated material and promotes cell detachment. As shown in Fig. 5, whereas VHL(−) cells detached almost immediately, VHL(+) cells required a much more prolonged incubation time. Interestingly, blockage of β1 integrin function and fibronectin assembly in VHL(+) 786-O cells with the antibody LIA1/2 (see below; Fig. 6*B*) dramatically reduced the time of detachment, which was then very similar to that of VHL(−) cells. On the other hand, the β1-activating antibody TS2/16, which increases the formation of β1 fibrillar adhesions and fibronectin fibrils (see below; Fig. 6*B*) prolonged the time of detachment of VHL(+) monolayers but had no effect on that of VHL(−) cells.

These results indicate that VHL(−) cells bear a major alteration in

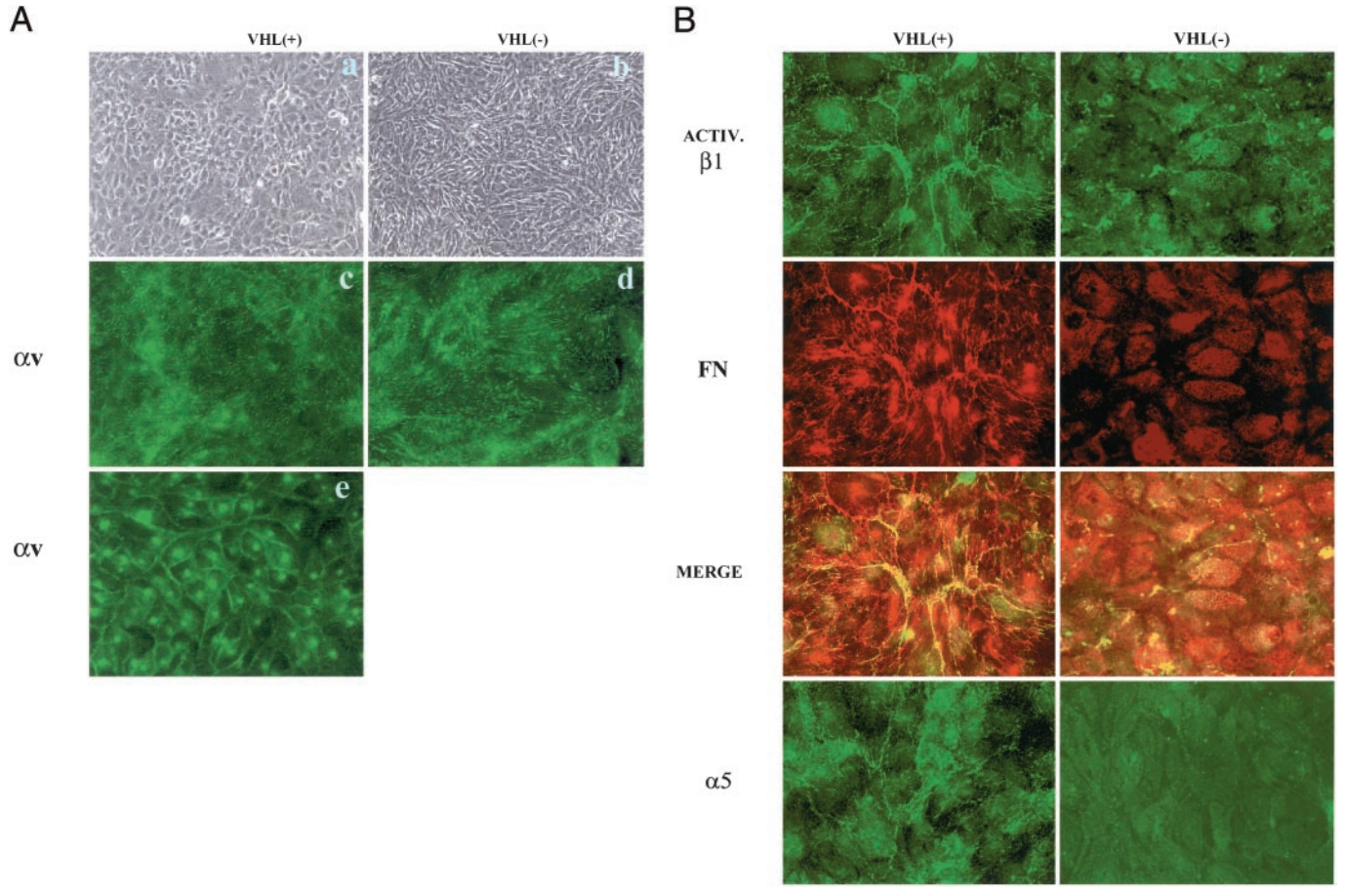

Fig. 4. Effect of VHL on cell morphology and the distribution of αv, fibronectin, and β1 integrins at late confluence. 786-O cells (125×10^3) were cultured on coverslips for 3–5 days and then fixed. *A*, cells were photographed with a phase-contrast microscope and stained with anti-αv. Two different fields, representing the area in contact with substrate or the apical region of the monolayer, respectively, are shown. Because VHL(−) cells display a much more flattened morphology than do VHL(+) cells, only one plane of the cell could be photographed. *B*, cells were stained with anti-$\alpha5\beta1$ or double stained with HUTS21 (activated β1 integrin) and antifibronectin. Similar results were obtained when cells were cultured on fibronectin (10 μg/ml), laminin (5 μg/ml), or vitronectin (5 μg/ml; data not shown).

the assembly of β1 fibrillar adhesions, which has functional consequences in defective fibronectin fibrillar organization and diminished adherence to the substrate.

