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Advances in Brief

β4 Integrin Transfection of UM-UC-2 (Human Bladder Carcinoma) Cells: Stable Expression of a Spontaneous Cytoplasmic Truncation Mutant with Rapid Loss of Clones Expressing Intact β4[1]

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Abstract

The α6β4 integrin is a component of the hemidesmosome, the anchoring structure in the basal membrane of epithelial cells. α6β4 expression is frequently altered in neoplastic cells. It is sometimes lost and sometimes overexpressed, which suggests that disruption of normal function is involved in neoplastic transformation. To examine the effect of this integrin on the growth and behavior of malignant cells that have lost β4, we transfected a full-length β4 cDNA into the UM-UC-2 cell line that expresses α6 but not β4. Although large numbers of clones were obtained when a control vector was used in the transfection, only 12 clones could be isolated that expressed β4. Of these, only two β4-positive clones, clones 8 and 11, persisted long enough for further study. Clone 8 cells initially expressed β4, but within 2 weeks, all positive cells were lost from the culture. Clone 11 persisted in culture and retained strong surface expression of α6β4. Biochemical analysis and Western blotting revealed that this clone contained a truncated form of β4 that had lost the distal cytoplasmic domain. We conclude that expression of wild-type β4 in UM-UC-2 inhibits cell growth, presumably by an integrin-mediated signaling pathway. Clone 11 escaped from normal signaling because the cytoplasmic domain, a region essential for basal polar localization, was lost. The α6β4 integrin appears to have tumor suppressor activity in epithelial tumors.

Introduction

The α6β4 integrin is a laminin/kalinin receptor (1, 2) and a component of the hemidesmosome (3, 4). The β4 subunit is a M_r 205,000 protein characterized by an unusually large cytoplasmic domain of M_r 118,000 that contains four type III fibronectin-like repeats in the COOH terminus (5, 6). This domain, unique among integrins, is essential for interactions with other cytoplasmic proteins and necessary for localization in hemidesmosomes (6–8) but not for association with α6 or adhesion to laminin (7). Furthermore, Clarke *et al.* (9) observed that the intact β4 integrin is part of a signaling pathway that up-regulates p21/Cip1 (cyclin-dependent kinase inhibitor) expression and induces apoptosis.

The α6β4 integrin was initially identified as a tumor-associated antigen by monoclonal antibodies raised to murine lung and human pancreatic, squamous cell, and bladder carcinomas (10–13). High expression is associated with tumor progression (14), poor prognosis (15), or metastasis (16). However, in some cases, expression of α6β4 is lost, suggesting that either overexpression or loss may contribute to malignant progression. The aim of this study is to analyze the effect of expression of the full-length β4 cDNA in a human bladder carcinoma cell line, UM-UC-2 (17), that expresses α6 but not β4 (13). Clones expressing the full-length β4 subunit did not persist in culture, but a clone expressing a spontaneously mutated β4 subunit, in which the distal cytoplasmic domain was lost, continued to proliferate and express the mutant form of β4 in association with the endogenous α6. These results suggest that the β4 integrin may function as a tumor suppressor gene.

Materials and Methods

Monoclonal Antibodies. Hybridoma supernatants containing the anti-β4 monoclonal antibody UM-A9 (12) and the anti-α6 antibody BQ-16 (13) were used undiluted. The rat anti-β4 monoclonal antibodies 439-9B, against the extracellular domain, and 450-11A, against the cytoplasmic domain, were generous gifts from Drs. S. J. Kennel and T. Langford of Oak Ridge National Laboratory (10, 18). Lyophilized ascites fluids were rehydrated and diluted to 1:1000 for use in Western blot analysis.

Cell Culture. Squamous carcinoma cell lines UM-SCC-22B and -38 were established in our laboratory (12, 14) and were used as α6β4-positive controls. The bladder carcinoma cell line, UM-UC-2 (13, 17), was used for transfection. Cells are maintained in Dulbecco's modification of Eagle's minimal essential medium supplemented with 2 mM L-glutamine, 1% nonessential amino acids (Sigma Chemical Co.), penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% FCS (Hyclone, Logan, UT; M10 medium). Confluent cultures are passaged using porcine trypsin (0.1% w/v; Sigma) and 0.02% EDTA in Puck's saline A.

