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Differential Regulation of a Novel Variant of the α_6 Integrin, α_{6p}[1]

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Abstract

We have reported previously the existence of an M_r 70,000 form of the α_6 integrin called α_{6p} in a variety of human epithelial cell lines. Four different experimental conditions were used to examine the regulation of α_6 and α_{6p} integrin. The production of the α_6 integrin was decreased by 45% using a protein translation inhibitor (2.25 μM puromycin), whereas production of the α_{6p} variant was unaffected. The α_{6p} variant was decreased 60% by actin depolymerization (10 μM cytochalasin D) corresponding to a decrease in its surface expression, whereas α_6 integrin production was unaffected. The α_{6p} variant was resistant to endoglycosidase H treatment, whereas the α_6 integrin was both sensitive and resistant to endoglycosidase H treatment, indicating retention in the endoplasmic reticulum and processing through the Golgi apparatus. Additionally, digestion by endoglycosidase F demonstrated both α_{6p} and α_6 integrin contained NH_2-linked glycosylations and both shifted M_r ~10,000 on enzymatic digestion. Finally, inhibition of serine/threonine phosphatases by either calyculin A (15 nM) or okadaic acid (62 μM) did not affect α_{6p}, whereas the production of α_6 integrin was decreased by 50%. These data suggest that the production of the α_{6p} variant is distinct from α_6 integrin and may involve a post-translational processing event at the cell surface.

Introduction

Integrins are signaling receptors that link the intracellular cytoskeleton to the extracellular matrix and play important roles in adhesion, migration, proliferation, signaling, differentiation, and cell survival (1–8). The α_6 integrin is a laminin receptor in epithelial cells (9–14). Previously, studies demonstrated a loss of the$\alpha_6\beta_4$ heterodimer during prostate tumor progression (15–17) and a persistent expression of the $\alpha_6\beta_1$ integrin (18). Additionally, expression of $\alpha_6\beta_1$ integrin is maintained in micrometastases (15,16,19–21).

Our previous studies identified a novel M_r 70,000 variant of the α_6 integrin, called α_{6p}, for the Latin word *parvus*, in prostate carcinoma cell lines (22). The variant paired with both β_1 and β_4 integrin subunits and was present in a number of epithelial carcinoma cell lines, as well as in a normal immortalized human keratinocyte cell line. Two-dimensional gel analysis and Western blotting data indicated the cytoplasmic light chain of the variant was identical to that of the full-length α_6 integrin and that the primary alteration was a shortened extracellular heavy chain. The shortened extracellular domain was missing the putative ligand-binding domain contained within the β-propeller (23–25).

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Adhesion to extracellular matrix proteins has been shown to play a role in cytoskeletal organization (26). The $\alpha_6\beta_1$ integrin localizes to the focal adhesion, functioning to link the extracellular matrix to the actin cytoskeleton via the β_1 cytoplasmic domain for both signal transduction and mechanical stability of the cell during migration (8,15,27–30). This interaction has been shown to be important for integrin signaling and recruitment of scaffolding molecules, such as paxillin and filamentous-actin (31,32).

The production of a variant form of the integrin, missing the ligand-binding region of the molecule, may influence these events. It is of particular interest to understand the circumstances surrounding the production of α_{6p} and whether it is subject to similar regulatory controls as the production of the α_6 integrin. We have extended our studies to examine the effect of known experimental perturbations of integrin function on the production of α_6 and α_{6p} integrin. The following experiments demonstrated that the α_6 and α_{6p} integrins responded differently to the inhibition of translation, the alteration of actin filaments, endoglycosidase digestion, and the action of serine/threonine phosphatase inhibitors. These data indicate that the mechanism of α_6 and α_{6p} production differs significantly. Furthermore, these data are consistent with the hypothesis that the α_{6p} integrin is produced by a processing event after the molecule reaches the cell surface.

Results

Production of α_6 Integrin, but not α_{6p}, was Translation Dependent

Recently, Alais *et al.* (33) demonstrated that the expression of α_1 integrins could be regulated through translation-dependent mechanisms. Our previous studies indicated that the α_{6p} variant was generated independent of a transcription event, such as an alternative mRNA splicing (22). We used the translation inhibitor, puromycin, to determine the importance of translation on the production of both α_6 and α_{6p} integrins. The human prostate carcinoma DU145H cells were exposed to 2.25 μM puromycin for 18 h or DMSO vehicle. The α_6 and α_{6p} integrin proteins were identified at M_r 160,000 and 70,000 respectively, from a whole cell lysate (Fig. 1*A*). Puromycin treatment resulted in a 45% reduction of the α_6 integrin as compared with the vehicle control (Fig. 1*B*). No effect on α_{6p} integrin protein levels was observed. Although the production of the α_6 integrin was dependent on translation, the level of the α_{6p} variant was not affected by the inhibition of translation.

