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Extracellular alpha 6 integrin cleavage by urokinase-type plasminogen activator in human prostate cancer

Manolis C. Demetriou^{a,b}, Michael E. Pennington^{a,b}, Raymond B. Nagle^c, and Anne E. Cress^{a,b,*}

^aDepartment of Cell Biology and Anatomy, Tucson, AZ 85724, USA

^bDepartment of Radiation Oncology, Tucson, AZ 85724, USA

^cDepartment of Pathology, University of Arizona, Tucson, AZ 85724, USA

Abstract

During human prostate cancer progression, the integrin $\alpha 6 \beta 1$ (laminin receptor) is expressed on the cancer cell surface during invasion and in lymph node metastases. We previously identified a novel structural variant of the $\alpha 6$ integrin called $\alpha 6p$. This variant was produced on the cell surface and was missing the β -barrel extracellular domain. Using several different concentrations of amiloride, aminobenzamidine and PAI-1 and the urokinase-type plasminogen activator (uPA) function-blocking antibody (3689), we showed that uPA, acting as a protease, is responsible for production of $\alpha 6p$. We also showed that addition of uPA in the culture media of cells that do not produce $\alpha 6p$, resulted in a dose-dependent $\alpha 6p$ production. In contrast, the addition of uPA did not result in the cleavage of other integrins. Using $\alpha 2$ -antiplasmin and plasmin depleted media, we observed that uPA cleaves the $\alpha 6$ integrin directly. Further, 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA) induced the production of $\alpha 6p$, and this induction was abolished by PAI-1 but not $\alpha 2$ -antiplasmin. Finally, the $\alpha 6p$ integrin variant was detected in invasive human prostate carcinoma tissue indicating that this is not a tissue culture phenomenon. These data, taken together, suggest that this is a novel function of uPA, that is, to remove the β -barrel ligand-binding domain of the integrin while preserving its heterodimer association.

Keywords

Integrin; Urokinase; Prostate cancer

Introduction

Integrins are heterodimeric proteins with α and β subunits, and each $\alpha \beta$ combination has its own binding specificity and signaling properties [1,2]. Integrins recognize several extracellular matrix (ECM) proteins and sense the extracellular matrix environment [1,2]. They are involved in many processes including cell migration, differentiation, blood clotting, tissue organization, and cell growth. Integrins generally contain a large extracellular domain (α subunit approximately 1000 residues, and β subunit, approximately 750 residues), a transmembrane domain, and a short cytoplasmic domain (approximately 50 residues or less) with the exception of $\beta 4$, whose cytoplasmic domain is large (more than 1000 residues) [3,4].

The structure of the extracellular segment of the alpha subunit has been determined using protein crystals of a soluble $\alpha v\beta 3$ integrin fragment [5]. The NH_2 terminal end of the alpha integrin subunit contains a seven-bladed β -propeller structure followed by a tail region composed of three β -sandwiched domains: an Ig-like “thigh” domain and two very similar domains that form the “calf” module [6]. The tail region can fold back at approximately 135° angle forming a V-shaped structure with a “genu” between the thigh domain and the calf module [6]. The profound bending suggests that a highly flexible site, the genu, exists in the integrin alpha subunit and results in a flexible 700 Å accessible surface region [6].

We have previously identified a structural variant of the $\alpha 6$ integrin called $\alpha 6p$ that is missing the extracellular β -propeller domain associated with ligand binding [7]. Integrin $\alpha 6p$ is a 70-kDa form, and mass spectrometry analysis showed that the NH_2 terminal end of the molecule contains at least amino acids starting at arginine 595 [7]. The analysis also showed that the rest of the carboxy terminus of $\alpha 6p$ was identical with the full-length $\alpha 6$ integrin [7]. Using a multiple sequence alignment tool, this position in $\alpha 6$ integrin lies within an accessible loop described for the αV integrin subunit [5]. The $\alpha 6p$ variant, produced while on the cell surface, remains paired with either the $\beta 1$ or $\beta 4$ subunit and has a 3-fold increase in biological half-life as compared to the full-length $\alpha 6$ integrin. [8]. The extracellular surface expression of the $\alpha 6$ integrin before cleavage suggested that an extracellular protease mediated the conversion.

