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ALTERATIONS OF THE α6β4 AND α6β1 INTEGRINS IN PROSTATE CARCINOMA

by

Tracy Lynn Davis

A Dissertation Submitted to the Faculty of the

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In Partial Fulfillment of the Requirements
For the Degree of

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Tracy Lynn Davis entitled Alterations of the alpha6beta4 and alpha6beta1al Integrins in Prostate Carcinoma

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

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SIGNED: Tracy L Davis

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DEDICATION

I dedicate this dissertation to those people whose lives have been forever altered by prostate cancer. For patients, families, survivors and victims alike, every year prostate cancer affects the lives of many people. Following diagnosis, patients and families are often faced with difficult decisions regarding treatment and therapy. It is my hope that continued research will bring forth better treatment options and improve diagnostic methods so that perhaps one day, the diagnosis of prostate cancer will no longer be a devastating event in the lives of so many.

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ABSTRACT

The (140 kD) α6 integrin is an essential gene product in epithelial cell maintenance and remodeling of the stratified epithelium. The prostate gland is an example of a glandular epithelium. In prostate cancer, alterations of integrins are observed. Specifically, a shift from α6β4 to persistent expression of α6β1 integrin occurs. Accompanying the loss of polarized α6β4 is loss of its extracellular ligand, laminin-5. Using immunofluorescence staining human prostate, breast and colon tissues, were examined for β4 integrin and laminin-5 expression. Loss of β4 and laminin-5 was apparent beginning in PIN lesions and was absent in prostate carcinoma, differing from retained expression in breast and colon carcinoma. These data suggested progressive loss of β4 integrin and laminin-5 occurs and that this combined defect is unique to prostate cancer progression.

A novel 70 kD (non-reduced) variant of the α6 integrin, called α6p for the latin word *parvus* (small), was identified on the cell surface of normal epithelial and carcinoma cell lines. The α6p variant paired with either β1 or β4 subunits and retained sequences corresponding to the extracellular 'stalk region' and the cytoplasmic tail of the α6 integrin. The β-propeller domain postulated to mediate ligand binding, was missing from this variant. Protein levels of α6p increased three fold during calcium-induced terminal differentiation in a normal mouse keratinocyte model system. Production of the α6p variant was dependent upon an intact actin cytoskeleton. Cell surface α6p was less responsive to changes in the actin cytoskeleton, relative to that observed for α6 and β1

integrins, suggesting α6p did not participate in the focal contact. Additionally, inhibition of serine/threonine phosphatases decreased α6 integrin protein levels, but not α6p integrin, again suggesting the variant functioned as an inactive receptor for signaling. Finally α6, but not α6p integrin co-immunoprecipitated with hemidesmosome components laminin-5 and CD151. Preliminary data demonstrated adhesion to synthetic peptide integrin antagonists resulted in a 65 kD form of the α6p variant with no alteration of α6 integrin. Together the presented data were consistent with differential regulation of α6 and α6p integrins and suggested the α6p variant functioned as an inactive receptor.

I. INTRODUCTION

Prostate cancer epidemiology

In the United States, prostate cancer is the most frequently diagnosed visceral cancer in men and is the second leading cause of death due to cancer. For the year 2000, the World Health Organization estimated there were 180,400 new cases of prostate cancer diagnosed in the United States and that nearly 31,900 men were expected to die of the disease (1), thus giving a man roughly a 1 in 6 chance of developing invasive prostate carcinoma during his lifetime. In 1999, the National Cancer Institute estimated the annual cost of prostate cancer to be nearly $15 billion in the United States, accounting for medical care, lost wages and loss of productivity from its victims (2).

The incidence rate for prostate cancer has varied considerably over the last 20 years. According to the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute, between 1987 to 1992 the incidence rate of prostate cancer dramatically increased 85% and then steadily decreased 29% from 1992 to 1996 (1). This fluctuation has been attributed to the approval by the Federal Drug Administration for use of the prostate-specific antigen (PSA) blood test for prostate cancer screening (3). Following the increased use of PSA screening, the mean age of diagnosis decreased by 2 full years (4,5) and the five year survival rate for all stages of prostate cancer increased from 67% to 92% (6-8). This 'screening effect' was initially attributed to detection of clinically inapparent cancers (7,9-11), but further scrutiny of the data showed the most rapid increase in incidence was due to detection of moderately

differentiated cancers, suggesting that PSA screening may detect more clinically relevant cancers than originally thought (12).

Two tools used to screen for prostate cancer are the prostate-specific antigen (PSA) blood test and the digital rectal examination (DRE) (13,14). The American Cancer Society and the American Urological Association recommend men aged 50 years and older, with a life expectancy of at least 10 years, have an annual examination using both the DRE and PSA tests. Many men will live past 10 or even 15 years after diagnosis of prostate cancer (6). However, relatively low incidence of prostate cancer in men aged 50 to 60 years has raised issues of cost effectiveness (15). Neither of these medical tests are ideal because while both are highly sensitive, they also both have low specificity (16). Prostate cancer is not the only condition which can elevate PSA in the blood. Benign conditions such as benign prostatic hyperplasia which often accompanies age, and prostatitis can cause elevated PSA levels (17,18). Thus, a positive or negative result from one test alone is insufficient to determine diagnosis and further confirmation is usually necessary (13,19). Furthermore, some prostate cancers may actually exhibit a normal PSA value, and thus be overlooked. One recent study demonstrated that as many as 24.5% of men aged 43 to 74 (median age 62) with normal PSA ranges, had histological evidence of prostate cancer upon biopsy (19).

The large majority of prostate cancers are diagnosed while still localized to the prostate. Yet it is now believed that there are at least two different phenotypes of prostate cancer. One is a clinically aggressive tumor which will quickly become life-threatening, while the other is a slow-growing, 'latent' form which may never present itself clinically

(20). How to distinguish between these two forms of cancer has become a real problem for men diagnosed with the disease. In one study, approximately 27% of men age 30 to 40 were found to have evidence of latent prostate cancer (21). Historically between the years 1974 and 1993, the percent of men who opted to have radical prostatectomies in hopes of curing prostate cancer increased from 9.2% to 29.2% (22). Epidemiological studies suggest that many of these men may have never needed surgery (20).

Unfortunately, current medicine is unable to accurately predict which biological behavior a given tumor will have based on its histology (23). Prostate cancer cells and stromal cells act in concert to modify the microenvironment, leading to metastasis (24). Poor predictions from current biomarkers may reflect their inability to assess the interaction of subpopulations of prostate cancer cells and their tumor microenvironments. There is a growing need for biomarkers which assess the tumor microenvironment as a prediction of metastasis (24,25) and recent evidence suggests assessment of cell motility and related factors maybe critical in improving both diagnosis and prognosis of human prostate cancer (26-28).

The causes of prostate cancer are at best, poorly understood. In order to gain insight into the biology of prostate cancer, we must first examine epidemiological studies. Examination of the world incidence of prostate cancer shows there is considerable variation of both incidence and mortality due to prostate cancer among international populations (8). Black American men suffer the highest incidence rate in the world with more than 222 cases diagnosed per 100,000 men per year compared to 147 cases per 100,000 white men per year. This rate far exceeds that of black men in

Africa which have very low incidence rates of prostate cancer (1), suggestive of genetic drift and environmental factors. Prostate cancer mortality rates for black men in the United States are nearly twice higher than that for whites (12). After adjusting for incidence, recent studies attribute this higher mortality in blacks to both later stage at diagnosis and higher grade of disease at diagnosis (29-33), more co-morbid disease (34), and less aggressive therapy (34,35), with later stage at diagnosis appearing to be the primary reason for the higher likelihood of prostate cancer mortality (33). While the U.S. has one of the highest incidence rates of prostate cancer in the world, not all populations within the country are at risk, suggesting certain genetic predispositions may play a role in the disease. Studies from SEER and the World Health Organization show Asian and American Indian men have very low rates of prostate cancer, 81.5 and 46.5 per 100,000 men respectively. While their mortality due to the disease is also quite low, 10.7 and 14.3 per 100,1000 men respectively. Hispanics have an intermediate incidence rate of 102.8 and a mortality of 16.7 per 100,000 men.

Examination of data gathered by the World Health Organization on prostate cancer incidence in other countries reveals Japanse men have one of the lowest incidence rates of prostate cancer (less than 10 per 100,000 men per year) worldwide. Immigration studies of men from Japan, China, Scandinavia and Italy to the United States demonstrate generational increases in prostate cancer incidence when compared to their place of origin (36-38). Although the rates in these immigrant populations do not equal those of American populations, they are consistently higher than their country of origin (6). These studies suggest a strong role for environmental factors, including diet in the

role of prostate cancer biology. Interestingly the number of latent cancers in the prostates of men from Japan and the United States are statistically very similar despite strikingly different incidence rates, suggesting a difference in promotion of initiated tumors in the prostate (39,40). This disparity may explain differences in clinical presentation of disease among the two populations.

Risk factors identified through epidemiological studies of prostate cancer include diet, age, genetics, socioeconomic status, hormone levels, sexually transmitted diseases, physical activity and chemical and carcinogen exposure risk (41) (4,42). There is a slightly decreased risk associated with increased physical activity, showing that physical activity may be protective against development of prostate cancer (41). At present, it appears the most important risk factor for developing prostate cancer is age. In men under the age of 60 years old, there is less than a 1 in 50 chance of developing the disease, but for men over the age of 60, there is a 1 in 7 chance of developing prostate cancer (1).

Immigrant epidemiological data suggest diet may partially explain differences in prostate cancer incidence in other countries. The typical western American diet has relatively high levels of animal fat and protein with low fiber content. Studies conclude high animal fat and red meat intake (4,43,44), as well as heterocyclic amines formed during cooking of meat (45,46), are directly associated with prostate cancer risk. However this association may not hold true for those of Japanese decent where no correlation between fat intake and prostate cancer risk has been demonstrated (47,48). Some dietary factors may actually be protective against development of prostate cancer,

such as diets high in vegetables (49), selenium (50-52), soy isoflavones (53-56), and lycopenes from tomatoes (57-59). Clearly, more research is warranted in this field to clarify the role of these compounds and the biology of prostate carcinoma (43).

Monozygous twin studies have identified both environmental and genetic influences contribute to development of prostate cancer (60). Much research has studied the role of genetics in prostate cancer. Observations that men with a father or brother who had prostate cancer were twice as likely to develop prostate cancer (61,62) lead to studies of familial prostate cancer. Researchers have now identified putative X linked alleles (63) and a susceptibility gene on chromosome 1q (64) which correlate with development of prostate cancer. This information may help in the early detection of prostate cancer among family members who are at a higher risk than the general population (65). Development of familial prostate cancer is now thought to occur due to familial sharing of alleles at many different loci, each contributing a small proportion of risk to developing prostate cancer. This may explain why prostate cancer is often clustered in certain families, but often not directly inherited as in Mendelian genetics (42).

In summary, prostate cancer is a national health problem for men in the United States with the highest incidence rate in the world occurring in the black population. Numerous epidemiological studies indicate the etiology of prostate cancer is multifactorial and variable in its clinical presentation. Unfortunately, current medical technology is insufficient to accurately distinguish between cancers localized to the prostate which will become life-threatening and those which may remain latent for long

periods of time. Evaluation of the tumor microenvironment is an emerging idea which may lead to improved prognostic accuracy. Therefore it is essential to investigate further the biological changes which accumulate during the progression of prostate cancer.

Alterations in prostate carcinoma progression

Prostate cancer is a disease which in its natural course, accumulates many alterations which differ from the normal prostate cell. Like many other progressively occuring epithelial cancers, prostate cancer is now generally accepted to occur through a precursor lesion (66). Prostatic intraepithelial neoplasia (PIN) lesions are graded histologically with gradations varying from I to III (66), although it is now thought that only high grade PIN lesions (III) are precursors to cancer. Post-mortem studies of prostates in men over age 20 who have died from other causes sometimes demonstrate early signs of intraepithelial neoplasia (67). Additionally black men in the United States, who have very high rates of prostate cancer, have been found to harbor increased numbers of PIN lesions compared to white men (68).

While it is rare for early PIN lesions (type I or II) to be associated with cancer, high grade PIN lesions (PIN-III) are frequently found in the presence of carcinoma elsewhere in the prostate (67). PIN is characterized by complex changes in both the secretory cells and the basal cells. In general, these lesions display abnormal proliferation/renewal activity and disordered cell differentiation (69). Histologically, high grade PIN lesions appear to have the characteristics of a neoplastic lesion including disordered small glands and signs of minimal nuclear atypia, (70,71) . Some consider

high grade PIN to be carcinoma *in situ* (69,72) based on observed genetic alterations such as bcl-2 and p53 accumulation in these lesions (73) and cytological studies (72). Recently, loss of the β4 integrin and its primary ligand laminin-5 were described in PIN lesions (74,75), which is necessary for the expression of the hemidesmosome adhesion complex (76,77). Thus the inability of transformed cells to express hemidesmosomes may be a crucial event in neoplastic progression from a high grade PIN lesion to early stromal invasion and could account for loss of basal cell differentiation in prostate cancer (78).

Dramatic genetic and protein alterations occur in prostate cancer which accumulate during the natural progression of the disease. The major findings which are consistently described in reviews are summarized below (26,79-81). Chromosomal instability is a hallmark of cancer. Many genetic alterations have been described in prostate cancer including gain of chromosomes 7, 12, 17, X, Y and deletion or loss of heterozygousity of 3p, 5q, 8p, 10p, 10q, 11p, 12q, 13q, 16q, 17p and 18q. The most frequently reported chromosomal alterations are gain of 7, and loss of 5q, 8p12-21, 10q23-24, 13q, 16q22.1-24 and 18q (79,81). Interestingly, examination of clinically latent prostate cancer has revealed examples of microsatellite instability (82), implying the latent phenotype may not be entirely innocuous.

Investigation of frequently lost alleles by micro-cell-mediated chromosomal transfer, located genes on human chromosomes 8, 10cen-q23, 11p11.2-13, and 17pter-q23 which, when introduced into rat prostatic cancer cells suppressed metastasis without affecting tumorigenicity or growth rate (83). One such gene on chromosome 11p11.2 was

identified as KAI1/CD82 which encodes a membrane glycoprotein, was recently identified as a member of a class of proteins known as tetraspanins (84-86). KAI1 was found to suppress metastasis in a rat prostatic carcinoma cell model (86). KAI1 levels are high in normal prostate but lost in tumors derived from metastatic lesions (83). Frequent loss of chromosome 11p in advanced prostate cancer results in downregulation of KAI1 (87-89) thus contributing to metastasis due to loss of negative regulation.

Mutations and deletions of tumor suppressors genes occur frequently in a variety of cancers. Alterations in tumor suppressor gene products such as Rb and p53 have been reported in late stage prostate cancer (90-97). In 1990, Bookstein and others demonstrated restoration of Rb in the human prostate carcinoma cell line DU145, suppressed tumorigenicity (98), but others have not shown a correlation between Rb and disease progression (99). Rb may also function as a suppressor of metastasis as demonstrated by addition to several carcinoma cell lines which resulted in inhibition of tumor cell invasion *in vitro* (100).

The bcl-2 protein is an inhibitor of apoptosis and a tumor suppressor. In prostate, increased bcl-2 levels strongly correlate with recurrence of disease and progression to androgen-independent growth (101). Increased expression of bcl-2 in prostate cancer and high grade PIN lesions has been observed (73,91,102). In a breast carcinoma cell line, overexpression of bcl-2 lead to increased metastatic and migratory potential (103), and restoration of bcl-2 to the DU145 prostate carcinoma cell line resulted in growth inhibition (104), however migratory potential was not tested in this cell line.

The recently identified tumor suppressor gene PTEN (phosphatase and tensin homolog deleted on chromosome 10, also known as MMAC1) is frequently mutated or deleted in prostate cancer resulting in an increased pool of phosphorylated proteins (105-109). In an experimental cell model system, overexpression of PTEN decreased migration, whereas inhibition of PTEN by anti-sense resulted in increased migration (110). Therefore, loss of PTEN from prostate cancer may be another mechanism which promotes cell motility and metastasis.

Alterations in proto-oncogenes have been described in high grade prostate tumors as well, for example mutations in c-myc have been noted (111,112). The intracellular signaling protein ras is rarely activated in prostate carcinoma (113), but mutations such as v-ras and Ha-ras lead to increased motility and metastasis in a rat prostatic carcinoma cell model (114,115). Another intracellular signaling protein, pp125 focal adhesion kinase (FAK) is a protein tyrosine kinase which co-localizes with integrins in focal adhesions. Upregulation of FAK was observed in protate cancer metastatic lesions and prostate cancer cell lines (116) and increased expression of FAK has previously been correlated with increased cell motility (117,118).

As with many different tumor types, numerous alterations in growth factors and growth factor receptors have been reported for prostate cancer (119,120). Mutations in fibroblast growth factor-2 (FGF-2) and its receptor frequently occur, resulting in loss of growth regulation (121-124). Increased serum levels of the insulin-like growth factor (IGF) have been correlated with prostate cancer risk and may function as a tool for early detection (125-127). Upregulation or alteration of growth factors and receptors can lead

to increased proliferation in prostate cancer. Also, loss of growth inhibitory factors such as transforming growth factor-β (TGF-β) and its receptor (128-133) contribute to increased proliferation by rendering prostate tumors insensitive to growth inhibition.

Prostate cancer frequently metastasizes to bone (134). Mutations in prostate tumor growth factors and receptors combined with native growth factors and hormones at bony sites may in part explain this propensity. For example, parathyroid hormone related peptide (PTHrP) is present in both prostate cancer and bone (135) and chemotactic for human prostate carcinoma cell lines DU145 and PC3 (136). Epidermal growth factor (EGF) is another chemoattractant found at bony sites and pelvic lymph nodes which is also upregulated in prostate cancer (137). Additionally, signaling through the EGF receptor enhances prostate cancer invasiveness both *in vitro* and *in vivo* (138,139). The insulin-like growth factor is produced by bone osteoclasts and can promote chemotactic migration of prostate cancer cells whereas TNF-α and IL-1β (also produced in bone) lead to decreased chemotaxis (140). Additionally it has been demonstrated that the serine protease PSA, which is often elevated by prostate cancer cells, can cleave the insulin-like growth factor binding protein, resulting in release of active growth factor (141). Together, these examples suggest the bone microenvironment may both positively and negatively regulate cell proliferation and motility.

The role of androgens is becoming more clear in the progression of prostate cancer. Androgens have been shown to increase both transforming growth factor-α (TGF-α), its receptor and epidermial growth factor (EGF) (142-145) resulting in increased growth stimulus for prostate tumor cells. Recent studies suggest keratinocyte

growth factor (KGF) may actually mediate androgen-induced activities in prostate cancer and benign prostatic hyperplasia (146,147). Thus androgens may initially facilitate growth and progression before the tumor ultimately becomes androgen-independent. Androgen receptor mutations and DNA silencing of the androgen receptor via methylation have been extensively reported and correlated with prostate cancer progression and failure of therapy (148-152). Of note, polymorphisms in the androgen receptor were recently identified in black men, which may potentially explain the epidemiological differences of prostate cancer incidence between white and black men in the United States (153).

While there are numerous alterations of chromosomes and growth factors in prostate cancer, these are relatively late events in the progression of the disease. Events which occur early in prostate cancer are alterations of cell adhesion molecules such as integrins and cadherins (26,80). Changes in integrin receptor expression have been associated with prostate cancer progression. In general, most of the integrins normally expressed in the prostate are down-regulated in prostate cancer. Currently, loss of $\alpha 2$, $\alpha 4$, $\alpha 5$, αv, $\beta 1C$ and $\beta 4$ integrins have been described in prostate carcinoma (154-156). Interestingly, expression of $\alpha 6$ and $\alpha 3$ are retained and the $\alpha 6\beta 1$ integrin is upregulated to become the predominant integrin pair observed in tumors (155,157).

In a cell culture model system, increased $\alpha 6$ integrin expression correlated positively with the progression of prostate carcinoma cells to a motile and invasive phenotype. DU145 cells selected for high and low $\alpha 6$ integrin expression demonstrated similar adhesion, but the high $\alpha 6$ expressors (four times higher $\alpha 6$ expression) were three

times more motile on laminin and were more invasive *in vivo* than low α6 expressors (158), strongly suggesting a role for the α6 integrin in tumor cell invasion. Other cell adhesion molecules, including cadherins are also altered in prostate carcinoma. E-cadherin expression can be silenced by DNA-methylation (159) and there is evidence of cadherin switching in prostate tumors from the epithelial (E-cadherin) to mesenchymal (N-cadherin) cadherin subtypes (160-163).

The extracellular matrix is also altered in prostate cancer. In normal prostate glands there is polarized distribution of laminin-5, collagen VII and α6β4 integrins. In tumors, there is loss of α6β4 integrin, BP180, the β3 and γ2 subchains of laminin-5, and collagen VII (75,157,164-166). Loss of the hemidesmosome cell adhesion complex has been reported in prostate cancer and is accompanied by downregulation of several hemidesmosome proteins including BP230, HD1/plectin (157,164,165). Alterations in the extracellular matrix may play an active role in promoting tumor cell migration due to altered receptor/ligand interactions (167). In addition to these extracellular matrix and integrin changes, tumors secrete a number of proteases which dramatically modify the extracellular matrix surrounding tumors.

Prostate cancer cells secrete high levels of matrix metalloproteases (MMPs) and low levels of endogenous inhibitors (TIMPS), leading to an imbalance of metalloprotease activity (168) which may facilitate proteolytic degradation of surrounding matrices and invasion. MMP-7, also known as matrilysin was first identified to be upregulated in prostate carcinoma (169) and was focally expressed by small groups of cancer cells and also localized to inflamed atrophic glands, dilated ducts and epithelial cancer cells

(164,170). More recently, MMP-2 and MMP-9 have been observed to have increased expression in prostate cancer (171). Not only does the metalloprotease levels increase, but the endogenous tissue inhibitors of metalloproteases (TIMPs) are concurrently decreased in high grade tumors (172-174) and invasive prostate carcinoma cell lines (175). These studies suggest that increased MMP and decreased TIMP expression correlates with prostate cancer progression. The most recent metalloprotease identified in abnormal distribution in prostate cancer was MT1-MMP (176), a member of a new class of membrane-anchored metalloproteases which can activate other metalloproteases, such as MMP-2 leading to a localized cascade of proteolysis in prostate tumors (177). Recently, MMP-2 and MT1-MMP were localized on invadopodia and play a large role in focal degradation of the extracellular matrix (178-180), thus facilitating increased tumor cell migration and metastasis via the integrin family (181) .