Outside-In Activation of β1 Integrins Stimulates Fibronectin Assembly in VHL(−) Cells. The ability of integrins to mediate fibronectin organization depends on their state of activation (34). Integrin activation is regulated by signals from inside the cell and can also be easily increased from outside with $MnCl_2$ or with activating antibodies (12, 13). To measure the integrin activation state in 786-O cells, we performed flow cytometry analysis with the antiactivated β1-integrin antibody HUTS21, and no significant differences between VHL(+) and VHL(−) cells were detected (Fig. 6*A*). Likewise, a short incubation with $MnCl_2$ before analysis increased β1-integrin activation very similarly in both groups of cells. Notably, when VHL(−) cells were cultured in the sustained presence of either the β1-activating antibody TS2/16 (Refs. 23, 35; Fig. 6*B*) or $MnCl_2$ (data not shown), an increase in fibronectin fibers was detected by immunofluorescence microscopy. This increment in matrix assembly, although modest, supports the notion that inappropriate β1-integrin function could play a role in the abnormal organization of β1 fibrillar adhesions and fibronectin fibers in VHL(−) cells. Finally, culturing of VHL(+) cells with the β1-blocking antibody LIA1/2 (23) completely abrogated the formation of fibronectin fibers (Fig. 6*B*), demonstrating that fibronectin assembly in 786-O renal cancer cells is completely dependent on β1 integrins (20, 36).

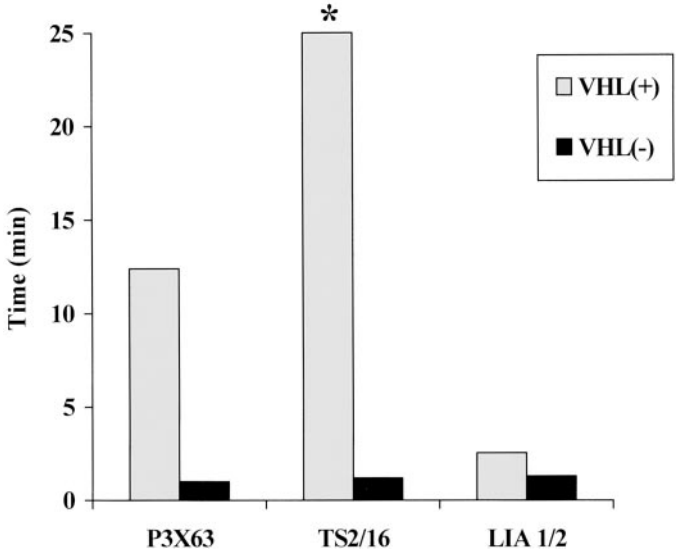

Fig. 5. Regulation of the strength of cell adherence by VHL. 786-O cells were grown in 24-well dishes in the presence of P3X63, TS2/16 (β1-activating mAb), or LIA1/2 (β1-blocking mAb). After 3–4 days, the culture medium was removed, and a solution containing 1% Triton X-100 was added to intact monolayers. Cell detachment was verified with a phase-contrast microscope, and the respective times were measured. Each condition was analyzed in triplicate. The *asterisk* above the *column* corresponding to VHL(+) cells cultured with TS2/16 indicates that the monolayers were not able to detach under this condition. One representative experiment is presented.