Urokinase-type plasminogen Activator (uPA) is a secreted 54-kDa serine protease which cleaves plasminogen as a primary substrate [9]. In addition, uPA has been shown to catalyze the proteolytic cleavage of the extracellular matrix protein fibronectin [10], hepatocyte growth factor/scatter factor (HGF/SF) [11], and macrophage-stimulating protein (MSP) [12]. There is no universal consensus site for uPA cleavage of its substrates. The enzyme uPA is synthesized and released by cells as an inactive, single chain proenzyme. When pro-uPA binds to the uPA-receptor (uPAR), it is cleaved primarily by plasmin, but also by kallikrein, blood coagulation factor XIIa, and cathepsin B [13], into its two-chain active form [14–16]. The binding of pro-uPA to the uPAR in effect targets the enzyme activity to areas of the cell surface containing the uPAR (reviewed in Refs. [17,18]).

The present study was prompted by the report of a binding site for uPAR on the $\alpha 6$ integrin in the extracellular β -propeller region [19] and the loss of this region during the $\alpha 6$ to $\alpha 6p$ conversion [7]. In addition, the knowledge that both uPA activity and the $\alpha 6$ integrin persist in invasive cancer led us to determine if the $\alpha 6p$ conversion via uPA was found in invasive cancer [20–24].

Material and methods

Cells

All human cell lines were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 . Cell lines DU145H and PC3-N were grown in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco BRL, Gaithersburg, MD, USA) plus 10% fetal bovine serum (FBS). Cell line MCF-10A, was grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM-F12) (Gibco BRL) plus 5% horse serum, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, and 500 ng/ml hydrocortisone. The MCF-10A (human breast cells) cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The DU145H cells were isolated by us as previously described [20] and contain only the $\alpha 6A$ splice variant [25]. The PC3-N cells are a variant of the PC3 prostate carcinoma cell line [26].

Antibodies and chemicals used in this study

Anti- $\alpha 6$ integrin antibodies include and were obtained as follows: J1B5, rat monoclonal was a generous gift from Dr. Caroline Damsky (University of California, San Francisco, USA) [27]. AA6A rabbit polyclonal antibody, which was raised and purified by Bethyl Laboratories Inc. (Montgomery, TX, USA). AA6A is specific for the last 16 amino acids (CIHAQPSDKERLTSDA) of the human $\alpha 6A$ sequence [28], as done previously [29]. The urokinase B-chain was recognized by No. 3689 murine IgG1 antibody and the uPAR antibody was No. 399R rabbit polyclonal antibody (American Diagnostica Inc., Greenwich, CT, USA). Rabbit polyclonal- $\alpha 3$ integrin antibody (Chemicon, Temecula, CA, USA) was used and is specific for the carboxy terminal end of the molecule in the cytoplasmic domain. The monoclonal- $\beta 4$ integrin antibody (3E1) (Chemicon). Urokinase and urokinase recombinant amino-terminal fragment were purchased from Chemicon. Amiloride hydrochloride hydrate, and 4-Aminobenzamidine dihydrochloride, plasmin, and 4-Aminophenylmercuric acetate (APMA) were obtained from Sigma, St. Louis, MO, USA. Plasminogen Activator Inhibitor-1 was obtained from Calbiochem-Novabiochem Corporation, La Jolla, CA. YO-2 and YO-4 inhibitors [30] were donated by Dr. Yoshio Okada, Kobe Gakuin University, Japan.