Expression Constructs and Transfection. The pRc-CMV[5] eukaryotic expression vector containing the full-length β4 subunit cDNA, pCMV-β4, was obtained from Dr. Filippo Giancotti (6, 7). This vector (InVitrogen, San Diego, CA) contains the CMV promoter, which drives high level expression of mammalian genes, and the neomycin resistance gene as a selectable marker. UM-UC-2 grown in M10 was transfected with 2–10 μg of pCMV-β4 or pRc-CMV by the calcium phosphate precipitation method (6–9). Neomycin-resistant clones were selected in 600 mg/ml geneticin (G418; Life Technologies, Inc., Grand Island, NY), a concentration previously determined to kill the parental UM-UC-2 cells within 7–10 days. Resistant colonies were transferred to 24-well plates and then to coverslips in 6-well plates and tested for β4 expression using immunofluorescence. The clones were subcultured and tested by immunoprecipitation and Western blot assays. Clones transfected with vector alone were selected and analyzed in parallel. Flow cytometry was used to assess intensity of surface α6β4 expression. While in culture, the cells were fed periodically with G418-containing medium to prevent outgrowth of revertant clones. All of the clones were frozen at early passage for subsequent study.

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[5] The abbreviations used are: CMV, cytomegalovirus; IF, immunofluorescence; RIPA, radioimmunoprecipitation assay.

IF Staining and Flow Cytometry. The relative level of expression of β4 was compared by IF staining and flow cytometry. For IF, cells were grown on coverslips or in eight-well Lab-Tek chamber slides (Nunc, Naperville, IL). The cells were washed in cold PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$, incubated with 3% (v/v) normal goat serum (Vector Laboratories, Burlingame, CA) for 45 min, washed three times, and incubated with primary antibodies for 2 h. After washing, specimens were incubated for 45 min with trimethylrhodamine isothiocyanate-conjugated affinity purified $F(Ab')_2$ fragment goat antimouse IgG (Accurate Chemical & Scientific Co., Westbury, NY) diluted 1:200. All incubations were at 4°C on a rotating platform. After fixing the cells in cold acetone for 3 min, coverslips were mounted using Vectashield medium (Vector).

For flow cytometry, cells were detached in trypsin/EDTA, collected in M10, counted, aliquoted at 10^6 cells/tube, and washed once in PBS containing 0.5% (w/v) BSA (PBS-B). The cells were incubated with 100 μl of primary antibody for 45 min on ice. After washing in PBS-B, 100 μl of fluorescein isothiocyanate-conjugated affinity purified $F(Ab')_2$ fragment goat antimouse IgG (Accurate; 1:20) were added for 30 min on ice. Then cells were washed, fixed in 200 μl of 1% paraformaldehyde, and analyzed by flow cytometry (Coulter Epics Co., Hialeah, FL) calibrated with standard beads at an excitation wavelength of 488 nm.

Immunoprecipitation. Cells were metabolically labeled for 4 h with 100 mCi/ml of [^{35}S]methionine (DuPont NEN, Wilmington, DE) in methionine-free medium as described previously (12). Labeled cells were rinsed with PBS and harvested by scraping in 200 μl of lysis buffer (1% NP40 in PBS containing protease inhibitors (13). After 30 min on ice, the lysates were clarified by centrifugation for 5 min at 2000 rpm at 4°C, mixed with 100 μl PBS-B (1% w/v) and 300 μl of RIPA [1% NP40, 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.1% sodium deoxycholate, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride], precleared twice with protein A-Sepharose beads (Sigma), and incubated overnight at 4°C with primary antibodies UM-A9 (anti-β4) and BQ-16 (anti-α6). Antigen-antibody complexes were incubated with 30 μl of protein A-Sepharose beads for 2 h at 4°C, and washed three times in high salt RIPA (500 mM NaCl) and once in 150 mM saline RIPA. The precipitated samples were boiled for 3 min in 60 μl reducing buffer (5% v/v 2-mercaptoethanol, 2% SDS, 10% glycerol, 0.0625 M Tris-Cl, and 0.005% bromphenol blue) and the proteins were separated by electrophoresis in 7% polyacrylamide.