Production of α_{6p}, but not α_6 Was Dependent on the Actin Cytoskeleton

The actin cytoskeleton influences integrin behavior on the cell surface, such as integrin clustering, dispersal from focal adhesions, and integrin-mediated adhesion to extracellular matrix proteins (34). Disruption of the actin cytoskeleton, but not the tubulin network, has been previously shown to inhibit $\alpha_6\beta_1$-mediated cell adhesion to laminin (35). If the α_{6p} variant was produced on the cell surface, one would expect the production of the variant to be dependent on the actin cytoskeleton. The human prostate carcinoma DU145H cell line was used for these studies because of the abundance of the $\alpha_6\beta_1$ and α_6pβ_1 integrins (22).

The actin staining in the DU145H cells revealed primarily a cortical staining pattern surrounding the periphery of the cells with few stress fibers, and treatment with cytochalasin D resulted in a loss of cortical actin replaced with perinuclear distribution of disorganized actin (data not shown). Microtubule networks were observed to radiate throughout the cytoplasm, originating from the microtubule organization centers near the nuclei of the DU145H cells, and treatment with nocodazole resulted in a loss of the tubulin network (data not shown). The total production of α_6 and α_{6p} integrins was examined after the addition of cytochalasin D. A time-dependent decrease in totalα_{6p} protein levels to ~60% of the control level at 18 h was observed, whereas the total α_6 integrin protein levels were relatively unaltered (Fig. 2, *A* and

B). The differential change in the α_6 and α_{6p} integrin proteins was apparent by 12 h postaddition of cytochalasin D, and by 18 h, the α_{6p} integrin form had decreased to 60% of the vehicle control (DMSO; Fig. 2, *A* and *B*). The microtubule network was disrupted using 8 μM nocodazole, and the total amount of α_6 and α_{6p} integrins was examined to determine whether nonspecific effects on the cytoskeleton were responsible for the altered production of α_{6p} variant (Fig. 2, *C* and *D*). No significant difference was observed in the total amount of the α_6 and α_{6p} integrin forms on depolymerization of the microtubules, suggesting that the tubulin network was not important for production of either α_6 or α_{6p} integrins.

Cytochalasin D Reduced Cell Surface Expression of α_6, α_{6p}, and β_1 Integrins

Because the data suggested that the α_{6p} was produced on the cell surface, we next determined if the loss of the α_{6p} production by cytochalasin D could be accounted for by the loss of α_{6p} cell surface expression. To distinguish between surface and cytoplasmic integrin subunits, cell surface proteins were labeled using biotin before adding either cytochalasin D (10 μM) or nocodazole (8 μM) to the cells. Depolymerization of actin by cytochalasin D resulted in a significant loss of α_6 and β_1 integrins from the cell surface to 36 and 30% of vehicle controls, respectively (Fig. 3, *A* and *B*), whereas the total production of α_6 integrin was not affected (Fig. 2). In contrast, the surface protein levels of the α_{6p} decreased to 67% of the control value (Fig. 3, *A* and *B*), and the total production of the α_{6p} integrin was reduced to ~65% of the control value (Fig. 2). No change in cell surface α_6, β_1, or α_{6p} integrins was observed in cells treated with nocodazole (Fig. 3, *A* and *B*). These data indicated that cytochalasin D decreased the cell surface expression of α_6, whereas the total level of α_6 integrin was unaffected (Figs. 2 and 3). In contrast, both the α_{6p} cell surface expression and the total α_{6p} production was significantly decreased (Fig. 3). These data taken together suggested again that the α_{6p} variant was produced at the cell surface, dependent on the actin cytoskeleton.

Differential Intracellular Processing of the α_6 and α_{6p} Integrin

It is known that the integrins can be modified after translation by glycosylation (2). There are nine potential NH_2-linked glycosylation sites contained in the α_6 integrin (36,37); five are contained within exons 13–25, the region present within the α_{6p} integrin (22). The enzyme endoH[3] is frequently used in combination with endoF to distinguish between complex and high-mannose oligosaccharides. Proteins sensitive to cleavage by endoH are not fully processed, *i.e.*, retained in the Golgi apparatus, whereas proteins sensitive to endoF cleavage are fully processed by the Golgi (38). We determined whether or not the α_{6p} integrin variant was differentially glycosylated compared with the full-length α_6 integrin. Human prostate carcinoma DU145H cells were lysed, immunoprecipitated with anti-α_6 integrin antibody J1B5, and subjected to digestion with either endoH or endoF as detailed in "Materials and Methods." EndoH digestion resulted in the appearance of at least three α_6 integrin intermediates, indicating the retention of these forms within the ER (Fig. 4). The majority of the α_6 integrin was both endoH and endoF resistant, indicating successful passage through the ER and entrance into the medial Golgi compartment. In contrast, the α_{6p} variant was not sensitive to endoH digestion but was sensitive to endoF (Fig. 4). No ER-retained forms of the α_{6p} integrin were detected, although the α_{6p} does contain high mannose type oligosaccharides. The data suggested that the variant may be produced after the molecule arrives at the cell surface, because the α_{6p} was not processed through the ER and was not dependent on active protein translation.

