Identification of a Novel Structural Variant of the α6 Integrin

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Abstract

The α6 integrin is a 140-kDa (nonreduced) laminin receptor. We have identified a novel 70-kDa (nonreduced) form of the α6 integrin called α6p for the latin word parvus, meaning small. The variant was immunoprecipitated from human cells using four different α6-specific monoclonal antibodies but not with α3 or α5 antibodies. The α6p integrin contained identical amino acid sequences within exons 13–25, corresponding to the extracellular “stalk region” and the cytoplasmic tail of the α6 integrin. The light chains of α6 and α6p were identical as judged by α6A-specific antibodies and electrophoretic properties. The α6p variant paired with either β1 or β4 subunits and was retained on the cell surface three times longer than α6. Reverse transcription/polymerase chain reaction analysis revealed a single polymerase chain reaction product. The α6p variant was found in human prostate (DU145H, LnCaP, PC3) and colon (SW480) cancer cell lines but not in normal prostate (PrEC), breast cancer (MCF-7), or lung cancer (H69) cell lines or a variant of a prostate carcinoma cell line (PC3-N). Protein levels of α6p increased 3-fold during calcium-induced terminal differentiation in a normal mouse keratinocyte model system. A novel form of the α6 integrin exists on cell surfaces that contains a dramatically altered extracellular domain.

Integrins are cell surface receptors that are involved in cell-matrix adhesion and signaling (recently reviewed in Ref. 1). The α6 integrin is a laminin receptor and contains 1050 amino acids present as a heavy (110 kDa) and a light (30 kDa) chain that are linked by a disulfide bond (2). The heavy chain of α6 integrin contains an 875-amino acid extracellular region and interacts with the β subunit to form the heterodimer (3). All the described integrin α subunits contain seven weak sequence repeats in the N-terminal region that are thought to be important in ligand binding and have been predicted to fold cooperatively into a single β-propeller domain with seven β-sheets (4,5). The minimum essential elements of the extracellular domains for subunit pairing and ligand binding are of considerable interest in understanding integrin regulation (6). Therefore, we have extended our previous studies, which indicated that a smaller variant of the α6 integrin exists (7,8).

Two alternatively spliced forms of α6 exist, containing identical heavy chains and different light chains known as α6A and α6B (3,9). The light chain of α6 integrin contains 170 amino

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acids composing an extracellular region, the transmembrane region, and the cytoplasmic domain (3). The α6A or α6B integrin subunit can pair with either the β1 or the β4 subunit (10) and is found on a variety of normal cell types. It is found on platelets (11), epithelia (12–15), endothelia (10,16,17), proximal and distal tubules of the kidney (18,19), astrocytes (20), Schwann and perineural cells (17,21), and lymphoid follicles (22). Large alterations of the α6 integrin heavy chain have not been reported.

Various disease states involving epithelial cells have been associated with alterations in α6 integrin-containing heterodimers. Mice lacking the α6 integrin completely will develop to birth but die shortly thereafter because of severe blistering of the skin and other epithelia (23). Alterations in the α6 integrin and/or a deficiency of its pairing subunit, β4 integrin, are associated with pyloric atresia-junctional epidermolysis bullosa, a human blistering disease of the epithelia (24–29).

Our work investigating a human epithelial cancer indicated a deficiency of the α6β4 heterodimer pairing during prostate tumor progression (30,31) and a persistent expression of the α6β1 integrin (32). Other groups also have observed the persistent nonpolarized expression of the α6 integrin during human tumor progression in cancers arising within the breast (33), kidney (34), endometrium (35), and pancreas (36,37), in addition to micrometastases from solid epithelial tumors (38).

Isolating the α6 integrin from human prostate cancer cells using α6-specific monoclonal antibodies retrieved not only the expected β1 and β4 subunits but also a predominant protein with an apparent molecular mass of 70 kDa (7,8). In this study we show that the protein is a novel and smaller form of the α6 integrin that is capable of pairing with either the β1 or β4 integrin subunit, referred to as α6p for the latin word parvus, meaning small.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**

All human cell lines were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Cell lines DU145H, HaCaT, and PC3-N were grown in Iscove’s modified Dulbecco’s medium (Life Technologies, Inc.) plus 10% fetal bovine serum. Cell lines MCF-7, PC3-ATCC, LnCap, and H69 were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) plus 10% fetal bovine serum. SW480 cells were grown in super medium (Dulbecco’s modified Eagle’s medium plus 5% nonessential amino acids, 5% L-glutamine, 5% sodium pyruvate, 10% fetal bovine serum). Normal prostate cells, PrEC, were grown in PrEGM bullet kit medium (Clonetics, San Diego, CA). The following cell lines were obtained from the American Type Culture Collection (Manassas, VA): MCF-7 (human breast tumor), PC3 (human prostate tumor), LnCap (prostate carcinoma cell line), H69 (human lung carcinoma), and SW480 (human colon carcinoma). The DU145H cells were isolated by us as described previously (8) and contain only the α6A splice variant (30). The PC3-N cells are a variant of PC3 prostate carcinoma cell line (39). The HaCaT cells (normal immortalized keratinocyte cell line) (40) were obtained from Dr. Norbert E. Fusenig (German Cancer Research Center, University of Heidelberg, Heidelberg, Germany). PrEC (a normal prostate cell line) was obtained from Clonetics. The calcium-induced terminal differentiation assay, cell culture techniques, and preparation of calcium medium used for mouse 291, 03C, and 03R cells have been described previously (41,42). Cells were maintained in 0.04 mM calcium (low calcium) and switched to medium with 0.14 mM calcium (medium calcium) or 1.4 mM calcium (high calcium) by 60% confluency. After 24 h treatment, cells were collected in phosphate-buffered saline, centrifuged, frozen in a dry ice bath, and kept at −80 °C in a freezer until used.
Antibodies Used in This Study

Anti-α6 integrin antibodies include and were obtained as follows. GoH3, a rat IgG2a, was from Accurate Chemicals (Westbury, NY) (43); J1B5, a rat monoclonal antibody, was a generous gift from Dr. Caroline Damsky, (University of California, San Francisco, CA) (44); 4F10, a mouse IgG2b, was from Chemicon (Temecula, CA) (43); BQ16, a mouse IgG1 that recognizes an external epitope of the α6 integrin, was a generous gift from Dr. Monica Leibert (Department of Urology, University of Texas, M.D. Anderson Cancer Center, Houston, TX) (45); 4E9G8, a mouse IgG1 that is specific for the unphosphorylated α6A cytoplasmic tail, was from Immunotech (Marseille, France) (11,46); AA6A, a rabbit polyclonal antibody that was raised and purified by Bethyl Laboratories Inc. (Montgomery, TX) specific for the last 16 amino acids (CIHAQPSDKERLTSDA) of the human α6A sequence (2) as done previously (47), and A33, a rabbit polyclonal antibody that was raised against amino acids 1–500 of the α6 integrin (48), were generous gifts from Dr. Arnoud Sonnenberg (The Netherlands Cancer Institute). Anti-β4 integrin antibodies were obtained as follows. 3E1, a mouse ascites IgG1, was from Life Technologies, Inc. (49); 439.9b, a rat IgG2bK, was from Pharmingen (San Diego, CA) (50); ASC-3, a mouse IgG1K, was from Chemicon (Temecula, CA) (51); and A9, a mouse IgG2a, was from Ancell (Bayport, MN) (52). Other anti-integrin antibodies used include anti-integrin antibody P1D6, a α5 mouse IgG3 (Life Technologies, Inc.) (53), and anti-β1 integrin P4C10, a mouse ascites IgG1 (Life Technologies, Inc.) (54).