Immunoblotting and ECL Detection. UM-A9 immunoprecipitates were separated by SDS-PAGE and were transferred to nitrocellulose filters in Western blot buffer (20 mM Tris, 150 mM glycine, and 15% methanol; Ref. 12). The filter strips were blocked for 1 h at room temperature in 3% nonfat dried milk dissolved in PBS with 0.1% Tween (PBS-T) and 0.1% antirat IgG (Sigma), washed, and then incubated for 1 h with the rat anti-β4 antibodies 439-9B or 450-11A diluted 1:1000 in PBS-T. After PBS-T washes, the strips were incubated for 1 h with biotinylated goat antirat antibody (1:1000) (Vector Labs, Burlingame, CA), washed, incubated with avidin-biotin horseradish peroxidase complex (Vectastain ABC-HRP reagent; Vector Labs) for 30 min, washed in PBS-T, and immersed in enhanced chemiluminescence (ECL) detection reagent (Amersham Corp., Arlington Heights, IL) for 1 min; chemiluminescence was detected on Kodak X-OMAT AR film.

Results

Selection of Clones Expressing Integrin β4 Subunit. Clones were selected for G418 resistance and tested for β4 surface expression using IF on unfixed cells. Forty-two G418-resistant clones were obtained from the pCMV-β4 transfection. Of these, 12 were positive for β4 by IF. Ten clones were randomly selected from the numerous (>150) pRc-CMV-transfected cells; all were negative for β4 IF. The pCMV-β4-transfected clones demonstrated a range of β4 intensity, as illustrated in Fig. 1. Examples of the IF results are shown for one control pRc-CMV-transfected clone, pCMVc1 (clone 1P; Fig. 1*a*), and three of the β4-positive clones, pCMVβ4c1 (clone 1; Fig. 1*b*), pCMVβ4c8 (clone 8; Fig. 1, *c* and *d*), pCMVβ4c11 (clone 11; Fig. 1, *e* and *f*). Most of the clones were weakly positive like that shown in Fig. 1*b*. Two pCMV-β4 clones, clones 8 and 11, exhibited strong IF staining with UM-A9 antibody. However, within 2 weeks, all positive cells were lost from the clone 8 culture. Clone 11 retained strong

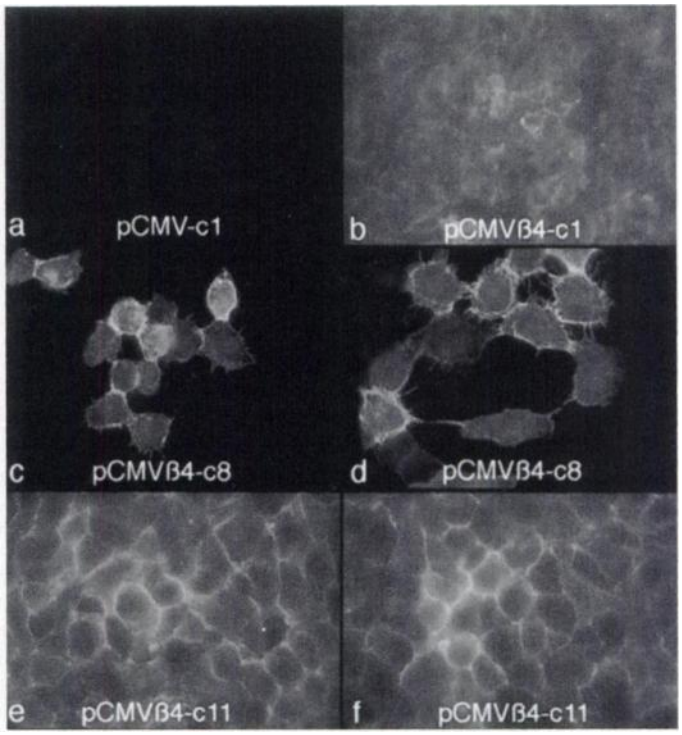

Fig. 1. IF photomicrographs showing expression of β4 integrin in transfected clones derived from the β4-negative UM-UC-2 bladder carcinoma cell line. *a*, pCMV-c1 plasmid control clone 1; *b-f*, β4-transfected clones (*pCMV-β4-c1*, *pCMV-β4-c8*, and *pCMV-β4-c11*).

expression of α6β4. A vial of clone 8 cells that had been previously frozen was thawed and retested. It was positive for β4 expression by IF, but in spite of selection of the 5% most positive cells by flow cytometry, again, within 2 weeks positive cells could no longer be detected.