[3]The abbreviations used are: endoH, endoglygosidase H; endoF, endoglycosidase F; ER, endoplasmic reticulum; IMDM, Iscove's modified Dulbecco's medium; RIPA, radioimmunoprecipitation assay; PVDF, polyvinylidene fluoride; HRP, horseradish peroxidase.

The α_6, but not α_{6p} Integrin, Was Altered by Serine/Threonine Phosphatase Inhibitors

Inhibition of serine/threonine phosphatases using pharmacological inhibitors has been shown previously to regulate integrin phosphorylation (39,40) and integrin function (41–43). Calyculin A is a potent inhibitor of protein phosphatase type 1 and 2A, whereas okadaic acid inhibits both, but it preferentially inhibits type 2A (44,45).

To examine the role for protein phosphatase inhibitors on α_6 and α_{6p}integrins, calyculin A and okadaic acid were tested. Using 15 nM calyculin A to inhibit serine/threonine phosphatases, the total amount of α_6 and α_{6p} integrins was examined. After treatment for 6 h with 15 nM calyculin A, we observed a 50% decrease in total protein production of α_6 integrin but only a 10% decrease in the variant α6p form (Fig. 5, *A* and *B*). Cells also were treated with 62 μM okadaic acid for 18 h. Two α_6 integrin forms were observed after treatment (Fig. 5*C*). The molecular weight shift observed in the lower form was consistent with a dephosphorylated α_6 integrin protein similar to that observed for α_4 integrin (39). There was a 2-fold increase of the faster migrating form of α_6 integrin, with a corresponding 50% decrease in the slower migrating form (Fig. 5*D*). No alteration in electrophoretic mobility of α_{6p} integrin was observed under the same experimental conditions. The pharmacological inhibitors used in this study were not toxic to the cells (data not shown).

Discussion

Previous studies have indicated that the α_6 integrin-containing heterodimer is altered in prostate carcinoma progression, shifting from the $\alpha_6\beta_4$ to $\alpha_6\beta_1$ integrin. Previously, we identified a novel variant of the α_6 integrin, called α_{6p}, which paired with both β_1 and β_4 subunits (22). The variant was missing a large portion of the extracellular domain, including the postulated ligand-binding region, but retained an identical cytoplasmic light chain. Four different experimental strategies were used here to determine whether α_{6p} and α_6 were regulated in a similar or distinct manner. It was found that the response of the α_{6p} and the α_6 integrin to the experimental conditions was distinct, indicating a disassociation between the appearance of these two integrin forms.

The most striking difference in the forms was the susceptibility of the α_6 integrin and the resistance of the α_{6p} integrin to the inhibition of protein translation using puromycin. Our previous studies identified only one mRNA transcript for the α_6 integrin in the DU145H cells (22). One formal possibility to explain the production of a smaller version of α_6 (α_{6p}) was that an altered translation of the α_6 mRNA occurred. Previous work has shown that isoforms of cell surface receptors can be generated by the selective use of internal ribosome entry sites or alternative translational start sites (46). Recently, expression of β_1 integrin was altered by a translation-dependent mechanism (33). Our studies indicated that production of the α_6 integrin could be suppressed using an inhibitor of translation but that expression of the α_{6p} variant was unaltered by translation inhibition. These data suggested that the α_{6p} variant was generated through a post-translational mechanism. These data also may indicate that a larger “pool” of the wild-type α_6 integrin exists relative to the α_{6p} form. Inhibition of α_6 production by puromycin may trigger processing of the α_6 to the α_{6p} form.

The integrin $\alpha_6\beta_1$ is processed after translation in the ER. The intracellular processing of the integrin can be monitored by determining the susceptibility of the protein to cleavage by endoH. Our results are similar to the findings of others that the α_6 integrin contains both endoH-sensitive and -resistant forms, consistent with passage of the molecule through the ER and the Golgi compartment (47). In contrast, the α_{6p} integrin was resistant to endoH cleavage, indicating that it was not resident within the ER. Because the α_{6p} is on the cell surface and does not traffic through the ER, it suggests that the protein was produced by a post-translational event occurring at the cell surface.