Surface Biotinylation of Cell Lines

Previous protocols (55,56) were slightly modified. Briefly, cells were grown to confluency in 100-mm tissue culture dishes and washed three times with HEPES buffer (20 mM HEPES, 130 mM NaCl, 5 mM KCl, 0.8 mM MgCl2, 1.0 mM CaCl2, pH 7.45). The cells were then incubated with 2 ml of HEPES buffer supplemented with sulfosuccinimidyl hexanoate-conjugated biotin (500 μg/ml; Pierce), which is impermeant to cell membranes (57), to label cell surface proteins for 30 min at 4 °C. The cells were washed three times and lysed in cold radioimmune precipitation buffer plus protease inhibitors (phenylmethylsulfonyl fluoride, 2 mM; leupeptin and aprotinin, 1 μg/ml). The lysate was briefly sonicated on ice before centrifugation at 10,000 rpm for 10 min, and the supernatant was collected for immunoprecipitations.

Immunoprecipitations

For immunoprecipitations, 200 μg of total protein lysate was used for each reaction and incubated with 35 μl of protein G-Sepharose and 1 μg of antibody. The final volume of the lysate was adjusted to 500 μl with radioimmune precipitation buffer (150 mM NaCl, 50 mM Tris, 5 mM EDTA, 1% (v/v) Triton X-100, 1% (w/v) deoxycholate, 0.1% (w/v) SDS, pH 7.5). The mixture was rotated for 18 h at 4 °C. After incubation, complexes were washed three times with cold radioimmune precipitation buffer and eluted in 2× nonreducing sample buffer. Samples were boiled for 5 min prior to loading onto a 7.5% polyacrylamide gel for analysis. The proteins resolved in the gel were electrotransferred to Millipore (Bedford, MA) Immobilon-P polyvinylidene fluoride membrane, incubated with either peroxidase-conjugated streptavidin or Western blotting antibodies plus secondary antibody conjugated to horseradish peroxidase, and visualized by chemiluminescence (ECL Western blotting detection system; Amersham Pharmacia Biotech).

Two-dimensional Nonreduced/Reduced Gel Electrophoreses

Nonreduced/reduced two-dimensional electrophoresis was done as described previously (58). The samples were incubated in 0.625 M Tris-HCl, pH 6.8, 10% glycerol, 10% SDS, and applied to SDS-polyacrylamide gel electrophoresis (7.5% acrylamide) without reduction. The excised lanes were incubated in reducing sample buffer for 15 min and horizontally loaded at the top.
of a second dimension slab gel (also 7.5% acrylamide). The proteins were electrotransferred
to polyvinylidene fluoride membrane (Millipore), incubated with either peroxidase-conjugated
streptavidin or Western blotting primary antibodies followed by secondary antibody
coujugated to horseradish peroxidase, and visualized by chemiluminescence (ECL Western
blotting detection system; Amersham Pharmacia Biotech).

**Amino Acid Sequencing by Matrix-assisted Laser Desorption Ionization Mass Spectrometry**
and Liquid Chromatography-Tandem Mass Spectrometry

Amino acid sequencing of $\alpha_6\beta_4$ was performed using two different analytical core services. For
analytical core service at Deutsches Krebsforschungszentrum (Heidelberg, Germany), the
$\alpha_6\beta_4$ protein was immunoprecipitated using J1B5, and the proteins were separated by SDS-
polyacrylamide gel electrophoresis (7.5%, 3 mm). After staining with Coomasie Blue, the
$\alpha_6\beta_4$ bands were excised, cut into small pieces (1 × 1 mm), washed, dehydrated (twice for 30
min with $H_2O$, twice for 15 min with 50% acetonitrile, and once for 15 min with acetonitrile),
and incubated with 0.5 $\mu$g of trypsin in 20 $\mu$l of digest buffer (40 mM NH$_4$HCO$_3$, pH 8.0) at
37 °C for 16 h. The supernatant was subsequently analyzed by MALDI$^1$ mass spectrometry
(Deutsches Krebsforschungszentrum) using thin film preparation technique. Aliquots of 0.3
$\mu$l of a nitrocellulose containing saturated solution of $\alpha$-cyano-4-hydroxycinnamic acid in
acetone were deposited onto individual spots on the target. Subsequently, 0.8 $\mu$l of 10% formic
acid and 0.4 $\mu$l of the digest sample were loaded on top of the thin film spots and allowed to
dry slowly at ambient temperature. To remove salts from the digestion buffer, the spots were
washed with 5% formic acid and with $H_2O$. Sequence analysis was performed on a Procise
494 protein sequencer using a standard program supplied by Applied Biosystems. The FastA
data base searching program of Pearson and Lipman (59) was used for data base searching.

For sequence analysis at the Proteomics Core of the Arizona Cancer Center and Southwest
Environmental Health Sciences Center of the University of Arizona, the $\alpha_6\beta_4$ protein was
immunoprecipitated using J1B5, and proteins were separated by SDS-polyacrylamide gel
electrophoresis (7.5%, 3 mm). After staining with Coomassie Blue, the $\alpha_6\beta_4$ bands were excised,
cut into small pieces (1 × 1 mm), and subjected to in gel digestion using trypsin as described
previously (60). The extracted peptides following digestion were analyzed by liquid
chromatography-tandem mass spectrometry using a quadrupole ion trap Finnigan LCQ classic
mass spectrometer equipped with a quartenary pump P4000 HPLC and a Finnigan electrospray
ionization source (ThermoFinnigan, San Jose, CA). The peptides were eluted from a reverse-
phase C18 micro-column (Vydac 250 × 1 mm, Hesperia, CA) with a gradient of 3–95%
acetonitrile in 0.5% formic acid and 0.01% trifluoroacetic acid over 150 min at a flow rate of
15 $\mu$l/min. Tandem mass spectrometry spectra of the peptides were analyzed with the
SEQUEST program (Turbo Sequest) to assign peptide sequence to the spectra (61). SEQUEST
analyses were performed against the nonredundant data base.

**RT-PCR Analysis**

Total cellular RNA was isolated by guanidium isothiocyanate cell lysis and cesium chloride
purification (62). RNA was quantitated from spectrophotometric absorbance measurements at
260 nm. First strand cDNA was synthesized in a 30-$\mu$l reaction comprised of 1 × PCR buffer
(10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl$_2$); 1 mM each dATP, dCTP, dGTP, and
dTTP; 100 pmol random hexamer, 20 units RNAsin; 200 units SuperScript reverse
transcriptase II (Life Technologies, Inc.), and 3 $\mu$g of total cellular RNA incubated at 42 °C
for 60 min. The reaction was terminated by incubation at 99 °C for 10 min. Integrin $\alpha_6$-specific
PCR was performed by adding 80 $\mu$l of amplification reaction buffer (1× PCR buffer, 25 pmol

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$^1$The abbreviations used are: MALDI, matrix-assisted laser desorption ionization; HPLC, high pressure liquid chromatography; RT,
reverse transcription; PCR, polymerase chain reaction; bp, base pair(s).

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of integrin α₆-specific primers, and 2.5 units of Taq DNA polymerase) to the cDNA reaction, followed by incubation at 94 °C for 5 min and then 40 cycles of 94 °C for 1 min, 60 °C for 3 min, and 72 °C for 10 min, with a final extension at 72 °C for 5 min and a quick chill to 4 °C. The PCR primers were derived from the integrin α₆ cDNA sequence reported by Tamura et al. (GenBank™ accession number X53586); the upstream primer sequence was from nucleotides 160 to 179, and the downstream primer was from nucleotides 3404 to 3423. The PCR product identity was confirmed by diagnostic restriction enzyme digests and size separation of the products through a 1X TBE, 1.5% agarose gel. The products were visualized by ethidium bromide staining and UV fluorescence.

