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INTEGRIN CLIPPING. A NOVEL ADHESION SWITCH?

by

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DEDICATION

This dissertation is dedicated to all the people whose lives have been affected by cancer. One of these people was my grandfather who passed away from lung cancer. Without him it would have been impossible for me to have accomplished what I have in my life today. It is my hope and expectation that the ongoing cancer research will result in the development of better diagnostic procedures and improved treatments that will lessen the suffering of cancer patients and their families.
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ABSTRACT

During human prostate cancer progression, the integrin α6β1 (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 α6 integrin called α6p. 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 α6p. We also showed that addition of uPA in the culture medium of cells that do not produce α6p, resulted in a dose dependent α6p production. In contrast, the addition of uPA did not result in the cleavage of other integrins. Using α2-antiplasmin and plasmin depleted medium, we observed that uPA cleaves the α6 integrin directly. Further, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) induced the production of α6p, and this induction was abolished by PAI-1 but not α2-antiplasmin. Using site directed mutagenesis we have identified the site of cleavage, and by comparing the sequence to the crystal structure of the αv integrin the cleavage site was shown to be located in an accessible loop that is upstream of the genu region. We have also shown that while a fraction of α6 integrin is normally associated with CD151, the α6p form is not associated with CD151. These data may indicate either that CD151 is released during α6p production or that CD151 association with α6 prevents the clipping of those molecules. In order to determine whether α6 integrin clipping occurs in tissue, we have found that
α6p is present in human prostate cancer tissue, in normal mouse epidermis, in mouse papillomas and squamous cell carcinomas induced by DMBA, TPA and MNNG treatments and in mouse melanomas induced by activated ras. Interestingly, subcutaneous injection into athymic nude mice of a malignant mouse keratinocyte derived cell line (6M90) that is α6p negative, results in the development of tumors that contain α6p integrin. Furthermore, we have shown that PC3N cells transfected with an uncleavable mutant of the α6 integrin grew smaller tumors when injected subcutaneously in SCID mice compared to wildtype α6 transfected cells. In addition, the tumors from the uncleavable mutant α6 transfected PC3N cells had higher levels of activated caspase 3 indicating higher levels of apoptosis. This finding suggests that the α6 integrin clipping is important for integrin signaling for survival. Collectively, all these data suggest that the cell surface clipping of the α6 integrin is a novel mechanism for altering integrin-laminin interactions during skin tissue remodeling and skin and prostate carcinogenesis.
I. INTRODUCTION

Prostate cancer epidemiology

Prostate cancer affects people all over the world but different ethnic populations and different countries show different rates of the disease [1]. North America and Scandinavia have the highest rates in the world, whereas in Asia the rates are the lowest [2]. In the United States alone, prostate cancer accounts for 33% of all newly diagnosed malignancies among men, and African-Americans have the highest rate of prostate cancer among the different US ethnic groups[2]. Prostate cancer is a disease that mostly affects elderly men. It is very uncommon for men younger than 50 years of age to be diagnosed with prostate cancer [1]. Approximately 85% of patients diagnosed with prostate cancer are older than the age of 65 [1]. Evidence suggests that most men that are older than 85 years old have histological prostate cancer [3].

There are many factors that could account for the differences in the rates of prostate cancer. For example, genetic factors, access and quality of health care, dietary factors, hormone factors and others [1]. It has been shown that prostate cancer patients with family history are diagnosed at an earlier age than those without family history [4]. In addition, it is estimated that 5-10% of all prostate cancer cases and about 40% of those occurring at earlier than the age of 55 may have a hereditary basis [4, 5]. The familial form of prostate cancer could be caused by inheritance of a susceptibility gene. Also exposure to the same environmental factor(s) could play an important role[1].
Dietary factors have also been implicated in prostate cancer. For example, although Japanese men have lower numbers of prostate cancer cases than US men, studies have shown that Japanese men who relocated to the United States increased their risk of prostate cancer to levels comparable to the ones in United States men [6-8]. Diets rich in red meat have been implicated in prostate cancer risk [9-11]. Others have suggested that it is the cooking and preparation, such as grilling, of the meat that is important for the disease [12]. Asian men, who show lower risk of prostate cancer than US men, consume dietary phyto-estrogens (found in soybean products) at high levels and these phyto-estrogens have been suggested to have a prophylactic effect on prostate cancer [13, 14]. Other dietary factors such as tomato-based products, and micronutrients such as selenium and vitamin E have also been associated with lower risk of prostate cancer (summarized in [1]).

Hormones have been shown to be implicated in prostate cancer. Castration of men before puberty or men with congenital abnormalities in androgen metabolism do not develop prostate cancer[15]. In addition, testosterone ablation is a well known treatment for prostate cancer.

A very important factor in prostate cancer treatment outcome is the early detection of the disease. The American Cancer Society recommends annual prostate cancer screening starting at the age of 50 [16]. The two most common methods for prostate examination are the digital rectal examination (DRE) and prostate-specific antigen (PSA) testing. Men with higher risk of prostate cancer such as African-Americans or those with first degree relatives that have prostate cancer should be
screened starting at the age of 45. If there are multiple first degree relatives with prostate cancer, then screening should start at the age of 40. If in any case there are any abnormal findings, definitive diagnosis should be done by needle biopsy.

In summary, prostate cancer is a major disease problem in the United States, especially for African-American men. There are several factors affecting the incidence of prostate cancer including genetic factors, access and quality of health care, dietary factors, hormone factors and others. Because of the complexity of the disease more research should be done in order to understand the development of the disease so that new diagnostic tools and new therapeutic approaches are developed.

**Alterations in prostate carcinoma progression**

There are several alterations that occur during prostate cancer progression. The contemporary model of prostate cancer progression suggests that genetic predisposition, oxidative damage and inflammatory changes are involved in the first stages of prostate cancer development termed proliferative inflammatory atrophy (PIA)[17]. Subsequently, downregulation of caretaker genes may lead to neoplastic transformation and the development of prostatic intraepithelial neoplasia (PIN) [17]. Loss of chromosomal regions and telomere shortening may then lead to localized cancer [17]. Finally, further genetic changes are associated with metastasis and androgen independence [17].

Genetic predisposition has been recognized in prostate cancer. For example, a study in prostate cancer families identified a prostate cancer susceptibility locus located on chromosome 1q24-25 and it was designated as hereditary prostate cancer 1 (HPC1)
Another susceptibility gene was later identified on chromosomal location 17p11 and it was termed HPC2/ELAC2 [19]. This gene has been shown to bind to tubulin and it might be involved in G2 cell cycle delay [20]. Also, in 2002 another candidate gene was identified on chromosome 8p called macrophage scavenger receptor 1 (MSR1) [21]. This gene encodes a transmembrane protein that functions as a receptor for a number of polyionic ligands including certain bacteria [22]. Moreover, BRCA2 mutation carriers have been shown to be at an increased risk of prostate cancer [23]. In addition to genetic predisposition, somatic gene alterations also have been identified in prostate cancer.

A homeobox gene termed NKX3.1 mapped on chromosomal region 8p21 has been suggested to be a prostate tumor suppressor [17]. A study showed that loss of 8p was identified in 80% of metastatic cancers [24]. In addition, loss of heterozygosity at chromosome 10q was associated with prostate cancer. The phosphate and tensin homologue (PTEN) gene located on chromosome 10q23 has been shown to be frequently mutated or deleted in prostate cell lines and prostate tumors [25]. The PTEN protein is a phosphatase that is active against protein and lipid substrates. Also, p27 expression has been shown to be reduced in early stage invasive prostate cancer [26]. This protein regulates the G1 to S cell cycle progression. Additionally, mutations in the retinoblastoma (Rb) gene located on chromosome 13q have been reported in 20-50% of prostate cancers [27]. Telomerase, the protein that is responsible for telomere lengthening, is frequently upregulated in prostate cancer [28]. Prostate stem cell antigen (PSCA) expression has been shown to be increased in more than 80% of prostate cancer specimens [29] and loss of heterozygocity of Kruppen-like factor 6 (KLF6) has been
observed in 77% of primary prostate tumors [30]. Accumulation of any of these genetic alterations and not just individual changes are involved in different prostate cancer stages, and understanding the development of these genetic alterations may help the development of diagnostic and therapeutic approaches for prostate cancer.

Another important player during prostate cancer progression is the androgen receptor (AR). The androgen receptor has been shown to be upregulated in 30% of hormone refractory prostate cancer patients [31]. In addition, mutations in the AR gene have been identified in 10-20% of prostate cancer specimens. These mutations seems to be higher in hormone refractory prostate cancers rather than untreated cancers [32-36]. Interestingly, DNA methylation of AR was higher in hormone refractory prostate cancer than from untreated primary tissues [37]. But, other ways to activate the receptor independent of androgens have been identified. For example, in cell lines AR was activated by growth factors such as the epidermal growth factor (EGF) and others [38].

During prostate cancer progression there are several changes in proteinase expression. For example, changes in matrix metalloproteinase (MMP) expression have been associated with prostate cancer progression. MMPs are zinc dependent endopeptidases whose substrates include different ECM molecules [39]. When tumor cells proliferate they secrete these proteases to degrade the basement membrane and the surrounding stroma [40]. It has been shown that primary human prostate tumors express higher levels of matrilysin and gelatinase A than normal prostate does [41]. It was later shown that matrilysin was expressed on epithelia in prostate carcinoma and that in normal prostate it was restricted to dilated ducts and atrophic glands [42]. Other studies
have shown that MMP-9 expression is higher in prostate cancer tissues compared to normal tissues [43]. In addition, it has been shown that prostate carcinoma cells expressing high levels of MT1-MMP, such as PC3N and PPC, demonstrated enhanced migration on human Ln-5-coated substrate, and this migration was inhibited using blocked antisense MT1-MMP oligonucleotides [44]. Recently, a study using a human ex vivo model has shown that MMP1, MMP7 and urokinase-type plasminogen activator (uPA) play an important role in the establishment of prostatic epithelial cells within bone marrow [45].

Another important protease system that is altered in prostate cancer is the urokinase-type plasminogen activator system. Urokinase-type plasminogen activator is a 54 kDa serine protease whose primary substrate is plasminogen [46]. The cleavage of plasminogen leads to the release of plasmin which is a serine protease with broad substrate specificity [47]. The most well studied functions of plasmin is to cleave fibrin polymers of the clot in thrombolysis [48, 49] and to degrade different extracellular matrix molecules [50, 51]. Plasmin has been shown to activate MMPs as well [52].

In addition to plasmin, uPA has also been shown to cleave other molecules directly. One of these molecules is the hepatocyte growth factor/scatter factor (HGF/SF) [53]. Another molecule is the macrophage-stimulating protein (MSP) [54].

Secretion of uPA is in the form of a single chain zymogen (pro-uPA) without any protease activity [47]. The pro-uPA binds to its receptor (uPAR) and is cleaved, primarily by plasmin, to a two chain polypeptide [48, 49, 55]. The A chain of uPA,
which is the N-terminal fragment, binds to uPAR; and the B chain, which is the C-terminal fragment, has the catalytic activity [47].

The receptor of uPA (uPAR) is a GPI-anchored protein [56, 57]. uPAR has been shown to interact with different β1 and β2 containing integrin heterodimers [58]. The reported uPAR binding site on the α6 integrin resides at positions 272-298 in domain IV of the molecule [59]. In addition, uPAR has been shown to be involved in many processes including signaling, adhesion and others (summarized in [60]). uPAR has been shown to be involved in cell adhesion indirectly through its interactions with integrins, but also directly by binding vitronectin [61-63].

There are inhibitors of the urokinase-type plasminogen system. These include the plasminogen activator inhibitors type 1 (PAI-1) and type 2 (PAI-2), and maspin [64-67]. The PAI-1 and PAI-2 inhibitors inhibit the uPAR bound uPA more efficiently than the soluble uPA, whereas maspin only inhibits the cell surface associated uPA and not the soluble uPA [64-67].

The serum levels of uPA or uPAR in prostate cancer patients can be used as a predictor of poor prognosis [47]. Although the serum levels of these molecules are elevated in patients with benign prostatic hyperplasia (BPH), they are only moderately elevated as compared to patients with prostate cancer [68-70]. In addition, the elevated serum levels of uPA and uPAR in prostate cancer patients also correlate with serum levels of prostate specific antigen (PSA) and prostate cancer metastasis [71, 72]. Finally, increased serum levels of uPA and uPAR inversely correlate with prostate cancer patient survival [69, 71].
Other events which occur early in prostate cancer are alterations of cell adhesion molecules [73, 74]. For example, during prostate cancer the adherens junction can be changed in different ways. One way is simply reduction of the levels of E-cadherin, or abnormal location of E-cadherin [75]. Other ways include mutation of E-cadherin, or proteolytic degradation of E-cadherin [75]. Also mutations in α-catenin, or abnormalities of β-catenin, or changes in β-catenin partners could affect adherens junction [75]. Changes also occur in other cell-ECM adhesion molecules, the integrins.

**Background on integrins**

Integrins compose a large family of transmembrane glycoproteins that are found in a wide range of animal species [76]. They are heterodimeric proteins with α and β subunits and each αβ combination has its own binding specificity [76]. In addition, each heterodimer has its own signaling properties [76]. Moreover, many integrins recognize several extracellular matrix (ECM) proteins [76]. A recent article reported that there at least eight different β subunits and eighteen α subunits that associate to form at least twenty-four different receptors [77]. Integrins generally contain a large extracellular domain (α subunit ~1000 residues, and β subunit ~750 residues), a transmembrane domain, and a short cytoplasmic domain (~50 residues or less) with the exception of β4, whose cytoplasmic domain is large (more than 1000 residues) [78, 79]. A current model suggests that the α subunit cytoplasmic domain inhibits certain functions of the β cytoplasmic domain. Binding of the extracellular domain to the ligand relieves this inhibition by allowing the two subunits to swing apart like a hinge [80] [81].
extracellular domains of integrins bind to the ECM or to counter receptors on other cells, whereas the cytoplasmic domains bind to cytoskeletal elements, therefore forming a transmembrane bridge between the ECM and the cytoskeleton [78].