Integrins on the cell surface are known to be regulated by the cytoskeleton (26,32,48–50), *e.g.*, the actin cytoskeletal attachment to integrins is important for modulation of integrin clustering and dispersal from focal contacts and "inside-out" signaling. In this study, we observed that the cell surface abundance of the α_6 and β_1 integrins was dependent on the actin cytoskeleton, whereas the total cellular production of the α_6 integrin was unaltered. In contrast, both the production and the cell surface expression of the α_{6p} form of the integrin were uniquely susceptible to actin depolymerization. These data combined with the resistance of the α_{6p} to endoH suggests that the α_{6p} variant formed after the integrin arrived on the surface of the cell.

The processing of cell surface receptors has been described previously as ectodomain shedding and plays an essential role in mammalian development (51). We note with interest that collagen XVII/BP180, an epidermal adhesion molecule, exists as a full-length transmembrane protein and is processed into a M_r 120,000 ectodomain that is shed from the keratinocyte surface (52). In addition, CD44, a specific adhesion receptor for hyaluronan, can be shed in a process that can be reduced by disruption of actin assembly with cytochalasin D (53). Current work is under way to determine whether the α_{6p} form of the integrin is generated in a manner similar to the process of ectodomain shedding. The data presented are consistent with a proteolytic processing of the α_6 integrin on the cell surface to the α_{6p} form. Experiments are under way to determine the nature of the protease activity involved. At the present, we know that broad-based metalloproteinase inhibitors are ineffective in blocking the α_{6p} production (data not shown). This is in contrast to recent findings that the integrin α_V can be processed by MT1-MMP (54).

A final experimental approach to examine α_6 and α_{6p} function was the use of phosphatase inhibitors. Inhibition of serine/threonine phosphatases has been shown previously to decrease cell-cell adhesion (55,56) and integrin-dependent adhesion and motility (40–43). Inhibitors of serine/threonine phosphatases, such as okadaic acid and calyculin A, resulted in dephosphorylation of α_4 integrin, resulting in high-avidity binding of VCAM-1 (39). In our experiments, treatment with calyculin A and okadaic acid resulted in a differential alteration of the electrophoretic properties of the α_6 integrin (Fig. 5). The α_6 integrin in treated cells existed as a protein doublet. The α_{6p} variant was not altered by treatment with serine/threonine phosphatase inhibitors. Although the significance of phosphorylation of the α_6 integrin cytoplasmic domain is understood incompletely, it has been shown to induce tyrosine phosphorylation of paxillin and other unknown proteins on ligand binding (57,58). Our results were suggestive that the cytoplasmic domain of the α_6 integrin was responsive to a signaling event, whereas the α_{6p} variant was not, despite having identical cytoplasmic domains (22). In this instance, the α_{6p} variant may play a dominant negative role. However, we note that ectopic expression of the α_6 cytoplasmic domain alone in myoblasts is active in suppressing proliferation, induction of differentiation, and suppression of focal adhesion signaling (59, 60). These data would support the notion that an integrin lacking the extra-cellular ligand-binding domain may still retain a role in altering the cellular response to growth. Experiments are underway currently to determine the role of the α_{6p} variant in the alteration of cellular adhesion and proliferation.

Materials and Methods

Cell Culture

Human prostate carcinoma cell line, DU145H, was isolated by us as described previously (19). Cells were grown in IMDM (Life Technologies, Inc., Gaithersburg, MD) plus 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Antibodies and Reagents

Anti-α_6 integrin antibodies were obtained as follows: GoH3, rat IgG2a (Accurate Chemicals, Westbury, NY; Ref. 61), J1B5, rat monoclonal was a generous gift from Dr. Caroline Damsky (University of California, San Francisco, CA; Ref. 62), and AA6A rabbit polyclonal, which was raised and purified using Bethyl Laboratories, Inc. (Montgomery, TX) specific for 16 amino acids (CIHAQPSDKERLTSDA) at the COOH terminus of the human α6A sequence (9) as done previously (11). Cytoskeletal inhibitors cytochalasin D and nocodazole were obtained from Sigma Chemical Co. (St. Louis, MO). Serine/threonine phosphatase inhibitors were obtained as follows: calyculin A, Okadaic acid (Alexis Biochemicals, San Diego, CA), and inactive analogue 1-nor-okadaone (LC Laboratories, Woburn, MA). For inhibition of translation, puromycin was obtained (Sigma Chemical Co.).

Immunoprecipitations/Western Blot Analysis

For immunoprecipitations, 200 μg of total protein lysate were 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 RIPA buffer [150 mM NaCl, 50 mM Tris, 5 mM EDTA, 1% (volume for volume) Triton X-100, 1% (w/v) deoxycholate, and 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 RIPA and eluted in 2 × nonreducing sample buffer. Immunoprecipitation and whole cell lysate samples were boiled for 5 min before loading onto a 7.5% SDS-polyacrylamide gel for analysis. Proteins resolved in the gel were electrotransferred to Millipore Immobilon-P PVDF membrane (Millipore, Bedford, MA), incubated with either peroxidase-conjugated streptavidin or Western blotting antibodies plus secondary antibody conjugated to HRP and visualized by chemiluminescence (ECL Western Blotting Detection System; Amersham, Arlington Heights, IL), and exposed to film. Protein bands were quantitated using Scion Image Analysis software as described previously (63) and graphed using Excel software.