In summary, prostate cancer progressively accumulates numerous genetic and protein alterations which contribute to the malignant phenotype. While commonly observed in other epithelial cancers, changes in chromosomes, tumor suppressors and proto-oncogenes also arise in prostate cancer, however they are relatively late events in the progression of the disease. Early events which are now becoming more apparent include alterations in expression of adhesion molecules, extracellular matrix proteins and proteolytic enzymes. We have focused our attention on the alterations of $\alpha 6\beta 1$ and $\alpha 6\beta 4$ integrins in these studies.

The integrin family

Integrins are a family of heterodimeric, glycosylated transmembrane receptors which connect the extracellular matrix to the cytoskeleton and have a wide variety of functions including adhesion, migration, proliferation, signaling, differentiation and cell survival (182-189). They are composed of an α and β subunit. To date, 8 different β subunits have been identified and 17 α subunits. Together, they pair to form at least 24 different heterodimers with pairing specificity being observed (184). Ligand binding is modulated by metal ions such as calcium and magnesium (184,190). The hallmark of integrins is the ability of individual family members to recognize multiple ligands (183). The genes for the α6, β1 and β4 subunits are mapped to chromosomes 2, 10 and 17, respectively. The integrins α6β1 and α6β4 bind different isoforms of laminin with overlapping specificities and preferences. The primary ligand for the α6β1 integrin is laminin 10/11 and for α6β4 it is laminin-5 (191-195).

Integrin α6 ligand binding and subunit heterodimerization

Much work has gone into understanding integrin ligand binding and subunit heterodimerization. The current structural model of the α subunit proposes that the seven N-terminal repeats adopt the fold of a β-propeller domain (196,197). These domains contain seven four-stranded β-sheets and are arranged in a torus around a pseudosymmetric axis. Structural homology studies of enzymes with known β-propeller folds have identified active sites at the top of the β-propeller, typically where adjacent

loops run in opposite directions (198-201). Recent studies of the β-propeller domain in integrins have demonstrated folds 1 and 3 in the α4 integrin subunit are important for ligand binding (202), whereas the α5 integrin ligand binding site is determined by amino acid sequences in repeats 2 and 3 of the N-terminal domain of the α subunit (203). The ligand binding site on the α6 integrin has not been extensively studied, however based on structural analyses, it is likely to be mediated by amino acids within the loops of the β-propeller folds in the N-terminal region.

Investigation of the minimal regions necessary for integrin subunit heterodimerization has been elusive due to the conformational nature of the integrins. Recently however, progress has been made in understanding how the α6A subunit dimerizes with the β1 subunit. There is a cytoplasmic membrane-proximal region of the α6 subunit which contains a conserved five amino acid sequence, GFFKR (204,205). This sequence is encoded on the last exon of the gene (exon 25). Deletion of this sequence prevented α6β1 heterodimerization and the α6 subunit translocation from the endoplasmic reticulum. Point mutational analysis has demonstrated the two phenylalanine residues are necessary for α6β1 pairing and function (206). The cytoplasmic C-terminal domain is involved in regulation of bidirectional signaling of the α6β1 integrin (207,208) and may play a role in differentiation (209,210).

The GFFKR motif is not essential for α6β4 pairing, but it increases the stability of the pair (207). The cytoplasmic domain of α6 is essential for inside-out and outside-in signaling via the α6β1 integrin receptor (208). De Melker and others examined the effect

of truncation of the α6 cytoplasmic domain on pairing with the β4 subunit. Loss of the GFFKR sequence did not prevent pairing of α6β4 integrin and only slightly affected ligand binding. Essentially, truncations of the cytoplasmic domain affect the ability of the α6 integrin to pair with β1 more strongly than that of β4 integrin. It may be that α6 subunit has a greater affinity for β4 than for β1 integrin, thus making pairing with β4 less susceptible to loss of the α6 cytoplasmic domain (207).

The role of α6 integrin in prostate cancer metastasis

Metastasis is defined as the formation of a progressively growing secondary tumor foci which is at a site that is discontinuous from the primary lesion (27). Few people die from cancer due to a primary tumor. It is the metastatic lesion which complicates cancer therapy and increases mortality. After the tumor has spread to distant places in the body, it is much more difficult to effectively treat and remove, compared to a primary tumor lesion. Because of this, patients with metastatic lesions have a much higher mortality than patients treated for a primary tumor (211,212). Primary tumors of the prostate often leave the gland via invasion of nerves, specifically along the perineural space (213). In one study of prostate capsule penetration, as many as 50% of the tumors were found within the perineural space (214). Once out of the prostate, the local lymph nodes become involved and the the primary site of distant metastasis is the bone, particularly the lumbar region (lower back) of the spinal column (134,215).

Cell motility is crucial for metastasis. One of the earliest correlations between cell motility and metastasis was demonstrated using the Dunning rat-R-3327 prostate cancer

cell model system (216,217). Thus, alterations which lead to increased tumor cell motility are likely to result in increased metastatic capacity. As described earlier, many alterations which occur in prostate cancer, result in modulation of cell motility which may contribute to migration and metastasis. The integrins play a significant role in metastasis (218). The α6 integrin is an adhesion receptor for laminin. Previous studies have identified the α6 integrin as being upregulated in prostate carcinomas and micrometastatic lesions (155,219-222). Therefore, the role of the α6 integrin becomes very interesting in understanding how prostate cancer progresses and metastasizes. The α6 integrin plays a central role in several of the primary steps of tumor cell metastasis, including detachment of cells from the primary tumor, vascular invasion and extravasation, migration to a conducive growth site and establishment of new growth.

Detachment of cells from the primary tumor site involves alteration of several different types of cell adhesion molecules (163,223). As previously mentioned, the integrin family is involved in mediating cell adhesion to the extracellular matrix (182,224) and many of the integrin subunits are down-regulated in prostate cancer. Specifically, there is loss of the α6β4 integrin and its primary ligand, laminin-5 (74,75,155,157,165) which facilitate stable anchorage of the cell to the collagen network of the extracellular matrix. Subsequently, there is an increase in the laminin receptor, α6β1 integrin in prostate cancer. Recently, it was demonstrated that laminin-5 could be proteolytically cleaved by plasminogen activator, resulting in alteration of cell motility (225), presumably due to loss of the ability of laminin-5 to bind integrin. Tumor cells secrete many proteolytic enzymes which promote degradation of the surrounding basal

lamina (226). A new membrane-bound metalloprotease called, MT1-MMP has been observed on the cell surface of prostate tumor cells in an abnormal distribution (176,226,227). This protease is capable of activating MMP-2 (also referred to as gelatinase A) by cleavage. Additionally, both MT1-MMP and MMP2 have been shown to cleave the γ subunit of rat laminin-5 and enhance human cell migration on cleaved rat laminin-5 (228,229). Thus focal proteolytic cascades result in altered extracellular matrix proteins and lead to increased cell motility along a changing substrate (167).

The extracellular matrix is a dynamic structure (167). After digestion via proteolytic enzymes, the basement membranes of tumors differ dramatically from that of normal tissues (78,164,230), thus promoting tumor cell migration. The α6 integrin is important for adhesion on laminin 10/11 and laminin 5 which are both components of the basal lamina, via the α6β1 and α6β4 integrins, respectively (191-195). The α6 integrin has been observed to be upregulated in various epithelial tumors and metastatic tumor lesions which will be discussed in the next section. This change in integrin profile is one likely mechanism to facilitate tumor cell detachment and motility from a primary tumor site.

While gaining access to the blood or lymphatic circulation is usually the next step of cancer metastasis, prostate cancer often initially leaves the prostate via migration along the peripheral nerves (213,214). The nerves are surrounded by Schwann cells which produce a laminin-rich basement membrane (214,231,232) which would support cell adhesion via the α6 integrin (191,194,195). Recently, Dubovy and others demonstrated α6 integrin and laminin co-associated in Schwann cells migrating along on nerves (233),

suggesting that α6 integrin expression on tumor cells would also contribute to cell migration.

Prostate tumors may also metastasize via the lymphatic and blood vessels. Once the tumor cell approaches a vessel, it may intravasate through a defined process by attaching to the endothelial cells, enzymatic digestion and movement through the basement membrane, retraction and/or attachment to the denuded basement membrane, and finally, escape into the interstitial space, e.g. The lumen of the vessel. Numerous studies have shown the α6 integrin is important in leukocyte tethering to laminin along denuded basement membranes in vessels, facilitating normal extravasation of leukocytes (194,234-236). It has been postulated that leukocyte intravasation into blood vessels is a good model system to study tumor cells which presumably use a similar mechanism (237,238).

Once attached to the vessel wall, the invadopodia contains proteases and adhesion molecules which facilitate the overall process of intravasation (and extravasation) (178-180). Invadopodia are cytoplasmic extensions which act as sites of extracellular matrix degradation and demonstrate localization of integrins and metalloproteases such as MMP-2 and MT1-MMP with low levels of TIMP-2 (179). Thus the tumor cell attaches to the vessel wall, focally degrades the basement membrane via proteolysis and extends invadopodia inbetween the endothelial cell junctions inducing endothelial cell retraction and facilitating passage through the basement membrane via cell migration (239). Once the tumor cell has entered the blood stream and extravasated into a distant secondary environment, it must survive and proliferate in order to give rise to a metastatic lesion.

One of the primary sites of prostate tumor metastasis is bone (134,215). The bone marrow is rich in laminin 10/11 protein (240), a ligand for the α6β1 integrin (191). Among other factors, both the extracellular matrix and integrins including α6β1 and α6β4 have been shown to be important in promoting cell survival (76,241-245).

In summary, the α6 integrin plays an important role in the process of metastasis in concert with other coordinating proteins. Initial loss of α6β4 integrin and upregulation of α6β1 integrin in prostate cancer may facilitate tumor cell detachment from the basement membrane. Secondly, the expression of α6β1 may assist tumor cell migration away from the primary site via the peripheral nerves within the laminin-rich perineural space. Additionally, the α6 integrin has previously been shown to play a role in both intra- and extravasation of the vessel walls which could also promote extracapsular penetration of the prostate gland. Lastly, among other factors, integrins and extracellular matrix proteins have been shown to promote cell survival of epithelial cells.

The α6 and β4 integrins in cancer

The role of the α6 integrin in metastasis and migration is underscored by studies which demonstrate an increase in the laminin receptor among a variety of cancers and micrometastatic lesions. The α6 integrin was originally identified in platelets (195,246) and has two forms (A and B) which are generated by alternative splicing. Each form contains an identical heavy chain with a similar light chain which differs by 18 amino acids at the C-terminal cytoplasmic region (247,248). The α6A form is a 1050 amino

acid protein with a 991-amino-acid extracellular, a 23-amino-acid transmembrane and a 36-amino-acid cytoplasmic domain (249). The α6A form has wide distribution in normal tissues throughout the body, while the α6B form is more restricted in its distribution found in heart, brain and embryonic stem cells (247,250,251). Previously, Delwel and others described two splice variants of the extracellular domain, resulting in minor amino acid alterations of both α6A and α6B (252), however no effect on adhesion or ligand binding was demonstrated (253). Of interest in this work is the α6A integrin, which is found on platelets (195,248), epithelia (254-257), endothelia (251,258,259), proximal and distal tubules of the kidney (260,261), astrocytes (262), Schwann and perineural cells (259,263) and lymphoid follicles (264).

In general the α6 integrin is found to be overexpressed in various epithelial carcinomas and implicated in invasion and metastasis (219,222,223,258,265). For example, upregulation of the α6 integrin is important for progression in metastatic breast carcinomas (266-269) and increased α6 integrin expression in the presence of laminin-5 correlated strongly with progression (266,270,271), suggesting that the α6 integrin is important for progression in some breast cancers. Although there are some breast carcinomas which have been shown to down-regulate α6 integrin (272,273). The α6 integrin also plays a role in metastasis and progression of sarcomas; including malignant fibrous histiocytoma, malignant peripheral nerve sheath tumors and synovial sarcomas (219). Additionally, examination of micrometastases from epithelial tumors demonstrated high α6 integrin expression (274). Multiple studies demonstrate the α6 integrin is important for adhesion, migration, and invasion (275-278), yet it is probably one of many

mechanisms which exist to facilitate migration and invasion, for instance, invasive basal cell carcinomas show a loss of the α6 integrin (279).

Yet a recent literature search revealed numerous reports of increased α6 integrin expression in epithelial carcinomas compared to normal cells. Normally epithelial cells contain both the α6β1 and α6β4 integrins (251). In tumors, increased expression of both of these laminin receptors has been reported. Prostate cancer however shows loss of α6β4 with persistent expression of α6β1. In some studies, expression of the α6 integrin was examined independent of its β subunit partner. In those studies, increased expression of α6 integrin was reported in lung carcinoma (280), endometrial carcinoma (281) and esophageal carcinoma (282). Previously increased expression of α6 integrin was also associated with neoplastic transformation of fibroblasts (283).

Reports which examined α6 paired with the β1 subunit reported overexpression in squamous cell carcinomas (284-287), variable in melanoma (288,289), highly metastatic melanoma (290,291), hepatocellular carcinoma (292,293), pancreatic carcinoma (294-296) and prostate carcinoma (155,157,158,221).

Observations of β4 integrin expression in normal prostate and its subsequent loss in neoplastic prostate, support the hypothesis that the β4 integrin functions as a stable adhesion receptor within the hemidesmosome. However observations in other epithelial cancers suggest the role of the β4 integrin may also facilitate migration in the absence of hemidesmosome components (220,297-302). The α6β4 integrin was observed to be highly expressed in bladder cancer (299,303), gastric carcinoma (301), head and neck

cancers (286,302), Lewis lung carcinoma (304), skin carcinoma (300), thyroid carcinoma (305) and pancreatic carcinoma (296). Whereas the α6β4 integrin was inversely correlated with differentiation in colorectal carcinoma (306) and decreased in basal cell carcinoma (300), oral squamous cell carcinoma (307,308), breast carcinoma (273,309,310), bladder cancer (299,303) and angiosarcoma (311).

In summary, the α6 integrin is upregulated in a number of different epithelial carcinomas in either the α6β1 or α6β4 heterodimers. Evidence suggests both adhesion receptors can facilitate migration and invasion, presumably dependent on whether or not the hemidesmosomal components are also present for α6β4 integrin. In the prostate however, studies suggest the α6β4 integrin primarily functions to anchor the cell to the extracellular matrix in a stable adhesion complex (the hemidesmosome), whereas the α6β1 integrin appears to be more important for motile functions of the cell as a component of the focal adhesion complex. It is likely that the functions of these receptors are cell-type specific and behave differently from that observed in prostate carcinomas.

The α6 integrin-containing complexes in normal and neoplastic prostate

The α6 integrin participates in at least two well-studied protein complexes at the cell surface in epithelial cells: the hemidesmosome (α6β4) and the focal adhesion (α6β1) (312,313). Normal prostate tissue contains both of these structures. Western blot analysis in Figure 1 shows retrieval of the α6 integrin by immunoprecipitation with components of the focal contact; including β1 integrin, paxillin and talin (panel A) hemidesmosome including β4, laminin-5, and CD151 (panel B) from normal prostate PrEC cells. The α6

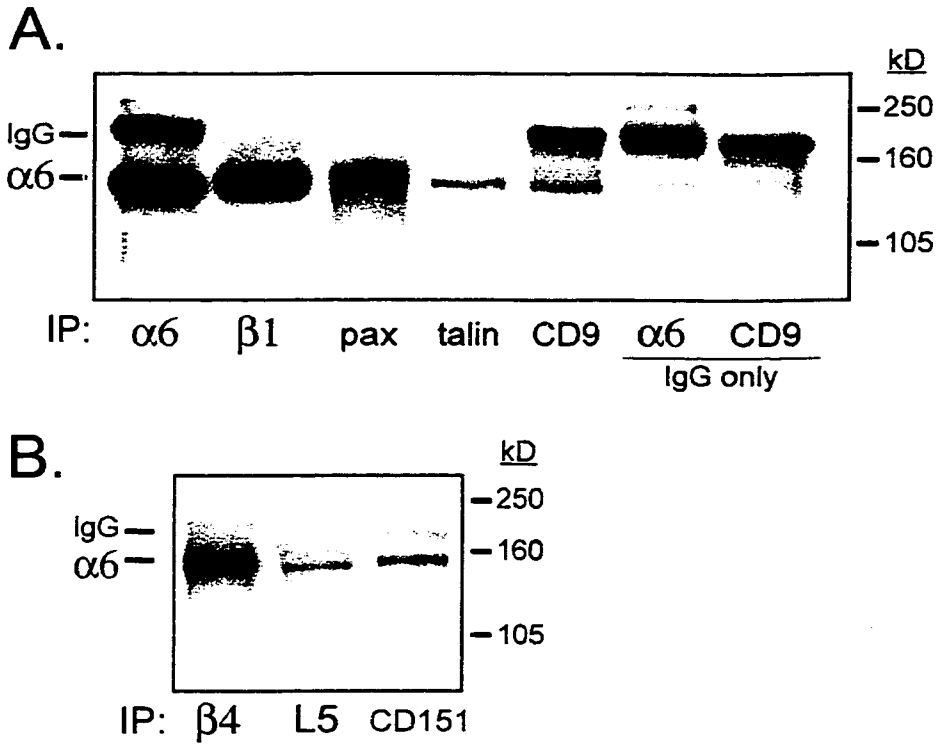

Figure 1: The α6 integrin is a component of two cell adhesion protein complexes.

Normal prostate epithelial PrEC-Hahn cells form both focal adhesions and hemidesmosomes. The α6 integrin associates with components from both protein complexes. In panel A, immunoprecipitations for α6 integrin (J1B5), β1 integrin (P4C10), paxillin (mAb349), talin (TD77) and CD9 (DUALL-1) were done. Samples were electrophoresed on a 7.5% non-reducing SDS-PAGE analysis and Western blotted for α6 integrin. Note α6 integrin associates with several focal adhesion proteins and also the tetraspanin, CD9, which has not been found to be associated with the focal adhesion. In panel B, three hemidesmosomal component immunoprecipitations are shown from the PrEC-Hahn cells. Immunoprecipitations for β4 (ASC3), laminin-5 (BM165) and CD151 (14A2.H1) are shown. Samples were electrophoresed on a 7.5% SDS-PAGE under non-reducing conditions and the membrane was probed for α6 integrin using the Western blotting antibody, AA6A.

integrin can also associate with CD9, which has not been found to associate with either focal contacts or hemidesmosomes (panel A).

In the neoplastic prostate there is a shift in the α6 integrin which occurs from α6β4 to the 'default' receptor, α6β1 integrin (158,314). Increased expression of α6β1 has been associated with a migratory and invasive phenotype in prostate cancer cells (158). In addition to this integrin change, there is also complete loss of the hemidesmosome complex in the neoplastic prostate, whereas there is an increase in the number of focal adhesion structures which contain α6 (74,75,78,155,157,165,230). The focal contact may facilitate a more pro-migratory tendency for prostate cancer cells which have lost the anchoring α6β4 integrin-containing complex, as has been demonstrated in keratinocyte and epithelial wound healing models (315).

Genetic studies in human blistering skin diseases may be an analagous model system for understanding the changes which occur in the hemidesmosome in the neoplastic prostate. These studies reveal loss or mutation of the individual components of the hemidesmosome result in dissociation of the entire complex (313). In prostate cancer, the mechanism which results in loss of the hemidesmosome is not understood. One possibility is simply that the normal basal cell population is lost in prostate cancer, as is progressively observed in PIN and cancer lesions. Since the basal cells normally express hemidesmosomes to facilitate anchorage to the extracellular matrix, loss of this population of cells could explain the loss of hemidesmosomes in cancer. Alternatively, the basal cells may play an important proliferative role in the normal and neoplastic human prostate (316). In 1996, Bonkhoff described a stem cell model in which stem cells

in the basal cell population acquire secretory luminal characteristics after androgen stimulation. Thus prostate cancer ultimately may be derived from these transformed stem cells (317,318).

The role of α6β4 integrin and laminin-5 in the hemidesmosome

The hemidesmosome is a specialized structure which acts as an anchor between the intracellular cytokeratin network and the extracellular matrix. Figure 2 shows a schematic of the hemidesmosome illustrating nine of at least eleven different proteins which are observed in the hemidesmosome including, keratin 5 and 14, α6β4 integrin, laminin-5, plectin, IFAP300, LAD-1, BP180, BP230, collagen VII (224,313,319-322). Mutation or loss of many of these proteins result in disruption of the hemidesmosome and blistering skin diseases known as epidermolysis bullosa. Specifically, loss of laminin-5 (323-329), α6 integrin (330,331) and β4 integrin (324,332-334) result in genetic blistering disorders of the skin which cause disruption of the entire structure and often lead to neonatal death due to secondary infections. It remains to be determined if similar mechanisms occur in prostate cancer, where components of the hemidesmosome are also missing and the ultrastructure is completely lost.

By electron microscopy, the basement membrane zone of the skin can be divided into four ultrastructurally distinct areas. The α6β4 integrin is contained in the hemidesmosome/upper lamina lucida along with the bullous pemphigoid antigens (BP180 and BP230) and plectin/HD1. Laminin-5 (also known as kalinin, epiligrin, nicein), is contained within the lower lamina lucida along with laminin-6 (k-laminin) and

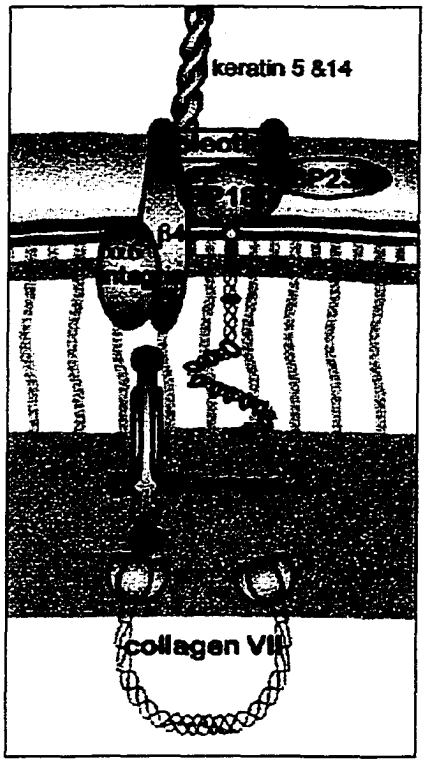

Figure 2: Schematic of the hemidesmosome

The schematic illustrates several components of the hemidesmosome including keratins 5 and 14, plectin/HD1, BP230, BP180, α6β4 integrin, laminin-5 and collagen VII.

nidogen/entactin. The lamina densa contains type IV collagen and perlecan, and the sub-lamina densa includes type VII collagen (335).