Cell treatments

For inhibitor and blocking antibody experiments, DU145H cells were grown to approximately 40% confluency and the growth media were replaced with fresh media containing different concentrations of the inhibitors or 10 $\mu\text{g/ml}$ antibody or normal IgG of the same subclass. Fresh media with inhibitors/antibodies were replaced every day for a total of 3 days. For uPA and amino-terminal fragment (ATF) treatments, MCF10A and PC3N cells were plated for 3 days and were then treated with different concentrations of uPA and ATF in serum-free or serum-containing media for 90 min. For DU145H conditioned media treatments, serum-containing media from confluent DU145H cells were collected and filtered through 0.2 μm filter and applied to MCF10A cells overnight. In experiments with plasminogen and plasmin-depleted media, medium supplemented with serum was chromatographed on a column of α -lysineagarose that had been washed with 0.1 M NaCl, 1 mM EDTA, and 50 mM Tris-HCl, pH 7.5, as previously described [31]. The uPA activity was analyzed using the uPA activity assay kit (Chemicon).

Surface Biotinylation of cell lines

Biotinylation was similar to other protocols [32,33]. 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 MgCl_2 , 1.0 mM CaCl_2 , pH 7.45). To label the cell surface proteins, the cells were incubated for 30 min at 4°C with 2 ml of HEPES buffer supplemented with sulfosuccinimidyl hexanoate conjugated biotin (500 $\mu\text{g/ml}$) (NHS-LC-Biotin, Pierce, Rockford, IL, USA). They were then washed three times and lysed in cold RIPA 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) plus protease inhibitors (PMSF, 2 mM; leupeptin and aprotinin, 1 $\mu\text{g/ml}$). The lysate was briefly sonicated on ice before centrifugation at 14,000 RPM for 10 min, and the supernatant was collected for immunoprecipitations.

Immunoprecipitations

Cells were grown to confluency and then washed three times with HEPES buffer and lysed in cold RIPA buffer plus protease inhibitors (PMSF, 2 mM; leupeptin and aprotinin, 1 $\mu\text{g/ml}$). The lysate was briefly sonicated on ice before centrifugation at 14,000 RPM for 10 min, and the supernatant was collected for immunoprecipitations. Each reaction contained 200 μg of total protein lysate, 35 μl of protein G sepharose and 5 μg of antibody. The final volume of the lysate was adjusted to 500 μl with RIPA buffer. The mixture was rotated for 18 h at 4°C, and

then complexes were washed three times with cold RIPA and pellets were suspended in 2× non-reducing sample buffer. Samples were boiled for 5 min, and after a quick chill on ice, they were loaded onto 7.5% SDS-PAGE. Proteins resolved in the gel were electrotransferred to Millipore Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA), 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, Arlington Heights, IL, USA).

Cleavage of the $\alpha 6$ integrin in vitro

The $\alpha 6$ integrin was immunoprecipitated from biotinylated cells as described above and after the last wash with the RIPA buffer, the pellets were resuspended in 10 μ l of 1× buffer from the uPA activity assay kit (Chemicon) containing 1 μ g uPA, or 1 μ g uPA and 2.5 mM EDTA, or 0.5 μ g/Al APMA, or 0.5 μ g/ μ l APMA and 2.5 mM EDTA, or 4 μ g plasmin. The mixtures were incubated overnight on ice and then 10 μ l of 2× non-reducing sample buffer were added to each tube. Samples were boiled for 5 min and after a quick chill on ice, they were loaded onto 7.5% SDS-PAGE. Proteins resolved in the gel were electrotransferred to Millipore Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore), incubated with peroxidase-conjugated streptavidin and visualized by chemiluminescence (ECL Western Blotting Detection System, Amersham).

Zymography

Immunocomplexes were analyzed on gelatin and casein gels, Invitrogen Corporation (Carlsbad, CA). Proteolytic activity was analyzed as described by the manufacturer.

Human tissue studies

Snap frozen normal and cancerous tissues from human prostates were obtained, and approximately 125 mm³ of tissue was homogenized in RIPA buffer by the use of a polytron homogenizer. The samples were then centrifuged at approximately 14000 RPM at 4°C for 15 min. The supernatants were collected, sonicated, and immunoprecipitated using the J1B5 antibody and Western blotted using the AA6A antibody as described above.