Alteration of α_6 and α_{6p} Integrins by Pharmacological Inhibitors

Human prostate carcinoma DU145H cells were treated in serum-free IMDM media containing 0.1% BSA with drug (10 μM cytochalasin D, 8 μM nocodazole, 15 nM calyculin A, 62 μM okadaic acid, 62 μM 1-nor-okadaone, and 2.25 μM puromycin) for 18 h in the dark. For time courses, media were exchanged for serum-free IMDM containing 0.1% BSA at the start of the time course, and drug was added at appropriate time points. Cells were then collected by scraping, centrifuged for 5 min at 800 × *g*, and washed two times in HEPES buffer. Cell pellets were lysed in RIPA buffer with protease inhibitors and sonicated. Whole cell lysate (10 – 15 μg) was loaded and electrophoresed on a 7.5% SDS-polyacrylamide gel under nonreducing conditions. Proteins were transferred to PVDF membrane followed by Western analysis for α_6 integrin with anti-α_6 integrin antibody, AA6A. Protein bands for α_6 and α_{6p} were scanned and quantified using Scion Image Analysis software as described previously (63) and graphed using Excel software.

Surface changes of α_6, β_1, and α_{6p} were determined by surface biotinylation of DU145H cells followed by 18 h of drug treatment for cytochalasin D. For nocodazole studies, DU145H cells were labeled after the 18-h drug treatment. Biotinylated DU145H cells were lysed, and 200 μg of total protein were used for immunoprecipitations with anti-α_6 integrin antibody, J1B5. Samples were analyzed as above, and PVDF membrane was incubated with HRP-streptavidin. Resulting protein bands for α_6, β_1, and α_{6p} from treated or vehicle samples were quantitated and graphed.

EndoH and EndoF Digestions

For digestions, 200 μg of whole cell lysate were first immunoprecipitated overnight with anti-α_6 integrin antibody, J1B5, in microcentrifuge tubes. The following day, the beads were washed three times with RIPA buffer, and the sample was resuspended in 35 μl of 2 × nonreducing sample buffer containing 4 mM $CaCl_2$ plus 1 mUnit either endoH or endoF (obtained from Sigma Chemical Co.), which had been diluted in 10% glycerol. Tubes containing reactions were placed in a shaking hot water bath at 37°C overnight. The following morning, the samples were analyzed by 7.5% SDS-PAGE under nonreducing conditions followed by Western blot analysis using anti-α_6 integrin antibody, AA6A.

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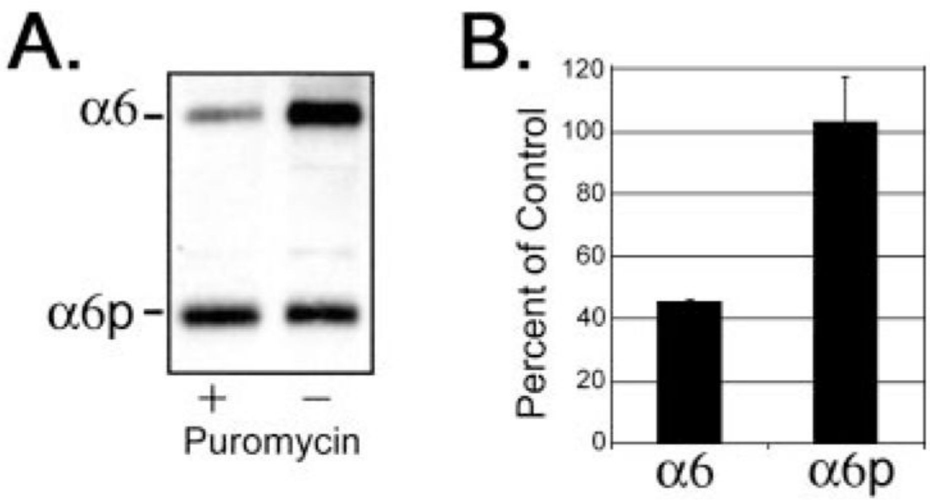

Fig. 1.

Expression of the α_6 integrin, but not α_{6p}, was dependent on translation. Human prostate carcinoma DU145H cells were treated with either 2.25 μM puromycin or DMSO vehicle for 18 h. Whole cell lysate (10 –15 μg) was loaded and electrophoresed on a 7.5% polyacrylamide gel under nonreducing conditions. Proteins were transferred to PVDF membrane followed by Western blot analysis with anti-α_6 integrin antibody, AA6A (*A*). Protein bands in *A* were quantified and graphed in Excel (*B*). Data shown were representative of three independent experiments.