Integrins are involved in many processes including cell migration, differentiation, blood clotting, tissue organization, and cell growth [78]. In addition to the well-known function of integrins in cell adhesion, they are also involved in signal transduction processes. Binding to the ECM triggers the so called ‘outside-in signaling’, whereas intracellular signaling can trigger Ecm binding ‘inside-out signaling’ [82]. Furthermore, integrins can mediate signals in two distinct ways. They can directly trigger signaling by clustering, during which adhesion to ECM is the only stimulus and this leads to activation of cytoplasmic kinases and lipid metabolism [83]. The second way of integrin signaling is ‘collaborative signaling’ during which integrins can modulate signaling events initiated through other types of receptors such as receptor tyrosine kinases [83].

Although integrins have been known to be involved in cell adhesion for a long time, it was fairly recently that they were found to be involved in signaling for cell growth and motility. Integrins can contribute to motility by modulating the affinity for ECM molecules by intracellular signaling (inside-out signaling) [82]. They can also contribute to motility by the ‘outside in’ signaling [82]. Finally, they can bridge the ECM with the actin cytoskeleton thus allowing the cells to exert force to the surrounding environment [82] and this force is important in cell motility [84].

A study on integrin subunit pairing showed that the pairing of the α and the β subunits is mapped on the amino-termini of the α and β subunits [85]. In this study
GPIIb-IIIa was bound to an affinity matrix and was treated with chymotrypsin. Two resulting fragments remained bound to the matrix and were identified as GPIIb and GPIIIa. In the presence of Ca and Mg the two fragments were maintained as a heterodimer. In addition, other sites of $\alpha\beta$ contacts are present in these molecules [86]. Such sites were summarized by [86] and include the propeller and hybrid domains ( $\alpha$ and $\beta$ subunits respectively), the calf-1 and EGF-3 ($\alpha$ and $\beta$ subunits respectively), and calf-2 and EGF4 ($\alpha$ and $\beta$ subunits respectively).

The $\alpha$ subunit of integrins is thought to be involved in the regulation of the integrin activation. More specifically, the GFFKR sequence, which is just a few amino acids after the transmembrane domain in the cytoplasmic site, is thought to maintain the inactive state of the integrin [87]. When this site is mutated, the integrin is constitutively active [88, 89]. In addition, other reports, present evidence that the $\alpha$ subunit cytoplasmic domain modulates proliferative signals occurring through the $\beta_{1A}$ integrin subunit [90].

The $\beta$ subunit of integrins has been shown to be involved in signaling. There are a variety of cytoskeletal proteins that interact with the $\beta$ subunit. These include $\alpha$ actinin, talin and others [91]. Regulatory and signal transducing proteins also interact with the $\beta$ subunit. Examples include Integrin Linked Kinase (ILK), $\beta_3$ endonexin and others [91]. Focal Adhesion Kinase (FAK) has been shown, in vitro, to bind to peptides derived from the cytoplasmic domains of $\beta_{1A}$, $\beta_2$ and $\beta_3$ integrin subunits [91]. In vivo studies failed to show a direct association of FAK with the integrin $\beta$ subunit, but
deletion of the carboxy terminal amino acids of the β integrin results in loss of localization of FAK to microbeads coated with anti-integrin antibodies [92].

There are a number of membrane-anchored proteins that can affect integrin function. For example CD98 is involved in regulation of ligand binding activity of integrins [93]. Another example is Integrin Associated Protein (IAP/CD47). IAP is involved in modulating integrin function in β2 mediated neutrophil migration [94, 95]. Another family of proteins, tetraspanins, are also involved in modulating integrin function [96].

Tetraspanins are membrane-spanning proteins that have been proposed to be involved in the organization of different proteins in the membrane. This organization is required so that these proteins can perform their functions [97]. In other words, tetraspanins have been proposed to be molecular facilitators, able to group cell surface proteins leading to the development of functional signaling complexes [97]. These proteins have four membrane spanning domains. Other domains include a small (20-28 aa) and a large (76-131 aa) extracellular loop, short intracellular amino (10-13 aa) and carboxy (7-15 aa) tails and a small (4 aa) cytoplasmic loop [97]. The functions that tetraspanins could be modulating include cell motility and metastasis, cell proliferation, cell morphology and possibly others [96]. Tetraspanins have been shown to be associated with a number of proteins including integrins. Studies showing that both integrins and tetraspanins regulate cell adhesion, tumor cell growth and metastasis suggest these associations are relevant [96]. Evidence suggests that tetraspanins associate with integrins via the extracellular domain of the α subunit of the integrin [96].
Experiments showed that deletion or exchange of the integrin α chain cytoplasmic or transmembrane domains did not result in loss of integrin-tetraspanin associations [96]. Some members of the tetraspanin family, CD9, CD53, CD63, CD81, CD82, and CD151, have been shown to be associated with the α6β1 integrin [98].

**The integrin α6 in prostate cancer progression**

The α6 integrin pairs with either the β4 or the β1 subunit but in cells that produce α6, β1 and β4, the dominant heterodimer is α6β4 [99]. Both of the α6 integrin heterodimers are laminin receptors [99]. The two possible α6 heterodimer pairs form two functionally distinct structures. The α6β4 heterodimer is part of a stable adhesion structure called hemidesmosome which forms a connection between the ECM (laminin-5) and the cytoskeleton (intermediate filaments). Hemidesmosomes are found in most epithelia but mainly in stratified epithelia. Mice that are deficient in α6 or β4 die shortly after birth (summarized in [100]). Furthermore, mutations in the α6 or β4 subunits result in a group of inherited skin and digestive tract disorders called epidermolysis bullosa [101, 102]. The α6β1 heterodimer is not part of the hemidesmosomes but it is part of focal adhesions and is found in several types of tissues including prostate. Focal adhesions are sites of cell adhesion to the ECM where bundles of actin filaments are bound to integrins through a multimolecular complex of junctional plaque proteins [103]. Focal adhesions come in different forms and are thought to be important not only in
adhesion but also in cell signaling [103]. The α6β1 integrin is widely expressed and is involved in many biological phenomena [99, 104].

The prostate glands are composed of basal cells and luminal cells. The normal basal cells express various integrins including the α6β4 heterodimer. During prostate cancer progression, a lot of the integrins are downregulated with the exception of the α6 and the α3 subunits [105-107].

Previous studies indicated that the α6β4 integrin is downregulated in prostate carcinoma [106-109]. Also, we have shown that the β4 integrin and its ligand, laminin 5, are present both in normal prostate glands and prostatic intraepithelial neoplasia (PIN) lesions [110]. Both β4 and laminin 5 were absent from prostate carcinomas [110]. The alpha 6 integrin persists in cancer tissues but it is associated with the β1 subunit and it is still fully functional in binding to laminin [107]. This could probably be explained because, although the α6 preferentially associates with the β4 subunit, when β4 levels are reduced, α6 associates with β1 [111]. Other experiments have also shown that the alpha6 integrin is important in prostate cancer. Experiments showed that DU145 prostate cancer cells selected for high α6 integrin expression exhibited significant increase in migration on laminin and increased invasion in a SCID mouse model [112]. Furthermore, a different study showed increased expression of α6β1 in metastatic clones of the prostatic epithelial cell line, NbE [113].
The α6p integrin

Our laboratory has recently shown that another form of the α6 integrin exists [114]. This is a structural variant of the α6 integrin called α6p that is missing the extracellular β-propeller domain. Integrin α6p is a 70 kDa form of the α6 integrin which has a three-fold increase in biological half-life as compared to the full length α6 integrin. This indicates that it is unlikely for α6p to be a degradation product [114, 115]. A previous study failed to identify an alternative transcript and it showed that the α6p integrin variant was found to be able to pair with either the β1 or β4 subunits [114]. In addition, using endoglycosidase treatments it was shown that α6p is produced while on the cell surface [115]. These pieces of evidence suggested that α6p was produced by proteolytic processing while on the cell surface.

Statement of the problem

As described previously in the introduction there are many integrin alterations in prostate cancer progression. It has not yet been fully explained what accounts for these changes nor what the complete effect of these changes has in prostate cancer progression. The following studies aimed to further investigate the production, the regulation and the functional role of the smaller variant of the α6 integrin (α6p). A better understanding of the role of the α6p variant in prostate carcinoma may lead to the development of new methods of diagnosis and therapy.
II. MATERIALS AND METHODS

Antibodies and chemicals used in this study

The rat monoclonal anti-α6 integrin antibody J1B5 was a generous gift from Dr. Caroline Damsky, (University of California San Francisco, USA)[116]. The AA6A rabbit polyclonal antibody was raised and purified by Bethyl Laboratories Inc. (Montgomery, TX, USA). AA6A is specific for the last 16 amino acids (CIHAQPSDKERLTSDA) of the human α6A sequence [117], as done previously[118]. The urokinase B-chain was recognized by No. 3689 murine IgG1 antibody and the uPAR antibody used was No. 399R rabbit polyclonal antibody (American Diagnostica Inc.: Greenwich, CT, USA). The rabbit polyclonal-α3 integrin antibody (Chemicon: Temecula, CA, USA) was used and is specific for the carboxy terminal end of the molecule, located in the cytoplasmic domain. The monoclonal-β4 integrin antibody (3E1) was used (Chemicon: Temecula, CA, USA). The anti-CD151 antibody 5C11 was a generous gift from Dr. Martin Hemler (Dana-Farber Cancer Institute, USA), and the anti-α6 integrin antibody A33 was a generous gift from Dr. Arnoud Sonnenberg (The Netherlands Cancer Institute, The Netherlands). Urokinase and urokinase recombinant amino-terminal fragment were purchased from Chemicon: Temecula, CA, USA. FAK was detected using the 4.47 mouse monoclonal antibody (Upstate Biotechnology Inc, Waltham, MA). 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 were donated by Dr. Yoshio Okada, Kobe Gakuin University, Japan [119].

**Cell treatments**

For inhibitor and blocking antibody experiments, DU145H cells were grown to approximately 40% confluency and the growth medium was replaced with fresh medium containing different concentrations of the inhibitors or 10μg/ml antibody or normal IgG of the same subclass. Fresh medium 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 medium for 90 minutes. For DU145H conditioned media treatments, serum containing media from confluent DU145H cells were collected and filtered through 0.2μm filter and applied to MCF10A cells overnight. In experiments with plasminogen and plasmin-depleted medium, medium supplemented with serum was chromatographed on a column of L-lysine-agarose that had been washed with 0.1M NaCl, 1mM EDTA, and 50mM Tris-HCl, pH7.5 as previously described [120].

**Biotin-labeling of tissue culture cell surface proteins:**

Cells were grown to confluence in 100 mm tissue culture dishes and washed 3 times with HEPES buffer (20 mM HEPES, 130 mM NaCl, 5 mM KCl, 0.8 mM MgCl₂, 1.0 mM CaCl₂, pH 7.45). They were then incubated with 2 ml of HEPES buffer
supplemented with 500 μg/ml Sulfosuccinimidyl hexanoate conjugated biotin (NHS-LC-Biotin, Pierce, Rockford, IL, USA) to label cell surface proteins for 30 min at 4°C. The cells were then washed three times with HEPES buffer 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 μg/ml). The lysate was then sonicated on ice and it was used for immunoprecipitation analyses.

**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 μg/ml). The lysate was briefly sonicated on ice before centrifugation at 14,000 RPM for 10 minutes, and the supernatant was collected for immunoprecipitations. Each reaction contained 200 μg of total protein lysate, 35 μl of protein G sepharose and 5μg of antibody. The final volume of the lysate was adjusted to 500 μl with RIPA buffer. The mixture was rotated for 18 hours at 4 °C, and then complexes were washed three times with cold RIPA and pellets were suspended in 2X non-reducing sample buffer. Samples were boiled for 5 minutes, 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).

**Immunofluorescence**

PC3N cells transfected with α6p were either treated or not treated with doxycycline for 24 hours. The cells were analyzed for α6p expression using the anti-myc-FITC conjugated antibody (Invitrogen Corporation, Carlsbad, CA) according to the manufacturers specifications.

**Cleavage of the α6 integrin in vitro**

The α6 integrin was immunoprecipitated from biotinylated cells as described above and after the last wash with the RIPA buffer, the pellets were resuspended in 10ul of 1X buffer from the uPA activity assay kit (Chemicon: Temecula, CA, USA) containing 1ug uPA, or 1ug uPA and 2.5mM EDTA, or 0.5ug/ul APMA, or 0.5ug/ul APMA and 2.5mM EDTA, or 4ug plasmin. The mixtures were incubated overnight on ice and then 10ul of 2X non-reducing sample buffer were added to each tube. Samples were boiled for 5 minutes 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 peroxidase-conjugated streptavidin and visualized by chemiluminescence (ECL Western Blotting Detection System, Amersham, Arlington Heights, IL, USA).
Western Blot Analysis

Samples were boiled for 5 minutes and then loaded onto a 7.5% SDS-polyacrylamide gel for analysis under non-reducing or reducing conditions. Proteins resolved in the gel were electrotransferred to Millipore Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore: Bedford, MA, USA). The blots were blocked for 1 hour and then were incubated with the different antibodies overnight at 4°C. The blots were washed and then incubated with secondary antibody conjugated to horseradish peroxidase. Proteins were visualized by chemiluminescence (ECL Western Blotting Detection System, Amersham, Arlington Heights, IL, USA) and the blot was exposed to Kodak X-OMAT film (Eastman Kodak Company, Rochester, NY, USA).

Zymography

Immunocomplexes were run 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 125mm³ of tissue was homogenized in RIPA buffer by the use of a polytron homogenizer. The samples were then centrifuged at approximately 14000RPM at 4°C for 15 minutes. 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 [121] and the data were graphed using Sigma Plot software.