Protein band quantification

Protein bands were quantitated using Scion Image Analysis software as previously described [34] and the data were graphed using Sigma Plot software.

Results

Significant abundance of $\alpha 6$ p on the cell surface of DU145H cells

To investigate the levels of $\alpha 6$ p on the cell surface, we compared the levels of $\alpha 6$ and $\alpha 6$ p integrin on the surface of DU145H cells. The cells were surface-biotinylated and $\alpha 6$ and $\alpha 6$ p were isolated by immunoprecipitations. The resulting data (Fig. 1A) showed that the ratio of $\alpha 6$ p to $\alpha 6$ was 0.8, indicating significant abundance of $\alpha 6$ p on the cell surface.

Modulation of uPA alters the production of $\alpha 6$ p integrin

In our studies to identify the protease involved in the cleavage of the $\alpha 6$ integrin, we used different inhibitors including MMP inhibitors, general serine protease inhibitors, and others (data not shown). We have used four different conditions to inhibit uPA and three different conditions to stimulate uPA to investigate the involvement of uPA in the production of $\alpha 6$ p. Amiloride is a uPA inhibitor [35], and aminobenzamidine is broad-spectrum serine proteinase inhibitor [36]. The amiloride treatments of 25, 50, and 250 μ M and the aminobenzamidine

treatments of 0.1, 0.5, 1.0, and 1.5 mM (Fig. 1B) reduced the levels of $\alpha 6p$ relative to the untreated DU145H cells. The uPA-blocking antibody (Fig. 1C) reduced the levels of $\alpha 6p$ relative to the untreated control and the 10 $\mu\text{g/ml}$ unrelated IgG of the same subclass. In addition, we have used different concentrations of PAI-1. PAI-1 completely abolished the levels of $\alpha 6p$ in DU145H cells (Fig. 2C). Even at the lower concentration of 2.15 $\mu\text{g/ml}$, PAI-1 completely abolished $\alpha 6p$ levels (data not shown). Taken together these data indicate that the inhibition of uPA activity inhibits $\alpha 6p$ production.

To further investigate the involvement of uPA in $\alpha 6p$ production, exogenous uPA was added to a cell line (MCF10A) that does not have $\alpha 6p$, to determine if production of $\alpha 6p$ was stimulated. The cells were treated with 1, 3, 5, and 20 μg uPA for 90 min in serum containing media (Fig. 2A, lanes 2–5). The addition of uPA to the cells induced a dose-dependent production of $\alpha 6p$.

Previous reports showed that DU145 cells contain high levels of uPA relative to normal cells [37], and in addition, these cells have no plasminogen activator inhibitor (PAI-1 or PAI-2) proteins present [38]. Therefore, we replaced the media from MCF10A cells with DU145H-conditioned media and incubated them overnight and observed production of $\alpha 6p$ in MCF10A cells (Fig. 2A, lane 7) indicating that uPA secreted by DU145H cells could cleave the $\alpha 6$ integrin in MCF10A cells.

Production of $\alpha 6p$ by uPA is plasmin-independent

Many of the effects of uPA are mediated by plasmin. For example, uPA-mediated plasminogen activation resulted in the proteolytic processing of the globular region of laminin 5 in oral keratinocytes [39]. We therefore investigated whether the uPA-induced cleavage of the $\alpha 6$ integrin is plasmin mediated. First, we incubated DU145H cells with different concentrations of $\alpha 2$ -antiplasmin (data not shown). Even at the highest dose which was not toxic to the cells (10 $\mu\text{g/ml}$), $\alpha 6p$ levels were not affected. Higher concentrations of $\alpha 2$ -antiplasmin (20 $\mu\text{g/ml}$) were toxic to the cells.

