

Role of the von Hippel-Lindau Tumor Suppressor Gene in the Formation of β 1-Integrin Fibrillar Adhesions¹

Miguel A. Esteban-Barragán, Pilar Ávila, Miguel Álvarez-Tejado, M. Dolores Gutiérrez, Ángeles García-Pardo, Francisco Sánchez-Madrid, and Manuel O. Landázuri²

Servicio de Inmunología, Hospital de la Princesa, Universidad Autónoma de Madrid (UAM), 28006 Madrid, Spain [M. A. E.-B., P. A., M. A.-T., M. D. G., F. S.-M., M. O. L.], and Departamento de Inmunología, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, 28006 Madrid, Spain [A. G.-P.]

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 β 1 and α v 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” α v focal contacts. As the culture advanced and cells became confluent, α v integrins partly relocated to the intercellular junctions in VHL(+) transfectants, which then developed large β 1 fibrillar-type adhesions and anchored firmly to the substrate. In contrast, confluent VHL(−) cells were unable to assemble β 1 fibrillar adhesions, and α v focal contacts remained unchanged at all stages of the culture. Exogenous activation of β 1 integrins with either divalent cations or activating antibodies partly restored the capability of VHL(−) cells to assemble β 1 fibrillar adhesions and fibronectin fibers. Finally, pulse-chase studies of metabolically labeled 786-O cells revealed that the maturation of the common β 1-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 β 1 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 α v or β 1 integrins, and are rich in talin and vinculin. Conversely, fibrillar adhesions are elongated, contain exclusively β 1 integrins, and possess low levels of vinculin or talin. The formation of fibrillar adhesions involves cytoskeletal rearrangement of the previously formed β 1 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 β 1-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 μ g/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- α 1 integrin), Tea 1/41 (anti- α 2 integrin), and VJ1/18 (anti- α 3 integrin) have been described previously. mAb ABA-6D1 (anti- α v) and mAb PID6 (anti- α 5 β 1) were provided by Dr. C. Martínez-Alonso (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.

Received 8/13/01; accepted 3/19/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by Grants SAF2001/0215 and FEDER-2FD971870 from the Ministerio de Educación y Cultura. Miguel A. Esteban-Barragán, Miguel Álvarez-Tejado, and M. Dolores Gutiérrez were supported by fellowships from Comunidad Autónoma de Madrid.

² To whom requests for reprints should be addressed, at Servicio de Inmunología, Hospital de la Princesa, Diego de León 62, Madrid 28006, Spain. Phone: 34-91-5202662; Fax: 34-91-5202374; E-mail: mortiz@hlpr.insalud.es.

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 $\beta 1$ -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 $\mu\text{g}/\text{ml}$ 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 [³⁵S]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 [³⁵S]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.

¹²⁵I 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 [¹²⁵I]iodine (Amersham Pharmacia Biotech) using 1,3,4,6-tetrachloro-3 α ,6 α -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. 1A). 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. 1B, 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. 1B).

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 , $\alpha 3$, and $\beta 1$ were clearly up-regulated in VHL(–) cells compared with VHL(+) cells (Fig. 2). The levels of $\alpha 2$ and $\alpha 5$ integrins were also augmented in VHL(–) cells, but their expression