RESULTS

DU145H Cells Contained a Smaller Form of the α₆ Integrin

Our previous studies showed that anti-α₆ antibody GoH3 was able to immunoprecipitate a surface-biotinylated 70-kDa (non-reduced) protein from DU145H cells in addition to the expected 185-, 140-, and 120-kDa (nonreduced) proteins corresponding to the β₄, α₆, and β₁ integrins, respectively (7,8). In DU145H cells, which only contain the α₆ integrin (30), this 70-kDa variant was the predominant form of the α₆ integrin found on the cell surface.

Five different anti-α₆ antibodies immunoprecipitated α₆ and its smaller variant, α₆p, from surface-biotinylated DU145H cells (Fig. 1). Four of the antibodies used were specific for extracellular epitopes of the full-length α₆A integrin (GoH3, J1B5, 4F10, and BQ16), and one was specific for the cytoplasmic tail of the α₆A light chain (AA6A). The integrin α₆p was not found to co-immunoprecipitate upon incubation with an anti-α₃ antibody, P1B5 (7), or an anti-α₅ antibody, P1D6.

The α₆p Variant Contained a Light Chain That Was Identical to That Found in α₆ Integrin

The full-length α₆ integrin consists of two disulfide linked chains: a heavy chain (110 kDa) and a cytoplasmic light chain (30 kDa) that are observed upon reduction of the protein samples and analysis by SDS-polyacrylamide gel electrophoresis. Our data (Fig. 1) indicated that an anti-α₆ integrin antibody specific for the cytoplasmic tail of α₆A recognized α₆p and suggested that the light chain from the α₆p variant might be similar to that in the full-length α₆ integrin. To answer this question, we surface-biotinylated DU145H cells and then immunoprecipitated the sample using the anti-α₆ integrin antibody, GoH3. The resulting sample was then analyzed using two-dimensional nonreducing/reducing gel electrophoresis (Fig. 2). The sample was electrophoresed under nonreducing conditions in the first dimension and then under reducing conditions for the second dimension. The 160-kDa band (nonreduced) corresponding to the full-length α₆ integrin, contained a heavy (110 kDa) and light (30 kDa) chain, as described previously, under the reducing conditions of the second dimension (3). The reduced β₁ integrin was identified at 120 kDa. The α₆p integrin split into a heavy fragment (43 kDa) and a light chain (30 kDa). These results indicated that the α₆p integrin contained the same light chain as the full-length α₆A integrin but that the heavy chains were significantly different.

The α₆p Variant Associated with β₁ and β₄ Integrins

The α₆ integrin is known to associate with either the β₁ or β₄ subunit (10). Next we determined whether α₆p would co-immunoprecipitate with the β₄ integrin (Fig. 3A). Human HaCaT cells were chosen for this experiment because of their abundance of β₄ integrin (7). They were surface-biotinylated and subjected to immunoprecipitation with different anti-β₄ integrin antibodies. The α₆p variant co-immunoprecipitated upon incubation with four different anti-β₄ integrin antibodies: A9, 439.9b, ASC3, and 3E1. Of particular interest was the retrieval of
α6p with the anti-β4 integrin antibody, A9, whose epitope is present when α6 is coupled to β4 integrin (52).

Next we tested whether the novel 70-kDa (nonreduced) protein could be recovered by immunoprecipitation with the anti-β1 integrin monoclonal antibody, P4C10 (Fig. 3B). HaCaT cells were surface-biotinylated and immunoprecipitated with anti-α6 integrin antibody, J1B5, and used as a standard. Both DU145 and HaCaT cells were surface-biotinylated and subjected to immunoprecipitation using P4C10. Interestingly, the 70-kDa (nonreduced) α6p variant co-immunoprecipitated with the β1 integrin in DU145 cells but not in HaCaT cells. The results indicated that the novel α6p variant paired with either the β4 or β1 integrin subunits. Although the β1 integrin was readily present in HaCaT cells, the α6p integrin did not co-immunoprecipitate with the anti-β1 integrin antibody, P4C10. This may indicate that in some cell lines, there is preferential pairing of the α6p integrin subunit with β4.

The α6p Integrin Was Recognized by Light Chain-specific Anti-α6A Monoclonal Antibodies

Our data (Fig. 2) indicated that α6p contained a light chain identical to that contained in the full-length α6 integrin. Next we tested whether the novel 70-kDa (nonreduced) protein could be recognized by anti-α6 integrin antibodies via Western blotting. DU145H cells were biotinylated and immunoprecipitated with GoH3 for a standard to compare with a Western blot (Fig. 4). DU145H, HaCaT, and H69 cells were lysed and immunoprecipitated with either anti-α6 integrin antibodies GoH3 or J1B5 or anti-β1 integrin monoclonal antibody, P4C10. A 70-kDa band that co-migrated with the biotinylated standard was recognized in HaCaT and DU145H cells by Western blot analysis using two different anti-α6A antibodies, AA6A and 4E9G8, which recognize the cytoplasmic domain of the α6A integrin. Additionally the α6 integrin, but not the α6p variant, was detected by Western blot analysis using A33, which is specific for the N-terminal of the α6 integrin. A lung carcinoma cell line, H69, is a cell line that does not contain α6 integrin and was not found to express α6p.

The α6p Variant Was Present in Several Different Epithelial Cancer Cell Lines

We next determined the presence of the α6p variant in other tumor or normal cell lines. The presence of α6 and α6p was initially analyzed by using whole cell lysates (20 μg of total protein) followed by Western blot analysis (data not shown). The results were tabulated and confirmed by immunoprecipitation with anti-α6 antibody GoH3 followed by Western blot analysis using anti-α6A antibody, AA6A (Fig. 5). The α6p variant was present in several prostate cancer cell lines (DU145H, PC3, and LnCaP) and a colon cancer cell line (SW480). Additionally, α6p was present in a normal, immortalized keratinocyte cell line, HaCaT. The α6p variant was not found in several cell lines including normal prostate cells, PrEC; a variant of the prostate cell line PC3, called PC3-N (39); a breast carcinoma cell line, MCF-7; and a lung carcinoma cell line, H69. Interestingly, the α6p variant was only observed in cells that expressed the full-length α6 integrin. The α6p variant was not present in α6-negative cell lines. Two epithelial cell lines, one normal cell line (PrEC) and one cancer cell line (PC3-N), expressed the full-length α6 integrin but not the α6p variant.

The α6p Variant Contained Several Amino Acid Fragments Identical to the α6 Integrin

Although these data showed the presence of the α6A light chain in the protein, we next determined whether the α6 heavy chain was present utilizing a direct protein sequencing method. The α6p protein was immunoprecipitated with J1B5 and electrophoresed. The protein gel was stained with Coomassie Blue, and the 70-kDa protein was excised and digested with trypsin. Protein sequences were obtained using either MALDI mass spectrometry (Deutsches Krebsforschungszentrum) or liquid chromatography-tandem mass spectrometry (Proteomics Core of the Arizona Cancer Center and Southwest Environmental Health Sciences Center, University of Arizona) (Fig. 6). Ten noncontinuous amino acid fragments within the α6p variant

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were identified that corresponded exactly to predicted trypsin fragments located on exons 13–25 of the published α6 integrin sequence (2). The sequencing data confirmed that both the heavy and light chains of the α6p variant contained identical portions of the full-length α6 integrin (Fig. 7).