The α6β4 integrin is a transmembrane molecules contained within the hemidesmosome protein complex (78,164) which consists of two subunits, α6 is 140 kD and β4 integrin is 205 kD. The role of the α6β4 integrin in the hemidesmosome appears to be one of anchorage and stability to the basal lamina (336-338). In hemidesmosomes, it functions as a receptor to primarily bind laminin-5 (193), this tight adhesion complex supports mechanical stress and supports epithelial integrity (339). Of note, it has recently been reported that the hemidesmosome may form in the absence of the laminin-5 ligand (340,341), however adhesion via α6β4 integrin in the absence of laminin-5 would be severely compromised as is demonstrated by anti-β4 integrin antibodies which resulted in stimulation of migration in colon cancer cells (342). Additionally, the α6β4 integrin may play a role in tumor cell migration in the absence of hemidesmosomal proteins (220,342-345).

The β4 integrin is a unique integrin subunit due to an extraordinarily long cytoplasmic tail of approximately 1000 amino acid residues and two pairs of fibronectin type III repeats with an intervening segment with a tyrosine activation motif (TAM) (346). Phosphorylation of the TAM is necessary for hemidesmosome initiation and recruitment of BP180 (77,347-349). Also, 27 amino acids of the downstream TAM with the third fibronectin repeat are necessary for α6β4 integrin localization to the hemidesmosome. Several structural variants due to alternative splicing mechanisms of

the β4 integrin cytoplasmic domain have been identified which may function to modulate laminin binding in some cell types (346,350).

The β4 integrin initiates (77) and is necessary for hemidesmosome formation (76). Distinct domains in the NH2- and COOH- terminal regions of the β4 cytoplasmic domain are required for the localization of HD1/plectin and the bullous pemphigoid antigens, BP180 and BP230 in hemidesmosomes (77). Recently the tetraspanin protein CD151 was reported to associated with α6β4 integrin in the hemidesmosome and that its recruitment to the adhesion complex was dependent upon α6 integrin (351). The α6 integrin is widely distributed while the β4 integrin is more restricted in its distribution due to the ability of the α6 integrin to pair with the β1 subunit (251). Mutations in both the α6 and β4 integrin genes have been reported in humans leading to a severe blistering disease known as pyloric atresia-junctional epidermolysis bullosa (PA-JEB) due to disruption of the hemidesmosome protein complex. Genetic loss of either α6 or β4 integrin result in lethal phenotypes (324,332-334,352).

The α6β4 integrin has been shown to play a role in signal transduction. Ligand-induced phosphorylation of Tyr 1526 in the β4 cytoplasmic domain results in recruitment of secondary signaling proteins Shc and Grb2 (353). This phosphorylation event is necessary for α6β4 integrin-induced signaling to ERK, but not for the formation of hemidesmosomes (348) and site-directed mutagenesis demonstrated alternative regions within the β4 tail modulate signaling and hemidesmosome formation (353,354). Additionally, epidermal growth factor has been shown to inhibit recruitment of secondary

messengers to the β4 integrin, resulting in induction of α6β4-dependent cell migration (354). Clearly, more research is warranted to understand the subtle regulation and functions of the α6β4 integrin.

The primary ligand for α6β4 integrin is laminin-5. Together, these proteins interact within the hemidesmosome to ultimately connect the cellular cytoskeleton to the surrounding extracellular matrix. In both prostate cancer and PIN lesions, normal laminin-5 distribution and expression is altered (165). Laminin-5 is a heterotrimeric molecule with a cruciform shape consisting of the α3, β3 and γ2 subunits which is encoded by three different genes, LAMA3, LAMB3 and LAMC2, respectively (355). The C-terminal globular domain of the α3 subunit is the postulated recognition site for α6β4 integrin-mediated adhesion (356), whereas the N-terminals of the three laminin chains together bind the amino terminal (NC-1) of collagen type VII (357). Thus, laminin-5 functions to link the transmembrane components of the hemidesmosome to the extracellular collagen VII network of the basement membrane. Laminin-5 is assembled intracellularly, then secreted (358). It is unable to bind nidogen (a linking protein) due to truncated arm domains, and so it must crosslink with either laminin-6 and/or laminin-7 in order to bind nidogen and thus connect with the type IV collagen network in the basement membrane indirectly (359,360). While laminin-5 is not required for hemidesmosome formation (340), it appears to stabilize the α6β4 integrin clustering and quiescent phenotype in keratinocytes (340,341,361). Human blistering disease junctional epidermolysis bullosa (JEB) results from mutations in all three laminin-5 genes: LAMA3 (325,362), LAMB3 (326,327,329) and LAMC2 (328,363,364).

In summary, the normal distribution of the α6β4 integrin and laminin-5 is altered in prostate cancer. Loss of β4 integrin and laminin-5 is accompanied by a loss of the hemidesmosome ultrastructure and several of its components including BP180, BP230, HD1/plectin, and collagen VII. Loss of the β4 integrin is sufficient to disrupt the hemidesmosome, due to its centralized role in the formation of the complex. It is not yet clear what leads to loss of β4 and whether or not a cause and effect relationship exists among other components of the hemidesmosome as appears to be observed in epidermolysis bullosa skin diseases.

Model of hemidesmosome formation

The current model of hemidesmosomes formation is that the complex is formed in sequential steps. First, the α6β4 integrin clusters due to extracellular laminin-5. The other laminins (e.g. L-6, L-7) can self assemble in the matrix and form a network which binds nidogen and ultimately collagen IV and VII. Secondly, the β4 integrin cytoplasmic tail recruits a dimer of plectin/HD1 which stabilizes integrin clustering (365-367). Plectin/HD1 thus facilitates binding of the cytokeratins to β4 integrin (368). BP180 is then recruited to the hemidesmosome to bind both the β4 cytoplasmic tail and plectin/HD1 (77). There is some suggestion that BP180 may interact with the extracellular domain of α6 integrin (312,369), although it is unclear whether or not this is a direct or indirect association. Lastly, BP230 is recruited to the hemidesmosome by BP180 (370) and may also function to bind and stabilize the structure via BP180 and the intermediate filaments (370). Currently, loss of the hemidesmosome structure in prostate

carcinoma has been described by examination of individual protein components and electron microscopy. Further study is needed to understand the mechanisms which are responsible for loss of the hemidesmosome in prostate carcinoma.

The role of α6 integrin in the focal adhesion

Loss of normal β4 expression in prostate carcinoma is thought to lead to increased expression of the α6β1 heterodimer (221,230) which is a prominent component of the focal adhesion. It is now becoming clear that numerous subtypes of focal adhesions may exist and that these structures are dynamic in both size and composition throughout cell adhesion and migration processes (371). Unlike the hemidesmosome, the focal adhesion contains more than one integrin type. Focal adhesions (also known as focal contacts) are specialized cell adhesion structures (183,189,372) which serve both structural and functional roles in linking the extracellular matrix to the actin cytoskeleton during adhesion and migration. Loss of structural components of the focal adhesion resulted in defects in membrane blebbing, cell adhesion and spreading, loss of focal adhesion assembly and loss of stress fibers (373-375). The focal adhesion is composed of integrins, structural proteins, extracellular matrix components and numerous cytoplasmic proteins (187,376,377). The central structural proteins of the focal adhesion are illustrated in the schematic in Figure 3.

The α6 integrin, in addition to other integrins, is a prominent transmembrane component of the focal adhesion, pairing with the β1 integrin subunit. In this complex, α6β1 functions to link the extracellular matrix to the actin cytoskeleton for transmission

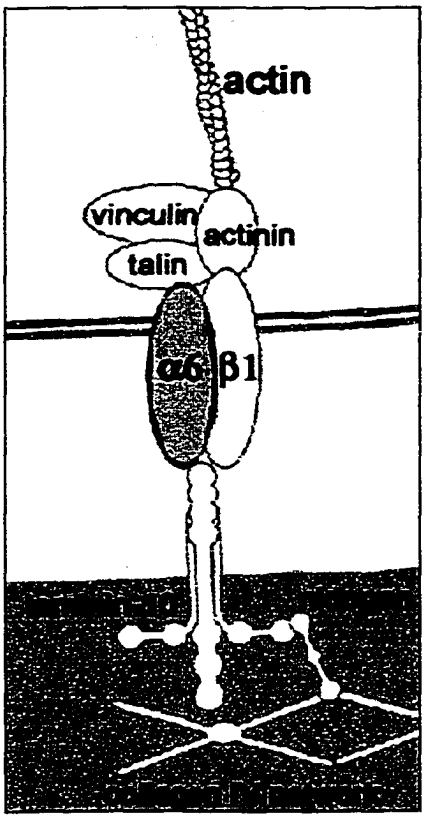

Figure 3: Schematic of the focal adhesion

The schematic illustrates several structural components of the focal adhesion including collagen IV, laminin10/11, α6β1 integrin, talin, vinculin, α-actinin and actin.

of signal and mechanical stability of the cell during matrix remodeling and migration (155,189,378). The primary extracellular matrix ligand for α6β1 is the recently described laminin 10/11 isoform, which also functions as a ligand for the α3β1 and α6β4 integrins (191). Like other laminins, laminin 10/11 is a cruciform complex composed of three subchains; α5, γ1, and β1. Binding of α6β1 integrin by laminin 10/11 facilitates integration of the focal adhesion complex via nidogen into the collagen IV network of the extracellular matrix (313).

The integrin α subunit cytoplasmic domains have been shown to be important for a diverse number of functions including: adhesion, motility, internalization, differentiation and cytoskeletal organization (204,205,379-388). Several studies have examined the role of the α6A cytoplasmic tail, when coupled to the β1 integrin subunit. Previously, ligand binding of α6A integrin was shown to induce tyrosine phosphorylation of paxillin and several other unknown proteins (389). As discussed above, the α6A integrin is an adhesion receptor for laminin which may facilitate cell migration via pseudopodial extensions. In comparison with the α6B splice variant, α6A is more migratory, more adhesive and induces more tyrosine phosphorylation upon ligand binding (275,276). Thus, the A and B variants of the α6 integrin differentially modulate the function of the α6β1 integrin. Sastry and others recently attempted to dissect the function of the α6A cytoplasmic domain from that of the full molecule. The α6A cytoplasmic domain was ectopically expressed in myoblasts and the resulting phenotype was suppression of proliferation, induction of differentiation and suppression of focal

adhesion signaling via focal adhesion kinase and mitogen-activated protein kinase (209,210).

The α6 integrin may function as a signaling receptor in the focal adhesion. The α6 cytoplasmic tail can be phosphorylated on serine residue 1041 (390,391), however physiological relevance of this event is still unclear. In other signaling receptors, phosphorylation often preceeds recruitment of secondary signaling molecules. Integrins are devoid of catalytic capability and depend upon recruitment of secondary proteins for signal transduction . Cytoplasmic proteins which have been reported to associate with the α6 subunit of the α6β1 integrin include calreticulin and paxillin. Calreticulin binds a conserved sequence, KXGFFKR in integrin α subunit cytoplasmic domains (392,393), including α6β1 (394). Therefore, α6 integrin can modulate cell adhesion, cell spreading and signaling via calreticulin (394-396).

Another cytoplasmic signaling protein which associates with α6β1 integrin in the focal adhesion is paxillin (397). Paxillin mediates protein—protein interactions (398) and has been demonstrated to bind to vinculin and focal adhesion kinase (116,398,399). Upon activation, the α6β1 integrin can alter paxillin phosphorylation (389) which is hypothesized to be important for coordination of signals from the focal adhesion to the cytoplasm and cytoskeleton (400-402). Recently, a conserved sequence for paxillin binding was identified on the α4 integrin cytoplasmic domain and a homologous sequence exists in the α6 integrin (403).

In thinking of how the focal complex is initially formed, it appears that the ligand laminin-10/11 (or other ligands for other integrins which are also associated with the

focal complex) may induce integrin clustering, talin is first recruited to the β cytoplasmic tail (404,405) and facilitates and/or stabilizes integrins clustering (377,406,407). Recruitment of α-actinin to the β integrin subunit facilitates binding to F-actin (408-411), however this is less well understood, and probably requires additional proteins. Vinculin appears to function as a molecular 'bridge' of the focal complex, thus stabilizing the structure and can be recruited to the complex by either talin or α-actinin (412-415).

Therefore, the α6 integrin is a integral component of at least two adhesion structures in epithelial cells, the hemidesmosome and the focal contact. Both α6β4 and α6β1 integrins facilitate signal transduction in their respective adhesion complexes. Observations of prostate cancer indicate loss of hemidesmosomes (and α6β4 integrin) and subsequent gain of focal adhesion structures which contain α6β1 integrin. The focal contact may facilitate a more pro-migratory tendency for prostate cancer cells which have lost the anchoring α6β4 integrin-containing complex. Therefore, alteration in α6 integrin-containing protein complexes may promote loosening of the cell from the basal lamina and use of a more migratory receptor, as has been demonstrated in keratinocyte and epithelial wound healing models.

Statement of the problem

Prostate cancer is a national health problem in the United States. The problem with mass screening for prostate cancer in the male population at large, is that the current screening tools available are suboptimal in their ability to accurately detect and predict prostate cancer behaviour. To complicate this, it is now known there are at least two

phenotypical categories of prostate cancer. One type may remain with the patient for a lifetime and not lead to any clinical symptoms, and the second type may quickly metastasize and become life-threatening. Because medicine is unable to histologically differentiate between these two types of prostate cancer, there is an urgent need for new diagnostic markers which would predict the capability of a given tumor to metastasize.

Previously, loss of the hemidesmosome structure and several of its components, including α6β4 integrin and laminin-5, have been observed in prostate carcinoma. Yet the α6β1 integrin is upregulated, unlike other integrins which are downregulated in their expression. As a result, there is a shift of the α6 integrin from α6β4 the hemidesmosome to the focal adhesion. Increased expression of α6β1 is also retained in micrometastatic lesions, suggesting that the α6 integrin may play a role in facilitating tumor migration and metastasis. The etiology of this integrin alteration in prostate cancer progression is not known, nor is it known whether this phenomenon occurs in other epithelial carcinomas. Additionally, previous studies indicated that a smaller variant of the α6 integrin existed. The following studies have extended this initial observation in order understand of the functional role of the smaller variant and its regulation. A better appreciation of the role of the α6 subunit in prostate carcinoma may lead to identification of novel targets for the improvement of diagnosis and prevention of progression.

II. MATERIALS AND METHODS

Amino Acid Sequencing by MALDI mass spectrometry and LC-MS/MS:

Amino acid sequencing of α6p was performed using two different analytical core services For analytical core service at DKFZ, Heidelberg, Germany, the α6p protein was immunoprecipitated using J1B5 and proteins were separated by SDS-polyacrylamide gel electrophoresis (7.5%, 3mm). After staining with Coomassie Blue, the α6p bands were excised, cut into small pieces (1 X 1mm), washed and dehydrated (2 X 30 min. with H_2O, 2 X 15 min. with 50% acetonitrile, and 1 X 15 min. with acetonitrile), and incubated with 0.5 μg of trypsin in 20 μl of digest buffer (40 mM NH_4HCO_3, pH 8.0) at 37°C for 16 hours. The supernatant was subsequently analysed by MALDI mass spectrometry (DKFZ, Heidelberg, Germany) using thin film preparation technique. Aliquots of 0.3 μl of a nitrocellulose containing saturated solution of alpha-cyano-4-hydroxycinnamic acid in acetone were deposited onto individual spots on the target. Subsequently, 0.8 μl 10% formic acid and 0.4 μl of the digest sample were loaded on top of the thin film spots and allowed to dry slowly at ambient temperature. To remove salts from the digestion buffer, the spots were washed with 5% formic acid and with H_2O. Sequence analysis was performed on a Procise 494 cLc protein sequencer using a standard program supplied by Applied Biosystems. The FastA data base searching program of Pearson and Lipman (416) was used for database searching.

For sequence analysis at the Proteomics Core of the Arizona Cancer Center and Southwest Environmental Health Sciences Center, University of Arizona, the α6p protein

was immunoprecipitated using J1B5 and proteins were separated by SDS–polyacrylamide gel electrophoresis (7.5%, 3mm). After staining with Coomasie Blue, the α6p bands were excised, cut into small pieces (1 x 1 mm) and subjected to in-gel digestion using trypsin as previously described (417). The extracted peptides following digestion were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a quadrupole ion trap Finnigan LCQ Classsic mass spectrometer equipped with a quarternary pump P4000 HPLC and a Finnigan electrospray ionization source (ThermoFinnigan, San Jose, CA). Peptides were eluted from a reverse-phase C18 micro-column (Vydac 250 x 1 mm, Hesperia, CA) with a gradient of 3-95% acetonitrile in 0.5% formic acid and 0.01% trifluroacetic acid over 150 min at a flow rate of 15 uL/min. Tandem MS spectra of peptides were analyzed with the SEQUEST program (v. Turbo Sequest) to assign peptide sequence to the spectra (418). SEQUEST analyses were performed against the non-redundant database.

Antibodies used in this study:

For ease of reference, anti-β4 integrin and anti-α6 antibodies used in these studies have been organized into charts (Table 1 and 2). The schematic (Figure 4) illustrates the relative position of the epitopes on the β4 integrin, modified from (419) . Epitope mapping for the anti-α6 integrin antibodies has proven more difficult, thus specific epitopes are indicated for two antibodies, AA6A and 4E9G8 in the table. The AA6A antibody worked well for detection of α6 and α6p integrin on both whole cell lysates and immunoprecipitations which had been stored for no more than two weeks at -20°C.

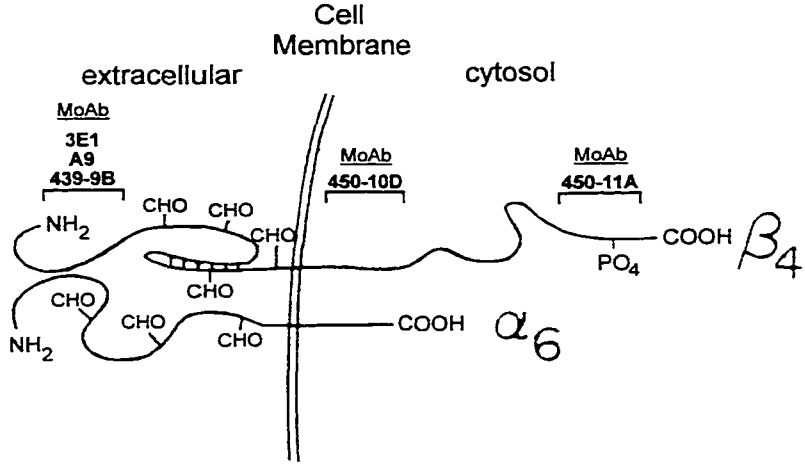

Figure 4: Relative positions of the epitopes of the β4 integrin antibodies

The schematic diagram of the α6β4 integrin illustrates the relative positions of the β4 integrin antibodies used in these studies. Extracellular β4 antibodies include 3E1, A9 and 439-9B. Intracellular β4 antibodies include 450-10D and 450-11A. The schematic was modified from (419).

Significant cross reaction of AA6A occurred with J1B5, GoH3 and DUALL-1, therefore antibody controls were necessary when using these for immunprecipitation. Western blot analysis with 4E9G8 worked best followed by immunoprecipitation with anti-α6 integrin antibodies, GoH3 or J1B5, and was not useful for detection of either α6 or α6p within whole cell lysates.

Other anti-integrin antibodies used include: anti-α5 integrin antibody, P1D6, mouse IgG3 (Gibco BRL: Gaithersburg, MD, USA) (420), and anti-β1 integrin P4C10, mouse ascites IgG1 (Gibco BRL: Gaithersburg, MD, USA) (421). Additional antibodies used in these studies are listed here. An anti-pan cytokeratin, polyclonal rabbit antibody (18A) which stains prostate basal cells more intensely than luminal cells (422) was used in dual immunofluorescence studies to locate normal and carcinomatous epithelial cells. GB3 is an anti-laminin-5 antibody whose epitope is recognized when all three chains of the molecule are assembled (423) (Seralab, Crawley Down, Sussex, England). Secondary antibodies used were adsorbed to reduce cross-reactivity against human tissues (Jackson Immuno Research Labs, West Grove, PA): goat anti-rabbit FITC, donkey anti-mouse Cy3, and donkey anti-rat Cy3. Other antibodies used are listed in Table 3.