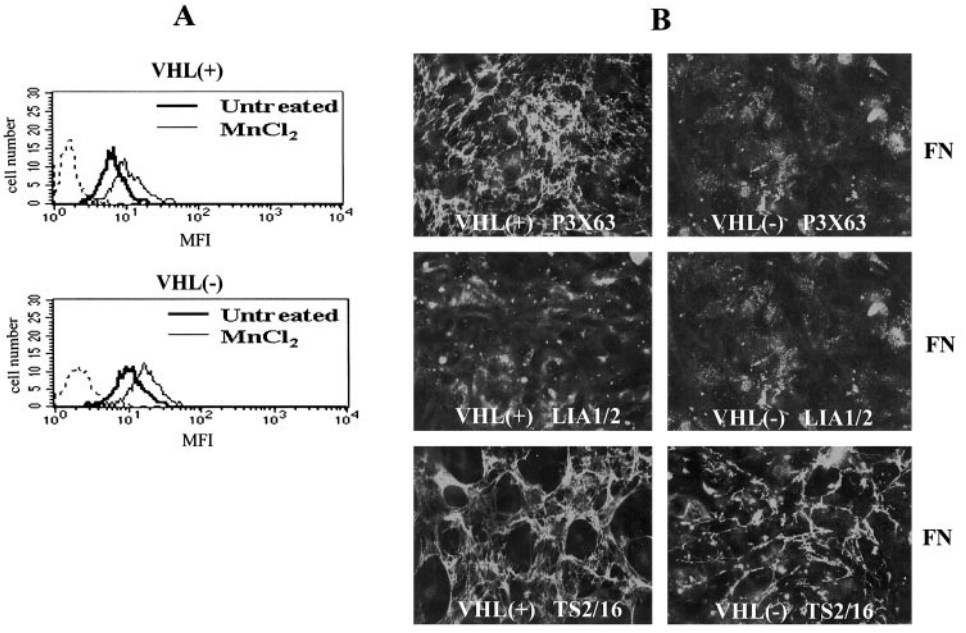

Fig. 6. Outside-in activation of β1 integrins increases fibronectin assembly in VHL(−) cells. *A*, 786-O (250×10^3) cells were cultured in the presence of P3X63, the β1-activating antibody TS2/16, or the β1-blocking antibody LIA1/2. Fibronectin organization was then studied by indirect immunofluorescence. The formation of β1 fibrillar adhesions was increased in VHL(−) cells treated with TS2/16 and abolished in VHL(+) cells treated with LIA1/2 (data not shown). *B*, comparative study by flow cytometry of the β1-integrin activation state in 786-O cells. One representative experiment is shown. *MFI*, mean fluorescence intensity.

Biosynthetic Maturation of β1 Integrins Is Delayed in VHL(−) Cells. Immature precursors of the different α- and common β1-integrin chains associate in the endoplasmic reticulum, where they are driven to the Golgi apparatus for further glycosylation and processing (37). Mature heterodimeric integrins are then translocated to the cell membrane, where they subsequently become functional. To determine whether the biosynthesis of β1 integrins could be affected in VHL(−) cells, we performed pulse-chase analysis of β1 integrins in metabolically labeled 786-O cells. Four bands could be typically observed in these experiments, representing β1 and associated α chains in different stages of maturation. Iodination of cell surface proteins was used to discriminate mature from immature integrin subunits (38). Interestingly, when compared with their VHL(+) counterparts, VHL(−) cells presented a significant delay in the maturation of the common β1-integrin chain during the first hours (Fig. 7). A comparable delay in β1-chain biosynthesis was also observed when $\alpha5\beta1$ integrins were immunoprecipitated (data not shown). However, α-chain maturation was unaffected in any case, showing that the defect is restricted to the β1 chain. Because the formation of integrin fibrillar adhesions is a dynamic process, a delay in the maturation of β1 integrins could affect the rate of assembly and maintenance of this complex. However, the organization of β1 fibrillar adhesions in VHL(+) 786-O cells is a slow process, and mature integrin surface levels are increased even in VHL(−) cells. Thus, the relative contribution of this delayed maturation to the defective matrix assembly may be modest.