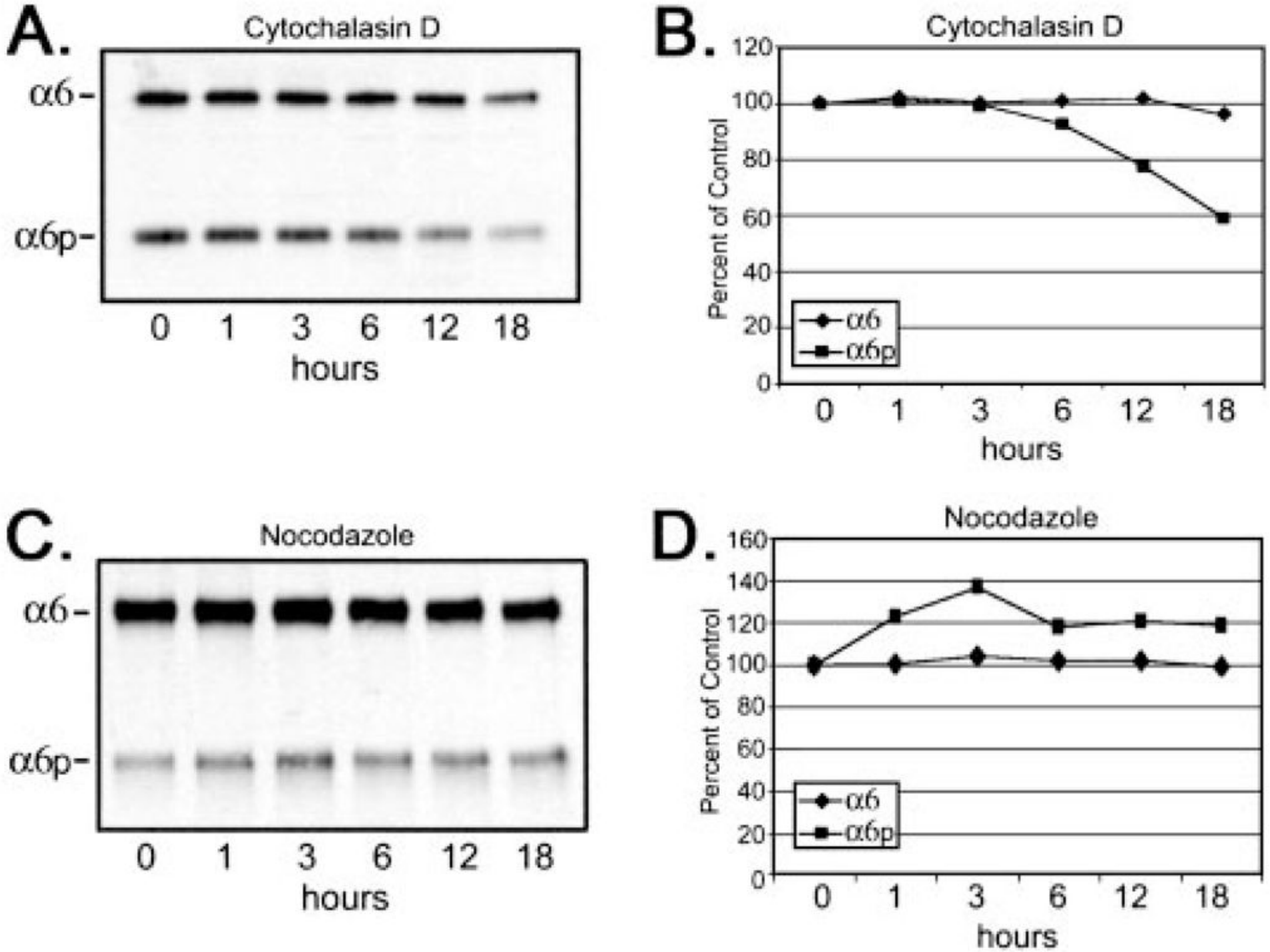

Fig. 2.

Disruption of the actin cytoskeleton reduced total protein expression of α_{6p} but not α_6 integrin. Human prostate carcinoma DU145H cells were treated with either 10 μM cytochalasin D (*A* and *B*) or 8 μM nocodazole (*C* and *D*) over a 24-h period of time. Identical amounts of whole cell lysates (10 μg) were loaded and electrophoresed on a 7.5% polyacrylamide gel under nonreducing conditions. Proteins were transferred to a PVDF membrane followed by Western analysis for α_6 integrin (*A* and *C*). The α_6 and α_{6p} protein bands were scanned and quantitated using Scion Image Analysis software and graphed in Excel (*B* and *D*). Data shown were representative of three experiments.