Table 1: Anti-human β4 Integrin Antibodies used in these studies:

Clone	Epitope	Source	Funct.	Dilution	Availability/Reference(s)
3E1	β4 integrin extracellular	Mouse Ascites IgG1	IF IP	1:2000 1:100	Gibco-BRL: Gaithersburg, MD (424)
439-9B	Human β4 integrin extracellular	Rat IgG2bK	IF IP WB	1:50 1:500 1:200	Pharmingen: San Diego, CA Steve Kennel (425)
450-11A	Human β4 integrin intracellular	Mouse IgG1	IF WB	1:50 ?	Research Diagnostics, Flanders, NJ Steve Kennel (419)
450-10D	Human β4 integrin intracellular	Mouse IgG2a	IF ?	1:50	Steve Kennel (419)
UM-A9	Human α6β4 integrin extracellular	Mouse IgG2a	IF IP	1:100 1:500	Ancell: Bayport, MN Art Mercurio, Tom Carey (426,427)
ASC3	Human β4 integrin	Mouse IgG1K	IF IP FC	? 1:100 ?	Chemicon International, Temecula, CA (428)

Abbreviations: IF = immunofluorescence, IP = immunoprecipitation, WB = Western Blot, FC = flow cytometry

Table 2: Anti-human α6 Integrin Antibodies used in these studies:

Clone	Epitope	Source	Funct.	Dilution	Availability/Reference(s)
GoH3	Human α6 integrin (extracellular)	Rat IgG2a	IF IP	? 1:100	Accurate Chemicals: Westbury, NY, USA (429)
BQ16	Human α6 Integrin (extracellular)	Mouse IgG1	IF IP WB	? 1:100 1:100	Monica Liebert, Dept. of Urology, UT, M.D. Anderson Cancer Center, Houston, TX (303)
UM-A9	Human α6β4 Integrin (extracellular)	Mouse IgG2a	IF IP	1:100 1:500	Ancell: Bayport, MN Art Mercurio, Tom Carey (426,427)
4F10	Human α6 Integrin	Mouse IgG2b	IP FC	1:100 ?	Chemicon International, Temcula, CA (429)
J1B5	Human α6 Integrin (activating?)	Rat IgG	IF IP	 1:100	Caroline Damsky UCSF, San Francisco, CA (430)
AA6A	Human α6A Cytoplasmic tail (last 16aa)	Rabbit affinity purified	IP WB	1:500 1:10,000	Bethyl Laboratories Inc. Montgomery, TX Anne E. Cress As done previously (248)

Abbreviations: IF = immunofluorescence, IP = immunoprecipitation, WB = Western Blot, FC = flow cytometry

Table 2: Anti-human α6 Integrin Antibodies – CONTINUED

4E9G8	Unphos. human α6A cytoplasmic tail	Mouse IgG1	WB	1:100	Immunotech: Marseille, France (195,246)
J8H	Human α6 Integrin (cytoplasmic)	Mouse IgG1k	IF IP FACS	Undilute 1:5 1:2	Arnoud Sonnenberg (351)
A33	Human α6 Integrin (a.a. 1-500)	Rabbit	WB	1:500	Arnoud Sonnenberg (351)

Abbreviations: IF = immunofluorescence, IP = immunoprecipitation, WB = Western Blot, FC = flow cytometry

Table 3: Other antibodies used in these studies:

Clone	Epitope	Source	Funct.	Dilution	Availability/Reference(s)
YL1/2	Human tubulin	Rat IgG2a	IF	1:250	Chemicon International, Inc. CA, USA (431)
349	Human paxillin	Mouse IgG1	IP WB	1:50	Transduction laboratories, Lexington, KY (432,433)
TD77	Human talin C-terminal	Mouse IgG1	IP WB	1:100	Autogen Bioclear UK Ltd. (434)
DU-ALL1	Human CD9	Mouse IgG1	IP	1:100	Sigma, St. Louis, MO (435)
BM165	Human Laminin-5 α3 chain	Mouse	IP WB Block	1:100 ?	RE Burgeson (436)
14A2.H1	Human CD151	Mouse IgG1k	IP	1:100	Research Diagnostics, Flanders, NJ, USA (437)

Abbreviations: IF = immunofluorescence, IP = immunoprecipitation, WB = Western Blot, FC = flow cytometry

Biotin-labeling of tissue culture cell surface proteins:

Previous protocols (438,439) were slightly modified. Briefly, cells were grown to confluency 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 $MgCl_2$, 1.0 mM $CaCl_2$, pH 7.45). Cells were then incubated with 2 ml of HEPES buffer supplemented with Sulfosuccinimidyl hexanoate conjugated biotin (500 μg/ml) (NHS-LC-Biotin, Pierce, Rockford, IL, USA) which is impermeant to cell membranes (440), to label cell surface proteins for 30 min at 4°C. Cells were washed three times and lysed in cold RIPA buffer (150 mM NaCl, 50 mM Tris, 5 mM EDTA, 1% (v/v) Triton X-100, 1% (w/v) deoxycholate, 0.1% (w/v) SDS, pH 7.5) plus protease inhibitors (PMSF, 2 mM; leupeptin and aprotinin, 1 μg/ml). The lysate was briefly sonicated on ice before centrifugation at 10,000 RPM for 10 minutes and the supernatent was collected for immunoprecipitations.

Cell lines and culture conditions:

All human cell lines were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cell lines DU145H, HaCaT and PC3-N were grown in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco BRL: Gaithersburg, MD, USA) plus 10% fetal bovine serum (FBS). Cell lines MCF-7, PC3-ATCC, LnCap, H69 were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL: Gaithersburg, MD, USA) plus 10% FBS. SW480 cells were grown in super media (DMEM plus 5% non-essential amino acids, 5% L-glutamine, 5% sodium pyruvate, 10% FBS). Normal prostate cells,

PrEC were grown in PrEGM bullet kit media (Clonetics: San Diego, CA, USA). The following cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA): MCF-7 (human breast tumor), PC3 (human prostate tumor), LnCap (prostate carcinoma cell line), H69 (human lung carcinoma), SW480 (human colon carcinoma). The DU145H cells were isolated by us as previously described (158,441) and contain only the α6A splice variant (155). The PC3-N cells are a variant of PC3 prostate carcinoma cell line (162). The HaCaT cells (normal immortalized keratinocyte cell line) (442) were obtained from Dr. Norbert E. Fusenig, German Cancer Research Center, University of Heidelberg, Germany. PrEC (a normal prostate cell line) was obtained from Clonetics (San Diego, CA, USA).

Calcium-induced terminal differentiation assay, cell culture techniques and preparation of calcium medias used for mouse 291, 03C and 03R cells have been described previously (443,444). Cells were maintained in 0.04 mM calcium (low calcium) and switched to medium with 0.14 mM calcium (medium calcium) or 1.4 mM calcium (high calcium) by 60% confluency. After 24 hours treatment, cells were collected in PBS, centrifuged and frozen in a dry ice bath and kept in -80°C freezer until used.

Endoglycosidase H and F digestions:

For digestions, 200 μg of whole cell lysate was first immunoprecipitated overnight with anti-α6 integrin antibody, J1B5 in microcentrifuge tubes. The following day, the beads were washed three times with RIPA buffer and the sample was

resuspended in 35µl of 2X non-reducing sample buffer containing 4 mM $CaCl_2$ plus 1 mUnit either endoglycosidase H or F (obtained from Sigma, St. Louis, MO, USA) which had been diluted in 10% glycerol. Tubes containing reactions were placed in a shaking hot water bath at 37°C overnight. The following morning the samples were placed on ice and examined by SDS-PAGE followed by Western blot analysis using anti-α6 integrin antibody, AA6A.

Immunofluorescence for Actin and Tubulin

Cells were grown optimally on glass coverslips for 1 to 2 days in IMDM containing 10% FBS. Media was exchanged for serum free media containing 0.1% BSA and drugs were added. Slides on glass coverslips were washed in Dulbecco's phosphate buffered-saline (PBS) (Gibco-BRL: Gaithersburg, MD), and fixed in 3.7% paraformaldehyde in PBS for 20 minutes. Cells were incubated in 50 mM ammonium chloride in PBS for 10 minutes and then permeabilized in PBS containing 0.2% Triton-X 100 for 10 minutes. Cells were placed in a humidified chamber and re-hydrated in 10% FBS/PBS for 30 minutes then incubated with either anti-tubulin antibody YL1/2 or Alexa Fluor 568 phalloidin in 10% FBS/PBS for 1 hour. For visualization of actin filaments, Alexa Fluor 568 phalloidin was obtained from Molecular Probes, Eugene, OR, USA, (445). Cells were washed in PBS 3 times for 5 minutes then appropriate secondary antibody conjugated for fluorochromes were incubated with cells in 10% FBS/PBS for 1 hour in the dark. Cells were washed 3 times in PBS and then affixed to slides and sealed with Mowiol. Coverslips were stored at 4°C for no longer than one week until examined

using a Zeiss 410 confocal scanning laser microscope. The resulting images were processed using Adobe Photoshop 5.5.

Immunohistochemistry for tissues:

For the colocalization of β4 or laminin-5 with cytokeratin, frozen sections were fixed in 4°C acetone for 5 minutes. Slides were rehydrated with PBS plus 0.1% BSA for 5 minutes, incubated with PBS plus 0.1% BSA and anti-β4 integrin antibodies or anti-laminin-5 monoclonal mouse antibodies and anti-cytokeratin polyclonal rabbit antibody for 1 hour at room temperature. Tissue samples were washed with PBS and incubated with appropriate secondary antibodies for 1 hour in the dark at room temperature and rinsed several times in PBS. Coverslips were placed on slides, sealed with Mowiol and stored at 4°C until examined using a Zeiss 410 confocal scanning laser microscope. The resulting images were processed using Adobe Photoshop 5.5.

Immunoprecipitations:

For immunoprecipitations, cells were grown to 90% confluency and then lysed in 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) containing protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM PMSF), sonicated and centrifuged at 14,000 RPM to remove the insoluble pellet. Cell lysate supernatent (10-500 μg) was used for each reaction and incubated with 35 μl of protein G sepharose and 1-5 μg of antibody. The final volume of the lysate was adjusted to 500 μl with RIPA buffer. The mixture was

rotated overnight at 4°C. After incubation, complexes were washed three times with cold RIPA and eluted in 2X non-reducing sample buffer. Samples were stored at -20°C for no longer than one week before analysis by SDS-PAGE. To avoid degradation of the α6p integrin, cell lysates were also stored for no longer than one week at -20°C before analysis.

For co-immunoprecipitations of α6 and α6p integrin from hemidesmosome and focal adhesion structures, cells were lysed as above but the insoluble pellet was not removed. Whole cell lysate (500 μg) per reaction was pre-cleared with 35 μl of protein G sepharose for one hour at 4°C in a total volume of 500 μl adjusted with RIPA buffer. Next, the pre-cleared lysate was collected into a new tube, incubated with 35 μl protein G sepharose and 1-10 μg of antibody and the final volume of the reaction was adjusted to 500 μl with RIPA buffer. The mixture was rotated on a Labquake shaker overnight at 4°C. After incubation, complexes were washed six times with cold RIPA and eluted in 2X non-reducing sample buffer.

Laminin-5 matrix preparation:

The laminin-5 matrix used for cell culture studies was prepared using HaCaT cells (442). Cells were plated onto glass coverslips and allowed to grow to confluency over a period of 1 to 2 days. Coverslips were then washed 3X with sterile phosphate buffered saline (PBS). Cells were removed by incubation in 0.04M NH_4OH for 15 minutes at room temperature. Matrix coated coverslips were then washed 3X in 10mM

HEPES buffer, pH 7.5. Human prostate carcinoma cells, DU145H were then plated and allowed to grow to 80% confluency overnight.

Peptide Assays using HYD1 and C1:

A series of D-amino acid peptides were originally identified using the "one-bead one-compound" combinatorial library method as previously described (446). A hybrid peptide, HYD1, (single-letter amino acid code, KIKMVISWKG) was postulated and tested for anti-adhesion (447). Another sequence called C1 (AKRNRIIIYAWKG) was postulated based on a homology model of the α6 integrin structure and a consideration of the binding loops. The peptides used in these experiments were synthesized with a biotin at the amino terminus and purified by Molecular Resources Core Facility, Colorado State University, Dept. of Biochemistry, Ft. Collins, Colorado. The HYD1 and C1 were active in inhibiting integrin-mediated cell adhesion. Two inactive, scrambled equivalents were also used in these experiments for controls, HYDS (WIKSMKIVKG) and CS (KAIKNAWYRIIRG). Lyophilized peptides were freshly dissolved in dH_20 for a final concentration of 1μg/μl. Each 3.5 cm diameter tissue culture-treated plate was coated with 250μg of peptide (HYD1, HYDS, C1, CS). Peptide was evenly distributed using the tip of a sterile pipette and was allowed to dry overnight at room temperature in the tissue culture hood. The following day, DU145H cells were seeded at 80% confluency and allowed to adhere overnight to immobilized peptide for 18 hours in serum free medium. All cells were collected with a cell scraper into a 5 ml centrifuge tube and collected from the media by centrifugation into a cell pellet. The cell pellet was washed 2 times in

HEPES buffer and then lysed in RIPA plus protease inhibitors. Equal amounts of protein (15 μg) were loaded and separated by SDS-PAGE under non-reducing conditions, transferred to PVDF membrane and blotted for α6 integrin using anti-α6 integrin antibody, AA6A.

For cell recovery from peptide experiments, DU145H cells were allowed to adhere to immobilized peptide under serum free conditions for 24 hours. Fetal bovine serum was added back to the cells growing on peptide under serum free conditions for a total concentration of 10%. Cells were allowed to recover for 24 hours post-addition of serum and then collected and lysed as described above.

Pharmacological inhibition of cytoskeletal and signaling components:

Pharmacological inhibitors used in these studies have been organized into a chart for reference, see Table 4. Human prostate carcinoma DU145H cells were grown to 90-95% confluency for 1 to 2 days. The following day, the media was replaced with serum free IMDM media containing 0.1% BSA and drug (10μM Cytochalasin D, 8μM Nocodazole, 15nM Calyculin A, 50μM Okadaic Acid, 50μM 1-nor-okadaone) or vehicle for 18 hours in the dark. For time courses, media was exchanged for serum free IMDM containing 0.1% BSA at the start of the time course and drug was added at appropriate time points. Cells were then collected, centrifuged for 5 minutes at 800 rpm and washed 2 times in HEPES buffer. Cell pellets were lysed in RIPA buffer with protease inhibitors and sonicated. Whole cell lysate (10-15μg) was loaded and electrophoresed on a 7.5%

Table 4: Pharmacological inhibitors used in these studies:

Drug	General Function	[Conc.]	Availability/Reference(s)
Calyculin A	Serine/Threonine Phosphatase inhibitor with high specificity for PP1 and PP2A	15 nM in DMSO	Alexis Biochemicals, San Diego, CA, USA
Okadaic Acid	Serine/Threonine Phosphatase Inhibitor higher specificity for PP1 than PP2A	62.1 μM in DMSO	Alexis Biochemicals, San Diego, CA, USA
1-nor-okadaone	Inactive analog of Okadaic acid	62.1 μM in DMSO	LC Laboratories, Woburn, MA, USA
Cytochalasin D	Inhibitor of actin Polymerization	10 μM in DMSO	Sigma, St. Louis, MO, USA
Nocodazole	Inhibitor of Microtubule Polymerization	8 μM in dH_2O	Sigma, St. Louis, MO, USA

SDS-polyacrylamide gel under non-reducing conditions. Proteins were transferred to PVDF membrane followed by Western analysis for α6 integrin with anti-α6 integrin antibody, AA6A Protein bands for α6 and α6p were scanned and quantified using Scion Image Analysis software as described previously (448) and graphed using Excel software.

Surface changes of α6, β1 and α6p were determined by surface biotinylation of DU145H cells for 3 hours at 4°C, followed by 18 hours of drug treatment for cytochalasin D. For nocodazole studies, DU145H cells were labeled for 1 hour after the 18 hour drug treatment. Biotinylated DU145H cells were lysed and 200μg total protein was used for immunoprecipitations with anti-α6 integrin antibody, J1B5. Samples were analysed as above and PVDF membrane was incubated with HRP-streptavidin. Resulting protein bands for α6, β1 and α6p from treated or vehicle samples were quantitated and graphed.

RNA extraction and northern blot analysis of pharmacologically inhibited DU145H cells:

Human prostate carcinoma DU145H cells were treated for 18 hours with drug (Calyculin A, 15nM; Okadaic Acid, 50μM; 1-nor-okadaone, 50μM; or Cytochalasin D, 10μM) or vehicle. Total RNA was extracted from cultured cells by acid guanidinium thiocyanate-pheno-chloroform extraction (449). Total RNA (15μg) was separated on a 1% agarose gel containing 1.85% formaldehyde, transferred to nylon membranes in 10X SSC for 48 hours and cross-linked by ultraviolet light. Blots were probed overnight as

previously described (162) at 42°C with a random primed ^{32}P-labeled probe of a 1098-bp fragment (generated by sst1 and nco1 digest) corresponding to the N-terminal of human alpha 6 integrin (GenBank Accession X53586), and washed for 30 minutes at temperatures ranging from 42°C to 56°C for 30 minutes using the following conditions: 2X SSC/0.1% SDS, 0.3X SSC/0.1% SDS, and 0.1X SSC/0.1% SDS. Blots were then exposed to X-OMAT AR film (Kodak, Rochester, NY). A 1.2-kb PstI fragment of human GAPDH (GenBank Accession J04038) was used for loading control.

RT/PCR Analysis:

Total cellular RNA was isolated by guanidium isothiocyanate cell lysis and cesium chloride purification (450). RNA was quantitated from spectrophotometric absorbance measurements at 260 nm. First strand cDNA was synthesized in a 30 µl reaction comprised of 1x PCR buffer (10 mM Tris, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$); 1 mM each dATP, dCTP, dGTP, and dTTP; 100 pmol random hexamer, 20 units RNAsin, 200 units SuperScript reverse transcriptase II (BRL, Bethesda, MD) and 3 µg of total cellular RNA incubated at 42°C for 60 minutes. The reaction was terminated by incubation at 99°C for 10 minutes. Integrin α6-specific PCR was performed by adding 80 µl of amplification reaction buffer (1x PCR buffer, 25 pmol of integrin α6-specific primers, and 2.5 units of Taq DNA polymerase) to the cDNA reaction, followed by incubation at 94°C for 5 minutes, then 40 cycles of 94°C for 1 minutes, 60°C for 3 minutes, 72°C for 10 minutes, with a final extension at 72°C for 5 minutes and a quick chill to 4°C. The PCR primers were derived from the integrin α6 cDNA sequence

reported by Tamura et al. (346), Genbank accession X53586; the upstream primer sequence was from nucleotide 160 to 179 and the downstream primer was from nucleotide 3404 to 3423. The PCR product identity was confirmed by diagnostic restriction enzyme digests and size separation of the products through a 1xTBE - 1.5% agarose gel. Products were visualized by ethidium bromide staining and UV fluorescence.

Tissue Samples

Surgical samples of normal, PIN and malignant human prostate, breast and colon tissues were embedded in O.C.T. medium (Miles, Elkhart, IN), and immediately snap frozen in an isopentane bath cooled by Freon. Cryostat sections were stained with hematoxylin and eosin, and examined in order to select areas for study. Sections used for immunohistochemistry (IHC) were fixed for 5 minutes in 4°C acetone, and stored at -20°C until used.

Tissue Grading and Analysis by Immunofluorescence Microscopy

The prostate carcinomas (n = 11) were graded 1-5 using the Gleason system (451). The breast carcinomas (n = 5) were classified as infiltrating, lobular or ductal and graded 1-3 based on Elston's criteria (452). The colon carcinomas (n = 6) were graded as well, moderate, or poorly differentiated.

Basal polarization of the β4 integrin was determined by direct comparison of the field to a normal gland within the same tissue, which showed a distinct linear

organization of protein along the basal aspect of the cells composing the gland. IHC staining was semi-quantitatively evaluated by the following scale of intensity: (0) no staining above background, (+/-) weak staining, (+1) positive staining up to intense staining (+2). A photograph which included these five levels of staining was used as a standard reference of comparison for scoring individual cases. Cases which scored (+1) to (+2) were recorded as positive.

Two-dimensional non-reduced/reduced gel electrophoresis:

Nonreduced/reduced two-dimensional electrophoresis was done as previously described (453). Samples were incubated in 0.625 M Tris-HCl, pH 6.8, 10% glycerol, 10% SDS, and applied to SDS-PAGE (7.5% acrylamide) without reduction. The excised lanes were incubated in reducing sample buffer for 15 minutes and horizontally loaded at the top of a second dimension slab gel (also 7.5% acrylamide). Proteins were electrotransferred to PVDF membrane (Millipore: Bedford, MA, USA), incubated with either peroxidase-conjugated streptavidin or Western blotting primary antibodies followed by secondary antibody conjugated to horseradish peroxidase and visualized by chemiluminescence (ECL Western Blotting Detection System, Amersham, Arlington Heights, IL, USA).

Western Blot Analysis

Immunoprecipitation and whole cell lysate samples were boiled for 5 minutes just prior to loading onto a 7.5% SDS-polyacrylamide gel for analysis under non-reducing

conditions. Proteins resolved in the gel were electrotransferred to Millipore Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore: Bedford, MA, USA). Blots were incubated with either peroxidase-conjugated streptavadin or Western blotting antibodies plus 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 exposured to Kodak X-OMAT film (Eastman Kodak Company, Rochester, NY, USA). Resulting protein bands were quantitated as previously described (448) and graphed using Excel software.

XTT Cell Proliferation Assay:

The viability of human prostate carcinoma DU145H cells treated with various cytoskeletal inhibitors was determined using a colorimetric 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). Cells were seeded overnight at a density of 10,000 cells per well in a 96 well plate. The following day, cells were treated with drug (Calyculin A, 15nM; Okadaic Acid, 50μM; Cytochalasin D, 10μM; Nocodazole, 8μM; or vehicle) in serum free IMDM media containing 0.1% BSA for 18 hours. 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 El_x800 microplate reader (Bio-Tek Instruments, Winooski, VT, USA) and 6 replicate wells were averaged for each treatment group. Cell survival was calculated as

a percentage by dividing the absorbance of the drug-treated group by the absorbance of the corresponding vehicle-treated group.

III. UNIQUE EXPRESSION PATTERN OF THE α6β4 INTEGRIN AND LAMININ-5 IN HUMAN PROSTATE CARCINOMA

Introduction

Prostate cancer, the most common visceral neoplasm in males (1) is variable in its clinical progression. Many cases present with slow-growing, clinically inapparent forms of the invasive carcinoma confined to the prostate, while others present with a rapidly growing, aggressive tumor that quickly metastasizes (20). The cause of this variability is still unknown, but is due in part to differences in the ability of a given carcinoma for cellular invasion and metastatic spread.

During invasion, tumor cells can make an extracellular matrix which differs from that found in the normal structures. The invading cells interact with the new basal lamina to promote migration (155,301). Prostate carcinomas synthesize a new basal lamina lacking the β3 and γ2 subchains of laminin-5 (165,454). Colorectal carcinomas produce a laminin-5 rich basal lamina, the presence of which was correlated with a high degree of metastasis to the liver. These metastatic lesions often had intact, well-defined basal lamina (455). Gastric carcinomas also have been shown to increase their expression of laminin-5 at the invasive edge (301,456).

Invasive prostate carcinoma also is associated with changes in cell adhesion receptors. In particular, loss of E-cadherin (457-460), gain of N-cadherin (162), loss of hemidesmosomes (157,165), and integrin alterations occur (74,154,221,230,459,461).

The α6β4 integrin which is expressed mainly in stratified epithelial tissues, is the predominant integrin pair found in normal prostate epithelium and is associated with the hemidesmosome, laminin-5 and intermediate filaments (76). The α6β4 integrin appears to be down-regulated in prostate carcinoma (74,155,157,230) and some breast carcinomas (271,310). Other studies indicate that it is persistent in head and neck tumors (426), colon carcinoma (306,344) and breast carcinoma (273,309).