**The α6p Variant Half-life Was Three Times Longer than α6**

Detection of the α6p band that was smaller than the full-length α6 integrin prompted us to ask whether this novel variant was a degradation product of the α6 integrin that would be rapidly cleared from the surface. To answer this question, the surface half-life of both integrins was determined. Previously we determined that it was possible to detect the surface half-life of the integrin by biotinylation strategy (63). The surface proteins of DU145H cells were biotinylated for 1 h, washed, and placed back in the incubator with medium. After 24, 48, or 72 h, the integrins were immunoprecipitated using the GoH3 antibody and analyzed under nonreducing conditions (Fig. 8A). The data indicated that the α6p form remained on the surface of the DU145H cells with a half-life of ~72 h, or almost 3 times longer than that of the full-length α6 integrin (Fig. 8B). The abundance of α6p was not influenced by exogenous protease inhibitors (BB94, leupeptin, aprotinin, 30% fetal bovine serum, ecotin), exogenous proteases (kallikrein), or activators of integrin function (12-O-tetradecanoylphorbol-13-acetate, 20 mM CaCl2) (data not shown).

**RT-PCR Analysis of the α6 Coding Region Revealed a Single Product**

RT-PCR was used to determine whether splice variants of the integrin α6 mRNA were potentially responsible for the production of the smaller integrin protein. Three micrograms of total cellular RNA from DU145H cells was reverse-transcribed into first strand cDNA and then PCR amplified with primers that essentially bracketed the entire integrin α6 protein coding region (all but the first four codons were amplified using these primers). The results of this experiment are shown in Fig. 9. A single PCR product consistent with a full-length RT-PCR product of 3263 bp was detected; the splicing out of coding exons would have been detected by the presence of smaller products in the PCR reaction.

To confirm the identity of the integrin α6 PCR product, diagnostic restriction enzyme digests were performed. Analysis of the integrin α6 sequence (2) revealed the presence of one EcoNI site (producing fragments of ~960 and 2300 bp), four SmaI restriction sites (producing fragments of 105, 150, 350, and 2650 bp), four EcoRI sites (producing fragments of 30, 680, 730, and 1780 bp), and one XhoI site (producing fragments of 420 and 2840 bp). Aliquots of the integrin α6 PCR product were digested with each of these restriction enzymes, and the results of this experiment are shown in Fig. 9. Each restriction digest produced the restriction fragments expected from the integrin α6 PCR product (the 30-bp EcoRI fragment and the 105-bp SmaI fragment could not be visualized on the gel shown in Fig. 9). Based on these results, it appears unlikely that the α6p variant is the result of the splicing out of exons in the known coding region.

**Calcium-induced Normal Keratinocyte Differentiation Increased α6p Integrin Protein Levels**

Mouse 291 normal keratinocyte terminal differentiation can be induced by calcium. O3C and O3R cells were derived from normal 291 mouse cell strains and are immortalized, nontumorigenic, and tumorigenic, respectively (64). Both cell strains are resistant to calcium-induced terminal differentiation. The presence of α6 and α6p integrins in normal 291 mouse keratinocytes was determined using whole cell lysates followed by Western blot analysis using anti-α6 integrin antibody, AA6A (Fig. 10A). The results for 291 cells were confirmed by immunoprecipitation with anti-α6 integrin antibody, GoH3 (data not shown). The α6 and α6p integrin protein bands were quantitated using Scion Image (65) and graphed (Fig. 10B). Calcium-induced terminal differentiation increased α6p integrin protein levels 3-fold in a dose-
dependent manner in 291 nontransformed mouse keratinocytes. The differing steady-state levels of \( \alpha_6 \) in proliferating O3C and O3R tumor cells under the same culture conditions suggested that the \( \alpha_6 \) integrin variant was responsive to terminal differentiation and not to calcium itself. Interestingly, \( \alpha_6 \) integrin levels were decreased in poorly differentiated squamous cell carcinoma O3R cells relative to initiated cell O3C precursors and terminally differentiated 291 keratinocytes.

**DISCUSSION**

Our previous work has shown that the \( \alpha_6 \) integrin is associated with an increased invasive potential of human prostate cancer cells in vitro and the progression of human prostate carcinoma in human tissue biopsy material. We have found the \( \alpha_6 \) integrin exists in the classical form (140 kDa, nonreduced) and in a novel smaller form (70 kDa) referred to here as \( \alpha_6p \). The \( \alpha_6p \) is related to the full-length \( \alpha_6 \) because it was immunoprecipitated with anti-\( \alpha_6 \) integrin antibodies (GoH3, J1B5, AA6A, 4F10, and BQ16) (Fig. 1). Two-dimensional gel analysis revealed that the light chain of the \( \alpha_6p \) integrin was the same size as that found in the full-length \( \alpha_6 \) form (Fig. 2). The \( \alpha_6p \) variant co-immunoprecipitated with both anti-\( \beta_4 \) (3E1, A9, 439.9b, and ASC3) and anti-\( \beta_1 \) (P4C10) integrin antibodies (Fig. 3) and was recognized by two anti-\( \alpha_6 \) integrin antibodies specific for the cytoplasmic domain (AA6A and 4E9G8) by Western blot analysis but not by a polyclonal antibody that was specific for the N-terminal domain (A33) (Fig. 4). The \( \alpha_6p \) variant was found in several different human prostate (DU145H, LnCaP, and PC3) and colon (SW480) cancer cell lines (Fig. 5). It was not found in several cell lines including normal prostate cells (PrEC), a breast cancer cell line (MCF-7), a lung cancer cell line (H69), or a variant of a prostate carcinoma cell line (PC3-N). MALDI mass spectrometry indicated multiple amino acid regions in the \( \alpha_6p \) variant that corresponded exactly to sequences contained within exons 13–25 of the published full-length \( \alpha_6 \) sequence (2) (Figs. 6 and 7). Calcium-induced terminal differentiation of normal mouse 291 keratinocytes resulted in a 3-fold increase of \( \alpha_6p \) protein levels (Fig. 10). It remains to be determined whether a cause and effect relationship exists between \( \alpha_6p \) and differentiation or whether production of the variant simply reflects a dynamic modulation of cell surface adhesion. Integrin modulation is known to occur in the differentiation of human keratinocytes (66). Modulation of calcium levels in 291 cell derivatives O3C and O3R cells that are both resistant to calcium-induced differentiation did not result in alterations of \( \alpha_6p \) integrin levels. Together, these data suggest that \( \alpha_6p \) integrin was responsive to the dynamic surface modulation induced by terminal differentiation and that the observed alteration of \( \alpha_6p \) protein levels was not solely due to calcium.

The 10 noncontinuous amino acid fragments obtained from the \( \alpha_6p \) variant corresponded exactly to sequences contained within exons 13–25 of the published full-length \( \alpha_6 \) sequence (2) (Figs. 6 and 7). No peptide fragments corresponding to exons 1–12 were obtained using this method, suggesting that the \( \alpha_6p \) variant is composed of exons 13–25 of the full-length \( \alpha_6 \) integrin.

The predicted molecular mass of exons 13–25 is 55 kDa; yet the \( \alpha_6p \) protein band had an apparent molecular mass of 70 kDa by gel analysis. This apparent contradiction may be due to a post-translational modification of the protein. The full-length \( \alpha_6 \) integrin has a predicted molecular mass of 140 kDa (2); yet experimentally, the protein band had an apparent molecular mass of 160 kDa under nonreducing conditions. The variation between predicted and apparent molecular mass in both proteins is likely due to the nine glycosylation sites predicted on the \( \alpha_6 \) protein and the five that would remain in \( \alpha_6p \). Previously, differences in N-linked glycosylation of the \( \alpha_6 \) integrin, revealed by endoglycosidase H and N-glycanase treatments, has accounted for the variation in the apparent molecular mass of the \( \alpha_6 \) integrin from platelets and carcinoma lines (10).
Our data indicated that the novel α6p variant contained a significant alteration in the heavy chain, which is entirely extracellular. The current structural model of the α subunit proposes that the seven N-terminal repeats adopt the fold of a β-propeller domain (4,5). These domains contain seven four-stranded β-sheets and are arranged in a torus around a pseudo-symmetric axis. Structural homology studies of enzymes with known β-propeller folds have identified active sites at the top of the β-propeller, typically where adjacent loops run in opposite directions (67–69). Recent studies of the β-propeller domain in integrins have demonstrated that folds 1 and 3 in the α4 integrin subunit are important for ligand binding (70), whereas the α5 integrin ligand binding site is determined by amino acid sequences in repeats 2 and 3 of the N-terminal domain of the α subunit (71). Based on our mass spectrometry data, which concluded that the α6p variant contained only exons 13–25, the entire proposed β-propeller domain would be missing. Thus, it would be likely that the α6p integrin variant would function as an inactive receptor for cellular adhesion to the extracellular ligand. The production of this α subunit variant on the cell surface may be a mechanism for regulation of extracellular adhesion.