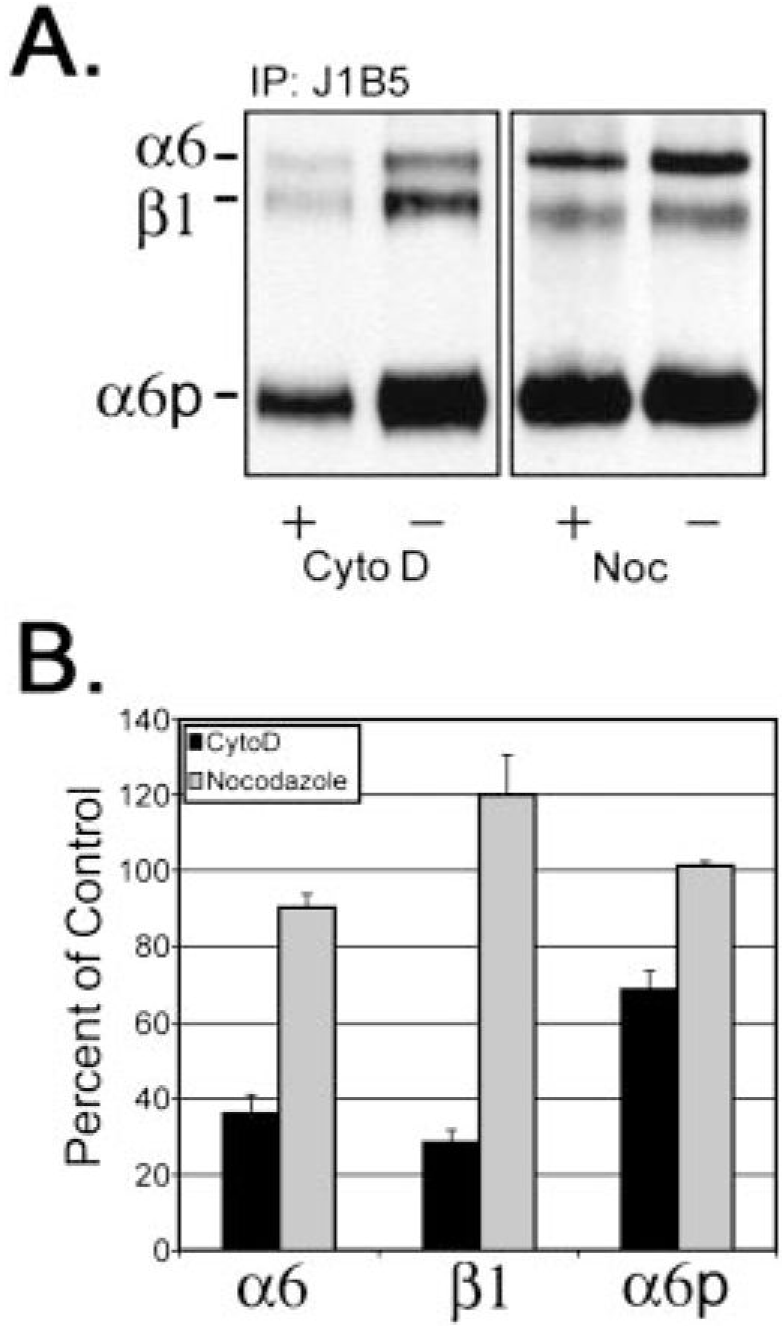

Fig. 3.

Disruption of the actin cytoskeleton significantly reduced cell surface expression of α_6, α_{6p}, and β_1 integrins. Surface changes of α_6, β_1, and α_{6p} were determined by surface of DU145H cells with biotin before treatment with either 10 μM cytochalasin D or 8 μM nocodazole for 18 h. Labeled cells were lysed, and 200 μg of total protein were used for immunoprecipitations with anti-α_6 integrin antibody, J1B5. Samples were separated on a 7.5% polyacrylamide gel under nonreducing conditions. Proteins were transferred to PVDF membrane followed by incubation with HRP conjugated to streptavidin (*A*). Resulting α_6, β_1, and α_{6p} integrin protein bands were quantified and graphed (*B*).