Although the loss of β4 integrin and laminin-5 are consistently observed in prostate cancer, it is unknown whether this occurs progressively or suddenly during the progression of prostate cancer. The goal of this study was to examine both α6β4 integrin and laminin 5, in human prostate carcinoma tissues including normal, PIN and cancer. Additionally, other epithelial carcinomas were investigated to determine whether or not this combined defect was unique to prostate.

Results

Tissue grading

Of the 11 prostate carcinomas examined, 10 were classified as Gleason grade 3 and one was Gleason grade 4. Four of the five breast carcinomas were infiltrating ductal, the remaining one was an infiltrating lobular carcinoma. Three of the four infiltrating ductal carcinomas were classified as Elston grade 2 lesion, and one was Elston grade 3. Of the six colon carcinomas examined, four were well-differentiated and two were moderately differentiated.

Expression of the β4 Integrin in Normal, PIN and Carcinoma of the Prostate

The dual IHC staining of normal, PIN and invasive prostate carcinoma tissues (Fig. 5A-C stained with 18A anti-cytokeratin; Fig. 1D-F stained with 3E1, anti-β4 integrin) indicated that the loss of the β4 protein epitope could be traced through these progressive lesions. In normal prostate glands, the β4 integrin was abundantly present in the basal cells (Fig.5 A, D). In PIN, an hypothesized premalignant lesion, the β4 integrin was retained by the residual basal cells, but was not present in areas in which the basal cells were lost or where transformed PIN cells abut the basal lamina of the gland (Fig.5 B, E). The β4 integrin also was observed in the vascular endothelium (v) (Fig. 1E) as previously described (462). In carcinomatous prostate glands, the β4 integrin was not expressed (Fig. 5C, F).

Expression of Laminin-5 in Normal, PIN and Carcinoma of the Prostate

Dual IHC staining of normal, PIN and carcinoma prostate tissues (Fig. 6A-C stained with 18A anti-cytokeratin; Fig. 2D-F stained with GB3, anti-laminin-5 monoclonal antibody) indicated that the loss of assembled laminin-5 protein could be traced through these progressive lesions. In normal prostate glands, the assembled laminin-5 was expressed by the basal cells (Fig. 6A, D). In the PIN lesion, assembled laminin-5 protein was localized within and adjacent to the residual basal cells, and was not seen elsewhere in the gland (Fig. 6B, E). In the carcinoma glands (Fig. 6C, F), assembled laminin-5 was no longer detectable. The punctate staining within these images in the tissue sections represents the autofluorescence of lysosomal pigments.

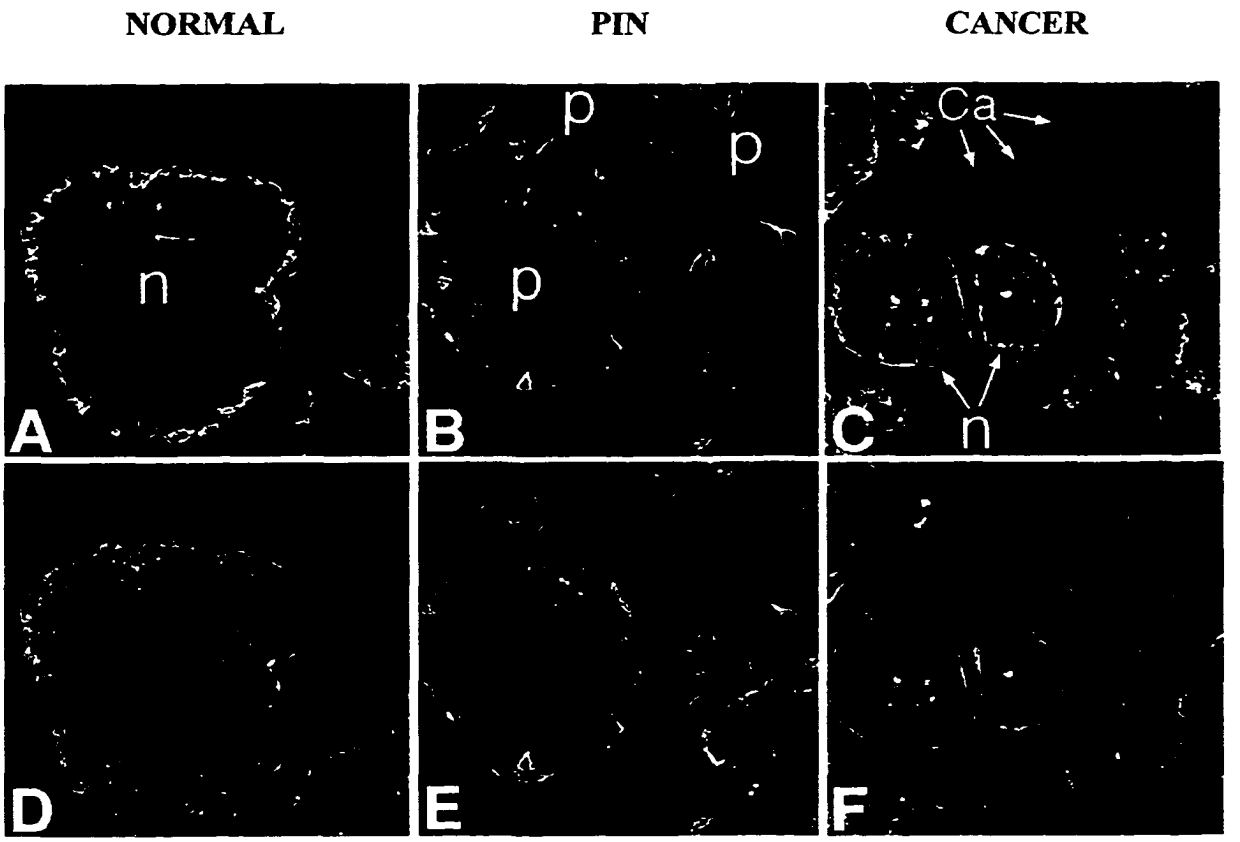

Figure 5: Expression pattern of the β4 integrin in normal, PIN and carcinoma of the prostate.

Dual immunofluorescence staining of prostate with 18A anti-cytokeratin (A,B,C) and 3E1 anti-β4 integrin (D,E,F). Panels A and D show staining of β4 in the basal cells of normal prostate gland (n) and adjacent microvasculature. There was a similar expression pattern for β4 in the residual basal cells in the PIN lesion (p) (Panels B and E). Panels C and F show the absence of basal cells in the invasive carcinoma (Ca) and the complete absence of β4 integrin staining with 3E1 anti-β4 antibody. Magnification, X400 for (A,D,C,F), X647 for (B,E).

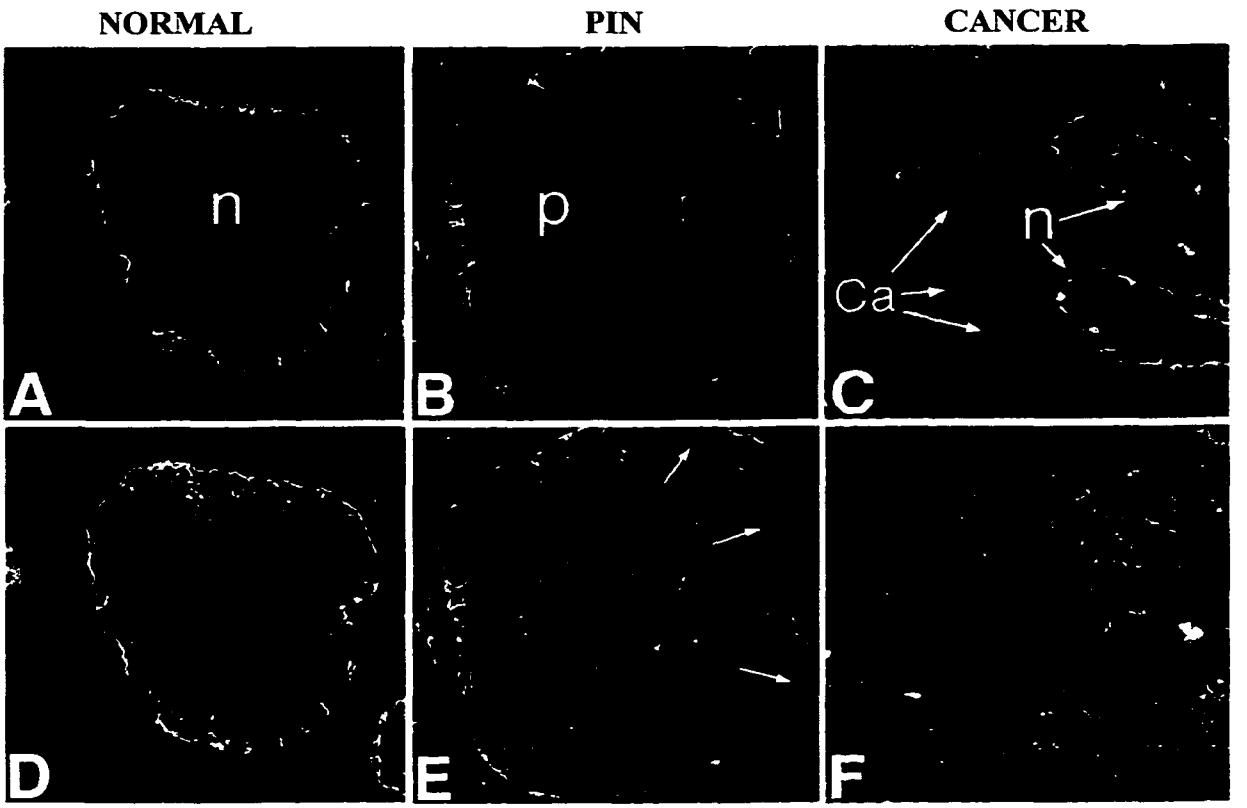

Figure 6: Expression pattern of assembled laminin-5 in normal, PIN and carcinoma of the prostate.

Dual immunofluorescence staining of prostate with 18A anti-cytokeratin (A,B,C) and GB3 anti-laminin (D,E,F). In A and D, the laminin-5 was polarized around the basal aspect of the normal gland (n) with some staining on the lateral aspect of the cells. In B and E, there was a similar pattern of expression at the basal cells that were attenuated in the PIN lesion (p). In places where basal cells were lost, there was no laminin-5 staining (arrows in E). In panels C and F showing the invasive carcinoma (Ca), note the complete absence of basal cells and the corresponding lack of laminin-5 staining. The punctate staining in panels D, E and F was due to autofluorescence of cytoplasmic lipofucsin. Magnification, X400.

Detection of the β4 Integrin Differed in Prostate, Breast and Colon Carcinoma

Human epithelial tissue samples were dual stained for cytokeratin and β4 integrin using IHC. Normal and carcinomatous cells were detected by an anti-cytokeratin polyclonal rabbit antibody, 18A (Figs. 7A-C) (422). The β4 integrin was detected using the 3E1 anti-human β4 integrin monoclonal antibody, which recognizes an extracellular epitope of the β4 protein (Fig. 7D-F). The β4 integrin was detectable at the epithelial stromal interface in normal prostate glands (Fig. 7D) and breast lobules (Fig. 7E). Invasive prostate and breast carcinoma (Fig. 7D, E) adjacent to normal tissue, did not contain detectable β4 integrin. In contrast, in the colon tissue, both normal crypts and carcinoma showed strong basal polarization of the β4 integrin (Fig. 7F) (data for normal colon not shown).

Expression of Laminin-5 Protein in Prostate, Breast and Colon Carcinoma

The presence of the ligand for the α6β4 integrin, laminin-5, was investigated using the three types of human epithelial tissue samples. The samples were dual stained for cytokeratin and assembled laminin-5 using IHC. Normal and carcinomatous cells were detected by an anti-cytokeratin polyclonal rabbit antibody, 18A (Fig. 8A-C). Laminin-5 was detected using the GB3 anti-laminin-5 antibody whose epitope is recognized when all three chains of the molecule are assembled (Fig. 8D- F) (423). Extracellular laminin-5 protein was detectable in normal prostate glands, breast lobules and colon crypts. Invasive prostate carcinoma adjacent to the normal structures did not contain detectable laminin-5 (Fig. 8D). Invasive breast carcinoma did not contain strong polar staining of

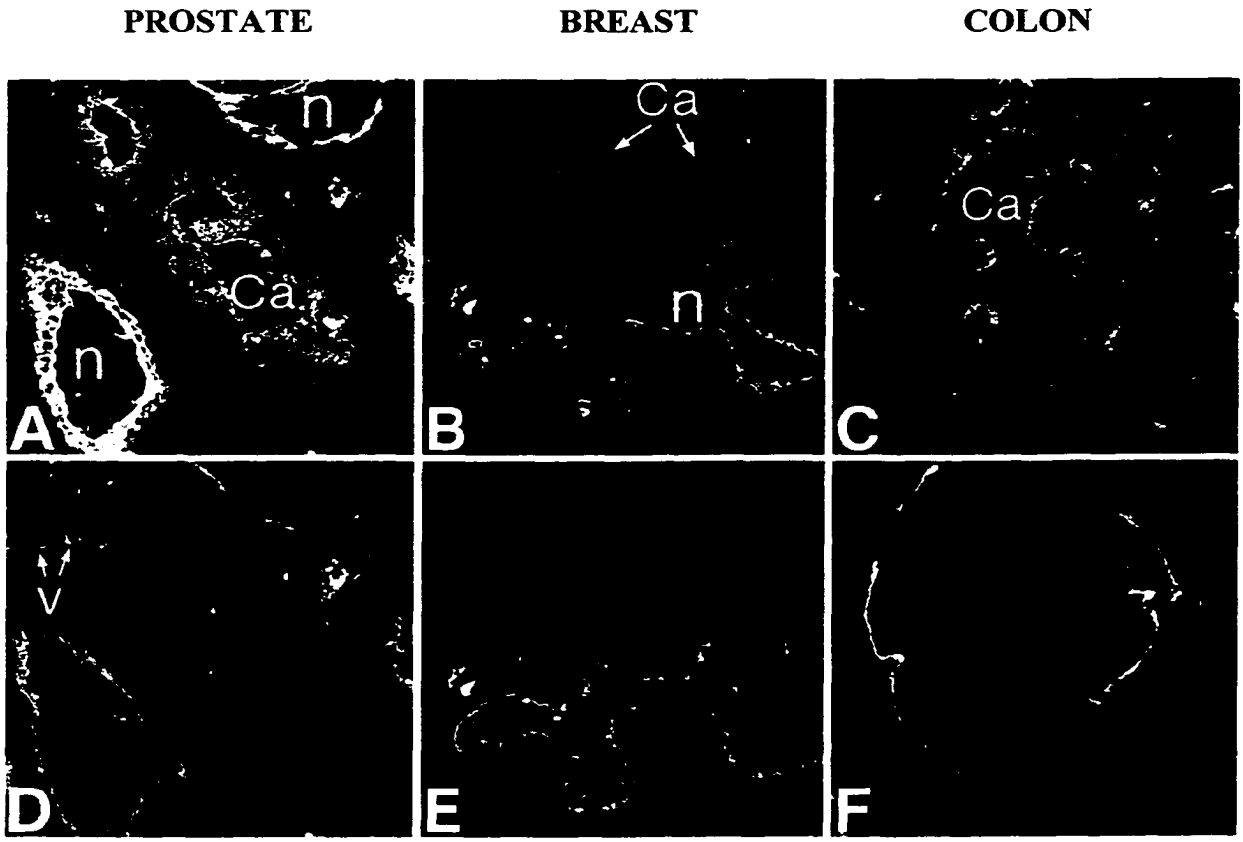

Figure 7: Expression of the α6β4 integrin in human prostate, breast and colon carcinoma.

Dual immunofluorescence staining with 18A anti-cytokeratin (A, B, C) and 3E1 anti-β4 integrin (D, E, F) of prostate (A, D), breast (B, E), and colon (C, F) carcinomas. In the prostate and breast tissues, normal (n) and malignant carcinoma (Ca) are present in adjacent areas within the same section. Normal colon is not shown. Note that the colonic carcinoma expressed β4 integrin polarized to the epithelial/stromal interface (F), but that the invasive prostate carcinoma (D) and the breast carcinoma (E) did not express this epitope. In addition to the β4 staining of the normal gland in D and E, cytokeratin negative vessels (V) revealed β4 staining. Magnification, X400.

the laminin-5 but did contain significant cytoplasmic staining (Fig. 8E). The breast carcinoma cases were observed to have diffuse cytoplasmic staining. In contrast, colon carcinoma showed strong extracellular polarized staining of the laminin-5 protein (Fig. 8F) (results for normal colon not shown).

Polarized Staining of β4 Integrin and Laminin-5 in Epithelial Carcinomas

Since the different types of carcinoma appeared to vary in the amount and distribution of β4 integrin and laminin-5 detectable, five different anti-β4 antibodies, representing epitopes present within the extracellular and cytoplasmic portions of the integrin were used (see Materials and Methods). Serial sections of normal and carcinoma tissues were examined by IHC. Normal prostate, breast tissue and colon tissue showed positive polarized staining for the five different β4 antibodies (data not shown), similar to the pattern shown in Fig. 7 D, E.

Polarized staining was recorded and distinguished from total positive staining within the carcinomas using the criteria set forth in the Materials and Methods. The results are shown for prostate carcinoma (n = 11) (Fig. 9A), breast carcinoma (n = 5) (Fig. 9B) and colon carcinoma (n = 6) (Fig. 9C). Using five different antibodies specific for the β4 integrin, no cases of prostate or breast cancer contained the β4 integrin basally polarized, in contrast to that found in colon carcinoma. Colon carcinoma consistently showed strong, basally polarized localization in all cases, independent of the antibody

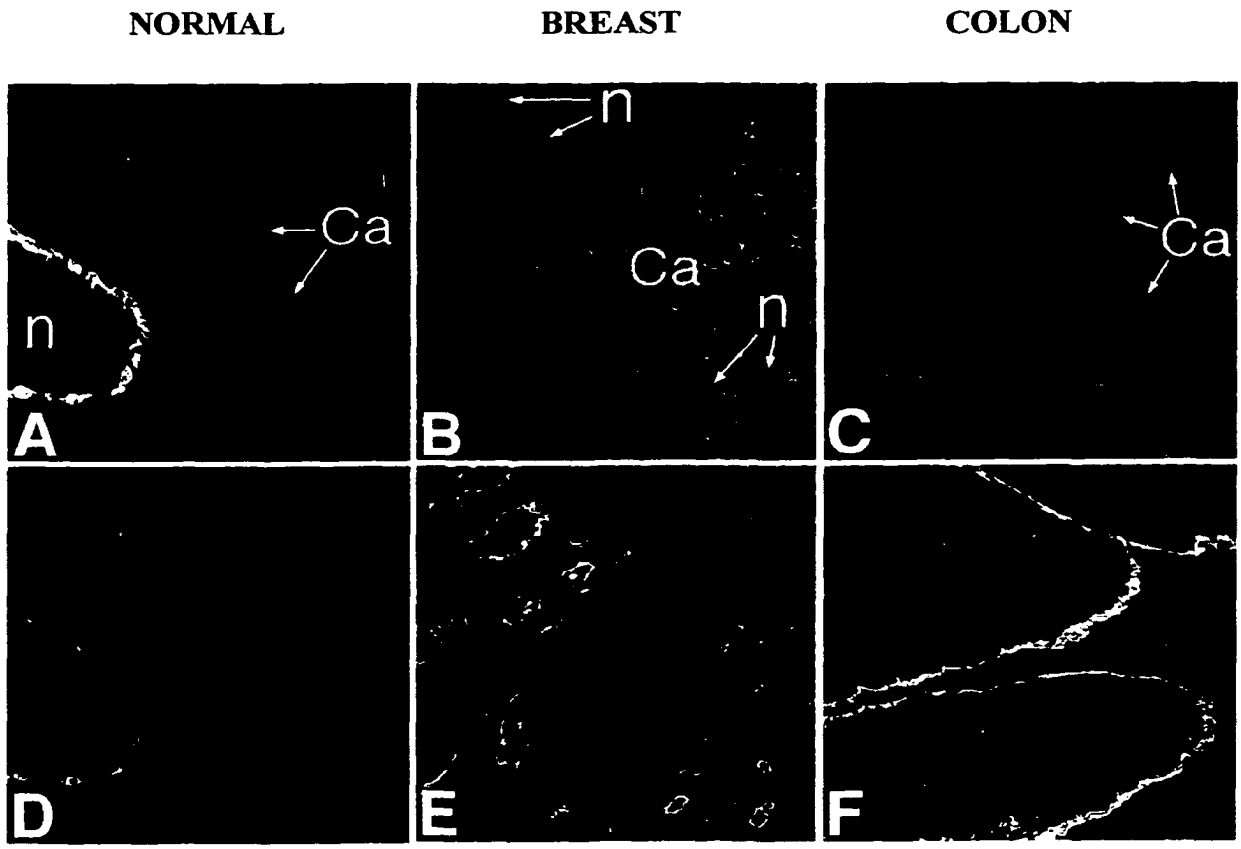

Figure 8: Expression of assembled laminin-5 in human prostate, breast and colon carcinoma

Dual immunofluorescence staining with 18A anti-cytokeratin (A,B,C) and GB3 anti-laminin-5 (D,E,F) of prostate (A, D), breast (B, E), and colon (C, F) carcinomas. Normal tissue components (n) are seen in prostate and breast images. Malignant components (Ca) were present in all three tissue types. Prominent laminin-5 staining was seen in normal prostate and breast. Note there was no polarization of the laminin-5 in prostate carcinoma (D). In breast carcinoma, there was significant cytoplasmic staining of laminin-5, but no polarization (E). In contrast, in colon carcinoma there was strong positive polarized staining surrounding the malignant glands (F). Magnification, X400 for (A, D, B, E), and X613 for C and F.