Then, we incubated MCF10A cells with purified uPA in the presence of serum-free (SF) media and observed that $\alpha 6p$ was produced (Fig. 2A, lane 6). Then DU145H cells were grown in plasminogen and plasmin-depleted media, and there was no reduction in the level of $\alpha 6p$ relative to the untreated control (Fig. 2B). In addition, we treated DU145H cells with $\alpha 2$ -antiplasmin or PAI-1 for 3 days and only PAI-1 abolished $\alpha 6p$ levels. Finally, we used chemical agents, YO-2 and YO-4, to determine if plasmin is involved in the $\alpha 6$ integrin processing. The YO-2 agent inhibits plasmin and uPA, whereas YO-4 inhibits plasmin alone and not uPA [30]. Only YO-2 reduced $\alpha 6p$ production (not shown), indicating that plasmin was not involved. Collectively, these data suggest that plasmin is not involved in the production of $\alpha 6p$.

Signaling through uPA receptor was not sufficient for $\alpha 6p$ production

Our next experiments investigated whether uPAR signaling was involved in $\alpha 6p$ production. There are several reports indicating that uPAR not only targets the uPA enzyme activity but that the uPA binding to uPAR also can initiate activation of signaling cascades [40–47]. Receptor-bound uPA does not become internalized and degraded rapidly unless associated with plasminogen activator inhibitor [48–51].

We first determined if uPAR associates with $\alpha 6$ or $\alpha 6p$ integrin since the amino acid sequences in the β -propeller domain involved in the $\alpha 6$ integrin-uPAR interaction have been identified [19]. Lysates from MCF10A and DU145H cells were immunoprecipitated with anti-uPAR, and the presence of $\alpha 6$ or $\alpha 6p$ was detected by Western blot analysis (Fig. 3A). As expected,

retrieval of uPAR retrieved the $\alpha 6$ integrin. The $\alpha 6$ p was not associated with uPAR from either cells induced to express $\alpha 6$ p or in cells containing constitutive expression of $\alpha 6$ p (Fig. 3A). This is in agreement with previous published results, which show that the uPAR-integrin α subunit interaction is mediated through the β -propeller region of the integrin α subunit (amino acid position 264–289) [19], and this region has been shown to be missing from $\alpha 6$ p [7].

Next, we used the amino-terminal fragment (ATF) of uPA, which has been shown to induce signaling through uPAR, to determine if the fragment alone was sufficient to induce $\alpha 6$ p production. We used different concentrations of ATF ranging from 10 to 400 nM and did not observe $\alpha 6$ p production (not shown). The highest dose (400 nM) of ATF did not produce $\alpha 6$ p in MCF10 A or PC3N cells, whereas purified uPA produced $\alpha 6$ p both in MCF10A and PC3N cells (Fig. 3B). Also, addition of uPA to MCF10A and PC3N cells did not induce a smaller variant of the $\alpha 3$ integrin, indicating that the $\alpha 6$ integrin cleavage by uPA is specific for $\alpha 6$ integrin (Fig. 3B). Moreover, addition of uPA did not result in cleavage of other integrins including $\alpha 2$, $\alpha 5$, and αV (data not shown).

Finally, we wanted to investigate the involvement of uPAR in $\alpha 6$ p production. We treated DU145H cells, twice daily, with ATF for 3 days to compete with uPA for binding to the receptor. We did not observe any effect on $\alpha 6$ p levels (data not shown). We also treated DU145H cells with *N*-acetyl-D-glucosamine, which was shown to inhibit the interaction of integrins with uPAR [52] and saw no effect on $\alpha 6$ p levels (data not shown). These results suggested that the $\alpha 6$ integrin-uPAR interaction was not important in the production of $\alpha 6$ p.