Additionally, because integrins are known to be conformationally dependent molecules with dynamic ligand interactions (72), alteration of the extracellular portion of the molecule could likely influence intracellular signaling (73). The integrin α subunit cytoplasmic domains have been shown to be important for a diverse number of functions including adhesion, motility, internalization, differentiation, and cytoskeletal organization (74–79). Recently, the role of the α6A cytoplasmic domain was examined in myoblasts and found to inhibit proliferation and promote differentiation. Interestingly, the cytoplasmic tail alone suppressed signaling through the focal adhesion kinase and mitogen-activated protein kinase pathways (80). A previous report indicated that post-translational processing of the α4β1 integrin can occur in leukocytes (81), but to our knowledge, this is the first description of a naturally occurring variation of this size in the extracellular domain of the α6 integrin subunit.

Interestingly, our data indicated that the altered extracellular region of the α6p variant did not affect its ability to remain paired with either β4 or β1 integrin subunits. The α6p variant was retrieved by immunoprecipitation using the anti-β4 integrin monoclonal antibody, A9, whose epitope is present when α6 is coupled to the β4 subunit (52). This finding suggests that the α6p subunit is able to heterodimerize with the β4 subunit in the same manner as the full-length α6 integrin. It remains to be determined whether the α6pβ4 integrin is functional with its cytoplasmic binding partners. It is also noteworthy that α6p co-immunoprecipitated with β1 integrin in DU145H cells but not in HaCaT cells, despite abundant levels of β1 integrin in the HaCaT cells (Fig. 3B). This finding may indicate a preferential pairing of α6p to β4 in some cell lines.

Previous studies suggested that integrins and TM4 tetraspan proteins could interact with one another to modulate integrin signaling and adhesion (82,83). Recently, it has been demonstrated that two members of this family, CD9 and CD81 can interact with the extracellular domain of the α6 integrin (84). It would be of interest to know whether the variant α6p retains the ability to bind to either of these tetraspan proteins.

In regard to the origin of the α6p variant, our data suggest several possibilities. Information obtained from cell surface retention half-life studies revealed that α6p (70 kDa) was almost three times more stable than that of the full-length α6 form (Fig. 8). From this data, we concluded the α6p protein was not a degradation product of the full-length α6 integrin because the protein was not preferentially cleared from the surface as might be expected for a protein targeted for degradation. The α6p protein was not generated after cell lysis, because multiple antiproteases and short immunoprecipitation times were unable to alter the presence of this variant. Although some integrins are highly susceptible to proteolytic processing, i.e. the β4
integrin (85), the fully processed $\alpha_6$ integrin has not yet been reported to be enzymatically cleaved by any enzymes in vivo. We were unable to induce proteolytic cleavage of the $\alpha_6$ integrin in vivo in our previous studies (85). Collectively, these findings argue against $\alpha_6\beta$ being a degradation product; however, they do not provide information as to whether $\alpha_6\beta$ was generated through a post-transcriptional processing event or alternative splicing of $\alpha_6$ message.

Our data does not suggest that $\alpha_6\beta$ originated from an alternative splicing event, because analysis by RT-PCR revealed that only one transcript for $\alpha_6$ was present within the known coding region (Fig. 9). Moreover, it has not previously been demonstrated in humans that alternative splicing plays a role in the regulation of the extracellular domain of integrins (6). Several integrins including $\alpha_6$ have been shown to have isoforms of the cytoplasmic domain generated by alternative splicing (2,9,47). Our data demonstrated a significant variation (a 70-kDa change) in the extracellular heavy chain of the $\alpha_6\beta$ integrin (Fig. 2). This large extracellular variation has not been described previously for other integrins.

Taken together, our data suggest that a post-transcriptional event is responsible for the generation of $\alpha_6\beta$. The $\alpha_6$ integrin subunit, in addition to other $\alpha$ subunits, normally undergoes endoproteolytic processing close to the C terminus after synthesis, resulting in the formation of a light and heavy chain (86). A previous report demonstrated that defective post-transcriptional processing of the pre-$\alpha_6$ transcript in carcinoma cells lead to loss of normal cleavage and a resulting larger 150-kDa single protein (87). Examples of normal post-transcriptional processing have been described in yeast via translational introns that can give rise to two different sized proteins from a single mRNA transcript (88). Alternatively, ribosomal scanning past the conventional initiation codon has been described for major histocompatibility class I molecules. In this process, the ribosome initiates translation further downstream (89). We note with interest that 12 alternative initiation codons are predicted within the $\alpha_6$ gene and one (position 1833) precedes exon 13. The mechanism for generating the $\alpha_6\beta$ heavy chain and the functional role of the variant in adhesion and signaling processes remain to be determined.