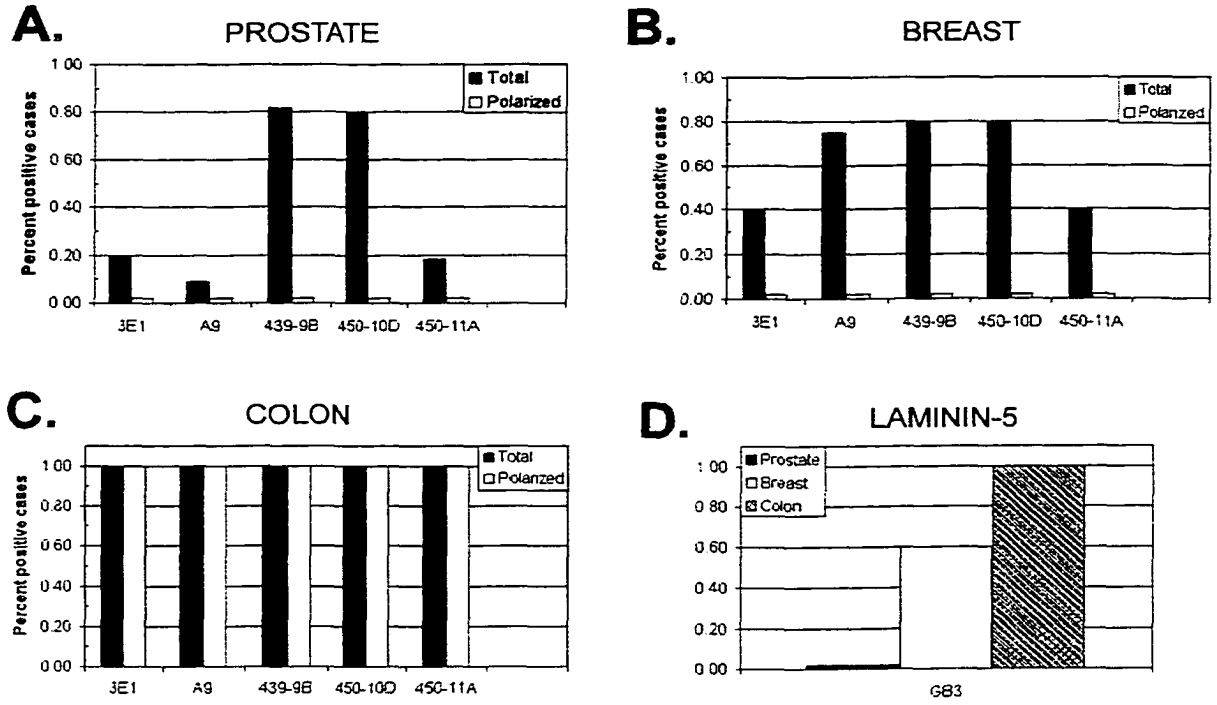

Figure 9: Tabulation of polarized and total β4 integrin and laminin-5 in prostate, breast and colon carcinoma.

Human prostate, breast and colon tissues containing both normal and carcinomatous regions were examined by immunohistochemistry using 5 different anti-β4 integrin antibodies. Cases were examined for total positive staining and total polarized staining. Immunofluorescence staining was semi-quantitatively evaluated by the following scale of intensity: (+2) very strong staining, (+1) positive staining, (+/-) weak staining, (-) no staining above background. Cases which scored a (+1) or a (+2) were scored positive, while cases scoring (+/-) or (-) were scored negative. The resulting data are tabulated for prostate (A), breast (B) and colon (C) carcinoma. Values are expressed as percent positive cases examined (prostate, n=11; for breast n=5; for colon n=6) for the two categories.

used to detect the β4 integrin (Fig. 9C). In prostate cancer, although none of the antibodies detected polarized staining of the β4 integrin, two antibodies (439-9B, 450-10D) detected diffuse cytoplasmic staining in 82% and 80% of the cases (Fig. 9A). These two antibodies recognized extracellular and intracellular epitopes of the integrin respectively. Diffuse cytoplasmic staining was also detected by three other β4 specific antibodies (3E1, A9, and 450-11A) in 20%, 9% and 18% of the cases, respectively. None of the 11 prostate cases retained all five β4 epitopes. Examples of negative and positive prostate cancer cases demonstrating either complete absence or diffuse cytoplasmic expression of β4 integrin is shown in Figure 10. One of the five breast cases retained all five β4 epitopes examined (data not shown). In breast cancer, three antibodies specific for β4 integrin (A9, 439-9B and 450-10D) detected 75% of the cases and two antibodies (3E1, 450-11A) detected 40% of the cases (Fig. 5B).

These same cases were also examined for laminin-5 expression using GB3, a monoclonal mouse antibody that recognizes the fully assembled laminin-5 protein. The results obtained for each of the three epithelial carcinomas are shown in Fig. 9D. Laminin-5 was absent in all nine cases examined of prostate carcinoma. There was variable cytoplasmic staining in three of five breast carcinomas and positive basally localized staining in all six colon carcinomas examined.

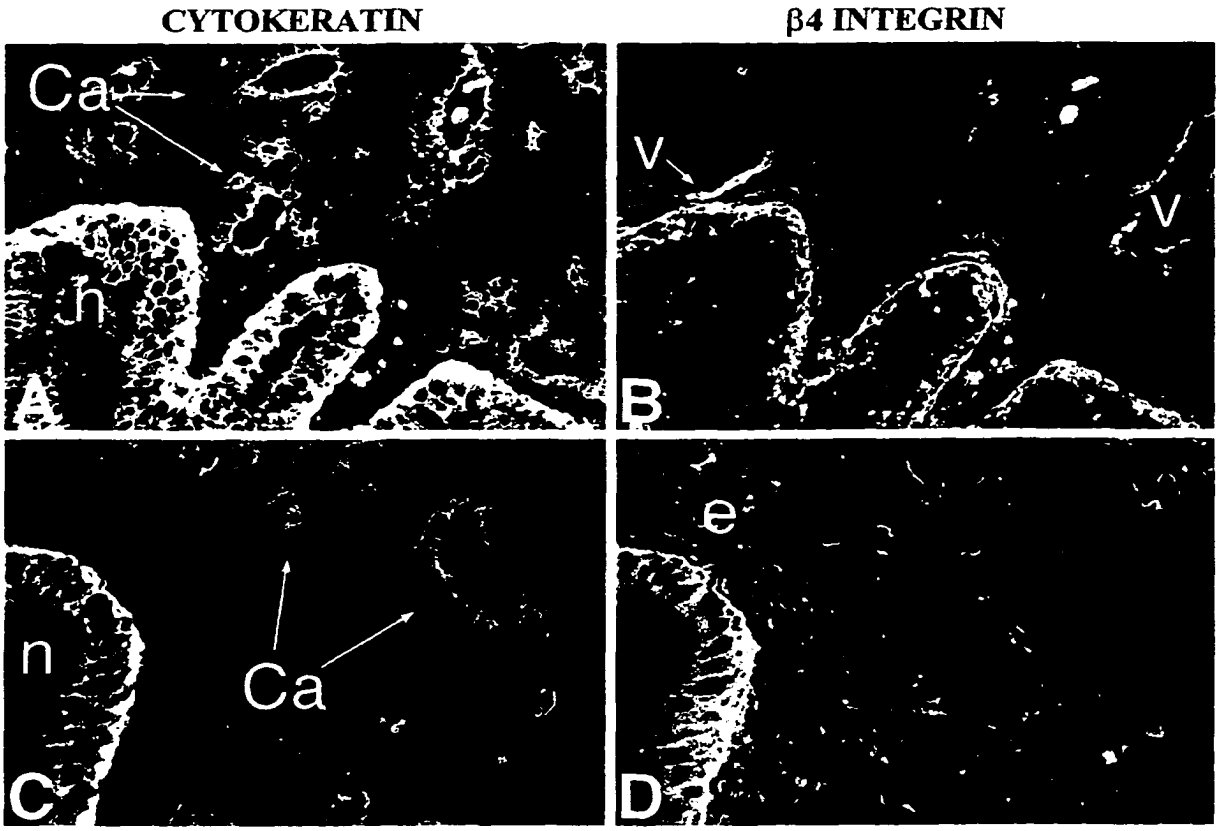

Figure 10: Expression of β4 integrin was variable in prostate carcinoma.

Dual immunofluorescence staining of example negative and positive cases of invasive prostate carcinoma with 18A anti-cytokeratin (Panel A,C) and A9 anti-β4 integrin (Panel B,D). The anti-cytokeratin antibody demonstrated intense staining of the basal cell layers of the normal glands (n) and also the weaker cytoplasmic staining of the invading carcinoma (Ca). The β4 integrin is also seen polarized at the epithelial stromal interface in the normal glands as well as in endothelia (V). Case one shows total absence of β4 expression in the invading carcinoma (Panel A,B). Case two shows normal localization at the epithelial stromal interface of the normal gland and weak cytoplasmic staining of the invading cancer (Panel C,D). Note the lack of polarized β4 integrin in either invading cancer (Panel B,D). In panel D, the punctate stromal staining is due to autofluorescence of intrinsic elastin tissue (e). Magnification, X400 for (A,B) X647 for (C,D).

Laminin-5-rich matrix induced focal β4 integrin clustering.

Previously the loss of laminin-5 in human prostate carcinoma tissues was demonstrated (165). In an effort to examine the effect of laminin-5 on β4 integrin expression, the human prostate carcinoma cell line, DU145H was used which expressed the β4 integrin, but expressed only low levels of the β2 chain of laminin-5 (165,463). DU145H cells were plated overnight on either laminin-5-rich matrix or serum covered glass. The following day, cells were prepared for immunocytochemistry and stained with anti-β4 integrin antibody, 3E1. When prostate carcinoma cells were grown on matrix, the β4 integrin organized into focal, punctate clusters (Figure 11A, high magnification 7D). In contrast, cells which were grown on the control, serum-covered glass coverslips demonstrated diffuse, weakly cytoplasmic staining of β4 integrin (Figure 11B, high magnification 11E). The laminin-5 matrix itself did not demonstrate staining for the β4 integrin when examined by immunohistochemistry in the absence of plated cells (Figure 11C). The β4 integrin focal clusters were quantitated as previously described (448) and graphed.

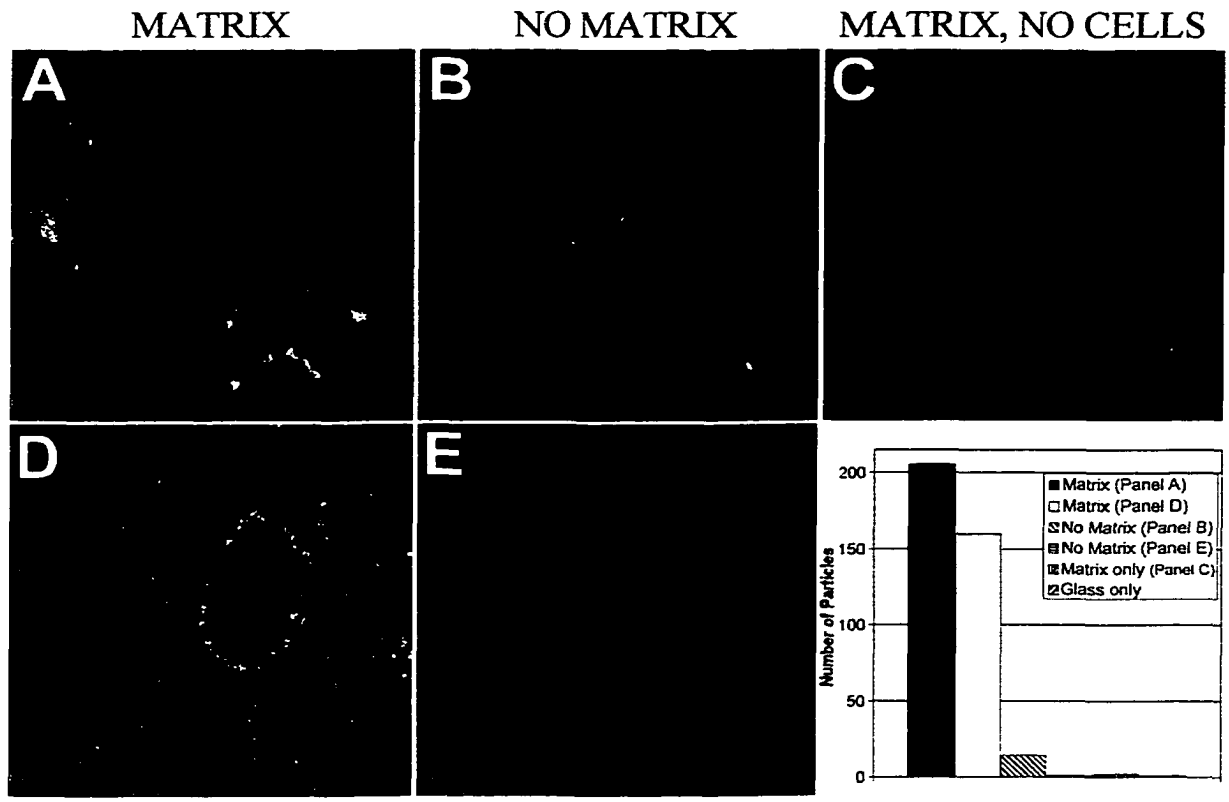

Figure 11: The β4 integrin clustered on a laminin-5-rich matrix.

The effect of a laminin-5-rich matrix on the β4 integrin was examined by immunocytochemistry using anti-human β4 integrin antibody, 3E1. Human prostate carcinoma DU145H cells were grown overnight in the presence (A,D) or absence (B,E) of a laminin-5-rich matrix derived from normal immortalized keratinocyte cell line, HaCaT. Note focal clustering of β4 integrin at the periphery of cells in panels A and D. In the absence of laminin-5 in the matrix, DU145H cells did not demonstrate focal clustering of the β4 integrin. Panel C shows that the β4 integrin was not present in the laminin-5-rich matrix when cells were not present. Shown in the remaining panel, the β4 integrin focal clusters were quantitated and graphed.

Discussion:

Previous studies have reported that in normal prostate glands, hemidesmosomes were present but in prostate carcinoma the structures were lost (157). Correlated with this ultrastructural observation was the finding that several proteins associated with the hemidesmosomes, including the β4 integrin as well as two chains of its ligand, laminin-5 and the anchoring filament collagen VII (74,155,165,166,230), were absent in prostate carcinoma. In the present study, the β4 integrin and laminin 5 expression pattern within normal, PIN lesions and prostatic carcinoma was investigated. The results indicate that the loss of the β4 integrin and laminin-5 expression occurs during the normal to PIN to carcinoma transition. Focal loss of the β4 integrin and laminin-5 was observed in PIN, consistent with the known loss of the basal cells which characterize these lesions. In particular, within the PIN lesion, the remaining basal cells often demonstrated extended processes containing β4 integrin that occurred between the luminal cells and was not confined to the normal location, i.e. at the basal aspect of the cells. In addition, β4 integrin epitope 'clustering' was restored in human prostate carcinoma DU145H cells by allowing the laminin-5 negative cells to adhere to a laminin-5-rich matrix (Figure 7), suggesting the presence of laminin-5 may stabilize β4 integrin at the cell surface in this model system.

The loss of α6β4 integrin in prostate carcinoma could be due to the loss of the normal basal cell population as is progressively seen through PIN and cancer lesions. Alternatively, previous studies indicate that the basal cells may play an important

proliferative role in the normal and neoplastic human prostate (316). Recently, a stem cell model has been described in which stem cells in the basal cell population acquire secretory luminal characteristics after androgen stimulation and thus prostate cancer ultimately may be derived from these transformed stem cells (317,318).

The reorganization of the α6β4 integrin within the PIN lesion is reminiscent of the loss of polarized α6β4 integrin observed in other epithelial disease states such as junctional epidermolysis bullosa with pyloric atresia (PA-JEB). The α6β4 integrin heterodimer is a major component of the hemidesmosome which is expressed at the basal surface of most stratified epithelial cells and serves to link the intermediate filaments of the cytoskeleton to the anchoring filaments of the basal lamina (251,337,338,464-466). The α6β4 integrin is required for hemidesmosome formation, adhesion to laminin-5 (76,467,468) and is present in normal prostate tissue (157), forming a link with the extracellular matrix protein, laminin-5. It remains to be determined if a structural rearrangement of other components of the hemidesmosome also occurs in PIN lesions.

The biological consequences of abnormal β4 integrin expression may involve increased migration and invasion either via actin containing motility structures (343), or via reorganization of the hemidesmosome. Normal keratinocyte maturation studies suggest that a loss of the α6β4 integrin attachments to the basal lamina was necessary for basal cell detachment and differentiation (469). Invasive tumors appear to show considerable variation in β4 expression. (For a review, see (297).) Tumors in which there is reduced expression of β4 include breast cancer (273,309,310), basal cell carcinoma (300), bladder cancer (299) and prostate cancer (74,155,157,230).

This study has emphasized that the β4 expression pattern is different in other epithelial cancers. Expression of β4 integrin and laminin-5 in prostate and breast carcinoma were distinctly different from the strong, polarized staining observed in colon carcinoma. In the absence of the ligand laminin-5, β4 is not polarized to the surface of breast and prostate carcinomas, but in the presence of laminin-5, has a polar distribution in colon carcinomas. Similarly when in the absence of laminin-5, human prostate carcinoma DU145H cells demonstrated diffuse β4 integrin stainining. Yet when provided with a laminin-5 matrix, the β4 integrin was localized in focal, punctate clusters at the edge of the cell. Together, these data suggest that the presence of the ligand may be linked to surface stabilization of the integrin. Interestingly the fibronectin receptor, α5β1 integrin, rapidly degrades in the absence of its specific extracellular ligand (470). In the case of α6β4, the absence of the major ligand laminin-5, may result in the failure of the α6β4 integrin to basally polarize with subsequent internalization and degradation. The β4 integrin has been shown to be specifically cleaved by calpain-like enzymes (471) and matrilysin (472) both of which are present in prostate carcinoma (170).

In prostate carcinoma, the loss of the β4 integrin and the continued expression of the α6 integrin, result in a predominance of the α6β1 integrin (155,441). This study suggests the hypothesis that loss of the α6β4 integrin in prostate carcinoma and retention of α6β1 may result in a loss of stable attachment to the basal lamina via the hemidesmosome and promote adhesion to laminin coated structures such as vessels and nerves (473).

Reorganization of adhesion structures in prostate cancer may be analogous to that observed in PA-JEB. In migration assays, the PA-JEB keratinocytes are more motile on laminin-5 than normal keratinocytes (332). Alteration of the hemidesmosome structure may facilitate invasion of prostate cancer by allowing migration through the stroma and along nerves. Extracapsular penetration of prostate cancer occurs along these structures and is correlated with a poor prognosis (474). It would be of particular interest to determine the dependence of the migration of $\alpha 6\beta 1$-containing prostate cancer cells along peripheral nerves using *in vitro* systems.

IV. IDENTIFICATION OF A NOVEL STRUCTURAL VARIANT OF THE α6 INTEGRIN

Introduction

Integrins are cell surface receptors that are involved in cell-matrix adhesion and signaling (recently reviewed in (475). The α6 integrin is a laminin receptor and contains 1050 amino acids present as a heavy (110 kD) and a light (30 kD) chain which are linked by a disulfide bond (346). The heavy chain of α6 integrin contains an 875 amino acid extracellular region and interacts with the β subunit to form the heterodimer (249). All the described integrin alpha subunits contain seven weak sequence repeats of approximately 60 amino acids in the N-terminal region which are thought to be important in ligand binding and have been predicted to fold cooperatively into a single β-propeller domain with seven β-sheets (196,197). The minimum essential elements of the extracellular domains for subunit pairing and ligand binding are of considerable interest in understanding integrin regulation (182). Therefore, this chapter extends previous studies which indicated that a smaller variant of the α6 integrin existed (158,441).

Two alternatively spliced forms of α6 exist, containing identical heavy chains and different light chains known as α6A and α6B (247,249). The light chain of α6 integrin contains 170 amino acids composing an extracellular region, the transmembrane region and the cytoplasmic domain (249). The α6A or B integrin subunit can pair with either the β1 or β4 subunits (251) and is found on a variety of normal cell types. It is found on

platelets (195), epithelia (254-257), endothelia (251,258,259), proximal and distal tubules of the kidney (260,261), astrocytes (262), Schwann and perineural cells (259,263) and lymphoid follicles (264). Although minor alterations exist (253), large variants of the α6 integrin heavy chain have not been reported.

Various disease states involving epithelial cells have been associated with alterations in α6 integrin-containing heterodimers. Mice lacking the α6 integrin completely will develop to birth but die shortly thereafter due to severe blistering of the skin and other epithelia (330). Alterations in the α6 integrin and/or a deficiency of its pairing subunit, β4 integrin, are associated with pyloric atresia-junctional epidermolysis bullosa, a human blistering disease of the epithelia (324,332-334,352,476).

Our work investigating a human epithelial cancer indicated a deficiency of the α6β4 heterodimer pairing during prostate tumor progression (155,157) and a persistent expression of the α6β1 integrin (230). Other groups also have observed the persistent non-polarized expression of the α6 integrin during human tumor progression in cancers arising within the breast (477), kidney (478), endometrium (281) and pancreas (295,296), in addition to micrometastases from solid epithelial tumors (274).

Isolating the α6 integrin from human prostate cancer cells using α6 specific monoclonal antibodies retrieved not only the expected β1 and β4 subunits, but also a predominant protein with an apparent molecular weight of 70 kD (158,441). In this study we show that the protein is a novel and smaller form of the α6 integrin which is capable

of pairing with either the β1 or β4 integrin subunit, referred to as α6p for the latin word *parvus*, meaning small.

Results

DU145H cells contained a smaller form of the α6 integrin.

Our previous studies showed that anti-α6 antibody GoH3 was able to immunoprecipitate a surface-biotinylated 70 kD (non-reduced) protein from DU145H cells in addition to the expected 185, 140 and 120 kDa (non-reduced) proteins corresponding to the β4, α6 and β1 integrins, respectively (158,441). In DU145H cells, which only contain the α6A splice variant of α6 integrin (155), this 70 kD variant was the predominant form of the α6 integrin found on the cell surface.

Five different anti-α6 antibodies immunoprecipitated α6 and its smaller variant, α6p, from surface biotinylated DU145H cells (Figure 12). Four of the antibodies used were specific for extracellular epitopes of the full length α6A integrin (GoH3, J1B5, 4F10 and BQ16), and one was specific for the cytoplasmic tail of the α6A light chain (AA6A). The integrin α6p was not found to co-immunoprecipitate upon incubation with an anti-α3 antibody, P1B5 (441) or an anti-α5 antibody, P1D6. The anti-α6 integrin antibody J8H also immunoprecipitated both α6 and α6p integrins (see Figure 15).