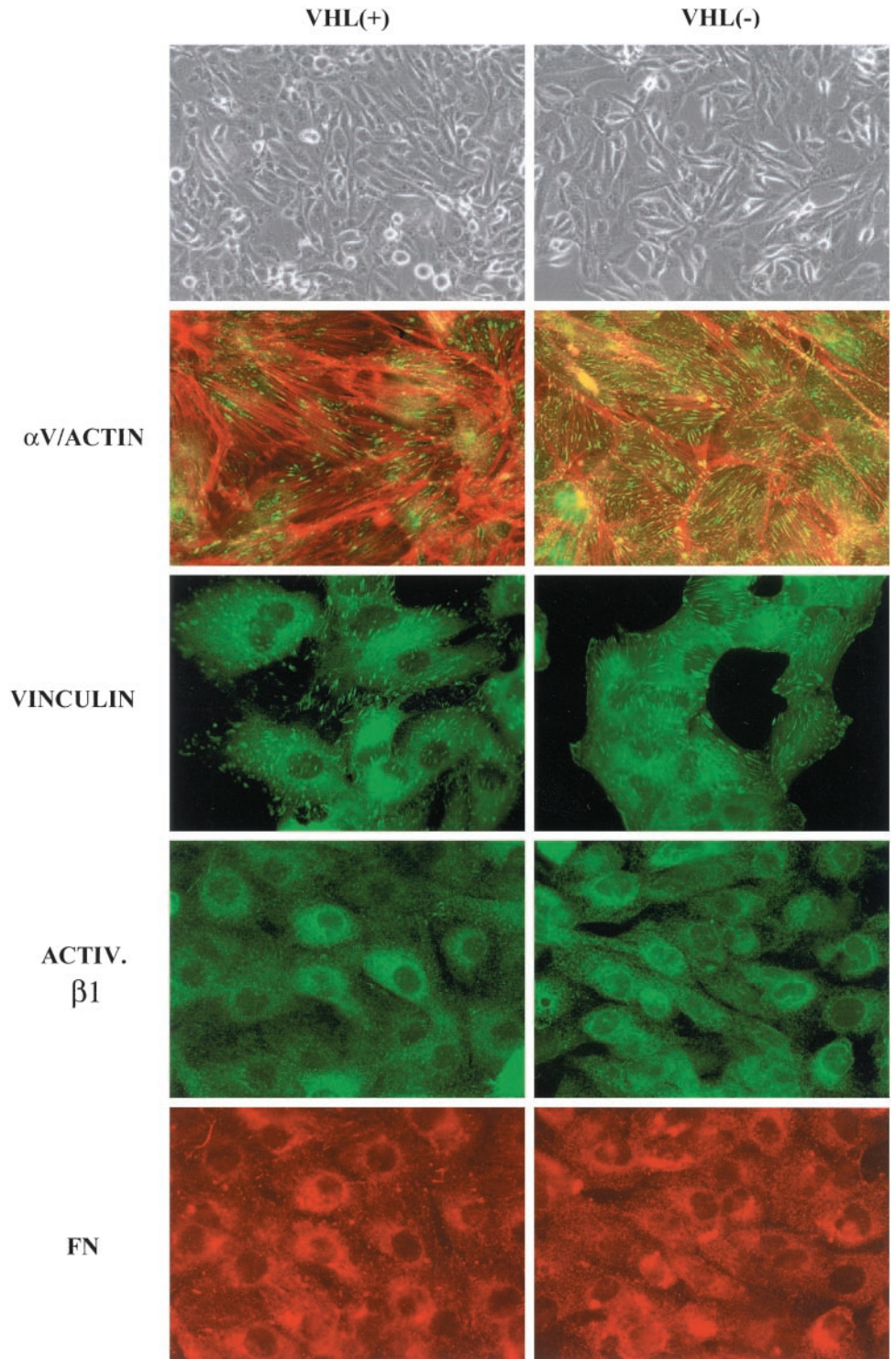


Fig. 3. Effect of VHL expression on cell morphology and the distribution of vinculin, α_5 , fibronectin, and β_1 integrins at early stages of culture. 786-O cells (125×10^3) were grown on coverslips for 10–15 h and fixed. Cells were photographed with a phase-contrast microscope and then double stained with anti- α_5 and Texas red-phalloidin (to visualize actin), double stained with antiactivated β_1 (HUTS21) and antifibronectin, or stained with antivinculin.

inadequate translocation of β_1 integrins to the mature fibrillar adhesion. As expected, $\alpha_5\beta_1$ integrins were diffusely distributed in VHL(-) cells.