Preparation of laminin 5 matrix

The laminin 5 matrix was prepared similar to a method described previously [122]. Briefly, MCF10A cells were grown in dishes for 3 days until confluent. They were then treated with 20mM ammonium hydroxide for 5 minutes (or until cells were lifted off) and then washed 3 times with water and 3 times with PBS. The cells were then plated on the matrix for different experiments.

XTT Cell Proliferation Assay:

The metabolic survival assay using sodium 3’-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) labeling reagent (XTT) (obtained from Roche Molecular Chemicals, Germany) was used. PC3N cells were seeded overnight at a density of 1500 cells (for 3 days culture), 3000 cells (for 2 days culture) and 6000 cells (for 1 day culture) per well in a 96 well plate. The XTT labeling reagent and electron coupling reagent were freshly prepared, added to wells according to the manufacturer’s directions and incubated for 4 hours in incubator. Absorbance was read at 490 nm using Elx800 microplate reader (Bio-Tek Instruments,
Winooski, VT, USA). The experiment was done in triplicate wells and were averaged for each treatment group.

**Mouse tissue analyses**

A single initiating dose of 100 µg DMBA (Sigma, St. Louis, MO) in 0.2 ml of acetone was applied topically to the shaved backs of female ICR mice (Harlan Sprague Dawley, Indianapolis, IN). It was followed by bi-weekly application of 5 nM TPA (Alexis Corp., San Diego, CA) in 0.2 ml of acetone for 21 weeks. At week 22, TPA applications were stopped and replaced with weekly application of 120 µg MNNG in 0.2 ml of acetone for a total of 40 weeks. For normal tissue, two of the untreated mice were shaved, and the whole skin was removed and frozen in liquid nitrogen. Another two untreated mice were shaved and the remaining hair was removed by hair removing cream. The skin was then removed, stretched out, and frozen in liquid nitrogen, and the epidermis was scraped and collected. The tumors were collected from the treated mice and were frozen in liquid nitrogen. The samples were homogenized by mortar and pestle and lysed in RIPA buffer. The samples were centrifuged at 14000RPM at 4°C for 15 minutes and the supernatants collected and sonicated. The resulting sample (50ug) was analyzed on a 7.5% SDS-PAGE gel, and a Western blot was performed using the AA6A antibody as described above.

**Cell lines and culture conditions**
All human cell lines were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The PC3 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco BRL: Gaithersburg, MD, USA) plus 10% FBS. The DU145H, and PC3-N cells were grown in Iscove’s Modified Dulbecco’s Medium (IMDM) (Gibco BRL: Gaithersburg, MD, USA) plus 10% fetal bovine serum (FBS). The DU145H cells were isolated by us as previously described [112, 123] and contain only the α6A splice variant [124]. The PC3N cells were isolated as a variant of PC3 prostate carcinoma cell line [125]. The MCF10A cells were grown in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F12 (DMEM-F12) (Gibco BRL: Gaithersburg, MD, USA) plus 5% horse serum, 20ng/ml epidermal growth factor, 100ng/ml cholera toxin, and 500ng/ml hydrocortisone. The MCF-10A (human breast cells) cell line was obtained from the American Type Culture Collection (Rockville, MD, USA).

**Time lapse video migration for α6 integrin mutants**

To produce a laminin 5 matrix, MCF10A cells were grown on glass Delta T dishes (0.15 mm; Bioptechs, Inc. Butler, PA) for 3 days until confluent. They were then treated with 20mM ammonium hydroxide for 5 minutes (or until cells were lifted off) and then washed 3 times with water and 3 times with PBS. The PC3N transfected cells were treated with 0.2ug/ml Doxycycline the previous day and the next day were lifted with 5mM EDTA in PBS and then resuspended in Dulbecco's modified Eagle's media containing 0.1%BSA and 50ug/ml uPA and incubated for 3 hours at 37C. They were washed with PBS and then incubated with 10ug per ml P1B5 blocking antibody in
Dulbecco's modified Eagle's medium containing 0.1%BSA, for 20 minutes at 37°C. The cells were plated at 70,000/dish in Dulbecco's modified Eagle's medium containing 0.1%BSA at 37 °C for 1 hour. Then they were transferred to the microscope and time lapse video microscopy was initiated. Images were captured using a grayscale CCD camera (ORCA-100, Hamamatsu, Japan) mounted on an inverted Olympus IMT2 microscope (Olympus America, Melville, NY) equipped with a BiopTechs Delta T live cell system (Bioptechs, Inc. Butler, PA) under a humidified (5% CO₂ balanced with ultrazero air) atmosphere. Data analysis was performed using SimplePCI 4.0 software (Compix Imaging Inc., Cranberry Township, PA).

**Time lapse video migration for the effect of the amino-terminal fragment of α6**

To produce a laminin 5 matrix, MCF10A cells were grown on glass Delta T dishes (0.15 mm; Bioptechs, Inc. Butler, PA) for 3 days until confluent. The cells were treated or untreated with 50µg/ml uPA for 3 hours at 37°C while on the plate. They were then treated with 20mM ammonium hydroxide for 5 minutes (or until cells were lifted off) and then washed 3 times with water and 3 times with PBS. After lifting the cells with EDTA solution PC3N cells were plated at 70,000/dish in Dulbecco's modified Eagle's medium containing 0.1%BSA at 37 °C for 1 hour. Then they were transferred to the microscope and the time lapse video started. Images were captured using a grayscale CCD camera (ORCA-100, Hamamatsu, Japan) mounted on an inverted Olympus IMT2 microscope (Olympus America, Melville, NY) equipped with a BiopTechs Delta T live cell system (Bioptechs, Inc. Butler, PA) under a humidified (5% CO₂ balanced with
ultrazero air) atmosphere. Data analysis was performed using SimplePCI 4.0 software (Compix Imaging Inc., Cranberry Township, PA).

**Cloning and transfections of α6p**

The cDNA for wildtype α6A was a generous gift from Dr. Isaac Rabinovitz. In order to clone α6p in the pcDNA3.1+ vector, PCR amplification was utilized using Platimun Pfx DNA polymerase (Invitrogen Corporation, Carlsbad, California). The primers used contained KpnI and ApaI sites: 5’ AAGGTACCCCGAGTGAATTCACTTCCA3’ and 5’ AAGGGCCCTTATGCATCAGAAGTAAGCC 3’. The PCR product was eluted from the gel and cut using KpnI and ApaI enzymes and was run on a gel and then eluted from the gel. In order to add a signal peptide to send the molecule to the surface and to be able to detect the transfected α6p we created overlapping and complementary oligos that would encode for the first 28 aminoacids of the α6 integrin and 10 aminoacids that encode for a myc tag. The 4 oligos used were (SIG 1-4):

SIG1
5’GATCATGGCCGCGCGCCGAGCTGTGCTTGGCTCTACCTGTCGGCGGGGCTCCTGTCCCGGCTCGGCGCAGC 3’,

SIG2
5’ CTTCAACTTGGACACTGAACAAAAACTCATCTCAGAAGAGGATCTGGTAC 3’

SIG3
5'CAGATCCTCTTCTGAGATGAGTTTTTGTTCAGTGTCCAAGTTGAAGGCTGCGCCGAGCCGGGACAG3'

SIG4
5'GAGCCCCGCCGACAGGTAGAGCAAGCACAGCTGCCCGGCGGCAGCCAT3'

In order to ligate the signal peptide oligos, 10μM of each oligo was added in a tube. Then the mixture was incubated for 5 min at 94°C, followed by 5 min at 85°C, 5 min at 75°C, 5 min at 65°C, 5 min at 55°C, 5 min at 45°C, 5 min at 35°C, and 5 min at 25°C. The sample was run on an agarose gel and then eluted to be used in ligations. The pcDNA3.1+ vector was cut using BamHI and ApaI restriction enzymes and it was run on an agarose gel and eluted. The cut pcDNA3.1+ vector and the cut PCR product along with the ligated oligos were mixed together for ligation using T4 DNA ligase (Invitrogen Corporation, Carlsbad, California) and were incubated at 14°C overnight. One ul of the ligation was used in DH5α bacteria transformation. Then different clones were screened by sequencing. In order to clone the α6p construct in the pcDNA4/TO vector the BamHI and ApaI sites were used. Plasmid DNA was purified using the QIAGEN plasmid midi kit (QIAGEN, Valencia California). The cells were transfected using Effectene (QIAGEN, Valencia California) according to the manufacturers protocol.

Site-directed mutagenesis

The cDNA for wildtype α6A was a generous gift from Dr. Isaac Rabinovitz. After cloning it into the pcDNA4/TO vector we used different sets of primers to induce
the desired mutations in the α6 integrin. We used QuikChange Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek Texas). The primers were designed using the recommendations of the manufacturer and were:

Primers for RR→AA mutation
5'GGAGATCCAAGAGCCAAGCTCTGCTGCGAGTGAATTCACTTCCAGAAG 3'
5'CTTCTGGAAGTGAATTCACTCGAGCAGCAGAGGCTTGCTTTGGATCTCC 3'

Primers for RV→AA mutation
5'GCCAAGAGCCAGCTCTCGAGGCGAGCAGAATTCAGTCAGAGCTTGGCTCTTGGATCTCC 3'
5'GGAAGAATCTCTGGAATTCTCTGCTGCCCTACGAGAGCTTGGCTTTGGC3'

Primers for R536→A mutation
5'AAAATCTGAGGCTATCTCCAGCACTCTTCAGTTTCCGAAACCAAG3'
5'CTTGGTTTTCGAAACTGAGACTGCTGTAGGATAGCCAGATT3'

Primers for R594→A mutation
5'GATCCAAGAGCCAGCTCTGCAAGGCAGGCTGAATTCACTTC3'
5'GAAGTGAATTCAGTCCTGGCTTTGCTGGATC3'
Primers for R595— A mutation

5'GAGCCAAGCTCTCGTGCGAGTGAATTCACTTCC3'
5'GGAAGTGAATTC ACTCGCGC ACGAGAGCTTGGCTC3'

The reactions were carried out as recommended by the manufacturer. The K562 cells were transfected by electroporation as described previously [126]. Briefly, 30ug of plasmid and 10ug of repressor plasmid (pcDNA6/TR) were added to 10 million cells in a final volume of 800ul of serum free RPMI media in a 0.4cm electroporation cuvette. The cells were electroporated at 960 microfarads and 320 V. After 10 minutes on ice the cells were plated in IMDM medium with 10% serum for 24 hours. The medium was replaced with fresh medium containing 0.2ug/ml doxycycline and the cells were incubated for another 24 hours. The cells were then analyzed for α6 integrin cleavage by immunoprecipitating the α6 integrin with J1B5 and adding 2ug of uPA in the immunocomplexes for 3 hours on ice. The samples were then run on a gel and immunoblotted using the anti-α6 integrin rabbit polyclonal antibody AA6A.

The PC3N cells were transfected using Effectene (QIAGEN, Valencia California) following the recommendations of the manufacturer.

**Cloning and expression of the amino-terminal fragment of the α6 integrin**

In order to clone the amino-terminal fragment of α6 in the pProEX vector, PCR amplification was performed using Platimun Pfx DNA polymerase (Invitrogen Corporation, Carlsbad, California). The first 500 amino-acids excluding the signal
peptide were amplified. The primers used contained BamHI and SalI sites: 5' AAGTCGACCCTATTTAACCTGG3' and 5' AAGGATCCTTCAACTTGGACAC3'. The PCR product was eluted from the gel and cut using BamHI and SalI enzymes and was then run on a gel. The sample was eluted from the gel and was used in ligations. The pProEX vector was cut using BamHI and SalI restriction enzymes and it was run on an agarose gel and eluted. The cut pProEX vector and the cut PCR product were mixed together for ligation using T4 DNA ligase (Invitrogen Corporation, Carlsbad, California) and were incubated at 14C overnight. One ul of the ligation was used in DH5α bacteria transformation. DNA was extracted from different clones and was screened by sequencing. Once a correct sequence was identified, the plasmid was used for BL21 bacterial transformation. The protein was expressed using the Probond Purification System (Invitrogen Corporation, Carlsbad, California) and purified according to the procedure recommended by the manufacturer.

**Mouse tumor growth studies**

PC3N cells or PC3N cells transfected with different α6 integrin constructs were subcutaneously injected in male SCID mice. As soon as measurable tumors developed, doxycycline diet begun. Tumors were measured every three to four days and the data were collected and graphed using scion image or Excel.
III. EXTRACELLULAR ALPHA 6 INTEGRIN CLEAVAGE BY UROKINASE-TYPE PLASMINOGEN ACTIVATOR IN HUMAN PROSTATE CANCER

Introduction

Integrins are heterodimeric proteins with α and β subunits, and each αβ combination has its own binding specificity and signaling properties [127, 128]. Integrins recognize several extracellular matrix (ECM) proteins and sense the extracellular matrix environment [127, 128]. 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 ~1000 residues, and β subunit ~750 residues), a transmembrane domain and a short cytoplasmic domain (~50 residues or less) with the exception of β4, whose cytoplasmic domain is large (more than 1000 residues) [129, 130].

The structure of the extracellular segment of the alpha subunit has been determined using protein crystals of a soluble αvβ3 integrin fragment [131]. The NH₂ 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 [132]. The tail region can fold back at ~135° angle forming a V-shaped structure with a “genu” between the thigh domain and the calf module [132]. The profound bending suggests that a highly
flexible site, the genu, exists in the integrin alpha subunit and results in a flexible 700Å accessible surface region [132].