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References

1. Giancotti FG, Ruoslahti E. Science 1999;285:1028–1032. [PubMed: 10446041]
2. Tamura RN, Rozzo C, Starr L, Chambers J, Reichardt LF, Cooper HM, Quaranta V. J Cell Biol 1990;111:1593–1604. [PubMed: 1976638]
3. Hogervorst F, Kuikman I, van Kessel AG, Sonnenberg A. Eur J Biochem 1991;199:425–433. [PubMed: 2070796]
4. Oxvig C, Springer TA. Proc Natl Acad Sci U S A 1998;95:4870–4875. [PubMed: 9560195]
5. Springer TA. Proc Natl Acad Sci U S A 1997;94:65–72. [PubMed: 8990162]
6. Sonnenberg A. Curr Top Microbiol Immunol 1993;184:7–35. [PubMed: 8313723]
7. Witkowski CM, Rabinovitz I, Nagle RB, Affinito KS, Cress AE. J Cancer Res Clin Oncol 1993;119:637–644. [PubMed: 7688749]
8. Rabinovitz I, Nagle RB, Cress AE. Clin Exp Metastasis 1995;13:481–491. [PubMed: 7586806]
9. Tamura RN, Cooper HM, Collo G, Quaranta V. Proc Natl Acad Sci U S A 1991;88:10183–10187. [PubMed: 1946438]
10. Sonnenberg A, Linders CJ, Daams JH, Kennel SJ. J Cell Sci 1990;96:207–217. [PubMed: 1698797]
11. Sonnenberg A, Modderman PW, Hogervorst F. Nature 1988;336:487–489. [PubMed: 2973567]
12. Miettinen M, Castello R, Wayner E, Schwarting R. Am J Pathol 1993;142:1009–1018. [PubMed: 7682756]
13. Mechtersheimer G, Munk M, Barth T, Koretz K, Moller P. Virchows Arch A Pathol Anat Histopathol 1993;422:203–210. [PubMed: 8493776]
14. Kourkoulis GK, Virtanen I, Moll R, Quaranta V, Gould VE. Virchows Arch B Cell Pathol Incl Mol Pathol 1993;63:373–383. [PubMed: 7686700]
15. Peltonen J, Larjava H, Jaakkola S, Gralnick H, Akiyama SK, Yamada SS, Yamada KM, Uitto J. J Clin Invest 1989;84:1916–1923. [PubMed: 2556449]
16. Terpe HJ, Stark H, Ruiz P, Imhof BA. Histochemistry 1994;101:41–49. [PubMed: 8026982]
17. Hogervorst F, Adriaal GI, Niessen H, Daams H, Sonnenberg A. J Cell Biol 1993;121:179–191. [PubMed: 7681434]
18. Patey N, Halbwachs-Mecarelli L, Droz D, Lesavre P, Noel LH. Cell Adhes Commun 1994;2:159–167. [PubMed: 7521761]
19. Simon EE, McDonald JA. Am J Physiol 1990;259:F783–F792. [PubMed: 2173420]
20. Wagner S, Tagaya M, Koziol JA, Quaranta V, del Zoppo GJ. Stroke 1997;28:858–865. [PubMed: 9099208]
21. Andrew SM, Edwards BD, Chalmers RJ, O’Driscoll JB. Br J Dermatol 1992;127:359–364. [PubMed: 1419755]
22. Jaspars LH, De Melker AA, Bonnet P, Sonnenberg A, Meijer CJ. Cell Adhes Commun 1996;4:269–279. [PubMed: 9117346]
23. Georges-Labouesse E, Messaddeq N, Yehia G, Cadabal F, Lhermier D, Le Meur M. Nat Genet 1996;13:370–373. [PubMed: 8673141]
24. Brown TA, Gil SG, Sybert VP, Lestringant GT, Tadini G, Caputo R, Carter WG. J Invest Dermatol 1996;107:384–391. [PubMed: 8751975]
25. Shimizu H, Suzuki K, Hatta N, Nishikawa T. Arch Dermatol 1996;132:919–925. [PubMed: 8712842]
26. Vidal F, Aberdam D, Miquel C, Christiano AM, Pulkkenin L, Uitto J, Ortonne JP, Meneguzzi G. Nat Genet 1995;10:229–234. [PubMed: 7545057]
27. Pulkkinni L, Kurtz K, Xu Y, Bruckner-Tuderman L, Uitto J. Lab Invest 1997;76:823–833. [PubMed: 9194858]
28. Niessen CM, van der Raaij-Helmer MH, Hulsman EH, van der Neut R, Ronkonak C, Sonnenberg A. J Cell Sci 1996;109:1695–1706. [PubMed: 8832392]
29. Gil SG, Brown TA, Ryan MC, Carter WG. J Invest Dermatol 1994;103:31S–38S. [PubMed: 7963682]
30. Cress AE, Rabinovitz I, Zhu W, Nagle RB. Cancer Metastasis Rev 1995;14:219–228. [PubMed: 8548870]
31. Nagle RB, Hao J, Knox JD, Dalkin BL, Clark V, Cress AE. Am J Pathol 1995;146:1498–1507. [PubMed: 7778688]
32. Knox JD, Cress AE, Clark V, Manriquez L, Affinito KS, Dalkin BL, Nagle RB. Am J Pathol 1994;145:167–174. [PubMed: 8030747]
33. Friedenrich K, Ruiz P, Franke F, Gille I, Terpe HJ, Imhof BA. Cancer Res 1995;55:901–906. [PubMed: 7850807]
34. Droz D, Patey N, Paraf F, Chretien Y, Gougougey J. Lab Invest 1994;71:710–718. [PubMed: 7526040]
35. Lessey BA, Albelda S, Buck CA, Castellbaum AJ, Yeh I, Kohler M, Berchuck A. Am J Pathol 1995;146:717–726. [PubMed: 8877452]
36. Weinel RJ, Rosendahl A, Neumann K, Chaloupka B, Erb D, Rheumund M, Santos S. Int J Cancer 1992;52:827–833. [PubMed: 1330897]
37. Weinel RJ, Rosendahl A, Pinschmidt E, Kisker O, Simon B, Santos S. Gastroenterology 1995;108:523–532. [PubMed: 7835595]
38. Putz E, Witter K, Offner S, Stosiek P, Zippelius A, Johnson J, Zahn RR, Rietmuller G, Pantel K. Cancer Res 1999;59:241–248. [PubMed: 9892213]
39. Tran NL, Nagle RB, Cress AE, Heimark RL. Am J Pathol 1999;155:787–798. [PubMed: 10487836]
40. Breitkreutz D, Schoop VM, Mirancea N, Baur M, Stark HJ, Fussenig NE. Eur J Cell Biol 1998;75:273–286. [PubMed: 9587059]
41. Kulesz-Martin MF, Koehler B, Hennings H, Yuspa SH. Carcinogenesis 1980;1:995–1006. [PubMed: 11272116]
42. Hennings H, Holbrook K, Steinert P, Yuspa S. Curr Probl Dermatol 1980;10:3–25. [PubMed: 7238094]
43. Sonnenberg A, Janssen H, Hogervorst F, Calafat J, Hilgers J. J Biol Chem 1987;262:10376–10383. [PubMed: 3301835]
44. Damsky CH, Librach C, Lim KH, Fitzgerald ML, McMaster MT, Janatpour M, Zhou Y, Logan SK, Fisher SJ. Development 1994;120:3657–3666. [PubMed: 7529679]
45. Liebert M, Wtemporary geometries STEIN, GEORGE MB, GRAM H. Bailey 1993;12:67–80. [PubMed: 8453405]
46. Hemler ME, Crouse C, Takada Y, Sonnenberg A. J Biol Chem 1988;263:7660–7665. [PubMed: 2967289]
47. Cooper HM, Tamura RN, Quaranta V. J Cell Biol 1991;115:843–850. [PubMed: 1833411]
48. Sterk LM, Geuijing CA, Oomen LC, Calafat J, Janssen H, Sonnenberg A. J Cell Biol 2000;149:969–982. [PubMed: 10811835]
49. Hesse H, Sakai LY, Hollister DW, Burgeson RE, Engvall E. Differentiation 1984;26:49–54. [PubMed: 6370774]
50. Falcioni R, Sacchi A, Resau J, Kennel SJ. Cancer Res 1988;48:816–821. [PubMed: 2448027]
51. Pattaramalai S, Skubitz KM, Skubitz AP. Exp Cell Res 1996;222:281–290. [PubMed: 8598215]
52. Van Waes C, Kozarsky KF, Warren AB, Kidd L, Paugh D, Liebert M, Carey TE. Cancer Res 1991;51:2395–2402. [PubMed: 1750876]
53. Wayner EA, Carter WG, Piotrowicz RS, Kunicki TJ. J Cell Biol 1988;107:1881–1891. [PubMed: 2846588]
54. Carter W, Wayner E, Bouchard T, Kaur P. J Cell Biol 1990;110:1387–1404. [PubMed: 1691191]