Biochemical Analysis and Flow Cytometry. Immunoprecipitation with the UM-A9 anti-β4 antibody followed by Western blot analysis with the 439-9B anti-β4 antibody directed against the extracellular domain of β4 (18) confirmed loss of β4 expression in clone 8 and revealed a change in the structure of the β4 protein in clone 11. In the first experiment (Fig. 2, *left panel*), both clones 8 and 11 expressed the β4 complex, with the expected β4 bands at M_r 205,000 (intact β4), M_r 185,000, and M_r 155,000 (proteolytic breakdown products; Refs. 12, 13, and 19). In clone 11, there was an additional prominent band of M_r 175,000. When this experiment was repeated 2 weeks later on the progeny of the same clones, the intact β4 protein could no longer be detected in either clone 8 or 11. In clone 11, the prominent band of M_r 175,000 and the M_r 155,000 bands were still detectable. In clone 8, only a very faint M_r 155,000 band could be observed. This suggested that the β4 gene had been truncated in clone 11, resulting in a smaller gene product. To further assess this possibility, cells were labeled with [^{35}S]methionine, immunoprecipitated with UM-A9, and analyzed by autoradiography (Fig. 3). UM-SCC-22B cells were used as a positive control. In clone 8 (Fig. 3, *left panel, Lane C8*), the α6β4 complex was undetectable, indicating that the cells in this clone were no longer expressing the transfected β4 protein. Immunoprecipitation with the BQ-16 anti-α6 antibody confirmed this. An α6 doublet of M_r 125,000 (processed heavy chain) and M_r 150,000 (α6 precursor; Refs. 12 and 13) was observed in clone 8 immunoprecipitates (Fig. 3, *right panel, Lane C8*). These α6 bands are also present in the parent UM-UC-2 (Fig. 3, *Lanes UC2*) and control plasmid transfected cells (Fig. 3, *Lanes C1P*). UM-A9 immunoprecipitates of clone 11 cells (Fig. 3, *left panel, Lane C11*) exhibited

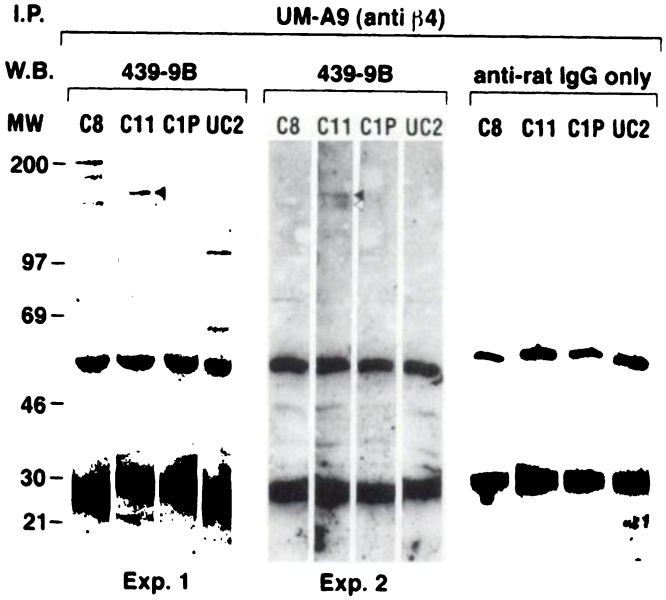

Fig. 2. Western blot of β4 integrin complexes immunoprecipitated from cell extracts of transfected clones of UM-UC-2. Cell extracts (*C1P*, pCMV-c1), a plasmid only control clone; *C8* (pCMV-β4-c8) and *C11* (pCMV-β4-c11), β4-transfected clones; *UC2* (UM-UC-2) parental nontransfected cells) were immunoprecipitated with the UM-A9 mouse monoclonal antibody to the extracellular domain of β4 and protein A-Sepharose beads as described in "Materials and Methods." The precipitated proteins were separated by SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes, and stained with the 439-9B rat antibody to the extracellular domain of the β4 protein. Two experiments are shown (*Exp. 1* and *Exp. 2*). In Exp. 1, the β4-transfected clones C8 and C11 each expressed the intact M_r 205,000 β4 protein and the proteolytic breakdown products of M_r 180,000 and M_r 150,000, but 2 weeks later (Exp. 2), both had lost the intact form. C11 also expressed a M_r 175,000 (*solid arrowhead*) and a M_r 155,000 (*open arrowhead*) doublet of β4-positive bands that were also present in Exp. 2. The lanes labeled antirat IgG are from Exp. 1 and are identical to the control lanes from Exp. 2. The dark bands of M_r 50,000 and M_r 28,000 in the control lanes and the experimental lanes are from cross-reactivity of the anti-rat IgG reagent with the heavy and light chains of the UM-A9 antibody used to precipitate β4.