We have previously identified a structural variant of the α6 integrin called α6p that is missing the extracellular β-propeller domain associated with ligand binding [114]. Integrin α6p is a 70 kDa form, and mass spectrometry analysis showed that the NH₂ terminal end of the molecule contains at least amino acids starting at arginine 595 [114]. The analysis also showed that the rest of the carboxy-terminus of α6p was identical with the full length α6 integrin[114]. Using a multiple sequence alignment tool, this position in α6 integrin lies within an accessible loop described for the αV integrin subunit[131]. The α6p variant is produced while on the cell surface, remains paired with either the β1 or β4 subunit and has a three-fold increase in biological half-life as compared to the full-length α6 integrin. [115]. The extracellular surface expression of the α6 integrin prior to 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 [46]. In addition, uPA has been shown to catalyze the proteolytic cleavage of the extracellular matrix protein fibronectin [133], hepatocyte growth factor/scatter factor (HGF/SF) [53] and macrophage-stimulating protein (MSP) [54]. 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 Xlla, and cathepsin B [134], into its two-chain active form [48, 49, 55]. 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[135, 136]).

Recently, a consensus site for urokinase binding has been identified [137]. This site was designated to be BXXSSXXB where X represents any aminoacid and B represents basic aminoacids [137]. This site was found in many proteins including some integrins [137]. After looking in the sequence of the α6 integrin we found a putative site starting at Q589 (QEPSSRRR) of the α6 integrin.

The present study was prompted by the report of a binding site for uPAR on the α6 integrin in the extracellular β-propeller region [59], the putative uPA binding site on the α6 integrin, and the loss of the extracellular β-propeller region during the α6 to α6p conversion [114]. In addition, the knowledge that both uPA activity and the α6 integrin persist in invasive cancer led us to determine if the α6p conversion via uPA was found in invasive cancer [112, 138-141].

**Results**

**Significant abundance of α6p on the cell surface of DU145H cells**

In order to investigate the levels of α6p on the cell surface, we compared the levels of α6 and α6p integrin on the surface of DU145H cells. The cells were surface biotinylated and α6 and α6p were isolated by immunoprecipitations. The resulting data (Figure 1A) showed that the ratio of α6p to α6 was 0.8, indicating significant abundance of α6p on the cell surface.
Modulation of uPA alters the production of \( \alpha 6p \) 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 \( \alpha 6p \). Amiloride is a uPA inhibitor [142], and aminobenzamidine is broad-spectrum serine proteinase inhibitor [143]. The amiloride treatments of 25, 50, and 250 uM and the aminobenzamidine treatments of 0.1, 0.5, 1.0, and 1.5 mM (Figure 1B) reduced the levels of \( \alpha 6p \) relative to the untreated DU145H cells. The uPA blocking antibody (Figure 1C) reduced the levels of \( \alpha 6p \) relative to the untreated control and the 10ug/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 (Figure 2C). Even at the lower concentration of 2.15ug/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 1ug, 3ug, 5ug, 20ug uPA for 90 minutes in serum containing media (Figure 2A, lanes 2-5). The addition of uPA to the cells induced a dose dependent production of \( \alpha 6p \).
Figure 1: Inhibitors and a blocking antibody of uPA reduce α6p levels in DU145H cells. (A) DU145H cells were surface biotinylated and α6 and α6p 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 α6 and α6p integrin levels using the AA6A rabbit polyclonal antibody. (C) DU145H cells were treated with a uPA blocking antibody or control IgG for 3 days. The samples were analyzed for α6 and α6p integrin levels using the AA6A rabbit polyclonal antibody.
Previous reports showed that DU145 cells contain high levels of uPA relative to normal cells [144], and in addition, these cells have no plasminogen activator inhibitor (PAI-1 or PAI-2) proteins present [145]. Therefore, we replaced the media from MCF10A cells with DU145H conditioned media and incubated them overnight and observed production of α6p in MCF10A cells (Figure 2A, lane 7) indicating that uPA secreted by DU145H cells could cleave the α6 integrin in MCF10A cells.

**Production of α6p 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 [146]. We therefore investigated whether the uPA-induced cleavage of the α6 integrin is plasmin mediated. First, we incubated DU145H cells with different concentrations of α2-antiplasmin (data not shown). Even at the highest dose which was not toxic to the cells (10μg/ml), α6p levels were not affected (Figure 2C). Higher concentrations of α2-antiplasmin (20μg/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 α6p was produced (Figure 2A, lane 6). Then DU145H cells were grown in plasminogen and plasmin-depleted media, and there was no reduction in the level of α6p relative to the untreated control (Figure 2B). In addition, we treated DU145H cells with α2-antiplasmin or PAI-1 for 3 days and only PAI-1
Figure 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 minutes with different concentrations of purified uPA or DU145H conditioned medium 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 three 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 three days. The samples were analyzed for α6 and α6p integrin levels using the AA6A rabbit polyclonal antibody.
abolished α6p levels. Finally, we used chemical agents, YO-2 and YO-4, to determine if plasmin is involved in the α6 integrin processing. The YO-2 agent inhibits plasmin and uPA, whereas YO-4 inhibits plasmin alone and not uPA [119]. Only YO-2 reduced α6p production (not shown) indicating that plasmin was not involved. Collectively, these data suggest that plasmin is not involved in the production of α6p.

**Signaling through uPA receptor was not sufficient for α6p production**

Our next experiments investigated whether uPAR signaling was involved in α6p 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 [147-154]. Receptor bound uPA does not become internalized and degraded rapidly unless associated with plasminogen activator inhibitor [155-158].

We first determined if uPAR associates with α6 or α6p integrin since the amino acid sequences in the β-propeller domain involved in the α6 integrin-uPAR interaction have been identified [59]. Lysates from MCF10A and DU145H cells were immunoprecipitated with anti-uPAR, and the presence of α6 or α6p was detected by western blot analysis (Figure 3A). As expected, retrieval of uPAR retrieved the α6 integrin. The α6p was not associated with uPAR from either cells induced to express α6p or in cells containing constitutive expression of α6p (Figure 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) [59], and this region has been shown to be missing from α6p [114]. 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 α6p production. We used different concentrations of ATF ranging from 10 to 400nM and did not observe α6p production (not shown). The highest dose (400nM) of ATF did not produce α6p in MCF10A or PC3N cells; whereas purified uPA produced α6p both in MCF10A and PC3N cells (Figure 3B). Also, addition of uPA to MCF10A and PC3N cells did not induce a smaller variant of the α3 integrin, indicating that the α6 integrin cleavage by uPA is specific for α6 integrin (Figure 3B). Moreover, addition of uPA did not result in cleavage of other integrins including α2, α5, and αV (data not shown).

Finally, we wanted to investigate the involvement of uPAR in α6p production. We treated DU145H cells, twice daily, with ATF for three days to compete with uPA for binding to the receptor. We did not observe any effect on α6p 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 [159], and saw no effect on α6p levels (data not shown). These results suggested that the α6 integrin-uPAR interaction was not important in the production of α6p.
Figure 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 three 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 three days and were then treated with 20ug/ml uPA or 400nM ATF for 1.5 hours. The samples were analyzed for $\alpha_6$ and $\alpha_6p$ integrin levels using the AA6A rabbit polyclonal antibody.
**uPA cleaves the α6 integrin in vitro**

We next determined whether uPA is able to cleave the α6 integrin directly in vitro or whether the cleavage of α6 integrin could be mediated by a contaminating matrix metalloproteinase (MMP). In order to distinguish between these possibilities, the MCF10A cell surface integrin was labeled by biotinylation, and resulting biotinylated α6 integrin retrieved by immunoprecipitation. The immunoprecipitated material, was incubated with uPA, or other compounds for 1 hour, and the resulting products analyzed by SDS-PAGE and detected via streptavidin-HRP (Figure 4A). The addition of uPA to the immunocomplexes produced α6p integrin in vitro. The presence of EDTA did not interfere with the cleavage of the α6 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 α6p indicating that MMPs are not involved in α6p 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 α6p (not shown). It is interesting that plasmin, while capable of cleaving α6 in vitro, produced cleavage products that are different from those generated by uPA (Figure 4B). These data show that while both uPA and plasmin can cleave α6 integrin in vitro, the products are distinct. The in vitro pattern of α6 integrin cleavage by uPA rather than plasmin matches the in vivo cleavage pattern of α6. Finally, to investigate if any other proteases were present in the
Figure 4: uPA can cleave the α6 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.
immunocomplexes, we performed casein and gelatin zymography. Immunocomplexes containing α6 integrin, α6β4 integrin or uPAR from different cell lines were analyzed. Co-precipitating protease activity was not detected in any sample (Figure 4 C and D).

**Phorbol ester induction of α6p**

It has been previously reported that 12-O-tetradecanoylphorbol-13-acetate (TPA) induces uPA activity in MCF10a cells [160]. We investigated whether TPA could induce α6p production. An induction of α6p relative to the vehicle controls was observed dependent upon different concentrations of TPA (Figure 5A). No α6p was detected in the untreated cells. In addition, to confirm that the production of α6p was induced by uPA we used PAI-1 inhibitor and showed that uPA was involved (Figure 5B). We also used the plasmin inhibitor α2-antiplasmin and found that the cleavage of the α6 integrin was plasmin independent (Figure 5B). Finally we analyzed the media from the cell treatments (described in Figure 5B) for uPA activity (Figure 5C). The levels of α6p from Figure 5B correspond to the uPA activity in Figure 5C.

**α6p is present in prostate cancer tissue**

Our results so far suggested that α6p was produced via uPA in tissue culture cell lines and that TPA could induce α6p production. Our next step was to determine whether α6p was present in human tissue. Since α6p was first identified in a prostate cancer cell line (DU145H), we obtained human normal and prostate cancer tissue
**Figure 5: TPA induces α6p in MCF10A cells.** (A) MCF10A cells were cultured for four days and were then treated with different concentrations of TPA for 18 hours. The samples were analyzed by SDS-PAGE and a Western blot was performed for the α6 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 hours. The samples were analyzed by SDS-PAGE and a Western blot was performed for the α6 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).
specimens. The tissue was obtained from frozen tissue blocks, and the resulting lysates were immunoprecipitated with the α6 antibody (J1B5); and then a western blot was performed using an anti-α6 antibody (AA6A) specific for the cytoplasmic domain. The full-length α6 integrin was detected in both the normal and the cancer tissue, while the α6p form was present predominantly in the prostate cancer specimen (Figure 6). These data suggest that α6p can be found in human prostate cancer tissue specimens as well as in prostate cancer tissue culture cell lines.
Figure 6: α6p is present in human prostate tissues. Normal and cancer human prostate tissues were analyzed for α6 and α6p levels by immunoprecipitation with the J1B5 rat monoclonal antibody and Western blotting using the AA6A rabbit polyclonal antibody.
Discussion

Our previous work has shown that the α6 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 [112, 138]. In addition, we have shown that during prostate cancer progression, many integrin receptors, with the exception of α3 and α6, are not expressed [107]. Finally, we have shown that the α6 integrin exists in the classical form (140 kDa, non-reduced) and in a novel smaller form (70 kDa), referred to as α6p [114]. Our previous studies in DU145H cells suggested that the production of the α6p variant involves a post-translational processing event at the cell surface [115].

Previous reports showed that DU145 cells contained five times the level of uPA compared to normal cells [144], and in addition, these cells had no PAI-1 or PAI-2 proteins present [145]. In addition, it has been shown that the α6 integrin interacts with the uPA receptor [159, 161]. Combined, these observations led us to investigate the possible involvement of uPA in the production of the α6p integrin variant.

We now have evidence indicating that the α6p integrin variant is produced by direct cleavage by uPA. Inhibitors of uPA and an uPA-blocking function antibody reduced the levels of α6p in DU145H cells suggesting that uPA is involved in the proteolytic cleavage of the α6 integrin. The addition of uPA to cell lines lacking α6p, induced α6p 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
α6 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 α6 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 α6 and α3 integrin have amino acid binding sites for uPAR, only the α6 integrin is cleaved. This implies that some additional factor(s) independent of uPAR regulates the cleavage. Our experiments indicate that the production of α6p is an inducible event dependent on uPA activity. Human breast epithelial cells (MCF10A) produce uPA after phorbol ester (TPA) stimulation [160], and our work indicates that TPA induces α6p production in a uPA dependent manner. Of particular interest is that the event appears highly regulated since only a portion of the α6 integrin on the cell surface is cleaved. In addition, the inhibition of α6p production does not increase the level of the α6 integrin. Current studies are to determine the regulatory features of the α6p production.

Studies indicated that α6p was present in human tissue and that it is not a tissue culture phenomenon. Since the α6p 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 α6p on the cell surface in remodeling epithelial tissues. Our working hypothesis is that the α6p 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 α6p form in tissue.

The exact site where uPA cleaves the α6 integrin resulting in the loss of the β-propeller domain is unknown. However, mass spectrometry analysis of the α6p form [114] indicates that the NH₂ 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 α6 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 [146], and that it cleaves hepatocyte growth factor/scatter factor (HGF/SF) [53], macrophage-stimulating protein (MSP) [54], and fibronectin [133] directly. To our knowledge this is the first report that uPA is involved in the cleavage of an integrin. The production of α6p may be a crucial step during tissue remodeling or toward uPA induced migration and invasion in cancer progression.
IV. IDENTIFICATION OF THE \( \alpha 6 \) INTEGRIN CLEAVAGE SITE AND TISSUE EXPRESSION OF \( \alpha 6 \)P

Introduction

Integrins are cell surface heterodimeric proteins composed of an \( \alpha \) and a \( \beta \) subunit. Each \( \alpha \beta \) combination has its own binding specificity and signaling properties [127, 128]. Integrins act as receptors for several extracellular matrix (ECM) proteins and sense the extracellular matrix environment [127, 128]. They have been implicated in many processes including cell migration, differentiation, blood clotting, tissue organization and cell growth. They are composed of a large extracellular domain (\( \alpha \) subunit \(~\)1000 residues, and \( \beta \) subunit \(~\)750 residues), a transmembrane domain and a short cytoplasmic domain (~50 residues or less) with the exception of \( \beta 4 \), whose cytoplasmic domain is large (more than 1000 residues) [129, 130].