uPA cleaves the $\alpha 6$ integrin in vitro

We next determined whether uPA is able to cleave the $\alpha 6$ integrin directly in vitro or whether the cleavage of $\alpha 6$ integrin could be mediated by a contaminating matrix metalloproteinase (MMP). To distinguish between these possibilities, the MCF10A cell surface integrin was labeled by biotinylation, and resulting biotinylated $\alpha 6$ integrin retrieved by immunoprecipitation. The immunoprecipitated material was incubated with uPA, or other compounds for 1 h, and the resulting products analyzed by SDS-PAGE and detected via streptavidin-HRP (Fig. 4A). The addition of uPA to the immunocomplexes produced $\alpha 6$ p integrin in vitro. The presence of EDTA did not interfere with the cleavage of the $\alpha 6$ integrin by uPA suggesting that uPA does not activate a contaminating MMP in the immunocomplex. Addition of APMA, an MMP activator, to the immunoprecipitates did not result in production of $\alpha 6$ p indicating that MMPs are not involved in $\alpha 6$ p production. The addition of EDTA, as divalent cation chelator, is also known to reversibly dissociate the heterodimer into its individual subunits; this treatment was shown to not produce $\alpha 6$ p (not shown). It is interesting that plasmin, while capable of cleaving $\alpha 6$ in vitro, produced cleavage products that are different from those generated by uPA (Fig. 4B). These data show that while both uPA and plasmin can cleave $\alpha 6$ integrin in vitro, the products are distinct. The in vitro pattern of $\alpha 6$ integrin cleavage by uPA rather than plasmin matches the in vivo cleavage pattern of $\alpha 6$. Finally, to investigate if any other proteases were present in the immunocomplexes, we performed casein and gelatin zymography. Immunocomplexes containing $\alpha 6$ integrin, $\alpha 6\beta 4$ integrin or uPAR from different cell lines were analyzed. Co-precipitating protease activity was not detected in any sample (Figs. 4 C, D).

Phorbol ester induction of $\alpha 6$ p

It has been previously reported that 12-*o*-tetradecanoylphorbol-13-acetate (TPA) induces uPA activity in MCF10A cells [31]. We investigated whether TPA could induce $\alpha 6$ p production. An induction of $\alpha 6$ p relative to the vehicle controls was observed dependent upon different concentrations of TPA (Fig. 5A). No $\alpha 6$ p was detected in the untreated cells. In addition, to confirm that the production of $\alpha 6$ p was induced by uPA we used PAI-1 inhibitor and showed

that uPA was involved (Fig. 5B). We also used the plasmin inhibitor $\alpha 2$ -antiplasmin and found that the cleavage of the $\alpha 6$ integrin was plasmin independent (Fig. 5B). Finally, we analyzed the media from the cell treatments (described in Fig. 5B) for uPA activity (Fig. 5C). The levels of $\alpha 6$ p from Fig. 5B correspond to the uPA activity in Fig. 5C.

$\alpha 6$ p is present in prostate cancer tissue

Our results so far suggested that $\alpha 6$ p was produced via uPA in tissue culture cell lines and that TPA could induce $\alpha 6$ p production. Our next step was to determine whether $\alpha 6$ p was present in human tissue. Since $\alpha 6$ p was first identified in a prostate cancer cell line (DU145H), we obtained human normal and prostate cancer tissue specimens. The tissue was obtained from frozen tissue blocks, and the resulting lysates were immunoprecipitated with the $\alpha 6$ antibody (J1B5); and then a Western blot was performed using an anti- $\alpha 6$ antibody (AA6A) specific for the cytoplasmic domain. The full-length $\alpha 6$ integrin was detected in both the normal and the cancer tissue, while the $\alpha 6$ p form was present predominantly in the prostate cancer specimen (Fig. 6). These data suggest that $\alpha 6$ p can be found in human prostate cancer tissue specimens as well as in prostate cancer tissue culture cell lines.

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 [20,21]. In addition, we have shown that during prostate cancer progression, many integrin receptors, with the exception of $\alpha 3$ and $\alpha 6$, are not expressed [25]. Finally, we have shown that the $\alpha 6$ integrin exists in the classical form (140 kDa, non-reduced) and in a novel smaller form (70 kDa) called $\alpha 6$ p [7]. Our previous studies in DU145H cells suggested that the production of the $\alpha 6$ p variant involves a post-translational processing event at the cell surface [8].