the truncated M_r 175,000 and the M_r 155,000 β4 protein bands observed previously in the Western blots. In addition, the M_r 125,000 processed α6 band was coprecipitated, indicating that α6β4 heterodimers were being formed in clone 11. Immunoprecipitation of the clone 11 extract with anti-α6 antibody confirmed this finding (Fig. 3, *right panel, Lane C11*). The truncated M_r 175,000 and M_r 155,000 β4 bands were coprecipitated with α6. A M_r 200,000 band was also present in this immunoprecipitate and in that from the parental UM-UC-2 cells (Fig. 3, *right panel, Lane UC2*); however, the nature of this band is not known since it was not precipitated or Western blotted by the anti-β4 antibodies.

To further examine the nature of the truncated protein in clone 11, β4 immunoprecipitates were Western blotted with either the 439-9B anti-β4 antibody to the NH_2-terminal extracellular domain of β4 or the 450-11A anti-β4 antibody to the COOH-terminal cytoplasmic domain of the β4 protein (18). UM-SCC-22B and UM-SCC-38 cells were used as positive controls. As shown in Fig. 4*A*, 439-9B stained the M_r 175,000 and the M_r 155,000 β4 bands in clone 11 extracts (Fig. 4*A*, *left panel, Lane C11*), as well as the M_r 205,000, M_r 185,000, M_r 175,000, and M_r 155,000 bands in the UM-SCC-22B extracts (Fig. 4A, *left panel, Lane 22B*). In contrast, the 450-11A antibody to the COOH-terminal domain failed to stain the M_r 175,000 band in β4 immunoprecipitates of clone 11 in either of two experiments (Fig. 4*A*, *center panel, Lane C11*, and *right panel, Lane C11*). Note that this antibody does stain the prominent M_r 205,000 band corresponding to the intact β4 protein in both UM-SCC-22B and UM-SCC-38 extracts. There was a slight overflow from the UM-SCC-22B lane during the loading of the gel, which resulted in a faint M_r 205,000 band at the top of the clone 11 lane. This was not present in a repeat experiment. Flow cytometry confirmed that clone 11 cells expressing the truncated β4 subunit do express β4 on the cell surface (Fig. 4*B*).

Discussion

In normal resting epithelia, the α6β4 integrin is found within the hemidesmosome, an organelle involved in anchoring the epithelium to the basement membrane (4). Many epithelial tumor cells fail to form hemidesmosome-like structures, suggesting that there may be loss of function associated with aberrant β4 expression. The function of the hemidesmosome can probably be disrupted by a variety of mechanisms, including loss of expression of the β4 subunit as in the UM-UC-2 cell line. When UM-UC-2 cells were transfected, abundant plasmid control clones were obtained, but very few β4-expressing clones were recovered. Of the clones that expressed β4, most ceased to express the integrin subunit within 2–3 weeks in culture. These clones retained the plasmid because they remained resistant to G418. These observations suggest that β4 gene expression suppresses growth and that those cells that lose the ability to express the intact β4 subunit have a growth advantage. This is consistent with the observations by Clarke *et al.* (9), who found that transfection of β4 into the β4-negative RKO colon carcinoma cells induced the expression of p21 and concomitant apoptosis. In contrast, clone 11 continued to express β4 on the surface, which seems to contradict the growth-inhibitory hypothesis. However, immunoprecipitation analysis of the β4 protein demonstrated that the clone 11 cells were producing a truncated protein of M_r 175,000. Western blot analysis of β4 immunoprecipitates with antibodies specific for the extracellular and distal cytoplasmic domains demonstrated that the distal cytoplasmic domain of β4 was lost. This spontaneous truncation is reminiscent of