To determine whether the formation of β_1 -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 β_1 -activating (TS2/16) or β_1 -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 β_1 integrin function and fibronectin assembly in VHL(+) 786-O cells with the antibody LIA1/2 (see below; Fig. 6B) dramatically reduced the time of detachment, which was then very similar to that of VHL(-) cells. On the other hand, the β_1 -activating antibody TS2/16, which increases the formation of β_1 fibrillar adhesions and fibronectin fibrils (see below; Fig. 6B) 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

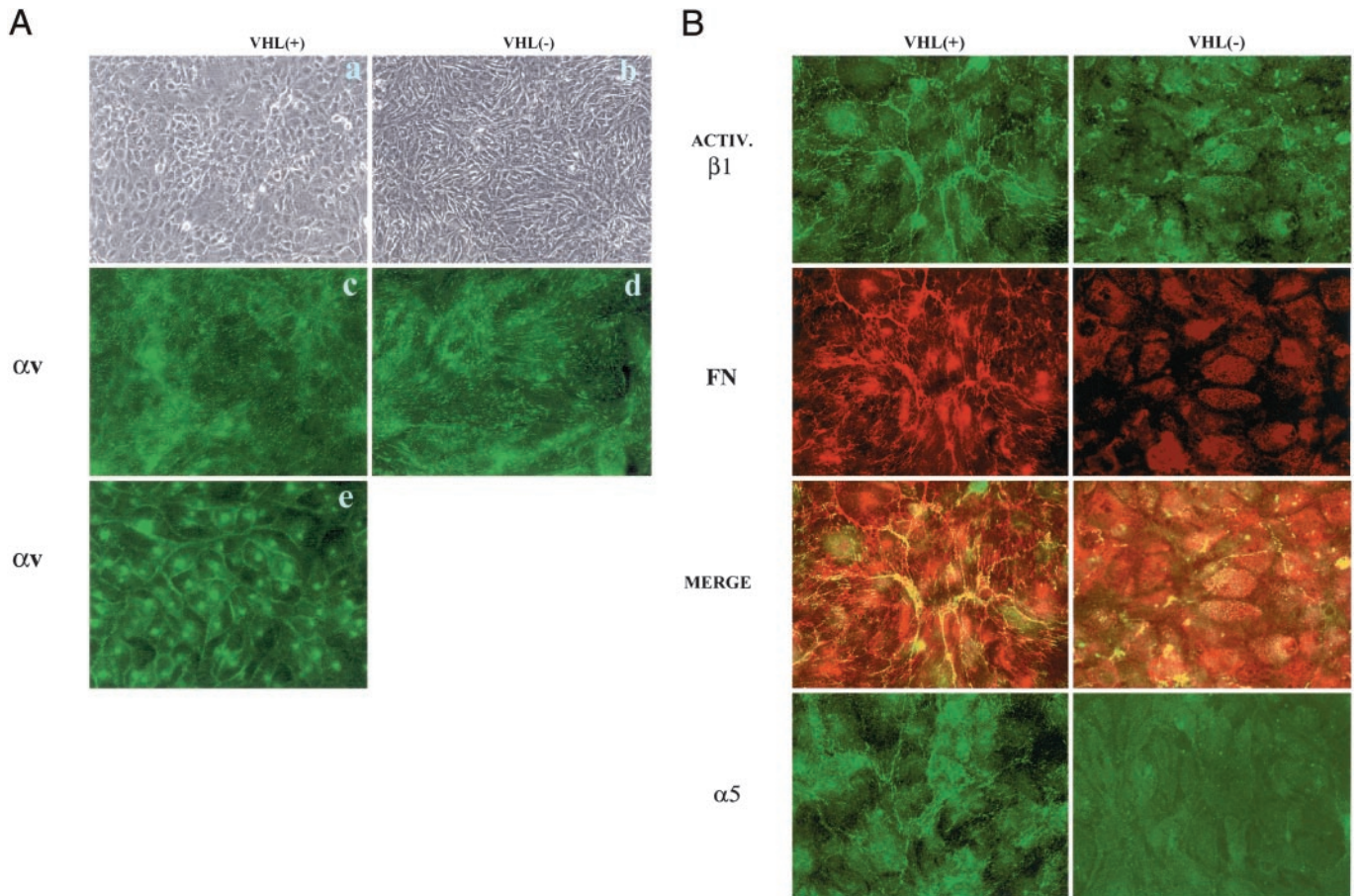


Fig. 4. Effect of VHL on cell morphology and the distribution of αv , fibronectin, and $\beta 1$ integrins at late confluence. 786-O cells (125×10^3) were cultured on coverslips for 3–5 days and then fixed. A, cells were photographed with a phase-contrast microscope and stained with anti- αv . Two different 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- $\alpha 5\beta 1$ or double stained with HUTS21 (activated $\beta 1$ integrin) and antifibronectin. Similar results were obtained when cells were cultured on fibronectin (10 $\mu\text{g/ml}$), laminin (5 $\mu\text{g/ml}$), or vitronectin (5 $\mu\text{g/ml}$; data not shown).