The \( \alpha 6 \) integrin pairs with either the \( \beta 4 \) or the \( \beta 1 \) subunit but in cells that produce \( \alpha 6 \), \( \beta 1 \) and \( \beta 4 \), the dominant heterodimer is \( \alpha 6 \beta 4 \) [99]. Both of the \( \alpha 6 \) integrin heterodimers are laminin receptors [99]. We have previously shown that the \( \alpha 6 \) integrin is persistent in prostate cancer [124]. In addition, we have previously identified a structural variant of the \( \alpha 6 \) integrin called \( \alpha 6 \)P that is missing the extracellular \( \beta \)-propeller domain associated with ligand binding [114]. This variant was later shown to be produced by proteolytic cleavage by uPA [162]. In this study we wanted to determine
the exact site of cleavage of the $\alpha_6$ integrin by uPA, and also to analyze the tissue
expression of this variant.

**Results**

**Site-directed mutagenesis of the $\alpha_6$ integrin**

We have tried to N-terminal-sequence $\alpha_6p$ by immunoprecipitating the protein
eluting it on from the gel and sending it out for analysis. Unfortunately, all of our
attempts indicated that the protein was blocked on the amino-terminus. Therefore, we
used an alternative approach to identify the cleavage site. We performed site-directed
mutagenesis upstream from the area we knew was present in $\alpha_6p$ by mass spectrometry
analysis. The diagram in Figure 7 shows the fragment that was identified by mass
spectrometry to be present in $\alpha_6p$ (below the striped bar). The sequence upstream of that
lies in an accessible loop based on sequence comparison with the crystal structure of the
$\alpha_v$ integrin. Knowing that uPA cleaves after an arginine we mutated several arginines by
site-directed mutagenesis, avoiding the ones that are conserved in other integrins, and
transfected the constructs in an $\alpha_6$ null erythroleukemia cell line (K562). After
immunoprecipitations of the mutated or wildtype $\alpha_6$ integrin, uPA was added to the
immunocomplexes and the samples were analyzed by SDS-PAGE analysis followed by
western blotting for the $\alpha_6$ integrin (Figure 8). The data showed that mutation of
arginine 594 to alanine significantly reduced the ability of uPA to cleave the $\alpha_6$ integrin
Figure 7: Schematic representation of the α6 and α6p integrins. 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 V and VI respectively. The site of uPAR interaction is at residues 272-298. The exposed loop (as indicated by triangle) in the thigh domain contains the predicted cleavage sites. The aminoacids under the striped bar were detected by mass spectrometry in α6p. Conserved amino acids (*), conservative substitutions (:) are as indicated. The membrane spanning region (shaded rectangle) occurs at residues 1012-1037.
Figure 8: Site-directed mutagenesis analysis of the α6 integrin. Different arginine residues were mutated to identify the cleavage site. The arginines were chosen first based on the fact that they had to be close but upstream from the site that was shown to be present in α6p by mass spectrometry. In addition, arginines that were present in other integrins (such as α3) were omitted. K562 cells were transfected with different α6 integrin constructs by electroporation. The cells were then plated O/N in a T75 flask and the next day they were stimulated with Doxycycline. After 24 hours the α6 integrin was immunoprecipitated O/N and the next day the immunocomplexes were washed three times with RIPA followed by one wash with uPA reaction buffer. Then 4ug of uPA was added to the immunocomplexes and left on ice for 3 hours, and non-reducing sample buffer was added to the samples which were boiled and then run on an SDS-PAGE gel. The α6 integrin was detected using the AA6A rabbit polyclonal antibody.
as compared to the wildtype α6 or control mutants (R536-A and R596V597-AA).

Interestingly mutation of both arginines 594 and 595 completely abolished the cleavage of the α6 integrin by uPA. In order to determine if the mutant and the wildtype α6 that were transfected in the K562 cell line were able to interact with the β1 subunit that is present in the 562 cells we performed immunoprecipitations using the β1 mouse monoclonal antibody TS2/16 and western blotting for the α6 integrin. The results showed that both the wildtype and mutant α6 interacted with the β1 subunit (Figure 9).

Tissue analyses for α6p expression

We have previously shown that α6p exists in human prostate cancer tissue. We wanted to investigate whether α6p is expressed in other tissues. ICR mice were used in a tumor promotion-progression model. A single initiating dose of 100 μg DMBA in acetone was applied topically to the shaved backs of female ICR mice. It was followed by bi-weekly application of 5 nM TPA in acetone for 21 weeks. At week 22, TPA applications were stopped and replaced with weekly application of 120 μg MNNG in acetone for a total of 40 weeks. The tumors were isolated and first analyzed by H&E staining and classified by a pathologist as papillomas and squamous cell carcinomas (SCC). The tumors or normal skin isolated from untreated control mice were analyzed by SDS-PAGE analysis for α6 and α6p integrin expression (Figures 10 and 11). The results showed that α6p was present in normal, papilloma and SCC tissue. Interestingly two of the papilloma tissue
Figure 9: The ectopically expressed α6 proteins interact with the β1 subunit. K562 cells were transfected with different constructs. The α6 and β1 integrins were immunoprecipitated using the anti-α6 rat monoclonal antibody J1B5 and the anti-β1 mouse monoclonal antibody TS2/16. After SDS-PAGE analysis the α6 integrin was detected using the anti-α6 rabbit polyclonal antibody AA6A.
samples showed complete conversion of the α6 integrin to α6p. This has never been observed before in any tissue or cell line.

We then analyzed tumors isolated from transgenic mice that express an activated Ha-ras gene in their pigmented cells that were described previously [163]. Melanoma tumors in different stages from these mice that were treated with DMBA were isolated and protein was extracted using RIPA lysis buffer. The samples were then analyzed on an SDS-PAGE gel and the α6 integrin was detected using the anti-α6 integrin rabbit polyclonal antibody AA6A (Figure 12). The results showed that all melanomas expressed α6p.

**Induction of α6p in vivo**

The next experiments were aimed to determine whether α6p can be induced in tumors established by subcutaneous injection of a tumorigenic cell line that does not contain α6p. The establishment of the tumorigenic keratinocyte cell line (6M90) was described previously [164]. These cells were injected subcutaneously in athymic nude mice and the tumors were isolated and protein was extracted using RIPA lysis buffer. In addition, RIPA lysis buffer was used to produce whole cell lysate from the 6M90 cell line. The samples were then analyzed on an SDS-PAGE gel and the α6 integrin was detected using the anti-α6 integrin rabbit polyclonal antibody AA6A. The results showed that α6p is present in all the tumors but it was absent from the parent cell line (Figure 13).
Figure 10: α6p is present in normal mouse skin and scraped epidermis. Normal ICR mouse skin was analyzed for α6 and α6p levels by western blotting. Briefly, two of the untreated mice were shaved, and the whole skin was removed and frozen in liquid nitrogen. Another two untreated mice were shaved and the remaining hair was removed by hair removing cream. The skin was then removed, stretched out, and frozen in liquid nitrogen, and the epidermis was scraped and collected. The samples were homogenized by mortar and pestle and lysed in RIPA buffer. After SDS-PAGE analysis the α6 and α6p integrins were detected using the AA6A rabbit monoclonal antibody.
Figure 11: \(\alpha 6p\) is present in mouse skin papillomas and squamous cell carcinomas. Papillomas and SCCs from ICR mice treated topically with a single initiating dose of DMBA, followed by twice weekly application TPA for 21 weeks and finally, at week 22, weekly application of MNNG for a total of 40 weeks, were collected and frozen in liquid nitrogen. The samples were homogenized by mortar and pestle and lysed in RIPA buffer. After SDS-PAGE analysis the \(\alpha 6\) and \(\alpha 6p\) integrins were detected using the AA6A rabbit monoclonal antibody.
Figure 12: $\alpha 6p$ is present in melanomas from transgenic mice expressing an activated Ha-ras. Transgenic mice that express an activated Ha-ras gene in their pigmented cells have been described previously [163]. Tumors in different stages from such mice that were treated with DMBA were isolated and protein was extracted using RIPA lysis buffer. The samples were then analyzed on an SDS-PAGE gel and the $\alpha 6$ integrin was detected using the anti-$\alpha 6$ integrin rabbit polyclonal antibody AA6A.
Discussion

We have previously shown that there is an α6 integrin variant, called α6p, and it was found in different cell lines and prostate cancer tissue. We have later shown that the α6p integrin is produced by uPA cleavage on the cell surface. In addition, we have shown that this cleavage does not involve plasmin and that α6p is present in human prostate cancer tissue. In this study we wanted to determine the cleavage site and to analyze α6p integrin expression in different tissues. Our attempts to N-terminal sequence the α6p protein indicated that the α6p N-terminus is blocked. Therefore we resorted to a different approach to determine the cleavage site.

Using site-directed mutagenesis we have shown that mutation of the R594 to alanine significantly reduced the levels of the α6 integrin cleavage. A double mutant R594R595-AA completely abolished the α6 integrin cleavage. Interestingly the R595-A mutant had minimal effect on α6 integrin cleavage. Also, other mutants, such as the R536-A single mutant or the R596V597-AA double mutant had no effect on α6 integrin cleavage. These results suggest that both arginines (R594 and R595) are important in α6 integrin cleavage. The site of α6 integrin cleavage was determined to be localized in an accessible loop upstream of the genu region (Figure 14). We also developed prostate cancer cell lines (PC3N) that overexpress either the wildtype α6, the uncleavable mutant α6, or a control mutant α6. These would be helpful tools for determining the function of the α6p integrin variant.

We also analyzed different tissues for α6p expression. The results showed that
Figure 13: \(\alpha 6p\) is induced \textit{in vivo}. The establishment of the tumorigenic keratinocyte cell line (6M90) was described previously [164]. These cells were injected subcutaneously in athymic nude mice and the tumors were isolated and protein was extracted using RIPA lysis buffer. In addition, RIPA lysis buffer was used to produce whole cell lysate from the 6M90 cell line. The samples were then analyzed on an SDS-PAGE gel and the \(\alpha 6\) integrin was detected using the anti-\(\alpha 6\) integrin rabbit polyclonal antibody AA6A.
Figure 14: Proposed α6 integrin structure with computer modeling. A proposed structure for the α6 integrin was modeled using the DeepView/SwissPdb Viewer software V3.7. The top panel shows the full structure and the bottom panel shows the genu region. R594 (at the cleavage site) and the proposed uPA binding site (QEPSSRRR) are also shown.
α6p is present in both normal mouse skin and scraped epidermis. This finding suggests that α6p production might be important in skin tissue remodeling. Our current hypothesis is that α6 integrin cleavage may be a step towards basal cell detachment, allowing them to push up the layers of the skin to replenish damaged skin. The results also showed that mice topically treated with DMBA, TPA followed by MNNG developed papillomas and squamous cell carcinomas that contained α6p. What was very interesting was the fact that two of the papillomas showed complete conversion of the α6 integrin to α6p. This phenomenon was never seen before in vivo or in vitro. These data suggest that α6p might be involved in tissue remodeling during skin tumor progression.

Experiments using transgenic mice that express an activated Ha-ras gene in their pigmented cells showed that melanoma tumors from these mice expressed α6p. This is in agreement with previously published data which show that uPA activity was not detected in premalignant stages and that it appeared early in tumorigenesis and became more prominent in later stages of tumors from melanoma tumors of transgenic melanoma-susceptible mice [165]. These data provide additional support to the hypothesis that α6p is important in tissue remodeling during tumor progression.

Finally, the α6p integrin is absent from the 6M90 mouse keratinocyte cell line in vitro. Upon subcutaneous injection of the 6M90 cell line into athymic nude mice, α6p is induced in the developed tumors. This suggests that the tissue microenvironment surrounding the 6M90 cells stimulates uPA activity resulting in α6p production.
Collectively, these data suggest that α6p might be involved both in normal processes in the skin and during carcinogenesis in the process of tissue remodeling. It will be important to use the α6 integrin mutants in experiments to understand the function of α6p.
V. TRANSFECTION OF α6P AND FUNCTIONAL ANALYSIS OF THE UNCLEAVABLE α6 INTEGRIN MUTANT

Introduction

During the course of our investigations, we discovered an α6 integrin variant called α6p [114]. This is a 70 kDa structural variant that is missing the extracellular β-propeller domain and can be found paired with either the β1 or β4 subunits [114]. The variant is also recognized by antibodies (J1B5 and GOH3) specific for the full length α6 [166, 167]. These antibodies are used for the immunohistochemical localization of the full length α6 integrin. Detection of the variant is accomplished using non-reducing PAGE, followed by western blotting techniques to detect the 70kD form using an antibody specific for the cytoplasmic light chain. Metabolic labeling experiments indicated that the α6p variant has a three-fold increase in biological half-life on the cell surface as compared to the full length α6 integrin and no precursor product type relationship exists between the α6 integrin forms [114, 115]. Taken together, these data indicated that α6p was not a degradation product.

Several experiments suggested that the α6p variant arises while on the cell surface. First, all the α6p within a cell can be surface biotinylated whereas approximately only one half of the full length α6 can be labeled in this fashion. Approximately 40% of the α6 integrin on the surface of DU145 cells existed in the α6p form. The α6p variant contained the expected glycosylations indicating it has been
trafficked through the endoplasmic reticulum and the golgi to reach the cell surface. [115].

The observation that $\alpha_6p$ is produced while on the cell surface led to the hypothesis that $\alpha_6p$ was produced by proteolytic processing after it is displayed on the cell surface. Several proteases were considered as likely candidates to mediate the cleavage, including matrix metalloproteinases (MMPs), ADAMS (Disintegrin and A Metalloproteinase) and the urokinase-type plasminogen activator (uPA).