55. Isberg RR, Leong JM. Cell 1990;60:861–871. [PubMed: 2311122]
56. Einheber S, Milner TA, Giancotti F, Salzer JL. J Cell Biol 1993;123:1223–1236. [PubMed: 8245127]
57. Staros JV. Biochemistry 1982;21:3950–3955. [PubMed: 7126526]
58. Parker CM, Pujades C, Brenner MB, Hemler ME. J Biol Chem 1993;268:7028–7035. [PubMed: 8463236]
59. Pearson W, Lipman D. Proc Natl Acad Sci U S A 1988;85:2444–2448. [PubMed: 3162770]
60. Shevchenko A, Wilm M, Vorm O, Mann M. Anal Chem 1996;68:850–858. [PubMed: 8779443]
61. Eng JK, McCormack A, Yates J. J Am Soc Mass Spectrom 1994;5:1994.
62. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ. Biochemistry 1979;18:5294–5299. [PubMed: 518835]
63. Wiskowski CM, Bowden GT, Nagle RB, Cress AE. Carcinogenesis 2000;21:325–330. [PubMed: 10657977]
64. Kulesz-Martin MF, Penetrante R, East CJ. Carcinogenesis 1988;9:171–174. [PubMed: 2446796]
65. Cress A. BioTechniques 2000;29:776–781. [PubMed: 11056807]
66. Watt FM, Jones PH. Dev Suppl 1993:185–192. [PubMed: 8049472]
67. Fulop V, Bocskei Z, Polgar L. Cell 1998;94:161–170. [PubMed: 9695945]
68. Callebaut I, Mornon J, Cell Mol Life Sci 1998;54:880–891. [PubMed: 9760994]
69. Paoli M, Anderson BF, Baker HM, Morgan WT, Smith A, Baker EN. Nat Struct Biol 1999;6:926–931. [PubMed: 10504726]
70. Irie A, Kamata T, Takada Y. Proc Natl Acad Sci U S A 1997;94:7198–7203. [PubMed: 9207068]
71. Mould AP, Askari JA, Humphries MJ. J Biol Chem 2000;275:20324–20336. [PubMed: 10764748]
72. Humphries MJ. Curr Opin Cell Biol 1996;8:632–640. [PubMed: 8939662]
73. Filardo EJ, Cheres EH. J Biol Chem 1994;269:4641–4647. [PubMed: 7508446]
74. Price AA, Cumberbatch M, Kimber I, Ager A. J Exp Med 1997;186:1725–1735. [PubMed: 9362532]
75. Shaw LM, Mercurio AM. J Cell Biol 1993;123:1017–1025. [PubMed: 8227138]
76. Kawaguchi S, Hemler ME. J Biol Chem 1993;268:16279–16285. [PubMed: 8344915]
77. Lu CF, Springer TA. J Immunol 1997;159:268–278. [PubMed: 9200463]
78. Gaietta G, Redelmeier TE, Jackson MR, Tamura RN, Quaranta V. J Cell Sci 1994;107:3339–3349. [PubMed: 7706390]
79. Honda T, Fujiwara H, Ueda M, Maeda M, Mori T. J Clin Endocrinol Metab 1995;80:2899–2905. [PubMed: 7559872]
80. Sastry SK, Lakonishok M, Wu S, Truong TQ, Huttenlocher A, Turner CE, Horwitz AF. J Cell Biol 1999;144:1295–1309. [PubMed: 10087271]
81. Bednarczyk JL, Szabo MC, McIntyre BW. J Biol Chem 1992;267:25274–25281. [PubMed: 1281155]
82. Ikeyama S, Koyama M, Yamaoko M, Sasada R, Miyake M. J Exp Med 1993;177:1231–1237. [PubMed: 8478605]
83. Berditchevski F, Odintsova E. J Cell Biol 1999;146:477–492. [PubMed: 10427099]
84. Berditchevski F, Zutter MM, Hemler ME. Mol Biol Cell 1996;7:193–207. [PubMed: 8688552]
85. von Bredow DC, Nagle RB, Bowden GT, Cress AE. Exp Cell Res 1997;236:341–345. [PubMed: 9344615]
86. Berthet V, Rigot V, Champion S, Secchi J, Fouchier F, Marvaldi J, Luis J. J Biol Chem 2000;275:33308–33313. [PubMed: 10913146]
87. Delwel GO, Kuikman I, van der Schors RC, de Melker AA, Sonnenberg A. Biochem J 1997;324:263–272. [PubMed: 9164866]
88. Engelberg-Kulka H, Benhar I, Schoulaker-Schwarz R. Trends Biochem Sci 1993;18:294–296. [PubMed: 8236443]
89. Bullock TNJ, Eisenlohr LC. J Exp Med 1996;184:1319–1329. [PubMed: 8879204]
Fig. 1. The $\alpha_6\beta$ integrin was immunoprecipitated from human cells
The DU145H cells were surface-biotinylated, and the $\alpha_6$ integrin was retrieved using either the GoH3, J1B5, AA6A, 4F10, or BQ16 antibodies, specific for human $\alpha_6$ integrin. The $\alpha_5$ integrin was retrieved from the lysate using the P1D6 antibody, specific for human $\alpha_5$ integrin. The immunoprecipitations were analyzed using a 7.5% polyacrylamide gel under nonreducing conditions, and the migration position of the biotinylated integrins are as indicated.
Fig. 2. The α₆β₃ integrin contained a light chain identical to the integrin
Surface-biotinylated proteins from DU145H cells were α₆ retrieved by immunoprecipitation using the GoH3 antibody and were analyzed first by 7.5% polyacrylamide gel electrophoresis under nonreducing conditions. The resulting lane was excised from the gel and placed on the top of a second 7.5% polyacrylamide gel. The position of the migration of the integrins in the first gel are indicated at the top of the figure. Electrophoresis was then performed under reducing conditions. The resulting migration of the heavy chain (HC) and light chain (LC) and the molecular masses are indicated. The asterisk indicates a biotinylated protein band that was variably seen and is of unknown identity.
Fig. 3. The $\alpha_6\beta$ integrin paired with either the $\beta_4$ or $\beta_1$ sub-units
The HaCaT cells were surface-biotinylated, and the $\beta_4$ integrin was retrieved using either A9, 439.9b, ASC3, or 3E1 antibodies, specific for human $\beta_4$ integrin (A). The DU145 cells were surface-biotinylated, and the $\beta_1$ integrin was retrieved using P4C10 antibody, specific for $\beta_1$ integrin (B). The immunoprecipitated proteins were analyzed using a 7.5% polyacrylamide gel under nonreducing conditions, and the migration positions of the biotinylated integrins are as indicated.
The α6β1 integrin and the β1-containing integrins were retrieved from the lysates of human DU145H, HaCaT, and H69 cells by immunoprecipitation with either anti-α6 integrin antibodies GoH3 or J1B5 or anti-β1 integrin antibody P4C10. The resulting proteins were analyzed using a 7.5% polyacrylamide gel under nonreducing conditions followed by Western blot (WB) analysis using the α6A-specific antibodies 4E9G8 or AA6A, which are specific for the cytoplasmic domain, or the anti-integrin antibody A33, which is α6 specific for the N terminus of α6 integrin. The migration position of a biotinylated integrin standard from DU145H cells are as indicated. The samples shown in the middle panel were electrophoresed on a separate gel, and the molecular mass of the α6A band is indicated relative to the adjacent panels by a solid bar. The asterisk indicates a biotinylated protein band that was variably seen and is of unknown identity.