the assembly of $\beta 1$ fibrillar adhesions, which has functional consequences in defective fibronectin fibrillar organization and diminished adherence to the substrate.

Outside-In Activation of $\beta 1$ 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 $\beta 1$ -integrin antibody HUTS21, and no significant differences between VHL(+) and VHL(-) cells were detected (Fig. 6A). Likewise, a short incubation with MnCl_2 before analysis increased $\beta 1$ -integrin activation very similarly in both groups of cells. Notably, when VHL(-) cells were cultured in the sustained presence of either the $\beta 1$ -activating antibody TS2/16 (Refs. 23, 35; Fig. 6B) 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 $\beta 1$ -integrin function could play a role in the abnormal organization of $\beta 1$ fibrillar adhesions and fibronectin fibers in VHL(-) cells. Finally, culturing of VHL(+) cells with the $\beta 1$ -blocking antibody LIA1/2 (23) completely abrogated the formation of fibronectin fibers (Fig. 6B), demonstrating that fibronectin assembly in 786-O renal cancer cells is completely dependent on $\beta 1$ integrins (20, 36).

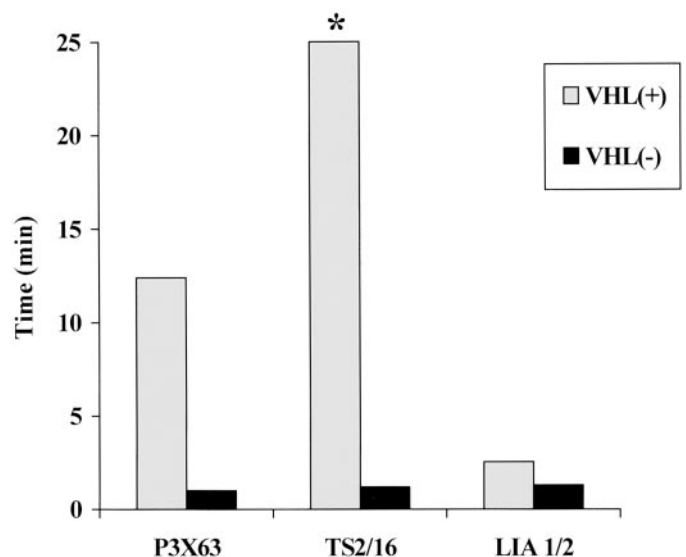
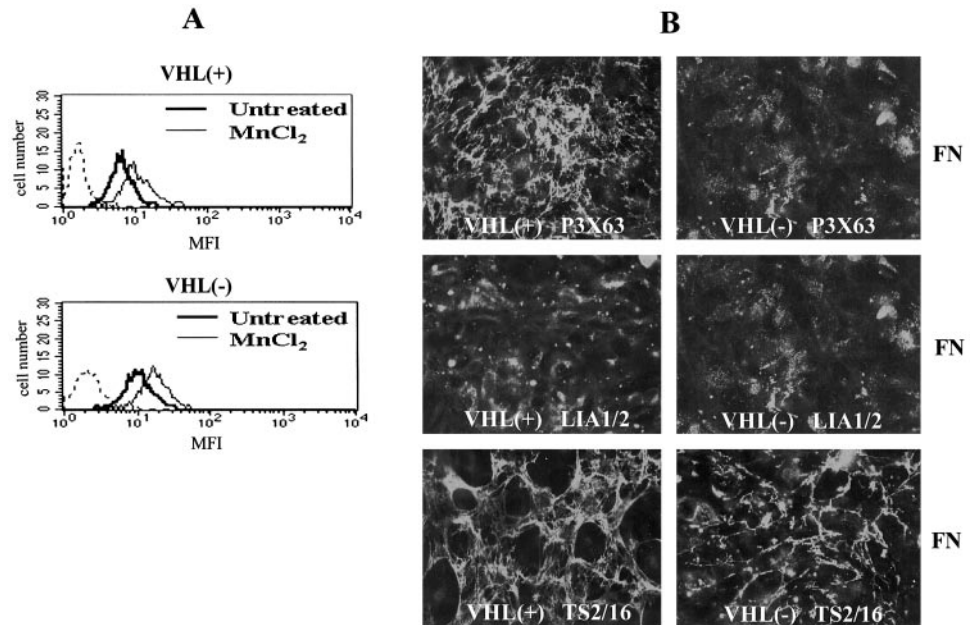