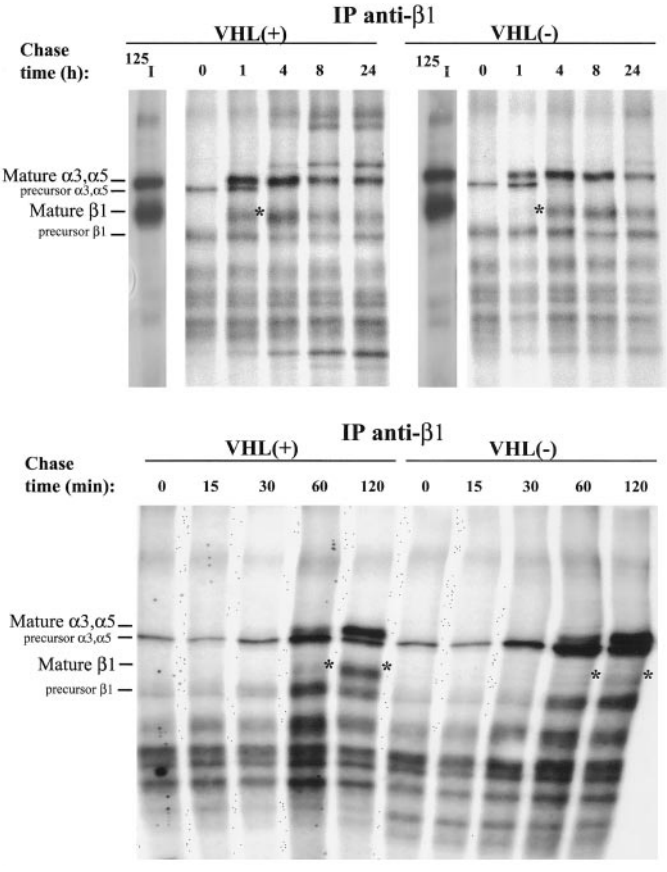

Fig. 7. Maturation of β1 integrins is delayed in VHL(−) cells. Shown is pulse-chase analysis of metabolically labeled 786-O cells (clones WT8 and MUT115). One representative experiment of each condition is presented. Cells were pulsed for 30 min (*Lanes 0*) and were chased either for 1, 2, 4, and 24 h (*top panel*) or for 15, 30, 60, and 120 min (*bottom panel*) to further emphasize the magnitude of the defect. Cell lysates were immunoprecipitated (*IP*) with the antibody TS2/16, which despite its activating effect on integrins in live cells recognizes all β1 integrins and is a well-suited antibody for immunoprecipitation studies. *Asterisks* indicate times at which differences between both groups of cells were more evident. Iodination of cell surface proteins and subsequent immunoprecipitation with anti-β1 was also used to distinguish mature from immature integrin subunits. The iodinated β1 band appears broader than the metabolically band because of the two different labeling methods. Protein G-Sepharose without immunoprecipitating antibody was used as a negative control (data not shown).

DISCUSSION

Fibronectin is a multifunctional component of the ECM that elicits intracellular signals that regulate cell adhesion, proliferation, differentiation, and tumorigenesis (11). Loss of fibronectin matrix organization has been recognized for many years as a very important event in cellular transformation and is a common feature in most cancer cells. A recent study by Ohh *et al.* (10) demonstrated that VHL interacts with fibronectin and is also responsible for the lack of extracellular fibronectin arrays in VHL(−) renal cancer cells and VHL −/− mouse fibroblasts. Nevertheless, the exact nature of such an association and the mechanism by which VHL regulates the assembly of this ECM protein are poorly understood. It was postulated