We have recently shown that $\alpha_6p$ was produced by proteolytic cleavage of the full length $\alpha_6$ integrin by urokinase-type plasminogen activator (uPA). This cleavage by uPA was specific to the $\alpha_6$ integrin since cleavage products are not produced for the $\alpha_3\beta_1$, $\alpha_5\beta_1$ or $\alpha\nu\beta_3$ integrins. Site-directed mutagenesis analysis determined that the site of cleavage of the $\alpha_6$ integrin is located in an accessible loop upstream of the genu region. The next studies were conducted to determine the function of $\alpha_6p$ by transfecting $\alpha_6p$ in prostate cancer cells, and by transfecting the uncleavable $\alpha_6$ integrin mutant in prostate cancer cells and analyzing tumor growth of these cells in SCID mice.

Results

Transfection of $\alpha_6p$ in PC3N cells

In order to clone $\alpha_6p$ we PCR amplified the region of the $\alpha_6$ integrin that corresponds to $\alpha_6p$ and also added a signal peptide to its amino-terminal end so that the protein is directed to the surface. We also included a myc tag between the signal peptide
and α6p so that we can detect the protein (Figure 15). We used a tetracycline inducible system in which doxycycline is used to turn on the gene of interest (Tet-on system). After transfection in PC3N cells we selected for stable clones using two antibiotics, namely blasticidin and zeocin. The repressor plasmid pcDNA6/TR contains blasticidin resistance while the pcDNA4/TO plasmid contains zeocin resistance. Therefore, only cells that were transfected with both plasmids would survive the double antibiotic selection.

After the selection process clones were isolated and were screened for α6p expression (Figure 16). The results indicated that 12 of the clones had inducible expression of α6p at different levels. In addition, 3 of the clones showed constitutive expression, meaning that α6p was expressed whether doxycycline was present or not. Immunostaining analysis using the anti-myc FITC antibody also showed the expression of α6p in the transfected cells (Figure 17).

Using biotinylation and immunoprecipitation analyses we found that the transfected α6p cannot pair with the β subunits confirming our previous studies that it can only be produced after it is on the cell surface (Figure 18). Consistent with this finding, other data indicate that the pairing of the α and β subunits is mapped functionally on the amino-termini of the α and β subunits [85]. In that study GPIIb-IIIa was bound to an affinity matrix and was treated with chymotrypsin. Two resulting fragments remained bound to the matrix and were identified as GPIIb and GPIIIa. In the presence of Ca^{++} and Mg^{++} the two fragment were maintained as a heterodimer. In
**Tetracycline inducible expression of α6p**

![Diagram](image)

**Figure 15: α6p transfection system.** The tetracycline inducible system used is composed of a repressor plasmid pcDNA6/TR and an expression vector pcDNA/4TO. The two plasmids confer blasticidin and zeocin resistance respectively. The α6p construct containing a signal peptide that directs the protein to the surface and a myc tag for detection was cloned in the HindIII and Apal sites. The systems works by having constitutive expression of the repressor by the pcDNA6/TR plasmid and then the repressor binds in the promoter of the pcDNA4/TO plasmid and prevents expression of the gene of interest. Upon doxycycline addition, the repressor is sequestered and the gene of interest is expressed.
Figure 16: Doxycycline induction of α6p in PC3N cells. PC3N cells were cotransfected with the pcDNA6/TR plasmid and the pcDNA4/TO-α6p plasmids and clones were isolated after 3 weeks of treatment with antibiotics. The transfected PC3N cells were either stimulated or not with doxycycline and whole cell lysate from each treatment was run on an SDS-PAGE gel and the α6 and transfected α6p integrins were detected by Western blotting using the AA6A rabbit polyclonal antibody.
Figure 17: Detection of doxycycline induction of α6p in PC3N cells. PC3N cells were contransfected with the pcDNA6/TR plasmid and the pcDNA4/TO-α6p plasmids and clones were isolated after 3 weeks of treatment with antibiotics. The transfected PC3N cells were either stimulated or not with doxycycline and the cells were fixed by methanol and stained with the anti-myc FITC conjugated antibody.
Figure 18: Surface biotinylation and immunoprecipitations analysis of the α6p transfected PC3N cells. Transfected PC3N cells were either treated or untreated with doxycycline for 24 hours and then biotinylated using the anti-α6 antibody AA6A or the anti-β1 antibody AIIB2. After SDS-PAGE analysis the proteins were detected using HRP-streptavidin.
addition, other sites of αβ contacts are present in these molecules [86]. Such sites were previously summarized [86] and include the propeller and hybrid domains (α and β subunits respectively), the calf-1 and EGF-3 (α and β subunits respectively), and calf-2 and EGF4 (α and β subunits respectively).

While characterizing the different α6p expressing PC3N clones we used the constitutive α6p expressing PC3N clone for tumorigenesis experiments. In a pilot experiment, untransfected PC3N or constitutive α6p expressing PC3N cells (PC3N-α6p) were injected subcutaneously in eight SCID mice (four for each treatment) and the tumors were monitored for approximately 38 days (Figures 19 and 20). The results showed that PC3N-α6p had smaller tumors that the untransfected PC3N cells. Knowing that the transfected α6p does not pair with the β subunit rendered these data not useful for our α6p functional studies. On the other hand, it is possible to use the PC3N-α6p cells for functional analyses of the α6 integrin. Although α6p is produced after α6 has paired with the β subunit and it is on the cell surface, in our transfections we used an α6p construct that we added a signal peptide to its amino-terminus in order to send it to the cell surface. It is reasonable to speculate that the transfected α6p that reaches the cell surface could compete with cytoplasmic partners of the wildtype α6 integrin. This could also explain the difference in tumor growth seen in the PC3N and PC3N- α6p induced tumors in SCID mice.
Figure 19: Tumor growth curve in SCID mice (Mean tumor volume). PC3N cells or PC3N cells constitutively expressing the transfected α6p were injected subcutaneously in SCID mice (4 for each cell line) and the tumor growth was monitored for approximately 40 days.
Figure 20: Tumor growth curve in SCID mice (individual mice). PC3N cells or PC3N cells constitutively expressing the transfected α6p were injected subcutaneously in SCID mice (4 for each cell line) and the tumor growth was monitored for approximately 40 days.
The α6p integrin variant does not interact with CD151 and is dependent on the lipid raft integrity

We wanted to investigate the effect of the α6 integrin cleavage on lateral associations with other proteins. We already knew from previous studies that only the full length α6 interacts with uPAR, so now we wanted to test if the interaction with the tetraspanin CD151 was affected by the cleavage.

We performed immunoprecipitation analyses in DU145H cells which contain both the full length α6 and the cleaved product α6p. Using the anti-α6 rat monoclonal antibody J1B5 we were able to immunoprecipitate both the full length α6 and α6p (Figure 21). However, using anti-CD151 mouse monoclonal antibody 5C11 we were only able to immunoprecipitate the full length α6 and not α6p. These data suggest that either cleavage of the α6 integrin abolishes existing CD151-α6 integrin interactions, or that cleavage of α6 integrin prevents it from binding to CD151. In addition, methyl-β-cyclodextrin, a compound that depletes cholesterol and disrupts rafts, reduced the levels of α6p in DU145H cells (Figure 22). This suggests that lipid rafts need to be intact for α6p to be produced.

Ectopic expression of the uncleavable α6 mutant in PC3N cells

After we have determined that the R594R595-AA double mutant does not get cleaved by uPA and it interacts with the β1 subunit, we wanted to transflect this mutant in PC3N cells. Using the tetracycline inducible system described above we cotransfected
Figure 21: CD151 interacts with the α6 but not the α6p integrin in DU145H cells. DU145H cells were lysed with RIPA buffer and proteins were immunoprecipitated for CD151 (using antibody 5C11), control IgG, or α6 (using antibody J1B5). Proteins were analyzed by SDS-PAGE and a Western blot was performed for the α6 integrin using the AA6A rabbit polyclonal antibody.
Figure 22: Methyl-β-cyclodextrin treatment reduces $\alpha 6 p$ levels in DU145H cells.

DU145H cells were treated with different concentrations of methyl-β-cyclodextrin (MCD) for 3 days. The media was replaced everyday with new media containing MCD. The cells were analyzed by SDS-PAGE analysis and the $\alpha 6$ and $\alpha 6 p$ integrins were detected using the anti-$\alpha 6$ rabbit polyclonal antibody AA6A.
PC3N cells with the wildtype α6, or the R594R595-AA double mutant, or a control mutant R536-A and the repressor plasmid. After 2 weeks of selection using the two antibiotics zeocin and blasticidin individual clones were isolated and screened for expression of the protein. In order to screen them we used the anti-α6 integrin antibody AA6A to look for overexpression of the protein (Figure 23). The data showed that the five PC3N-a6WT clones screened (1, 6, 7, 11, and 12) had significantly higher α6 integrin levels than the untransfected PC3N cells. In addition, from the PC3N R536A clones, five showed significant increase in the protein (1, 2, 5, 11, and 12). Finally, five PC3N-R594R595AA clones (1, 4, 9, 10, and 11) showed significant increase in protein levels as compared to the control. Equal loading was checked by stripping the blots and reprobing for FAK. Selected clones were screened for α6 integrin cleavage (Figure 24). The results verified that the transfectants expressed the proteins after doxycycline treatment and that only wildtype α6 was cleaved by uPA

Tumor growth analysis of PC3N cells expressing the wildtype or mutated α6 integrin in SCID mice

PC3N cells transfected with either the wildtype or mutated α6 integrin were injected subcutaneously in SCID mice (one site per mouse, 10 million cells per site). As soon as tumors were developed, doxycycline diet started and the tumor volumes were recorded. The data in Figure 25 show that the uncleavable mutant transfected cells showed significantly smaller tumors. In addition, immunoprecipitations and western blotting analyses of tumor lysates showed that the wildtype α6 integrin transfected cells
Figure 23: Stable transfection of PC3N cells with different α6 integrin constructs using a tetracycline inducible system. PC3N cells were transfected with different α6 constructs. To select for expressing clones, the cells were grown for 2 weeks in zeocin and blasticidin containing media. Individual clones were selected and were screened for expression. After 24 hour incubation with doxycycline the cells were lysed and SDS-PAGE analysis was performed and the α6 integrin was detected using the anti-α6 rabbit polyclonal antibody AA6A. As a loading control, the blots were stripped and FAK was detected using the 4.47 mouse monoclonal antibody (Upstate Biotechnology Inc, Waltham, MA).
Figure 24: Analysis of transfected PC3N cells for α6 integrin cleavage. PC3N cells were transfected with different α6 constructs. Stable clones were isolated and grown in the presence of Doxycycline or not and uPA was added in selected samples. The cells were lysed and analyzed for α6 and α6p integrins.
Figure 25: Tumor growth curve in SCID mice of PC3N cells transfected with wildtype or mutated α6 integrin. PC3N cells transfected with wildtype (α6) or mutated α6 (RR) integrin were injected subcutaneously in SCID mice and the tumor volume was monitored for several days.
Figure 26: Analysis of SCID mouse tumors for $\alpha 6$ and $\alpha 6p$ expression. PC3N cells were transfected with different $\alpha 6$ constructs. Stable clones were injected subcutaneously in SCID mice and tumor volume was monitored. The tumors were isolated and analyzed for $\alpha 6$ and $\alpha 6p$ integrins.
Figure 27: Analysis of SCID mouse tumors for caspase 3 activity. PC3N cells were transfected with different α6 constructs. Stable clones were injected subcutaneous in SCID mice and tumor volume was monitored. The tumors were isolated and analyzed for caspase 3 activity by staining using an anti-activated caspase 3 antibody. Lanes A-C show tumor sections from mice injected with PC3N cells transfected with wildtype α6. Lanes D-F show tumor sections from mice injected with PC3N cells transfected with mutant α6 (R594R595 → AA).
formed tumors that contained higher levels of the α6p integrin than the tumors form the α6 mutant transfected cells (Figure 26). Moreover staining different tumors with an antibody that detects activated caspase 3 indicated that the cells in the tumors from mutated α6 integrin transfected cells had higher levels of apoptosis (Figure 27). After consulting with Dr. James Ranger-Moore (University of Arizona) we decided to repeat the experiment with 12 mice per group to increase the statistical power of the results. This experiment is now in progress.

Discussion

We have previously shown that there is a smaller form of the α6 integrin called α6p. We also showed that uPA was the protease that cleaved the α6 integrin to produce α6p. This cleavage occurred on the cell surface after α6 had paired with the β subunit. In addition, we showed that α6p is present in different tissues. Our next goal was to determine the function of α6p by transfecting α6p in prostate cancer cells. The results showed that the transfected α6p did not pair with the β subunit. Although these data restricted the use of α6p transfections for determining the function of α6p, they further supported our previous data that α6p is produced while on the cell surface, after it was paired with the β subunit. However, the transfections could potentially be used as a dominant negative form of the α6 integrin. Although α6p is produced after α6 has paired with the β subunit and it is on the cell surface, in our transfections we used an α6p construct that we added a signal peptide to its amino-terminus in order to send it to the
cell surface. Preliminary data with α6p transfected cells subcutaneously injected in mice indicated that the α6p transfected cells showed slower growth than the untransfected cells. Our current hypothesis is that the transfected α6p that reaches the cell surface sequesters cytoplasmic signaling molecules from the wildtype α6 integrin in these cells.

We have also shown that α6p does not interact with the CD151 tetraspanin. This suggests that a functional role of α6p might be to control signaling induced by lateral associations. Tetraspanins have been shown to interact with integrins in tetraspanin-enriched microdomains [168]. These microdomains are similar but distinct from lipid rafts [168]. Lipid rafts are disrupted upon cholesterol depletion but tetraspanin complexes are more resistant [169]. It has been shown that cholesterol depletion caused shedding of tetraspanin complexes from intact cells, but the depletion of cholesterol did not dissociate the protein components [169]. Interestingly treatment of DU145H cells (the cells that constitutively express α6p) with methyl-β-cyclodextrin, a compound that has been shown to deplete cholesterol and disrupt lipid rafts, reduces the levels of α6p (Figure 22). This suggests that the CD151 interaction with the α6 integrin does not target the α6 integrin for cleavage, but this needs to be further investigated using CD151 mutants that do not interact with the α6 integrin. It also suggests that lipid rafts are important for the uPA dependent cleavage of the α6 integrin. Lipid rafts often contain caveolin and GPI-anchored proteins which are not found in the tetraspanin enriched microdomains [170-173]. One of these GPI-anchored proteins is uPAR. Knowing that uPAR is involved in uPA activation, disrupting the lipid rafts would result in reduction
of uPAR levels on the cell surface and therefore reduction of uPA activation. This would explain the reduction of α6p levels upon treatment with methyl-β-cyclodextrin.