Fig. 5. Regulation of the strength of cell adherence by VHL. 786-O cells were grown in 24-well dishes in the presence of P3X63, TS2/16 ($\beta 1$ -activating mAb), or LIA1/2 ($\beta 1$ -blocking mAb). After 3–4 days, the culture medium was removed, and a solution containing 1% Triton X-100 was added 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 $\beta 1$ integrins increases fibronectin assembly in VHL(-) cells. A, 786-O (250×10^3) cells were cultured in the presence of P3X63, the $\beta 1$ -activating antibody TS2/16, or the $\beta 1$ -blocking antibody LIA1/2. Fibronectin organization was then studied by indirect immunofluorescence. The formation of $\beta 1$ 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 $\beta 1$ -integrin activation state in 786-O cells. One representative experiment is shown. MFI, mean fluorescence intensity.



Biosynthetic Maturation of $\beta 1$ Integrins Is Delayed in VHL(-) Cells. Immature precursors of the different α - and common $\beta 1$ -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 $\beta 1$ integrins could be affected in VHL(-) cells, we performed pulse-chase analysis of $\beta 1$ integrins in metabolically labeled 786-O cells. Four bands could be typically observed in these experiments, representing $\beta 1$ 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 $\beta 1$ -integrin chain during the first hours (Fig. 7). A comparable delay in $\beta 1$ -chain biosynthesis was also observed when $\alpha 5\beta 1$ integrins were immunoprecipitated (data not shown). However, α -chain maturation was unaffected in any case, showing that the defect is restricted to the $\beta 1$ chain. Because the formation of integrin fibrillar adhesions is a dynamic process, a delay in the maturation of $\beta 1$ integrins could affect the rate of assembly and maintenance of this complex. However, the organization of $\beta 1$ 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.