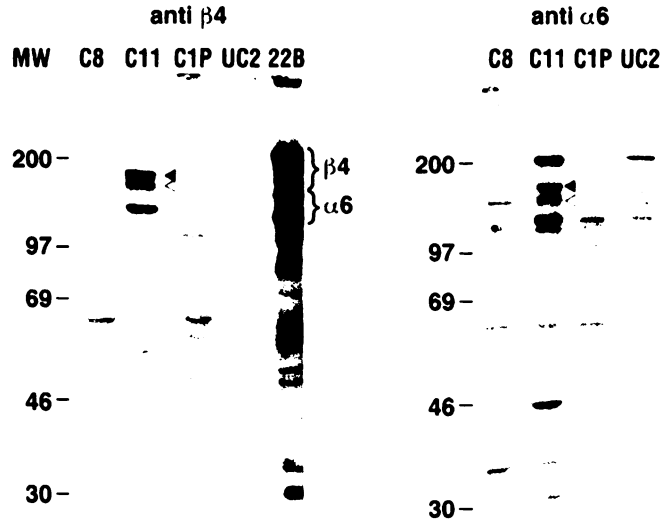

Fig. 3. Immunoprecipitation and SDS-PAGE analysis of α6β4 proteins from clones 8 and 11. Cells from clone 8 (*C8*), clone 11 (*C11*), plasmid control clone 1 (*C1P*), UM-UC-2, and UM-SCC-22B (β4-positive control) were labeled metabolically with [^{35}S]methionine, lysed, immunoprecipitated with the UM-A9 anti-β4 antibody (*left panel*) or the BQ16 anti-α6 antibody (*right panel*), resolved by gel electrophoresis under reducing conditions, and developed by autoradiography. The intact α6β4 integrin complex is indicated by *braces* next to the UM-SCC-22B lane. The M_r 175,000 truncated β4 protein expressed by clone 11 is indicated by an *arrowhead* in the *second lane* of the *left panel*. The M_r 155,000 protein of the doublet observed in Fig. 2, as well as a band corresponding to the α6 heavy chain at M_r 125,000, are also present. Similarly, in the C11 lane precipitated by the α6 antibody (*right panel*), the M_r 175,000 and M_r 155,000 β4 complex are present. These experiments indicate that the truncated β4 protein assembles into the α6β4 complex. The dark band at M_r 200,000 in the C11 lane precipitated by the α6 antibody is most likely a fibronectin band that is sometimes coprecipitated by anti-α6 antibodies and is also present in the UM-UC-2 precipitates.