that abnormally processed or misfolded fibronectin might accumulate inside cells in the absence of an intact VHL. These aberrant fibronectin species, if secreted, could at least potentially interfere with the assembly of normal fibronectin. In this study, we failed to detect any accumulation of endogenous fibronectin in VHL(−) cells, as assessed by Western blot of total and intracellular fibronectin. In addition, neither the use of exogenous normal fibronectin nor culture on fibronectin-coated coverslips improved the ability of VHL(−) cells to organize extracellular fibronectin. From these findings, we suggest that the abnormal ECM organization in VHL(−) renal cancer cells might require a different explanation. Moreover, our data showing that α3, β1, αv, and to a lesser extent, α2 and α5 integrins are up-regulated in VHL(−) cells demonstrate that the altered assembly of the fibronectin matrix is not attributable to decreased integrin expression. These results are also clearly consistent with the highly migratory phenotype that has been reported previously in VHL(−) cells (9).

Comparison of β1 and αv cell-matrix adhesions in VHL(+) and VHL(−) renal cancer cells at different stages of culture provides important clues concerning their distinct fibronectin matrix organization and migratory behavior. Early after plating, both VHL(+) and VHL(−) cells display proper αv classic FCs and patches of vinculin, but lack β1 fibrillar adhesions. However, as the culture advances and cells become confluent, VHL(+) transfectants progressively assemble large β1 fibrillar adhesions, and αv integrins partly relocate to the intercellular junctions. The presence of β1-integrin fibrillar adhesions in VHL(+) cells at late confluence allows them to firmly anchor to the substrate and might be an important mechanism in controlling cell migration. In contrast, late confluent VHL(−) cells are not capable of organizing adequate β1 fibrillar adhesions or fibronectin fibers and show weaker attachment to the substrate. The failure of the β1-activating antibody TS2/16 to delay the detachment of VHL(−) cells treated with Triton X-100 (see Fig. 5) contrasts with its effect of improving matrix assembly in the same cells. However, this improvement is modest when compared with fibronectin matrix assembly in VHL(+) cells.

VHL(−) cells displayed adequate αv classic FCs at any stage of culture. This implies that there is no general failure in the assembly of cell-matrix adhesions in VHL(−) cells and demonstrates that the defect is restricted exclusively to β1 fibrillar adhesions. In this regard, a recent report by Kamada *et al.* (39) showed that the organization of vinculin, an intracellular constituent of classic FCs, is diminished in VHL(−) UMRC6 cells compared with VHL(+) transfectants. Accordingly, the authors concluded that the formation of FCs was impaired in VHL(−) cells. However, in most adherent cells, vinculin distribution is not fully restricted to cell-matrix adhesions, and in their study, Kamada *et al.* (39) did not analyze the subcellular distribution of αv and β1 integrins, which are much more specific markers for classic FCs and fibrillar adhesions. Differences between both studies could be attributable to the cell type used.

The restored ability of VHL(+) transfectants to assemble extracellular fibronectin is mediated by β1 integrins: it is completely abrogated by β1-blocking antibodies. We propose that an abnormal organization of β1 fibrillar adhesions could help to explain the deficient fibronectin assembly of VHL(−) cells. Nevertheless, we cannot formally rule out the possibility that an undetectable amount of abnormal fibronectin is accumulated and secreted in VHL(−) cells, interfering with normal fibronectin assembly.

How does VHL regulate the formation of β1 fibrillar adhesions and subsequent fibronectin organization? VHL could regulate the expression or function of any of the intracellular constituents of the fibrillar adhesion. In this regard, tensin, which has been implicated in the translocation of β1 integrins from the classic FC to the mature fibrillar adhesion (17), is an excellent candidate to be regulated by VHL. In addition, VHL could be modulating other proteins, such as the small GTP-binding proteins Rho, Rac, and cdc42, which play an important role in the control of cytoskeleton and regulate the formation of cell-matrix adhesions (40). These possibilities are being addressed at present.

In conclusion, this report shows that, in renal cancer cells, the presence of VHL is required for the adequate assembly of β1-integrin fibrillar adhesions and demonstrates that VHL controls the strength of cell adhesion through this mechanism. The failure of VHL(−) cells to organize β1 fibrillar adhesions could explain their abnormal matrix assembly and may play a role in their highly migratory phenotype.

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