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

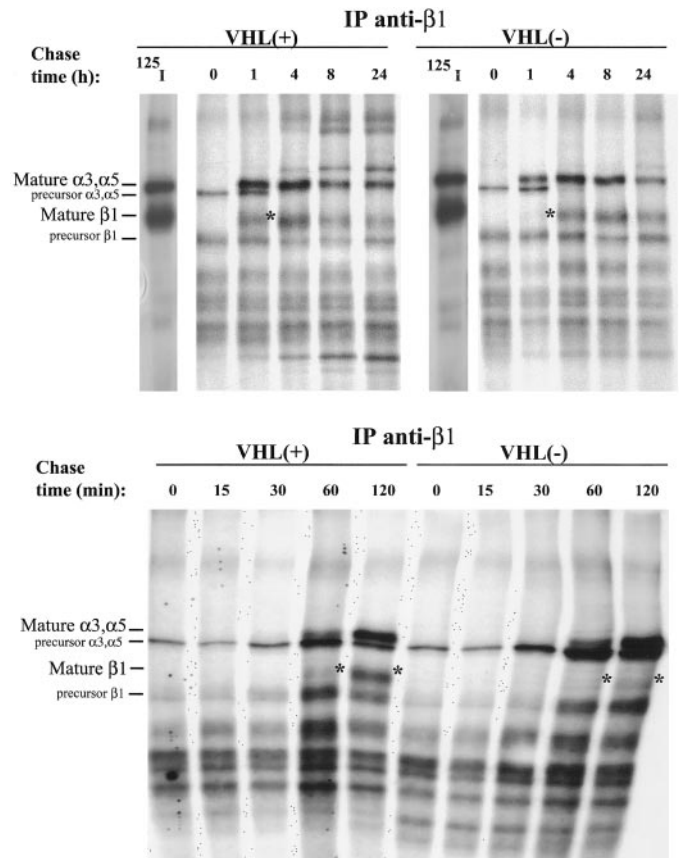


Fig. 7. Maturation of $\beta 1$ integrins is delayed in VHL(-) cells. Shown is pulse-chase analysis of metabolically labeled 786-O cells (clones WT8 and MUT115). One representative experiment of each condition is presented. Cells were pulsed for 30 min (Lanes 0) and were chased either for 1, 2, 4, and 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 $\beta 1$ 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- $\beta 1$ was also used to distinguish mature from immature integrin subunits. The iodinated $\beta 1$ 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).

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 $\alpha 3$, $\beta 1$, αv , and to a lesser extent, $\alpha 2$ and $\alpha 5$ 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 $\beta 1$ 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 $\beta 1$ fibrillar adhesions. However, as the culture advances and cells become confluent, VHL(+) transfectants progressively assemble large $\beta 1$ fibrillar adhesions, and αv integrins partly relocate to the intercellular junctions. The presence of $\beta 1$ -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 $\beta 1$ fibrillar adhesions or fibronectin fibers and show weaker attachment to the substrate. The failure of the $\beta 1$ -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 $\beta 1$ 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(-) UMR6 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 $\beta 1$ 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 $\beta 1$ integrins: it is completely abrogated by $\beta 1$ -blocking antibodies. We propose that an abnormal organization of $\beta 1$ 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 $\beta 1$ 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 $\beta 1$ 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 $\beta 1$ -integrin fibrillar adhesions and demonstrates that VHL controls the strength of cell adhesion through this mechanism. The failure of VHL(-) cells to organize $\beta 1$ fibrillar adhesions could explain their abnormal matrix assembly and may play a role in their highly migratory phenotype.

ACKNOWLEDGMENTS

We thank W. G. Kaelin and K. Plate for providing the 786-O and RCC10 cell lines, respectively; we also thank C. Martínez-A. and E. A. Wayner for providing antibodies and M. Vicente-Manzanares, J. Aragonés, and L. del Peso for critical reading of the manuscript.