Transfection of PC3N cells with the wildtype or the uncleavable mutant of the α6 integrin resulted in two cells lines that showed inducible expression of the two proteins. Different clones were isolated and screened for expression after doxycycline treatment. They were also tested for α6 integrin cleavage. Once the clones were screened, they were injected subcutaneously in SCID mice and the tumor volume was monitored for several days. The data showed that tumors formed by PC3N cells that were transfected with the uncleavable mutant were significantly smaller and showed less α6p protein and more caspase-3 activity. These results suggest that processing of the α6 integrin may be important in cell survival. We speculate that the inability of the α6 integrin to be cleaved in the tumors of the mutated α6 transfected cells is due to the inability of the integrins to crosstalk. It has been well established that integrins can crosstalk and that inhibition of one integrin can affect the function of another. By cleaving the α6 integrin we hypothesize that the α6 integrin function is altered and therefore other integrins could be influenced as well. In addition, we have shown that the cleavage of the α6 integrin abolishes lateral association. It has been well documented that these lateral associations are important in integrin signaling. By blocking the ability of cells to cleave the α6 integrin it is possible to affect the function of other integrins, or signaling through lateral associations, leading to activation of caspases and cell death. We are currently analyzing the signaling changes induced by the α6 integrin cleavage using the uncleavable mutant.
VI. STUDIES ON THE AMINO-TERMİNAL FRAGMENT OF THE α6 INTEGRIN

Introduction

Integrins are cell surface heterodimeric proteins involved in many processes such as cell adhesion, migration, and signaling. The α6 integrin is a laminin receptor whose expression is persistent in prostate cancer. We have previously shown that a smaller form of the α6 integrin called α6p is present in prostate cancer cell lines and prostate cancer tissue. Our work has shown that the α6p protein is produced on the cell surface by direct cleavage by uPA and that the α6p integrin remains paired with the β subunit.

Our previous studies have focused on the α6p protein trying to understand how the protein is produced and what the function of this protein is. In these studies we wanted to determine the fate of the amino-terminal fragment of the α6 integrin after the cleavage by uPA. The amino-terminal fragment contains the ligand binding region and part of the stalk region of the α6 integrin. We hypothesized that it would be found in the ECM and we wanted to determine if it was functional.

Results

The amino-terminal fragment is recoverable from the extracellular matrix

In these studies we wanted to determine the fate of the amino-terminal fragment of the α6 integrin after cleavage by uPA. Since this fragment contains the ligand binding
region, we wanted to determine if it remains in the extracellular matrix. MCF10A cells were grown for 3 days and were then treated with uPA to induce \( \alpha_6 \)p production. Using a previously described method [122], we isolated the ECM and we performed SDS-PAGE and western blot analysis to detect the amino-terminus of the \( \alpha_6 \) integrin using the anti-\( \alpha_6 \) integrin rabbit polyclonal antibody A33. The results showed that the amino-terminal fragment of \( \alpha_6 \) is recoverable from the ECM (Figure 28).

**Functional studies on the amino-terminal fragment of \( \alpha_6 \) and purification**

The study of the function of the amino-terminal fragment of the \( \alpha_6 \) integrin was prompted by work which has shown that shedding of fragment ECM molecules after cleavage by proteases causes binding to growth factor receptors and stimulation of signaling in cells [174] or induction of cell migration [44].

In order to determine the effect of the amino-terminal fragment of the \( \alpha_6 \) integrin on PC3N cells, we seeded the cells on an extracellular matrix that contained the amino-terminal fragment of the \( \alpha_6 \) integrin. This was accomplished by growing MCF10A cells for three days and treating or not treating the cells with uPA. The ECM was prepared by a previously described method [122], and PC3N cells were grown on this matrix. After performing an XTT cell proliferation assay, we determined that there was no significant difference in the growth of the cells plated on cleaved or uncleaved \( \alpha_6 \) in the ECM (Figure 29).

The effects of the amino-terminal fragment of the \( \alpha_6 \) integrin on migration was evaluated using time-lapse video migration. The PC3N cells were plated on cleaved \( \alpha_6 \)
or uncleaved α6 prepared matrix from MCF10A cells and were filmed during migration (Figure 30). Individual cells were analyzed for distance of migration. The results did not show any differences in the rate of linear migration or spreading morphology.

**In vitro expression of the amino-terminal fragment of α6 and purification**

In order to further study the function and localization of the amino-terminal fragment of the α6 integrin we decided to clone this fragment, express it *in vitro*, and purify it. Using PCR we cloned the fragment in a His-tag containing vector, pProEX, (Invitrogen) and expressed the protein in BL21 bacteria (Figure 31). The protein was then purified under denaturing conditions using a Nickel column (Figure 32). We are currently working to produce an antibody specific for this protein. This antibody will be used for immunohistochemistry analyses to determine the fate of this fragment *in vivo*. We expect that his antibody will detect both the amino-terminal fragment and the full length α6. In our immunohistochemistry analyses we will perform double staining using this antibody and another anti-α6 integrin antibody (J1B5) that does not detect the amino-terminal fragment.
Figure 28: The amino-terminal fragment of the cleaved α6 integrin is recoverable from the ECM. MCF10A cells were grown for three days and then treated with 50μg/ml uPA for three hours. The MCF10A matrix was prepared according to the procedure previously described [122]. The samples were analyzed by SDS-PAGE and a Western blot was performed for the α6 integrin using the A33 rabbit polyclonal antibody.
Figure 29: The amino-terminal fragment of the cleaved α6 integrin does not affect cell proliferation. MCF10A cells were grown for three days and then treated or not treated with 50μg/ml uPA for three hours. The MCF10A matrix was prepared according to the procedure previously described [122]. PC3N cells were plated on the matrix and the media was collected at different time points. The samples were analyzed with the use of the XTT proliferation kit (ROCHE, Basel Switzerland).
Figure 30: The amino-terminal fragment of the cleaved α6 integrin does not affect cell migration. MCF10A cells were grown for three days and then treated or not treated with 50μg/ml uPA for three hours. The MCF10A matrix was prepared according to the procedure previously described [122]. PC3N cells were plated on the matrix and the cells were analyzed for migration using time lapse video migration.
Figure 31: Recombinant amino-terminal fragment of the $\alpha 6$ integrin expression in BL21 bacteria. A recombinant fragment of the amino-terminus of the $\alpha 6$ integrin fused to a histidine tag was expressed in BL21 bacteria as described by the manufacturer (Invitrogen corporation, Carlsbad, California)
Figure 32: Purification of a recombinant amino-terminal fragment of the α6 integrin. A recombinant fragment of the amino-terminus of the α6 integrin fused to a histidine tag was expressed in BL21 bacteria as described by the manufacturer (Invitrogen corporation, Carlsbad, California). It was then purified using a Nickel column as described by the manufacturer (Invitrogen corporation, Carlsbad, California).
Discussion

We have previously shown that the α6 integrin exists in the classical form (140 kDa, non-reduced) and in a novel smaller form (70 kDa), referred to as α6p [114]. Our previous studies in DU145H cells suggested that the production of the α6p variant involves a post-translational processing event at the cell surface [115]. Additional experiments showed that uPA cleaves the α6 integrin directly to produce α6p. We later showed that α6p is present in different tissues, including human prostate cancer, normal mouse skin, mouse papillomas, squamous cell carcinomas and malignant melanomas. Finally, using site directed mutagenesis we have identified the α6 integrin cleavage site and determined it was localized in an accessible loop upstream from the genu region.

Our studies have shown that the amino-terminal fragment of the α6 integrin is recoverable from the ECM upon uPA cleavage. Since it has been shown that cleavage of ECM molecules such as laminin could have effects on cell growth and migration [44, 174] we wanted to determine if the cleaved fragment of the α6 integrin has similar effects. Our data showed that the amino-terminus fragment of α6 did not affect cell growth or migration. It is still possible that this fragment could act as an adhesion blocker since it resides in the ECM, and therefore may inhibit integrin function. Having purified a recombinant form of the amino-terminus of the α6 integrin, would allows us to produce an antibody for this fragment and therefore enable us to determine the localization of the fragment in tissues and also aid us in our functional analyses.
VI. CONCLUDING DISCUSSION

In the United States, one of every three cancers diagnosed is prostate cancer. This makes prostate cancer a major problem in the US. Metastasis is the leading cause of death from prostate cancer. Current methods of predicting the metastatic potential of prostate cancers are not reliable and new methods need to be developed. New proteins that are metastatic prostate cancer specific need to be identified so that metastatic prostate cancer can be detected early and therefore improve the therapeutic outcome of prostate cancer patients. Integrins are proteins involved in cell-ECM adhesion and their expression has been shown to be deregulated during prostate cancer. Our laboratory and others have shown that the α6 integrin is persistent in prostate cancer whereas expression of other integrins is downregulated. We have previously identified a smaller form of the α6 integrin, called α6p, which was present in the metastatic prostate cancer cell line DU145H but absent from the normal prostate cell line PrEC. The studies in this dissertation examined the production, tissue expression, and function of α6p.

Using different inhibitors and a uPA blocking antibody we have shown that the α6p integrin is produced by direct cleavage by uPA and this cleavage does not involve plasmin. This cleavage is a unique event for the α6 integrin since other α subunits that we tested do not get cleaved. In addition, biotinylation experiments did not show any evidence for cleavage of the β1 or β4 subunits. We have also shown that the site of cleavage of the α6 integrin lies within an accessible loop as described for the αv integrin. In addition, this site is located close to a potential uPA binding site on the α6 integrin.
The uncleavable mutants we have developed will be important tools in understanding the importance of the α6 integrin cleavage.

In order to study the function of α6p, we have transfected an α6 integrin construct which was missing a large portion of the amino-terminus but contained the signal peptide, the membrane proximal region of the extracellular domain, the transmembrane and cytoplasmic domains, in cells that expressed β1 and β4 integrins. Using biotinylation and immunoprecipitation experiments we have shown that the α6 construct was found on the cell surface but it was unable to pair with the β subunits. These data further supported the idea that the amino-terminus of α6 is required for the pairing of the α6 subunit with the β1 or β4 subunits.

The cleavage of the α6 integrin influences lateral membrane association (Figure 33). For example, it has been shown that uPAR, the GPI-anchored receptor for uPA, interacts with different integrins including α6 [59]. We have shown that although full length α6 interacted with uPAR in DU145H cells, α6p did not. In addition, the CD151 tetraspanin interacts with the full length α6 but not with α6p. This interaction has been shown to be important in integrin signaling. For example, CD151 has been shown to recruit signaling molecules such as PI-4K to mediate signaling [175]. It remains to be investigated whether other lateral associations are affected by this cleavage. Also it would be interesting to investigate the effects of α6 integrin cleavage on signaling through lateral association. It has been well documented that uPAR and CD151 mediate integrin signaling.

Another possibility for the functional significance of α6p is that it might be
Figure 33: Effect of the α6 integrin cleavage on lateral associations. The α6 integrin associates laterally with uPAR and CD151. CD151 is involved in recruiting the tetraspanin web which is involved in signaling. Cleavage of α6 results in loss of the lateral association leading to disruption of the tetraspanin web signaling.
involved in tissue remodeling. Tissue remodeling is important for many processes including differentiative processes in the adult such as repair processes, uterine and mammary gland changes, trophoblast invasion, neovascularization, menstrual cycle and pregnancy [176]. Tissue remodeling is also important in cancer progression.

During tissue remodeling, proteases including matrix metalloproteinases as well as the plasminogen activator/plasmin system become important [177]. Recently, E-cadherin, a cell-cell adhesion molecule, has been shown to be a substrate for matrilysin [178]. The shedding of E-cadherin has been shown to be required for epithelial repair [178]. Moreover, integrins have been shown to be involved in this process (summarized in [179]). In addition, it has been shown that integrins interact with the uPA receptor (uPAR) [58], and that integrin-dependent adhesion, spreading and migration can be modulated by the plasminogen activator system [177]. It is reasonable to hypothesize that the cleavage of α6 integrin by uPA is involved in tissue remodeling perhaps by releasing the adhesion form the ECM and allowing cell to migrate during remodeling processes.

We have shown that α6p was present in human prostate cancer tissue but not normal prostate tissue. Also, it was present in normal mouse skin, and mouse papillomas and squamous cell carcinomas induced by DMBA, TPA and MNNG treatments. In addition, α6p was present in melanomas induced in transgenic mice over-expressing activated Ha-ras in their pigmented cells. The fact that α6p was present in normal mouse skin gave rise to our hypothesis that α6p is important during induction of squamous differentiation. For basal cells to detach from the basal lamina and replenish damaged
skin, they have to abolish their adhesion structures which contain the α6 integrin (hemidesmosomes). Cleaving the α6 integrin extracellular domain which contains the ligand binding region may be an important step towards disassembly of the adhesion structure and induction of squamous differentiation. We hypothesize that the α6 integrin cleavage is an important step for cells to detach and move up to replace terminally differentiated keratinocytes.

It is important to note that α6p was found to be present in normal skin as well as different epithelial cancer tissues. This observation suggests that α6p has a role in normal skin homeostasis as well as the known remodeling architecture of glandular epithelium in cancer. Two models are proposed to accommodate the different adhesion structures in the structures in these tissues.