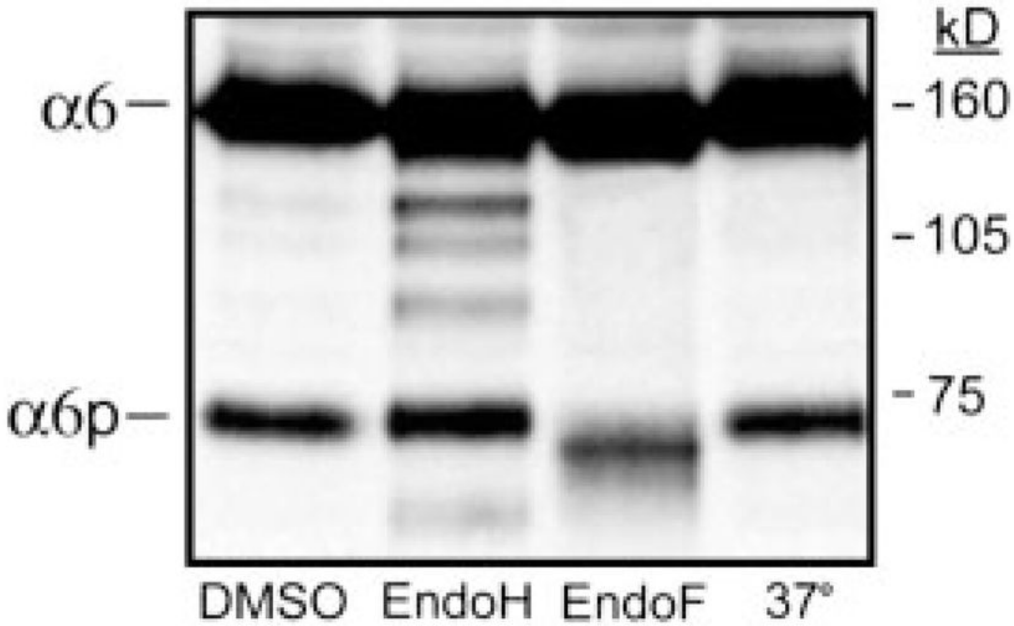

Fig. 4.

The α_6 integrin sensitivity to endoglycosidase treatment. Human prostate carcinoma DU145H cells were immunoprecipitated for α_6 integrin using J1B5 antibody and then subjected to digestion with either endoH or endoF overnight at 37°C. Resulting protein samples were analyzed by 7.5% SDS-PAGE under nonreducing conditions followed by Western blot analysis using anti-α_6 integrin antibody, AA6A. The migration of the molecular weight standards and integrins are indicated.

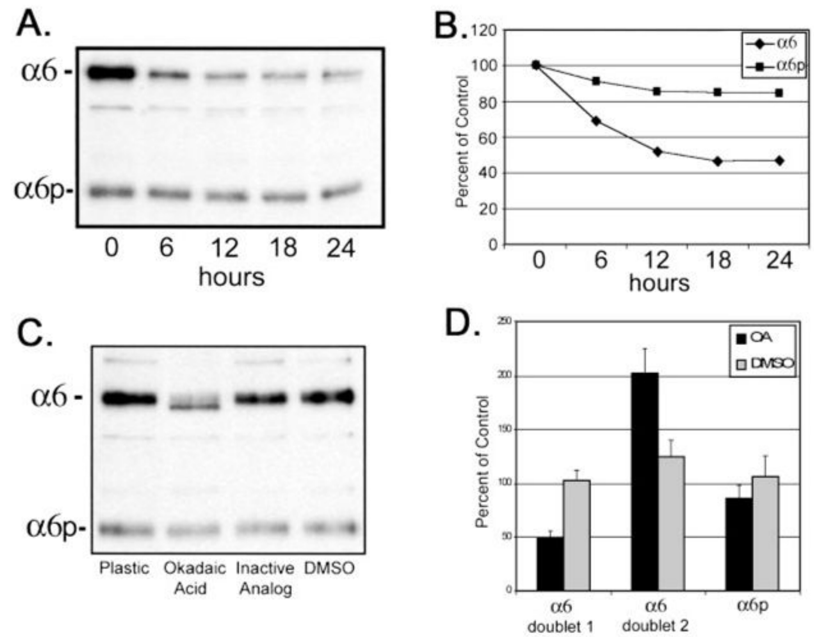

Fig. 5.

Calyculin A and okadaic acid treatment of DU145H cells decreased α_6 integrin protein levels but not α_{6p}. Human prostate carcinoma DU145H cells were treated with 15 nM calyculin A over a 24-h period of time. Identical amounts of whole cell lysate (10) were loaded and electrophoresed on a 7.5% polyacrylamide gel under nonreducing conditions. Proteins were transferred to PVDF membrane followed by Western analysis for α_6 integrin (*A*). Protein bands in *A* were scanned and quantified using Scion Image Analysis software and graphed in Excel (*B*). Data shown were representative of three independent experiments. DU145H cells were treated with 50 μM okadaic acid, the inactive analogue 1-nor-okadaone, or vehicle (DMSO) for 18 h. Whole cell lysates were examined for α_6 integrin protein expression as above (*C*). Resulting α_6 and α_{6p} bands from three independent experiments were quantified and graphed (*D*).