REFERENCES

- Iliopoulos, O., Kibel, A., Gray, S., and Kaelin, W. G., Jr. Tumour suppression by the human von Hippel-Lindau gene product. *Nat. Med.*, *1*: 822–826, 1995.
- Stebbins, C. E., Kaelin, W. G., Jr., and Pavletich, N. P. Structure of the VHL-ElonginB complex: implications for VHL tumor suppressor function. *Science (Wash. DC)*, *284*: 455–461, 1999.
- Iwai, K., Yamanaka, K., Kamura, T., Minato, N., Conaway, R. C., Conaway, J. W., Klausner, R. D., and Pause, A. Identification of the von Hippel-Lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex. *Proc. Natl. Acad. Sci. USA*, *96*: 12436–12441, 1999.
- Krek, W. VHL takes HIF's breath away. *Nat. Cell Biol.*, *2*: E121–E123, 2000.
- Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R., and Ratcliffe, P. J. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature (Lond.)*, *399*: 271–275, 1999.
- Ohh, M., Park, C. W., Ivan, M., Hoffman, M. A., Kim, T. Y., Huang, L. E., Pavletich, N., Chau, V., and Kaelin, W. G. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. *Nat. Cell Biol.*, *2*: 423–427, 2000.
- Davidowitz, E. J., Schoenfeld, A. R., and Burk, R. D. VHL induces renal cell differentiation and growth arrest through integration of cell-cell and cell-extracellular matrix signaling. *Mol. Cell Biol.*, *21*: 865–874, 2001.
- Lieubeau-Teillet, B., Rak, J., Jothy, S., Iliopoulos, O., Kaelin, W., and Kerbel, R. S. von Hippel-Lindau gene-mediated growth suppression and induction of differentiation in renal cell carcinoma cells grown as multicellular tumor spheroids. *Cancer Res.*, *58*: 4957–4962, 1998.
- Koochekpour, S., Jeffers, M., Wang, P. H., Gong, C., Taylor, G. A., Roessler, L. M., Stearman, R., Vasselli, J. R., Stetler-Stevenson, W. G., Kaelin, W. G., Jr., Linehan, W. M., Klausner, R. D., Gnarr, J. R., and Vande Woude, G. F. The von Hippel-Lindau tumor suppressor gene inhibits hepatocyte growth factor/scatter factor-induced invasion and branching morphogenesis in renal carcinoma cells. *Mol. Cell Biol.*, *19*: 5902–5912, 1999.
- Ohh, M., Yauch, R. L., Lonergan, K. M., Whaley, J. M., Stemmer-Rachamimov, A. O., Louis, D. N., Gavin, B. J., Kley, N., Kaelin, W. G., Jr., and Iliopoulos, O. The von Hippel-Lindau tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix. *Mol. Cell*, *1*: 959–968, 1998.
- Lukashev, M. E., and Werb, Z. ECM signalling: orchestrating cell behaviour and misbehaviour. *Trends Cell Biol.*, *8*: 437–441, 1998.
- Sánchez-Mateos, P., Cabañas, C., and Sánchez-Madrid, F. Regulation of integrin function. *Semin. Cancer Biol.*, *7*: 99–109, 1996.
- Schoenwaelder, S. M., and Burridge, K. Bidirectional signaling between the cytoskeleton and integrins. *Curr. Opin. Cell Biol.*, *11*: 274–286, 1999.
- Ben-Ze'ev, A. Cytoskeletal and adhesion proteins as tumor suppressors. *Curr. Opin. Cell Biol.*, *9*: 99–108, 1997.
- Critchley, D. R. Focal adhesions—the cytoskeletal connection. *Curr. Opin. Cell Biol.*, *12*: 133–139, 2000.
- Zamir, E., Katz, B. Z., Aota, S., Yamada, K. M., Geiger, B., and Kam, Z. Molecular diversity of cell-matrix adhesions. *J. Cell Sci.*, *112*: 1655–1669, 1999.
- Pankov, R., Cukierman, E., Katz, B. Z., Matsumoto, K., Lin, D. C., Lin, S., Hahn, C., and Yamada, K. M. Integrin dynamics and matrix assembly: tensin-dependent translocation of $\alpha 5 \beta 1$ integrins promotes early fibronectin fibrillogenesis. *J. Cell Biol.*, *148*: 1075–1090, 2000.
- Katz, B. Z., Zamir, E., Bershadsky, A., Kam, Z., Yamada, K. M., and Geiger, B. Physical state of the extracellular matrix regulates the structure and molecular composition of cell-matrix adhesions. *Mol. Biol. Cell*, *11*: 1047–1060, 2000.
- Cukierman, E., Pankov, R., Stevens, D. R., and Yamada, K. M. Taking cell-matrix adhesions to the third dimension. *Science (Wash. DC)*, *294*: 1708–1712, 2001.
- Sechler, J. L., Corbett, S. A., and Schwarzbauer, J. E. Modulatory roles for integrin activation and the synergy site of fibronectin during matrix assembly. *Mol. Biol. Cell*, *8*: 2563–2573, 1997.