The current working model for normal tissues (such as skin) states that the α6β4 integrin is associated with CD151 in hemidesmosomes (HDs) on the basal cells (Figure 34). When the basal cells divide and detach to replace terminally differentiated keratinocytes, the formation of HDs needs to be halted and already formed HDs require disassembly. The uPA dependent cleavage of the α6 integrin may be an important step in the hemidesmosome halting assembly or promoting disassembly both by cleaving the ligand-binding region of the α6 integrin, and also by allowing integrin switching. The release of CD151 from the clipped α6 integrin may allow CD151 to associate with a different integrin such as the α3β1 which has been shown to be present in epithelial tissues and has been shown to be important in cell migration. When the α6 integrin is
**Figure 34: Current working model for normal tissues.** The α6β4 integrins are part of the hemidesmosomes (HDs) that anchor the cell to the basement membrane (via laminin 5). Cleavage of α6 may be a step towards the disassembly of the HDs and 'integrin switching' to α3β1 in focal adhesions, when cells migrate to replace terminally differentiated keratinocytes. CD151 interacts laterally with the α6 subunit and during the integrin switching it may switch partners by pairing with the α3 subunit.
clipped and the hemidesmosome is disassembled, the $\alpha 3\beta 1$ integrin may be activated (integrin switching) thus allowing cells to migrate to replenish damaged skin.

The current working model for cancer tissues (such as the prostate) states that when cells receive a pro-migratory signal and migrate through tissues that contain ECM molecules other than laminin (such as collagen and fibronectin), they clip the $\alpha 6$ integrin (laminin receptor) and therefore allow for integrin switching to a different integrin such as the $\alpha 3\beta 1$ integrin (collagen, laminin, and fibronectin receptor) (Figure 35). The $\alpha 3\beta 1$ integrin is the only other integrin heterodimer that is maintained in prostate cancer tissues. This process may also involve the CD151 transition (from $\alpha 6$ to $\alpha 3$) as described in the model for normal tissues. Therefore, the integrin switch from $\alpha 6\beta 1$ to $\alpha 3\beta 1$ may allow for cell migration on substrates other than laminin.

A current model suggests that the $\alpha$ subunit cytoplasmic domain inhibits certain functions of the $\beta$ cytoplasmic domain. Binding of the extracellular domain to the ligand relieves this inhibition by allowing the two subunits to swing apart like a hinge [80] [81]. Therefore it is reasonable to hypothesize that the $\alpha 6\beta$ integrin variant will affect the signaling of the $\alpha 6$ integrin.

A previous report has shown that 11 amino acids at the cytoplasmic domain at the carboxy-terminus of the $\alpha 6A$ integrin inhibit proliferation and promote differentiation by regulating $\beta 1$ integrin signaling [180]. The $\beta 1$ integrin signaling regulation by the $\alpha 6$ subunit could possibly be affected by the uPA dependent cleavage of the $\alpha 6$. For example, $\alpha 6\beta$ could prevent signals from being activated by not allowing ligand binding
Figure 35: Current working model for cancer tissues. The α6β1 integrin associates laterally CD151 and binds to ECM (laminin 10/11). When cells receive a pro-migratory signal, and they migrate through ECM that contains molecules other than laminin, they ‘switch’ to a different integrin (α3β1) by clipping the α6 integrin. CD151 interacts laterally with the α6 subunit and during the integrin switching it may switch partners by pairing with the α3 subunit.
to the integrin (dominant negative). On the other hand, it might be allowing signals to continue being transmitted while allowing for release from the ECM adhesion (dominant active).

The α6p variant production might also be a mechanism for ‘integrin switching’ where cleaving of the α6 integrin causes cells to activate or inhibit other integrin receptors. Many cells express several integrins on their cell surface and many of them could be simultaneously engaged [181]. Activation of one integrin can affect the function of other integrins [181]. This phenomenon is called integrin crosstalk [181-184]. Examples of integrin crosstalk have been shown in different cells lines (summarized in [181]). A recent review describes how integrins can affect the function of other integrins [185]. The model proposed in this review suggests that the αvβ3 and αvβ5 integrins trans-inhibit the α5β1 integrins during angiogenesis [185]. It could be speculated that cleavage of the α6 integrin could stimulate cells to activate or inhibit other integrins to switch adhesion to a different substrate or to activate adhesion or signaling through other receptors on the same substrate, a process we call ‘integrin switching’. It has been recently shown that expression of β6 integrin in oral squamous cell carcinoma cells (SCC9) increased activation of uPA, MMP-3 and MMP-9 [186]. We are currently collaborating with this group to investigate the possibility of integrin switching in this model. Our working hypothesis is that by expressing the β6 integrin in these cells, there is an integrin switch and therefore we expect the α6 integrin to be clipped.
Furthermore, as described above, we have shown that the cleavage of the \( \alpha6 \) integrin abolishes lateral associations. By cleaving \( \alpha6 \) integrin, the CD151 tetraspanin and the associated tetraspanin web are released from the \( \alpha6 \) integrin and could potentially associate with another integrin such as the \( \alpha3\beta1 \) heterodimer. Current studies in the lab are investigating this possibility.

In addition, the amino terminal fragment that is being cleaved off could have a function. Ectodomain shedding is known as the release of the extracellular domain of transmembrane receptors by limited proteolysis [187]. This process is thought to regulate the function of the receptors. This process occurs near or at the cell surface and it can occur in non-stimulated or stimulated cells [187]. The best characterized way to stimulate ectodomain shedding is the use of phorbol esters [187]. Interestingly, the \( \alpha6p \) integrin is produced by cleavage close to the cell surface and we have shown that the phorbol ester 12-o-tetradecanoyl-phorbol-13-acetate (TPA) induces \( \alpha6p \) production. Ectodomain shedding leading to the generation of soluble domains of receptors can modulate the functions of the ligands [188]. We can speculate that the cleaved \( \alpha6 \) fragment can modulate the function of the laminin by binding to it and occupying (i.e. blocking) the site where other laminin receptors could bind, thus allowing "integrin switching". Ectodomain shedding has been shown to regulate adhesion and migration previously. For example, L-selectin shedding has been shown to modulate adhesion and migration of leukocytes [189]. In addition, ADAMS (a disintegrin and metalloproteinase) have been involved in ectodomain shedding [190].
Different reports suggested that in addition to the release of the extracellular domain of integrin in the ECM by proteolytic cleavage, alternative splicing leads to a similar phenomenon. For example, a previous report showed that an alternative splice variant of αIIb integrin produced a truncated form of αIIb which lacked the cytoplasmic and transmembrane domains [191]. This variant was expressed by human leukemia, prostate cancer, and melanoma cells, but was absent from normal breast and prostate epithelia or platelets. Importantly, this protein was secreted and deposited on the ECM. A different report showed that an alternative splice variant of the β3 integrin encodes a 60kDa truncated form of β3 which lacks the cytoplasmic and transmembrane domains and was secreted in the ECM. This protein was shown to block adhesion of cells plated on ECM containing the truncated form of β3 [192]. Finally, we have shown that the cleaved α6 fragment (the β-propeller region) is recoverable from the ECM (Figure 28). The cleaved α6 fragment and the truncated αIIb and β3 proteins could act as adhesion blockers and therefore inhibit integrin function. Producing a purified recombinant form of the amino-terminus of the α6 integrin would enable studies to understand the function of this fragment and also it will enable the production of an antibody for this fragment that could be used or immunohistochemistry studies to determine the localization of the fragment in different tissues. This can be accomplished by double staining using this antibody and another anti-α6 integrin antibody that does not detect the amino-terminal fragment.

Our data indicating that α6α was induced in vivo are very interesting. This phenomenon suggests that the tumor microenvironment is inducing α6p production. One
speculation is that signals from surrounding tissues are inducing uPA activation which in turn cleaves the α6 integrin. More tissue studies need to be done to further understand the importance of the α6 integrin cleavage. Although cell lines are invaluable tool for scientific research several pieces of evidence suggest that 3-dimensional models such as tissues behave differently in signaling cascades from the 2-dimensional tissue culture experiments. A transgenic mouse model overexpressing or knocking-in the uncleavable α6 integrin under the control of keratin 5 promoter would enable us to infer the function of the α6 integrin cleavage in both skin and prostate tissue, as well as other tissues.

Our studies comparing the wildtype or uncleavable α6 integrin mutant in PC3N cells showed that tumors from SCID mice that were injected with PC3N cells expressing the uncleavable mutant were significantly smaller than tumors from mice injected with PC3N cells expressing wildtype α6. These results are in agreement with evidence that shows that the uPA inhibitor p-aminobenzamidine inhibits growth of human prostate tumors (DU145 cells) in SCID mice [193]. In addition, in our studies using p-aminobenzamidine in DU145 cells we saw a reduction in α6p levels. These results suggest that the α6 integrin clipping by uPA may be important in tumor growth.

The majority of patients with advanced prostate cancers develop skeletal metastases [194]. Despite the efforts for cure of metastatic prostate cancer, there remains no effective long-term cure [194]. More than 95% of bone ECM is composed of collagen [195]. Coculture of PC-3 cells with osteoblasts results in TGFβ1 presence in the conditioned media and increased PC-3 migration and invasion [196]. In addition, PC-3 cells showed increased expression of the collagen receptors, the α2β1 and α3β1 integrins
Also, osteoblast-conditioned medium stimulates uPA production from prostate cancer cells [197]. The above evidence suggests a model for studying integrin switching. Our working model is that PC-3 cells in the presence of bone tissue would produce uPA to cleave the \( \alpha_6 \) integrin and upregulate the \( \alpha_2\beta_1 \) and \( \alpha_3\beta_1 \) integrins to enable them to adapt to the bone ECM. We are currently injecting PC3N cells that express wildtype or mutated \( \alpha_6 \) in the bone of SCID mice to investigate the growth ability of these cells in the bone ECM.

Concluding, prostate cancer affects many lives every day all over the world. It affects not only the patients but their families as well. Because of all the medical advances in the recent years the life expectancy of people has increased significantly from past years. This gave rise to an increase in prostate cancer in men which is a disease of older men. New methods for detection and treatment of prostate cancer need to be developed in order to improve the quality of life of older men. A better understanding of the role of the \( \alpha_6p \) variant in prostate carcinoma may lead to the development of new methods of diagnosis, prognosis, and therapy.
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23 April 2004  Our ref: HW/smc/April 2004.jl217

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APPENDIX B: ANIMAL PROTOCOL REVIEW FORM

The University of
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Institutional Animal Care and Use Committee

Verification of Review
By The Institutional Animal Care and Use Committee (IACUC)
PHS Assurance No. A-3248-01 - USDA No. 86-3

The University of Arizona IACUC reviews all sections of proposals relating to animal care and use. The following listed proposal has been granted Final Approval according to the review policies of the IACUC:

PROTOCOL CONTROL NUMBER/TITLE:
#01-124 - "Oncogene Activation During Skin Tumor Progression"

PRINCIPAL INVESTIGATOR/DEPARTMENT:
G. Tim Bowden & Manolis Demetriou - Cell Biology & Anatomy

GRANTING AGENCY:
For PhD Dissertation: "Integrin Clipping - A Novel Adhesion Switch"

SUBMISSION DATE: August 21, 2001
APPROVAL DATE: September 27, 2001 APPROVAL VALID THROUGH*: September 26, 2004

*When projects or grant periods extend past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.

REVIEW STATUS FOR THIS PROJECT WAS CONFIRMED ON: May 17, 2004

REVISIONS (if any):

MINORITY OPINIONS (if any):

Richard C. Powell, PhD, MS
Vice President for Research
DATE: May 17, 2004

This approval authorizes only information as submitted on the Animal Protocol Review Form, Amendments, and any supplemental information contained in the file noted as reviewed and approved by the IACUC.
REFERENCES


25. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J.,
Miliareis, C., Rodgers, L., McCombie, R., Bigner, S. H., Giovannella, B. C.,
PTEN, a putative protein tyrosine phosphatase gene mutated in human brain,

26. Cote, R. J., Shi, Y., Groshen, S., Feng, A. C., Cordon-Cardo, C., Skinner, D., and
Lieskovosky, G. (1998). Association of p27Kip1 levels with recurrence and

Tester, D. J., Qian, J., Takahashi, S., Jenkins, R. B., Bostwick, D. G., and
Thibodeau, S. N. (1996). Allelic imbalance and microsatellite instability in

M. J., and De Marzo, A. M. (2002). Telomere shortening is an early somatic DNA

29. Reiter, R. E., Gu, Z., Watabe, T., Thomas, G., Szigeti, K., Davis, E., Wahl, M.,
Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer.

30. Narla, G., Heath, K. E., Reeves, H. L., Li, D., Giono, L. E., Kimmelman, A. C.,
Glucksman, M. J., Narla, J., Eng, F. J., Chan, A. M., Ferrari, A. C., Martignetti, J.


The University of Arizona
Tucson Arizona

Verification of Review
By The Institutional Animal Care and Use Committee (IACUC)
PHS Assurance No. A-3248-01 -- USDA No. 86-3

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The following listed proposal has been granted Final Approval according to the review policies of the IACUC:

PROTOCOL CONTROL NUMBER/TITLE:
#02-152 - "Prostate Carcinoma: Invasion and Metastasis Factors"

PRINCIPAL INVESTIGATOR/DEPARTMENT:
Ray Nagle, MD, PhD - Pathology/AZCC

GRANTING AGENCY:
NIH

SUBMISSION DATE: August 15, 2002
APPROVAL DATE: October 24, 2002
APPROVAL VALID THROUGH*: October 23, 2005

*When projects or grant periods extend past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.

REVIEW STATUS FOR THIS PROJECT WAS CONFIRMED ON, October 29, 2002

REVISIONS (if any):

MINORITY OPINIONS (if any):

Richard C. Powell, PhD, MS
Vice President for Research

DATE: October 29, 2002

This approval authorizes only information as submitted on the Animal Protocol Review Form, Amendments, and any supplemental information contained in the file noted as reviewed and approved by the IACUC.