Previous reports showed that DU145 cells contained five times the level of uPA compared to normal cells [37], and in addition, these cells had no PAI-1 or PAI-2 proteins present [38]. In addition, it has been shown that the $\alpha 6$ integrin interacts with the uPA receptor [52,53]. Combined, these observations led us to investigate the possible involvement of uPA in the production of the $\alpha 6$ p integrin variant.

We now have evidence indicating that the $\alpha 6$ p integrin variant is produced by direct cleavage by uPA. Inhibitors of uPA and a uPA-blocking function antibody reduced the levels of $\alpha 6$ p in DU145H cells suggesting that uPA is involved in the proteolytic cleavage of the $\alpha 6$ integrin. The addition of uPA to cell lines lacking $\alpha 6$ p induced $\alpha 6$ p production in a manner that was dose-dependent on uPA. This unique action of uPA is not dependent upon plasmin. While plasmin is capable of cleaving the $\alpha 6$ integrin in vitro, the cleavage products do not correspond to those generated by uPA in vitro or in vivo. In addition, the cleavage of the $\alpha 6$ integrin occurs independently of the signaling functions of uPAR or integrin uPAR interaction as indicated by the uPA amino-terminal-fragment (ATF) studies and the *N*-acetyl-D-glucosamine studies, respectively. Furthermore, while both the $\alpha 6$ and $\alpha 3$ integrin have amino acid binding sites for uPAR, only the $\alpha 6$ integrin is cleaved. This implies that some additional factor(s) independent of uPAR regulates the cleavage. Our experiments indicate that the production of $\alpha 6$ p is an inducible event dependent on uPA activity. Human breast epithelial cells (MCF10A) produce uPA after phorbol ester (TPA) stimulation [31], and our work indicates that TPA induces $\alpha 6$ p production in a uPA dependent manner. Of particular interest is that the event appears highly regulated since only a portion of the $\alpha 6$ integrin on the cell surface is cleaved. In addition, the inhibition of $\alpha 6$ p production does not increase the level of the $\alpha 6$ integrin. Current studies are to determine the regulatory features of the $\alpha 6$ p production.

Studies indicated that $\alpha 6p$ was present in human tissue and that it is not a tissue culture phenomenon. Since the $\alpha 6p$ form of the integrin is missing the β -propeller domain while maintaining the heterodimer on the surface, it would be of interest to localize the expression of $\alpha 6p$ on the cell surface in remodeling epithelial tissues. Our working hypothesis is that the $\alpha 6p$ form of the integrin would allow disattachment of the cell from the extracellular matrix while preserving the connections of the beta subunit to the cytoskeleton within the cell. Current work is underway to develop reagents that are specific for the $\alpha 6p$ form in tissue.

The exact site where uPA cleaves the $\alpha 6$ integrin resulting in the loss of the β -propeller domain is unknown. However, mass spectrometry analysis of the $\alpha 6p$ form [7] indicates that the NH_2 terminal end of the molecule contains at least the 'genu' region and a portion of an exposed loop in the thigh domain of the molecule while amino acid residues corresponding to the β -propeller and most of the "thigh" region are not detectable. We are currently investigating the interesting possibility that $\alpha 6$ integrin is cleaved within the exposed loop in the thigh region.

Urokinase-type plasminogen activator has been shown to cleave a variety of extracellular molecules either through plasminogen activation or directly. For example, it has been shown that uPA cleaves laminin 5 through plasminogen activation [39], and that it cleaves hepatocyte growth factor/scatter factor (HGF/SF) [11], macrophage-stimulating protein (MSP) [12], and fibronectin [10] directly. To our knowledge, this is the first report that uPA is involved in the cleavage of an integrin. The production of $\alpha 6p$ may be a crucial step during tissue remodeling or toward uPA-induced migration and invasion in cancer progression.

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Fig. 1.