21. Schwarzbauer, J. E., and Sechler, J. L. Fibronectin fibrillogenesis: a paradigm for extracellular matrix assembly. *Curr. Opin. Cell Biol.*, *11*: 622–627, 1999.
22. Shaub, A. Unravelling the extracellular matrix. *Nat. Cell Biol.*, *1*: E173–E175, 1999.
23. Arroyo, A. G., Sánchez-Mateos, P., Campanero, M. R., Martín-Padura, I., Dejana, E., and Sánchez-Madrid, F. Regulation of the VLA integrin-ligand interactions through the beta 1 subunit. *J. Cell Biol.*, *117*: 659–670, 1992.
24. Luque, A., Gómez, M., Puzón, W., Takada, Y., Sánchez-Madrid, F., and Cabañas, C. Activated conformations of very late activation integrins detected by a group of antibodies (HUTS) specific for a novel regulatory region (355–425) of the common $\beta 1$ chain. *J. Biol. Chem.*, *271*: 11067–11075, 1996.
25. Bonifacino, J. S. Isolation and analysis of proteins. *In*: J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, W. Strober (eds.), *Current Protocols in Immunology*, Vol. 1. New York: John Wiley & Sons, 1992.
26. Meisenhelder, J., and Hunter, T. Radioactive protein-labelling techniques. *Nature (Lond.)*, *335*: 120, 1988.
27. Coligan, J. E., Gates, F. T. 3rd, Kimball, E. S., and Maloy, W. L. Radiochemical sequence analysis of biosynthetically labeled proteins. *Methods Enzymol.*, *91*: 413–434, 1983.
28. Pulido, R., and Sánchez-Madrid, F. Glycosylation of CD45: carbohydrate processing through Golgi apparatus is required for cell surface expression and protein stability. *Eur. J. Immunol.*, *22*: 463–468, 1992.
29. Markwell, M. A., and Fox, C. F. Surface-specific iodination of membrane proteins of viruses and eucaryotic cells using 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril. *Biochemistry*, *17*: 4807–4817, 1978.
30. Hynes, R. O. Fibronectins. *In*: A. Rich (ed.), *Springer Series in Molecular Biology*, p. 54. New York: Springer-Verlag, 1990.
31. Hanahan, D., and Weinberg, R. A. The hallmarks of cancer. *Cell*, *100*: 57–70, 2000.
32. Kuwada, S. K., and Li, X. Integrin $\alpha 5/\beta 1$ mediates fibronectin-dependent epithelial cell proliferation through epidermal growth factor receptor activation. *Mol. Biol. Cell*, *11*: 2485–2496, 2000.
33. Domínguez-Jiménez, C., Yañez-Mó, M., Carreira, A., Tejedor, R., González-Amaro, R., Alvarez, V., and Sánchez-Madrid, F. Involvement of $\alpha 3$ integrin/tetraspanin complexes in the angiogenic response induced by angiotensin II. *FASEB J.*, *15*: 1457–1459, 2001.
34. Wu, C., Keivens, V. M., O'Toole, T. E., McDonald, J. A., and Ginsberg, M. H. Integrin activation and cytoskeletal interaction are essential for the assembly of a fibronectin matrix. *Cell*, *83*: 715–724, 1995.
35. Arroyo, A. G., García-Pardo, A., and Sánchez-Madrid, F. A high affinity conformational state on VLA integrin heterodimers induced by an anti- $\beta 1$ chain monoclonal antibody. *J. Biol. Chem.*, *268*: 9863–9868, 1993.
36. Hocking, D. C., Sottile, J., and McKeown-Longo, P. J. Activation of distinct $\alpha 5\beta 1$ -mediated signaling pathways by fibronectin's cell adhesion and matrix assembly domains. *J. Cell Biol.*, *141*: 241–253, 1998.
37. Albigès-Rizo, C., Frchet, P., and Block, M. R. Down regulation of talin alters cell adhesion and the processing of the $\alpha 5\beta 1$ integrin. *J. Cell Sci.*, *108*: 3317–3329, 1995.
38. Hemler, M. E., Sánchez-Madrid, F., Flotte, T. J., Krensky, A. M., Burakoff, S. J., Bhan, A. K., Springer, T. A., and Strominger, J. L. Glycoproteins of 210,000 and 130,000 M.W. on activated T cells: cell distribution and antigenic relation to components on resting cells and T cell lines. *J. Immunol.*, *132*: 3011–3018, 1984.
39. Kamada, M., Suzuki, K., Kato, Y., Okuda, H., and Shuin, T. von Hippel-Lindau protein promotes the assembly of actin and vinculin and inhibits cell motility. *Cancer Res.*, *61*: 4184–4189, 2001.
40. Hall, A. Rho GTPases and the actin cytoskeleton. *Science (Wash. DC)*, *279*: 509–514, 1998.