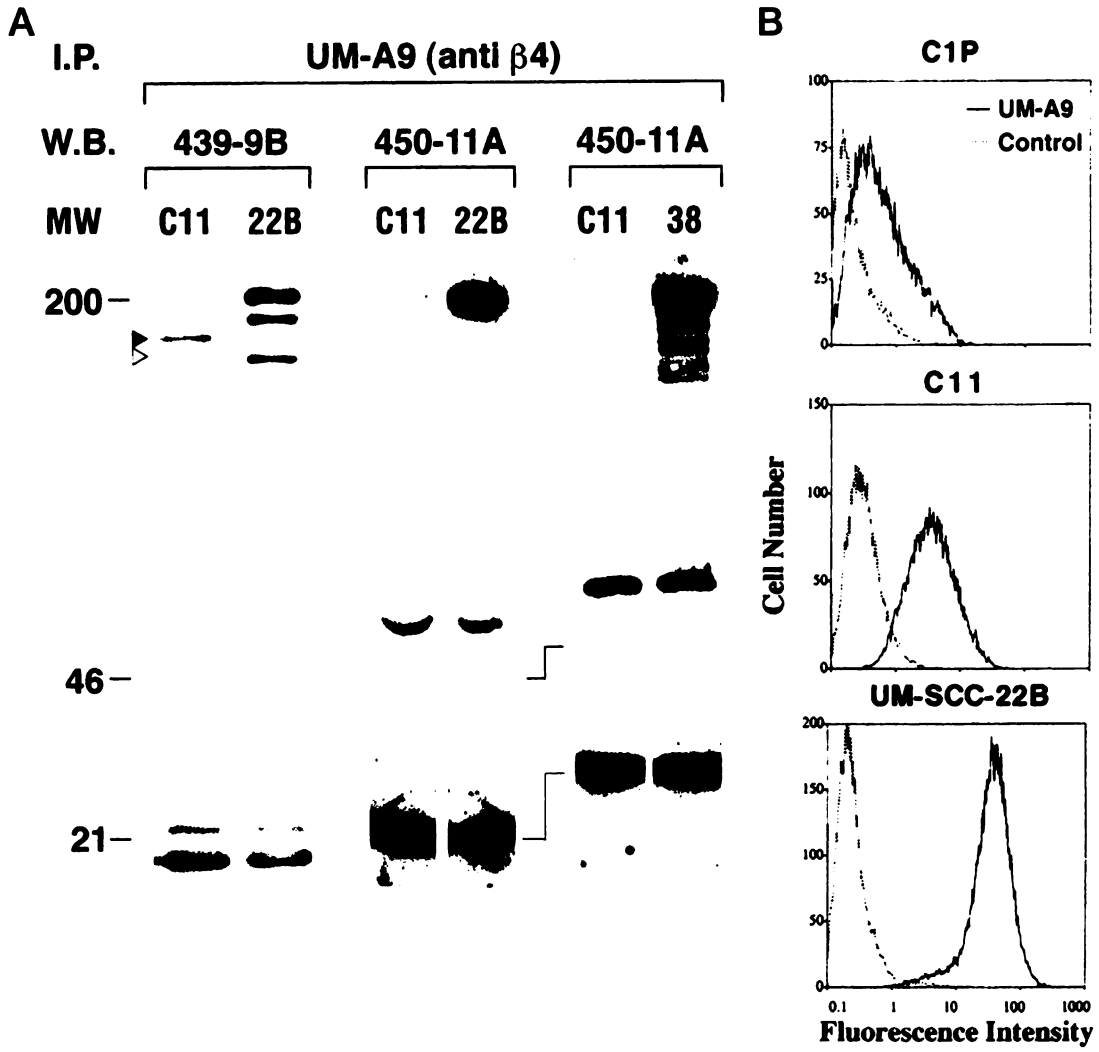

Fig. 4. Western blot and flow cytometric analysis of β4 integrin expression in clone 11 cells. *A*, Western blot of the β4 integrin immunoprecipitated from clone 11 and from positive control cell lines UM-SCC-22B and UM-SCC-38. Immunoprecipitated proteins were identified with the 439-9B antibody to the extracellular domain of β4 (*left panel*) or the 450-11A antibody to the distal cytoplasmic domain of β4 (*center* and *right lanes*). Both antibodies identify the β4 proteins identified in the control cell lines, but in clone 11, only the antibody to the extracellular domain can bind to the β4 protein of clone 11, indicating that the truncated β4 protein in these cells has lost the distal cytoplasmic domain. *B*, flow cytometric analysis of surface β4 expression in the vector-only transfected clone C1P, in the β4 transfected C11 clone, and in the positive control cell line UM-SCC-22B cell line. The results show that the truncated form of β4 expressed in clone 11 is present on the cell surface.

mutant constructs of β4 that have the ability to disrupt α6β4 polarization in rat bladder carcinoma cells (6–8). Similarly, Shaw *et al.* (21) showed that a cytoplasmic deletion mutant of β4 could act as an inhibitor of α6β1 function in breast carcinoma cells by competing for the α6 subunit and blocking α6β1-mediated attachment and migration. Taken together, these results indicate that the β4 cytoplasmic domain is required for integrin function.

We postulate that disrupted function of the hemidesmosome is a common characteristic of tumors. It appears that hemidesmosome function may be inhibited by loss of the β4 subunit as in the RKO colon carcinoma cells and UM-UC-2 bladder carcinoma cells. In these cells, when the β4 protein is restored by transfection, programmed cell death and/or growth inhibition ensues. However, in the hemidesmosome-forming 804G rat bladder carcinoma cell line, the α6 and β4 subunits are intact, and addition of β4 to these cells has no growth-inhibitory effects (6–8), suggesting that some other component of the anchoring signaling pathway has been compromised. The nature of these aberrations remains to be characterized. However, we recently found a poorly differentiated carcinoma of the parotid gland that expresses α6β4 but fails to express the BPAG2 or bullous pemphigoid-180 protein.[6] Just as with the E-cadherin/catenin interaction, a variety of disruptions in the α6β4 pathway may result in subversion of normal regulatory function.

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