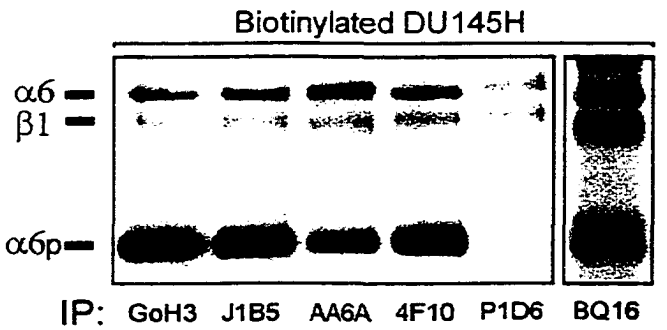

Figure 12: The α6p integrin was immunoprecipitated from human cells.

The DU145H cells were surface biotinylated and the α6 integrin retrieved using either the GoH3, J1B5, AA6A, 4F10, or BQ16 antibodies, specific for human α6 integrin. The α5 integrin was retrieved from the lysate using the P1D6 antibody, specific for human α5 integrin. The immunoprecipitations were analyzed using a 7.5% polyacrylamide gel under non-reducing conditions and the migration position of the biotinylated integrins are as indicated.

The α6p variant contained a light chain which was identical to that found in α6 integrin:

The full length α6 integrin consists of two disulfide linked chains; a heavy chain (110kD) and a cytoplasmic light chain (30 kD), which are observed upon reduction of the protein samples and analysis by SDS-PAGE. The data (Figure 12) indicated that an anti-α6 integrin antibody specific for the cytoplasmic tail of α6A recognized α6p and suggested that the light chain from the α6p variant might be similar to that in the full length α6 integrin. To answer this question, DU145H cells were surface labeled with biotin and then immunoprecipitated using the anti-α6 integrin antibody, GoH3. The resulting sample was then analyzed using two dimensional non-reducing/reducing gel electrophoresis (Figure 13). The sample was electrophoresed under non-reducing conditions in the first dimension and then under reducing conditions for the second dimension. The 140 kD band (non-reduced) corresponding to the full length α6 integrin, contained a heavy (110 kD) and light (30 kD) chain, as previously described, under the reducing conditions of the second dimension (249). The reduced β1 integrin was identified at 120. The α6p integrin split into a heavy fragment (43 kD) and a light chain (30 kD). These results indicated that the α6p integrin contained the same light chain as the full length α6A integrin but that the heavy chains were significantly different.

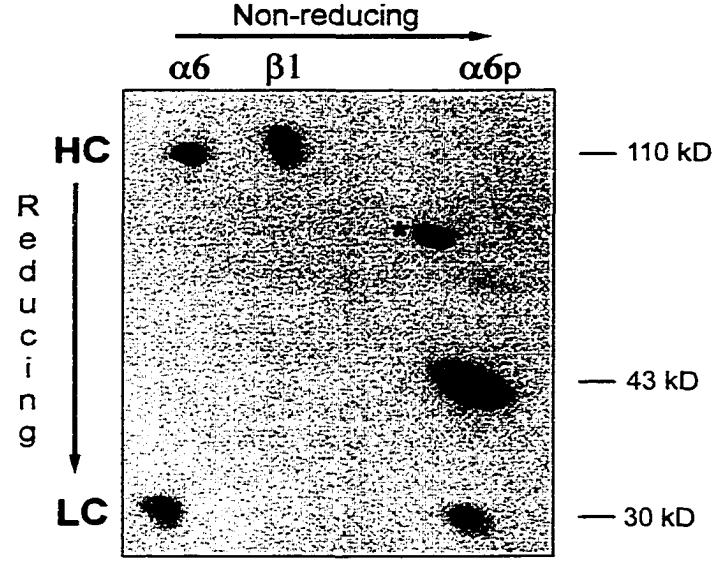

Figure 13: The α6p integrin contained a light chain identical to the α6 integrin.

Surface biotinylated proteins from DU145H cells were retrieved by immunoprecipitation using the GoH3 antibody and were analyzed first by 7.5% polyacrylamide gel electrophoresis under non-reducing conditions. The resulting lane was excised from the gel and placed on the top of a second 7.5% polyacrylamide gel. The position of the migration of the integrins in the first gel are indicated at the top of the figure. Electrophoresis was then performed under reducing conditions. The resulting migration of the heavy chain (HC) and light chain (LC) and the molecular weights are indicated. The asterisk indicates a biotinylated protein band which was variably seen and is of unknown identity.

The α6p variant associated with β1 and β4 integrins:

The α6 integrin is known to associate with either the β1 or β4 subunit (251). Next we determined if α6p would co-immunoprecipitate with the β4 integrin (Figure 14A). Human HaCaT cells were chosen for this experiment due to their abundance of β4 integrin (441). They were surface biotinylated and subjected to immunoprecipitation with different anti-β4 integrin antibodies. The α6p variant co-immunoprecipitated upon incubation with four different anti-β4 integrin antibodies; A9, 439.9b, ASC3 and 3E1. Of particular interest was the retrieval of α6p with the anti-β4 integrin antibody, A9, whose epitope is present when α6 is coupled to β4 integrin (426).

Next we tested whether the novel 70 kD (non-reduced) protein could be recovered by immunoprecipitation with the anti-β1 integrin monoclonal antibody, P4C10 (Figure 14B). HaCaT cells were surface biotinylated and immunoprecipitated with anti-α6 integrin antibody, J1B5, and used as a standard. Both DU145 and HaCaT cells were surface biotinylated and subjected to immunoprecipitation using P4C10. Interestingly, the 70 kD (non-reduced) α6p variant co-immunoprecipitated with the β1 integrin in DU145 cells, but not in HaCaT cells. The results indicated that the novel α6p variant paired with either the β4 or β1 integrin subunits. While the β1 integrin was readily present in HaCaT cells, the α6p integrin did not co-immunoprecipitate with the anti-β1 integrin antibody, P4C10. This may indicate that in some cell lines, there is preferential pairing of the α6p integrin subunit with β4.

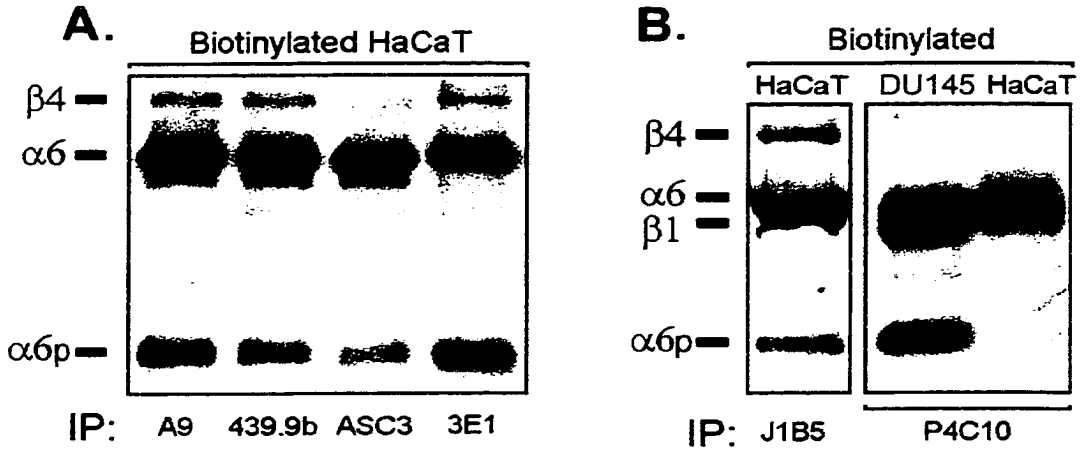

Figure 14: The α6p integrin paired with either the β4 or β1 subunits.

The HaCaT cells were surface biotinylated and the β4 integrin retrieved using either A9, 439.9b, ASC3 or 3E1 antibodies, specific for human β4 integrin (Panel A). The DU145 cells were surface biotinylated and the β1 integrin was retrieved using P4C10 antibody, specific for β1 integrin (Panel B). The immunoprecipitated proteins were analyzed using a 7.5% polyacrylamide gel under non-reducing conditions and the migration position of the biotinylated integrins are as indicated.

The α6p integrin was recognized by light chain-specific anti-α6A monoclonal antibodies:

The data (Figure 13) indicated that α6p contained an identical light chain to that contained in the full length α6 integrin. Next we tested whether the novel 70 kD (non-reduced) protein could be recognized by anti-α6 integrin antibodies via Western blotting. DU145H cells were biotinylated and immunoprecipitated with GoH3 for a standard to compare with a Western blot (Figure 15). DU145H, HaCaT and H69 cells were lysed and immunoprecipitated with either anti-α6 integrin antibodies GoH3 or J1B5, or anti-β1 integrin monoclonal antibody, P4C10. A 70 kD band which co-migrated with the biotinylated standard was recognized in HaCaT and DU145H cells by Western analysis using two different anti-α6A antibodies, AA6A and 4E9G8 which recognize the cytoplasmic domain of the α6A integrin. Additionally the α6 integrin, but not the α6p variant was detected by Western blot analysis using A33, which is specific for the amino terminal of the α6 integrin. A lung carcinoma cell line, H69, is a cell line which does not contain α6 integrin and was not found to express α6p.

The α6p variant was present in several different epithelial cancer cell lines:

We next determined the presence of the α6p variant in other tumor or normal cell lines. The presence of α6 and α6p was initially analyzed by using whole cell lysates (20 μg total protein) followed by Western blot analysis (data not shown). The results were

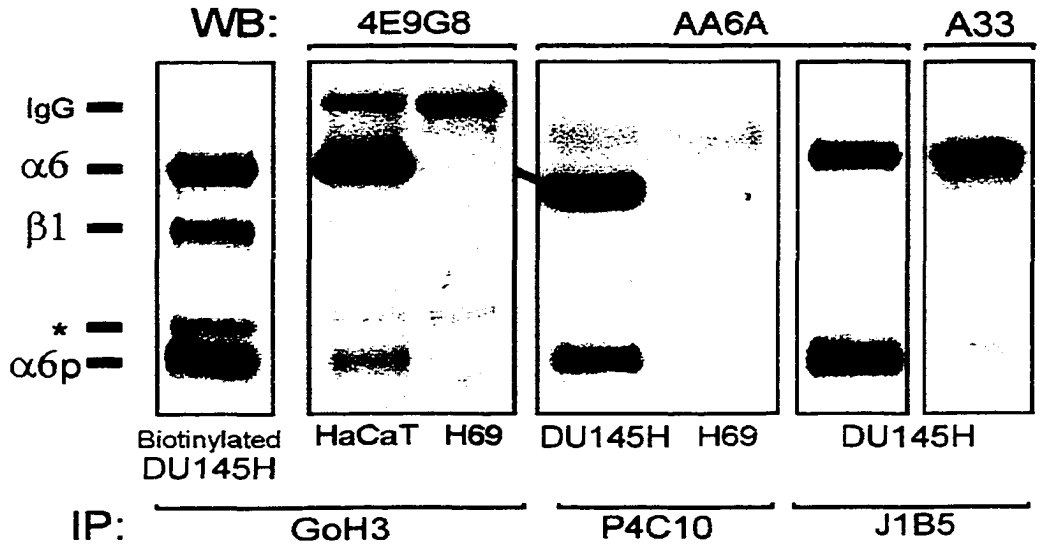

Figure 15: The α6p integrin was recognized by antibodies specific for the α6A light chain.

The α6 integrin and the β1 containing integrins were retrieved from the lysates of human DU145H, HaCaT and H69 cells by immunoprecipitation with either anti-α6 integrin antibodies GoH3 or J1B5, or anti-β1 integrin antibody, P4C10. The resulting proteins were analyzed using a 7.5% polyacrylamide gel under non-reducing conditions followed by Western blot analysis using α6A specific antibodies, 4E9G8 or AA6A, which are specific for the cytoplasmic domain, or anti-α6 integrin antibody A33, which is specific for amino terminal of α6 integrin. The migration position of a biotinylated integrin standard from DU145H cells are as indicated. Samples shown in the middle panel were electrophoresed on a separate gel and the molecular weight of the α6A band is indicated relative to the adjacent panels by a solid bar. The asterisk indicates a biotinylated protein band which was variably seen and is of unknown identity.

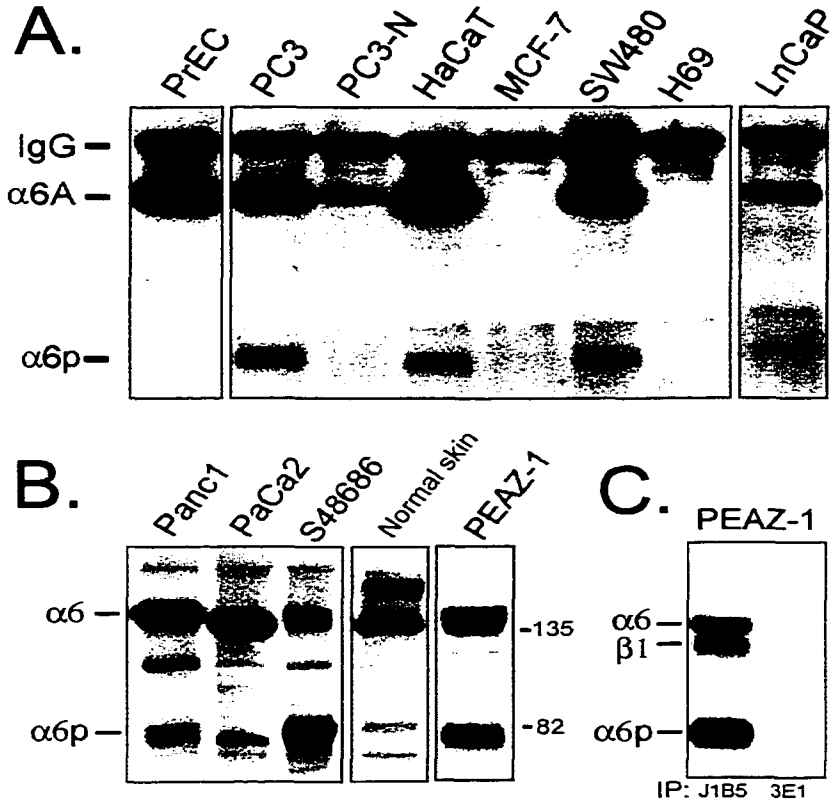

Figure 16: The α6p integrin was present in normal and tumor epithelial cell lines. The α6 containing integrins were retrieved from the lysates of normal immortalized keratinocytes (HaCaT) and prostate epithelial cells (PrEC), prostate cancer cell lines (PC3, PC3-N, LnCaP), breast cancer cell line (MCF-7), colon carcinoma cell line (SW480) and a lung carcinoma cell line (H69) by immunoprecipitation with the GoH3 antibody. The immunoprecipitated proteins were analyzed using a 7.5% polyacrylamide gel under non-reducing conditions. The presence of α6p was detected by Western blot analysis using the AA6A antibody, specific for the human α6A light chain (Panel A). The α6 and α6p integrins were also detected by 15 μg whole cell lysates analyzed as above in pancreatic cancer cell lines (Panc1, PaCa2, S48686), a human normal skin biospy and human prostate carcinoma cell line, PEAZ-1 (Panel B). Surface biotinylated PEAZ-1 cells were immunoprecipitated with anti-α6 integrin or anti-β4 integrin antibodies, J1B5 or 3E1, respectively (Panel C).

tabulated and confirmed by immunoprecipitation with anti-α6 antibody GoH3 followed by Western Blot analysis using anti-α6A antibody, AA6A (Figure 16). The α6p variant was present in several prostate cancer cell lines (DU145H, PC3, LnCaP) and a colon cancer cell line, SW480. Additionally, α6p was present in a normal, immortalized keratinocyte cell line, HaCaT. The α6p variant was not found in several cell lines including: normal prostate cells, PrEC; a variant of prostate cell line PC3, called PC3-N (162); a breast carcinoma cell line, MCF-7; or a lung carcinoma cell line, H69. Interestingly, the α6p variant was only observed in cells which expressed the full length α6 integrin. The α6p variant was not present in α6-negative cell lines. Two epithelial cell lines, one normal (PrEC) and one cancer (PC3-N), expressed the full length α6 integrin but not the α6p variant.

Whole cell lysates (15 μg) followed by Western blot analysis were used to detect the presence of α6p in three different pancreatic carcinoma cell lines (Panc1, PaCa2 and S48686), a human normal skin biopsy, and the human prostate carcinoma cell line, PEAZ-1 (Panel B). Surface biotinylated proteins from PEAZ-1 cells were immunoprecipitated with anti-α6 integrin antibody, J1B5 and anti-β4 integrin antibody, 3E1 (Panel C). The α6p variant was found to co-immunoprecipitate primarily with the β1 subunit in the PEAZ-1 cells and the β4 integrin subunit was not detected.

The α6p variant contained several amino acid fragments identical to the α6 integrin:

Although these data demonstrated the presence of the α6A light chain in the protein, we next determined whether the α6 heavy chain was present utilizing a direct protein sequencing method. The α6p protein was immunoprecipitated with J1B5 and electrophoresed. The protein gel was stained with Coomassie blue and the 70 kD protein was excised and digested with trypsin. Protein sequences were obtained using MALDI mass spectrometry (DKFZ, Heidelberg, Germany) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Proteomics Core of the Arizona Cancer Center and Southwest Environmental Health Sciences Center, University of Arizona) (Figure 17). Ten non-continuous amino acid fragments within the α6p variant were identified which corresponded exactly to predicted trypsin fragments located on exons 13 through 25 of the published α6 integrin sequence (346). The sequencing data confirmed that both the heavy and light chains of the α6p variant contained identical portions of the full length α6 integrin (Figure 18).

The α6 and α6p integrins contained N-linked glycosylations:

The predicted molecular weight of exons 13 through 25, the exons contained within the α6p variant is 55 kD, however the apparent molecular weight observed in our protein gels was approximately 70 kD. It is known that the integrins can by modified by

Figure 17: Sequences obtained from the α6p variant corresponded to exons 13 through 25 of the full length α6 integrin.

Sequences of exons 1 through 25 of the α6 integrin are indicated by their one letter amino acid abbreviation. MALDI mass spectrometry and HPLC coupled to mass spectrometry identified ten non-continuous amino acid fragments from the α6p variant. These corresponded exactly to sequences contained within α6 exons 13 through 25 and are indicated by a box. Five of eight putative glycosylation sites are retained within exons 13 through 25, and are indicated in bold and underlined.

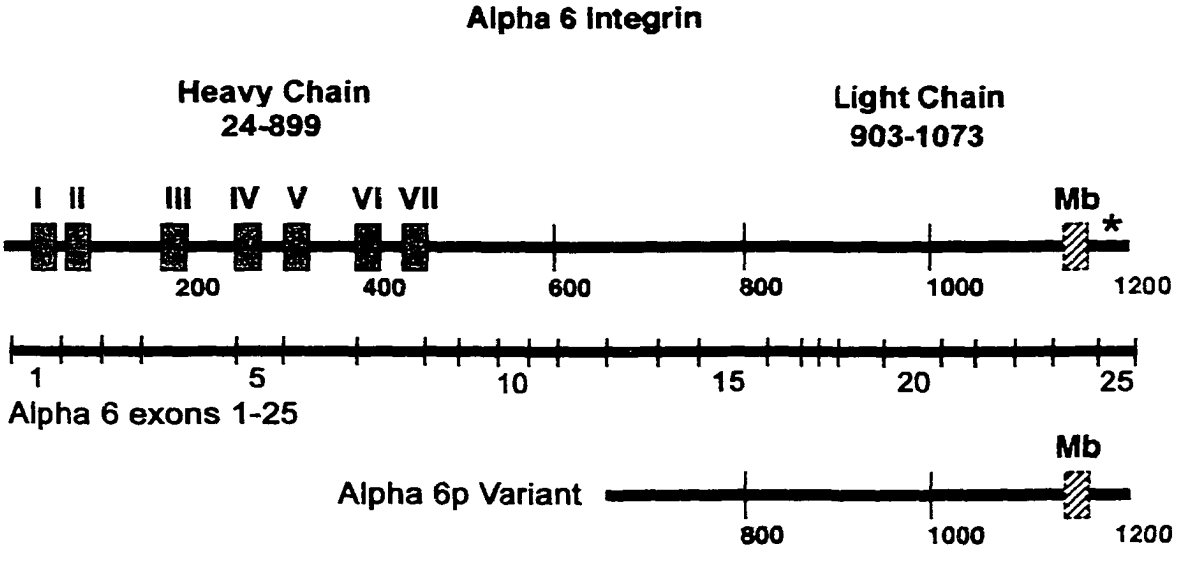

Figure 18: Schematic of the α6 and α6p integrins

A schematic of the full length α6 integrin and the smaller α6p variant is shown. Repeated domains (shaded rectangles) are indicated by Roman numerals I-VII (I = 42-79, II = 113-145, III = 185-217, IV = 256-292, V = 314-352, VI = 375-411, VII = 430-470). The putative ligand and cation binding domains are contained between repeated domains III and IV, and V and VI respectively. Exons 1 though 25 of the α6 integrin sequence are indicated. Ten non-continuous amino acid fragments obtained from the α6p integrin corresponded exactly to sequences contained within exons 13 through 25 of the full length α6 integrin. The mapped sequence positions of the two Western blotting anti-α6A antibodies (AA6A, 4E9G8) that recognize both α6 and the α6p variant are shown by an asterisk on the full length α6 schematic. Conformationally-dependent epitopes for anti-α6 integrin antibodies used for immunoprecipitation are not indicated on the schematic.

glycosylation (183). There are nine potential N-linked glycosylation sites contained in the α6 integrin (249,251), five of which are contained within exons 13 through 25. We determined whether or not the α6p integrin variant was differentially glycosylated compared to the full length α6 integrin by endoglycosidation digests. The enzyme endoglygosidase H (endoH) is frequently used in combination with endoglycosidase F (endoF) to distinguish between complex and high-mannose oligosaccharides. Proteins which are sensitive to cleavage by endoH are not fully processed, i.e. retained in the golgi apparatus, while proteins sensitive to endoF cleavage are fully processed by the golgi (479). Normal immortalized keratinocytes (HaCaT) were immunoprecipitated with anti-α6 integrin antibody and subjected to digestion with either endoH or endoF as described in materials and methods. Resulting samples were analysed on a 7.5% non-reducing gel followed by Western blot analysis with anti-α6 integrin antibody, AA6A (Figure 19).

The α6 and α6p integrins were retrieved by immunoprecipitation with anti-α6 integrin antibody, J1B5. A shift in the molecular weight of both α6 and α6p integrins was apparent in samples digested with endoF, but not with endoH. Several intermediate proteins which reacted with anti-α6 integrin antibody were observed in the endoH digestion. Interestingly, the α6p variant was more dramatically affected by endoF digestion than the full length α6 integrin, resulting in a molecular weight shift of approximately 10 kD.