Inhibitors and a blocking antibody of uPA reduce $\alpha 6p$ levels in DU145H cells (A) DU145H cells were surface biotinylated and $\alpha 6$ and $\alpha 6p$ were immunoprecipitated using the AA6A antibody. The samples were analyzed using streptavidin-HRP and the bands were quantified using the Scion Image software and the graph was plotted using Sigma Plot. (B) DU145H cells were treated with different concentrations of Amiloride or aminobenzamidine. The samples were analyzed for $\alpha 6$ and $\alpha 6p$ integrin levels using the AA6A rabbit polyclonal antibody and $\alpha 3$ integrin using a rabbit polyclonal antibody (Chemicon, Temecula, CA, USA). (C) DU145H cells were treated with a uPA blocking antibody or control IgG for 3 days. The samples were analyzed for $\alpha 6$ and $\alpha 6p$ integrin levels using the AA6A rabbit polyclonal antibody.

Fig. 2.

The production of α6p by addition of uPA in MCF10A cells is plasmin independent (A) MCF10A cells were cultured for 3 days and then treated for 90 min with different concentrations of purified uPA or DU145H conditioned media overnight. The samples were analyzed for α6 and α6p integrin levels using the AA6A rabbit polyclonal antibody. (B) DU145H cells were either untreated or treated with plasmin depleted media for 3 days. The samples were analyzed for α6 and α6p integrin levels using the AA6A rabbit polyclonal antibody. (C) DU145H cells were treated with α2-antiplasmin or PAI-1 for 3 days. The samples were analyzed for α6 and α6p integrin levels using the AA6A rabbit polyclonal antibody.

Fig. 3. uPAR associates with the $\alpha 6$ integrin but not $\alpha 6p$, and uPAR signaling is not sufficient to produce $\alpha 6p$ (A) DU145H and MCF10A cells were cultured for 3 days and immunoprecipitated with anti-uPAR antibody or with anti- $\alpha 6$ antibody J1B5. Samples were blotted for the $\alpha 6$ integrin using the AA6A-biotinylated antibody. (B) MCF10A and PC3N cells were cultured for 3 days and were then treated with 20 $\mu\text{g/ml}$ uPA or 400 nM ATF for 1.5 h. The samples were analyzed for $\alpha 6$ and $\alpha 6p$ integrin levels using the AA6A rabbit polyclonal antibody.

Fig. 4. uPA can cleave the $\alpha 6$ integrin directly in vitro (A and B) Surface biotinylated proteins from MCF10A cells were retrieved by immunoprecipitation using the AA6A antibody and the washed immunoprecipitates were either untreated or treated with the different compounds indicated. Samples were analyzed by SDS-PAGE, and blotted for HRP-streptavidin. (C and D) Whole cell lysates from different cell lines were immunoprecipitated with different antibodies and the immunocomplexes were run on gelatin gels (C) or casein gels (D) to detect proteolytic activity. The proteolytic activity of the controls is indicated by arrows.

Fig. 5.

TPA induces $\alpha 6$ p in MCF10A cells (A) MCF10A cells were cultured for four days and were then treated with different concentrations of TPA for 18 h. The samples were analyzed by SDS-PAGE and a Western blot was performed for the $\alpha 6$ integrin using the AA6A rabbit polyclonal antibody. (B) MCF10A cells were cultured for four days and were then treated with different concentrations of TPA or with TPA and different inhibitors for 18 h. The samples were analyzed by SDS-PAGE and a Western blot was performed for the $\alpha 6$ integrin using the AA6A rabbit polyclonal antibody. (C) Media from the samples from panel B was analyzed for uPA activity using the uPA activity assay kit (Chemicon International).

Fig. 6. $\alpha 6p$ is present in human prostate tissues. Normal and cancer human prostate tissues were analyzed for $\alpha 6$ and $\alpha 6p$ levels by immunoprecipitation with the J1B5 rat monoclonal antibody and Western blotting using the AA6A rabbit polyclonal antibody.