Fig. 4. The α6p integrin was recognized by antibodies specific for the α6A light chain
Fig. 5. The α6p integrin was present in normal and tumor epithelial cell lines
The α6-containing integrins were retrieved from the lysates of normal skin (HaCaT) and prostate epithelial cells (PrEC), prostate cancer cell lines (PC3, PC3-N, and LnCaP), breast cancer cell line (MCF-7), colon carcinoma cell line (SW480), and a lung carcinoma cell line (H69) by immunoprecipitation with the GoH3 antibody. The immunoprecipitated proteins were analyzed using a 7.5% polyacrylamide gel under nonreducing conditions. The presence of α6p was detected by Western blot analysis using the AA6A antibody, specific for the human α6A light chain.
Alpha 6p sequences within Alpha 6 exons 1-25

1  MAAAGQL\\LLYLSAGLSSRLGAAFNLDREDNVRKYGDPSLFGSFLAMHWQLQEDKR
2  LLLGAPORGEALPLQRANRRTGGLYSCDTARIPCTRIEFDND
3  ADPTESKEQDMGVTQSGGPPGKV
4  TCAHRYEKRQHVNTKQSGRDIFGR**R**YVLQLRLRDQMDGGGLSDFCDGRLRHEKFGSC**Q**QVAATFTKDFHYIVFGAPGTYNWK
5  GIVRVEQK**NT**FD**MNIF**ED**GP**YEV**GETEH**D**SL**PVPA**NSYL
6  GFS**LDS**GKGIVSKDEITVFSGAPRA**A**NHSQAVLL**K**RDMKSAH**L**LPEHIFDGEGLASSFGYD**VA**VDLNKD**G**
7  WQDIVGAPQYFDRD**GE**V**G**V**A**V**V**Y**M**N**Q**Q**G**R**WNNKRPI**L**A**T**K**D**S**M**F**G**I**A**V**K**N**G**D**N**Q**G**Y**P**
8  DIAVGAPYDDLGBKFYHGSANGINTK**P**Q
9  VLKG**P**FYFGS**A**GAMNLDRS**P**VDAVGLSDSVTF**R**
10 S**R**P**V**N**I**V**K**T**I**T**T**P**R**I**D**L**R**Q**T**A**C**G**PSG**I**C**
11 LQVKS\EF**Y**T**A**N**P**A**Y**N**PSI
12 SVG**T**L**E**A**E**K**ER**K**SRL**S**S**S**V**Q**R**N**Q**G**S**E**P**K**Y**T**Q**E**L**T**K**R**Q**K**QKV**C**M**E**E**T**W**L**Q**
13 DNIRD**K**L**R**P**P**T**S**A**V**E**Q**E**P**S**R**S**R**T**S**S**S**E**L**L**Y**L**N**S**E**E**P****A**
14 K**H**E**L**R**G**C**G**D**D**N**N**\C**N**S**L**K**L**E**Y**K**F**C**T**R**E**G**N**Q**D**K**F**Y**L**D**
15 O**K**G**V**P**E**V**L**E**Q**Q**I**A**L**E**I**V**T**I**V**N**S**P**S**P**P**N**P**T**K**Q**G**D**A**H**A**E**M**A**T**P**D**T**L**T**Y**S**A**Y**E**L**R**A**F**P**
16 EK**Q**L**S**C**V**A**N**Q**N**G**S**A**D**E**C**E**L**G**N**P**F**K**R**N**S**N**
17 VT**F**Y**V**L**S**T**T**E**V**T**F**D**T**P**Y**L**D**I**N**I**K**L**E**I**
18 IT**S**N**D**O**K**L**A**P**T**A**A**K**K**V**E**L**L**L**V**S**Y**G**C**
19 VK**P**C**N**V**T**C**G**O**Y**G**A**M**A**I**M**S**E**D**S**E**L**I**E**E**R**K**
20 V**I**N**L**K**H**E**I**L**T**N**E**I**A**G**L**N**P**E**K**S**C**W**M**W**W**L**Y**L**Y**L**Y**E**S**K**G**L**E**K**V**T**C**P**Q**E**K**I**N**S**L**I**T**
21 E**S**H**N**R**K**R**K**R**E**I**T**E**K**Q**I**D**D**N**R**K**F**S**P**A**E**R**K**Y**Q**T**L**
22 N**C**S**N**V**N**C**V**N**R**I**R**C**P**R**L**G**L**S**K**A**L**I**R**S**R**L**W**S**F**L**E**
23 E**Y**S**M**N**Y**L**D**L**M**R**A**F**D**Y**T**A**A**A**E**N**R**L**P**N**A**C**T**O**
24 V**R**Y**T**P**P**S**S**T**V**A**Q**Y**S**Q**G**P**P**W**W**V**L**V**A**I**G**L**M**A**L**L**L**F**L**W**K**
25 C**G**F**K**K**R**K**K**K**K**H**Y**D**T**Y**H**R**A**E**H**C**O**P**S**K**E**R**L**T**S**D**A**

Fig. 6. Sequences obtained from the α6p variant corresponded to exons 13–25 of the full-length α6 integrin.

The sequences of α6 exons 1–25 of the α6 integrin are indicated by their one-letter amino acid abbreviations. MALDI mass spectrometry and HPLC coupled to mass spectrometry identified 10 noncontinuous amino acid fragments from the α6p variant. These corresponded exactly to sequences contained within α6 exons 13–25 and are indicated by boxes. Five of nine putative glycosylation sites are retained within exons 13–25 and are indicated in bold and underlined type. 10 of 20 cysteine residues (indicated by closed circles) are retained within exons 13–25.
Fig. 7. Schematic of the $\alpha_6$ and $\alpha_{6p}$ integrins
A schematic of the full-length $\alpha_6$ integrin and the smaller $\alpha_{6p}$ variant is shown. Repeated domains (shaded rectangles) are indicated by Roman numerals I–VII. I, 42–79; II, 113–145; III, 185–217; IV, 256–292; V, 314–352; VI, 375–411; VII, 430–470. The putative ligand- and cation-binding domains are contained between repeated domains III and IV and domains V and VI, respectively. Exons 1–25 of the $\alpha_6$ integrin sequence are indicated. 10 noncontinuous amino acid fragments obtained from the $\alpha_{6p}$ integrin corresponded exactly to sequences contained within exons 13–25 of the full-length $\alpha_6$ integrin. The mapped sequence positions of the two Western blotting anti-$\alpha_6$A antibodies (AA6A and 4E9G8) that recognize both $\alpha_6$ and the $\alpha_{6p}$ variant are shown by an asterisk on the full-length $\alpha_6$ schematic. Conformationally dependent epitopes for anti-integrin $\alpha_6$ antibodies used for immunoprecipitation are not indicated on the schematic.
Fig. 8. The α6p variant had a longer surface retention time than α6 integrin
A, DU145H cells were surface-biotinylated and α6 incubated for 24, 48, or 72 h, followed by lysis and immunoprecipitation with anti-α6 antibody GoH3. The samples were analyzed by a nonreducing 7.5% polyacrylamide gel, transferred to polyvinylidene fluoride membrane, reacted with peroxidase-conjugated streptavidin, and visualized by chemiluminescence. The asterisk indicates a biotinylated protein band that was variably seen and is of unknown identity.
B, the film was digitized, and the densitometry values were analyzed for relative degradation rates of α6, β1, and α6p.
Fig. 9. RT-PCR of the integrin coding region revealed an α6 single PCR product
PCR primers that bracketed the integrin α6 coding region were used to amplify first strand
cDNA generated from cell line DU145H (lane 1). To confirm the identity of the integrin α6
PCR product, aliquots were digested with four diagnostic restriction enzymes (EcoNI, lane 2;
EcoRI, lane 3; SmaI, lane 4; and XhoI, lane 5), size separated on a 1× TBE-1.5% agarose gel,
and visualized by ethidium bromide staining. The molecular mass standard is EcoRI/HindIII-
digested λ DNA (lane M).
Fig. 10. Calcium-induced normal keratinocyte differentiation increased α6p levels

The presence of α6 and α6p integrins was determined in normal 291 mouse keratinocytes, immortalized O3C nontumorigenic, and O3R tumorigenic derivatives. The cells were maintained in 0.4 mM calcium (low, lanes L) and switched to 0.14 mM (medium, lanes M) or 1.4 mM (high, lanes H) calcium medium at 60% confluency for 24 h of treatment, then frozen in a dry ice bath, and kept at −80 °C in a freezer until use. Whole cell lysates (20 μg) were electrophoresed under nonreducing conditions on a 7.5% polyacrylamide gel and transferred to polyvinylidene fluoride membrane followed by Western blot analysis using anti-α6 integrin antibody, AA6A (A). The α6 and α6p integrin protein bands were scanned and quantitated using Scion Image and graphed (B).