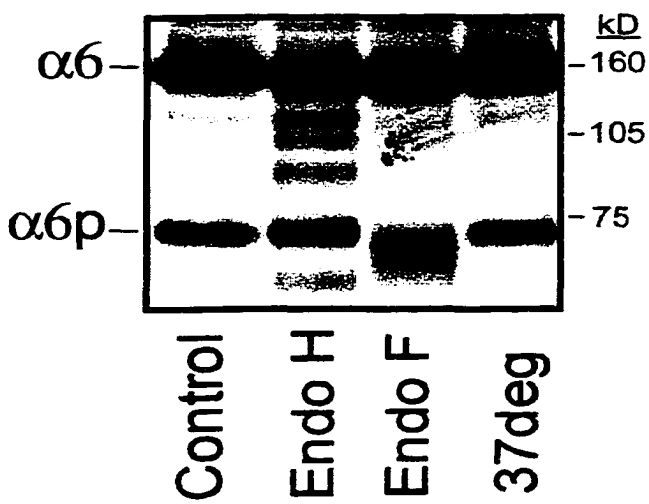

Figure 19: The α6 and α6p integrins demonstrated N-linked glycosylation

Normal immortalized keratinocyte cells (HaCaT) were immunoprecipitated for α6 integrin using J1B5 antibody and then subjected to digestion with either endoglycosidase H or endoglycosidase F. Resulting protein samples were separated on a 7.5% polyacrylamide gel under non-reducing conditions followed by Western blot analysis using anti-α6 integrin antibody, AA6A. The migration of the molecular weight standards and integrins are indicated.

The α6p variant surface half-life was three times longer than α6:

Detection of the α6p band which was smaller than the full length α6 integrin prompted us to ask whether this novel variant was a degradation product of the α6 integrin which would be rapidly cleared from the surface. To answer this question, the surface half-life of both integrins was determined. Previously we determined it was possible to detect the surface half-life of the integrin by biotinylation strategy (314). The surface proteins of DU145H cells were biotinylated for one hour, washed and placed back in the incubator with media. After 24, 48 or 72 hours, the integrins were immunoprecipitated using the GoH3 antibody and analyzed under non-reducing conditions (Figure 20A). The data indicated that the α6p form remained on the surface of the DU145H cells with a half-life of approximately 72 hours, or almost 3 times longer than that of the full length α6 integrin (Figure 20B). The abundance of α6p was not influenced by exogenous protease inhibitors (BB94, leupeptin, aprotinin, 30% fetal bovine serum, ecotin), exogenous proteases (kallikrein) or activators of integrin function (TPA, 20mM $CaCl_2$) (data not shown).

RT/PCR analysis of the α6 coding region revealed a single product:

RT/PCR was used to determine if splice variants of the integrin α6 mRNA were potentially responsible for the production of the smaller integrin protein. Three micrograms of total cellular RNA from DU145H cells was reverse-transcribed into first

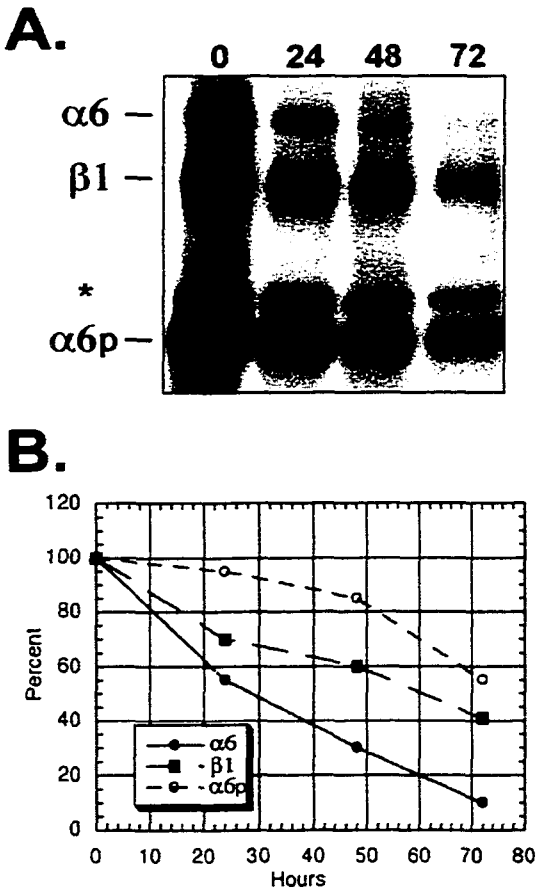

Figure 20: The α6p variant had a longer surface retention time than α6 integrin.

Panel A: DU145H cells were surface biotinylated and incubated for 24, 48 or 72 hours, followed by lysis and immunoprecipitation with anti-α6 antibody GoH3. Samples were analyzed by a non-reducing 7.5% polyacrylamide gel, transfered to PVDF membrane and reacted with peroxidase-conjugated streptavadin and visualized by chemiluminescence. The asterisk indicates a biotinylated protein band which was variably seen and is of unknown identity. Panel B: The film was digitized and the densitometry values analyzed for relative degradation rates of α6, β1 and α6p.

strand cDNA, and then PCR amplified with primers that essentially bracketed the entire integrin α6 protein coding region (all but the first 4 codons were amplified using these primers). The results of this experiment are shown in Figure 21. A single PCR product consistent with a full length RT/PCR product of 3263bp was detected; the splicing out of coding exons would have been detected by the presence of smaller products in the PCR reaction.

To confirm the identity of the integrin α6 PCR product, diagnostic restriction enzyme digests were performed. Analysis of the integrin α6 sequence (346), revealed the presence of one EcoNI site (producing fragments of ~960 and 2300bp), four SmaI restriction sites (producing fragments of 105, 150, 350, and 2650bp), four EcoRI sites (producing fragments of 30, 680, 730, and 1780bp), and one XhoI site (producing fragments of 420 and 2840). Aliquots of the integrin α6 PCR product were digested with each of these restriction enzymes, and the results of this experiment are shown in Figure 21. Each restriction digest product produced the restriction fragments expected from the integrin α6 PCR product (the 30 bp EcoRI fragment and the 105 bp SmaI fragment could not be visualized on the gel shown in Figure 21). Based on these results, it appears unlikely that the α6p variant is the result of the splicing out of exons in the known coding region.

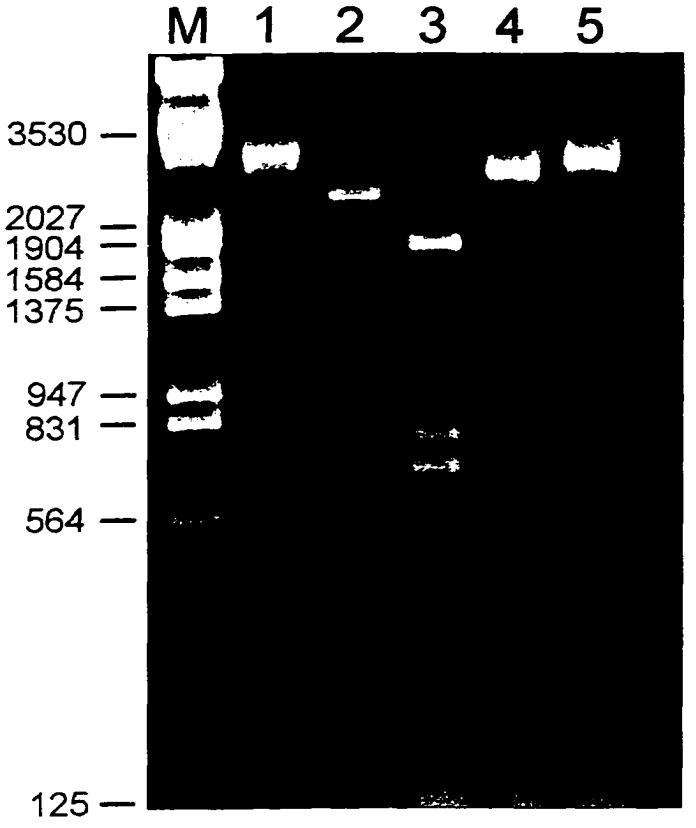

Figure 21: RT/PCR of the α6 integrin coding region revealed a single PCR product.

PCR primers that bracketed the integrin α6 coding region were used to amplify first strand cDNA generated from cell line DU145H (lane 1). To confirm the identity of the integrin α6 PCR product, aliquots were digested with four diagnostic restriction enzymes (EcoNI, lane 2; EcoRI, lane 3; SmaI, lane 4; and XhoI, lane 5), size separated on a 1x TBE-1.5% agarose gel, and visualized by ethidium bromide staining. The molecular weight standard is EcoRI/HindIII digested lamda DNA, lane M.

Calcium-induced normal keratinocyte differentiation increased α6p integrin protein levels:

Mouse 291 normal keratinocyte terminal differentiation can be induced by calcium. O3C and O3R cells were derived from normal 291 mouse cell strains and are immortalized, non-tumorigenic and tumorgenic, respectively (480). Both cell strains are resistant to calcium-induced terminal differentiation. The presence of α6 and α6p integrins in normal 291 mouse keratinocytes was determined using whole cell lysates followed by Western blot analysis using anti-α6 integrin antibody, AA6A (Figure 22A). Results for 291 cells were confirmed by immunoprecipitation with anti-α6 integrin antibody, GoH3 (data not shown). The α6 and α6p integrin protein bands were quantitated using Scion Image (448) and graphed (Figure 22B). Calcium-induced terminal differentiation increased α6p integrin protein levels three-fold in a dose-dependent manner in 291 non-transformed mouse keratinocytes. The differing steady-state levels of α6p in proliferating O3C intitated and O3R tumor cells under the same culture conditions suggested that the α6p integrin variant was responsive to terminal differentiation and not to calcium itself. Interestingly, α6p integrin levels were decreased in poorly-differentiated squamous cell carcinoma O3R cells relative to initiated cell O3C precursors and terminally differentiated 291 keratinocytes.

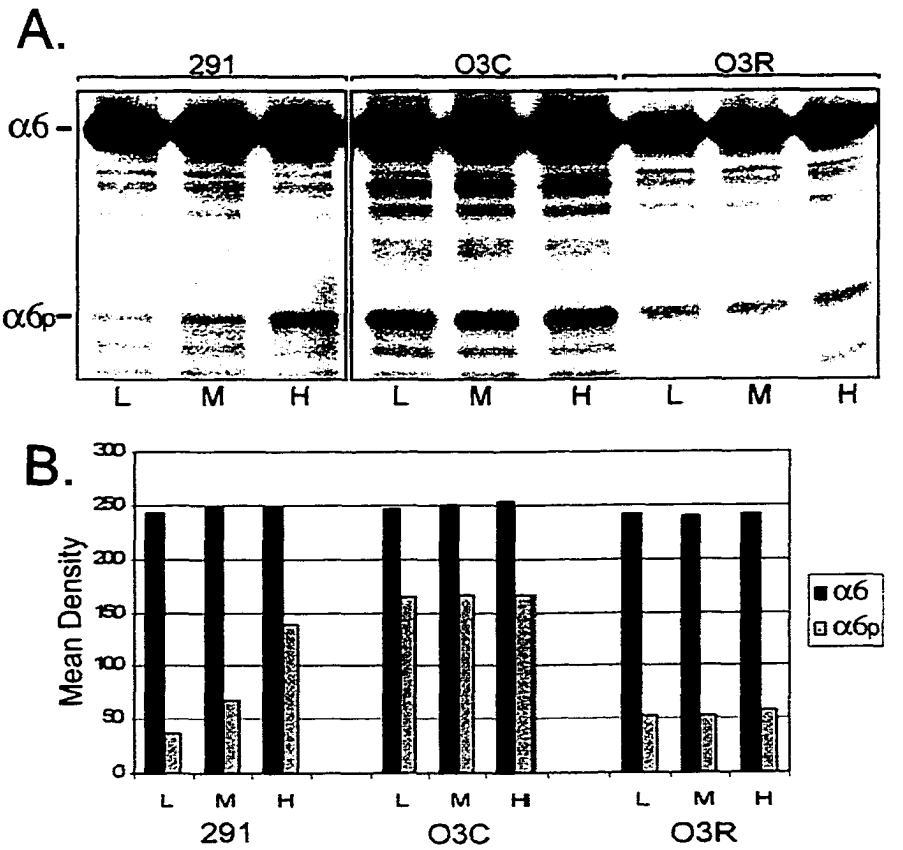

Figure 22: Calcium-induced normal keratinocyte differentiation increased α6p levels.

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

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. We have found the α6 integrin exists in the classical form (140 kD, non-reduced) and in a novel smaller form (70 kD), referred to here as α6p. The α6p is related to the full length α6 because it was immunoprecipitated with anti-α6 integrin antibodies (GoH3, J1B5, AA6A, 4F10, BQ16, J8H) (Figure 12). Two dimensional gel analysis revealed that the light chain of the α6p integrin was the same size as that found in the full length α6 form (Figure 13). The α6p variant co-immunoprecipitated with both anti-β4 (3E1, A9, 439.9b, ASC3) anti-β1 (P4C10) integrin antibodies (Figure 14) and was recognized by two anti-α6 integrin antibodies (AA6A, 4E9G8) by Western blot analysis (Figure 15). The α6p variant was found in several different human prostate (DU145H, LnCaP, PC3, PEAZ-1), colon (SW480), and pancreatic (Panc1, PaCa2, S48686) cancer cell lines and a human normal skin biopsy (Figure 16). It was not found in several cell lines including normal prostate cells (PrEC), a breast cancer cell line (MCF-7), a lung cancer cell line (H69) or a variant of a prostate carcinoma cell line (PC3-N). MALDI mass spectrometry indicated multiple amino acid regions in the α6p variant which corresponded exactly to sequences contained within exons 13 through 25 of the published full length α6 sequence (346) (Figure 17,18). Calcium-induced terminal differentiation of normal mouse 291 keratinocytes resulted in a three-fold increase of α6p protein levels (Figure 19). It remains to be determined if a cause and effect relationship exists between α6p and differentiation.

Integrin modulation is known to occur in the differentiation of human keratinocytes (481). Modulation of calcium levels in 291 cell derivatives O3C and O3R cells which are both resistant to calcium-induced differentiation, did not result in alterations of α6p integrin levels. Together, these data suggest α6p integrin was responsive to terminal differentiation and the observed alteration of α6p protein levels was not solely due to calcium.

The ten non-continuous amino acid fragments obtained from the α6p variant corresponded exactly to sequences contained within exons 13 through 25 of the full length α6 integrin (Figure 17,18). No peptide fragments corresponding to exons 1 through 12 were obtained using this method, suggesting the α6p variant is composed of exons 13 through 25 of the full length α6 integrin.

The predicted molecular weight of exons 13 through 25 is 55 kD, yet the α6p protein band had an apparent molecular weight of 70 kD by gel analysis. This apparent contradiction may be due to a post-translational modification of the protein. The α6 integrin has a predicted molecular weight of 140 kD (346); yet experimentally, the protein band has an apparent molecular weight of 160 kD under non-reducing conditions. The variation between predicted and apparent molecular weight in both proteins is likely due to the nine glycosylation sites predicted on the α6 protein and the five that would remain in α6p. Incubation of the proteins with endoglycosidase F to remove N-linked glycosylation only slightly affected the α6 integrin but significantly shifted the α6p integrin approximately 10 kD (Figure 19).

Our data indicated the novel α6p variant contained a significant alteration in the heavy chain, which is entirely extracellular. The current structural model of the α subunit proposes that the seven N-terminal repeats adopt the fold of a β-propeller domain (196,197). These domains contain seven four-stranded β-sheets and are arranged in a torus around a pseudosymmetric axis. Structural homology studies of enzymes with known β-propeller folds have identified active sites at the top of the β-propeller, typically where adjacent loops run in opposite directions (198-200). Recent studies of the β-propeller domain in integrins have demonstrated folds 1 and 3 in the α4 integrin subunit are important for ligand binding (202), whereas the α5 integrin ligand binding site is determined by amino acid sequences in repeats 2 and 3 of the N-terminal domain of the α subunit (203). Based on our mass spectrometry data which concluded the α6p variant contained only exons 13-25, the entire proposed β-propeller domain would be missing. Thus, it would be likely that the α6p integrin variant would function as an inactive receptor for cellular adhesion to the extracellular ligand.

Additionally, because integrins are known to be conformationally dependent molecules with dynamic ligand interactions (482), alteration of the extracellular portion of the molecule could influence intracellular signaling (483). The integrin α subunit cytoplasmic domains have been shown to be important for a diverse number of functions including: adhesion, motility, internalization, differentiation and cytoskeletal organization (204,205,379-382). Recently, the role of the α6A cytoplasmic domain was examined in myoblasts and found to inhibit proliferation and promote differentiation. Interestingly, the

cytoplasmic tail also suppressed signaling through the focal adhesion kinase and mitogen-activated protein kinase pathways (210). A previous report indicated that post-translational processing of the α4β1 integrin can occur in leukocytes (484), but to date, this is the first description of a naturally occurring variation in the extracellular domain of the α6 integrin subunit of this magnitude.

Interestingly, these data indicated the altered extracellular region of the α6p variant did not affect its ability to remain paired with either β4 or β1 integrin subunits. The α6p variant was retrieved by immunoprecipitation using the anti-β4 integrin monoclonal antibody, A9, whose epitope is present when α6 is coupled to the β4 subunit (426). This finding suggests the α6p subunit is able to heterodimerize with the β4 subunit in the same manner as the full length, α6 integrin. It is also noteworthy that α6p co-immunoprecipitated with β1 integrin in DU145H cells but not in HaCaT cells, despite abundant levels of β1 integrin in the HaCaT cells (Figure 14B). This finding may indicate a preferential pairing of α6p to β4 in some cell lines.

Previous studies suggested that integrins and TM4 tetraspan proteins could interact with one another to modulate integrin signaling and adhesion (485,486). Recently, it has been demonstrated that two members of this family, CD9 and CD81 can interact with the extracellular domain of the α6 integrin (487). It would be of interest to know whether the variant α6p retains the ability to bind to either of these tetraspan proteins.

In regard to the origin of the α6p variant, the data suggest several possibilities. Information obtained from cell surface retention half-life studies revealed that α6p (70 kD) was almost three times more stable than that of the full length α6 form (Figure 20). Conclusions from these data, were that the α6p protein was not a degradation product of the full length α6 integrin since the protein was not preferentially cleared from the surface as might be expected for a protein targeted for degradation. In addition, brefeldin A, an inhibitor used to indicate golgi-dependent membrane-trafficking, did not influence α6p production (488). The α6p protein was not generated after cell lysis, because multiple antiproteases and short immunoprecipitation times were unable to alter the presence of this variant. While some integrins are highly susceptible to proteolytic processing, i.e. the β4 integrin (472), the fully processed α6 integrin has not yet been reported to be enzymatically cleaved by any enzymes *in vivo*. Previously studies were unable to proteolytically cleave the α6 integrin *in vivo* (472). Collectively, these findings argue against α6p being a degradation product, however they do not provide information as to whether α6p was generated through a post-transcriptional processing event or alternative splicing of α6 message.

The data do not suggest that α6p originated from an alternative splicing event, because analysis by RT/PCR revealed only one transcript for α6 was present within the known coding region (Figure 21). Moreover, it has not previously been demonstrated in humans that alternative splicing plays a role in the regulation of the extracellular domain of integrins (182). Several integrins, including α6 have been shown to have isoforms of

the cytoplasmic domain generated by alternative splicing (247,248,346). The data demonstrated a significant variation (a 70 kD change) in the extracellular heavy chain of the α6p integrin (Figure 13). This large extracellular variation has not been previously described for other integrins.

Taken together, the data suggest a post-transcriptional event is responsible for the generation of α6p. The α6 integrin subunit, in addition to other α subunits, normally undergoes endoproteolytic processing close to the carboxyl terminus after synthesis, resulting in the formation of a light and heavy chain (489). A previous report demonstrated that defective post-transcriptional processing of the pre-α6 transcript in carcinoma cells lead to loss of normal cleavage and a resulting larger 150 kD single transcript (490). Examples of normal post-transcriptional processing have been described in yeast via translational introns which can give rise to two different sized proteins from a single mRNA transcript (491). Alternatively, ribosomal scanning past the conventional intiation codon has been described for MHC class I molecules. In this process, the ribosome initiates translation further downstream (492). Interestingly there are 12 alternative initiation codons which are predicted within the α6 gene using an open reading frame finder program (http://www.ncbi.nlm.nih.gov/gorf). It remains to be determined the mechanism for generating the α6p heavy chain and the functional role of the variant in adhesion and signaling processes.

V. DIFFERENTIAL REGULATION OF α6 AND α6p INTEGRINS SUGGESTS α6p MAY FUNCTION AS AN INACTIVE SUBUNIT

Introduction

Integrins are signaling receptors which link the intracellular cytoskeleton to the extracellular matrix and play important roles in adhesion, migration, proliferation, signaling, differentiation and cell survival (182-189). The α6 integrin is a laminin receptor in epithelial cells (191-193,248,346,493). Previously studies demonstrated a loss of the α6β4 heterodimer during prostate tumor progression (75,155,157) and a persistent expression of the α6β1 integrin (230). Additionally, expression of α6β1 integrin is maintained in micrometastases (155,157,158,221,274).

The studies in the previous chapter identified a novel 70 kD variant of the α6A integrin, called α6p, for the latin word *parvus*, in prostate carcinoma cell lines (494). The variant paired with both β1 and β4 integrin subunits and was present in a number of epithelial carcinoma cell lines, as well as a normal immortalized human keratinocyte cell line. Two dimensional gel analysis and Western blotting data indicated the cytoplasmic light chain of the variant was identical to that of the full length α6 integrin and that the primary alteration was in the extracellular heavy chain, including the putative ligand binding domain contained within the β-propeller (196,197,203).

The α6β4 integrin is a component of the hemidesmosome of prostate epithelial cells which forms a tight adhesion complex primarily with laminin-5 (193) (339), thus supporting mechanical stress and stable anchorage to the basal lamina (336,337) (338). This large protein complex is composed of at least eleven different proteins including; keratins 5 and 14, α6β4 integrin, laminin-5, HD1/plectin, IFAP300, LAD-1, BP180, BP230, collagen VII and CD151 (224,319-321,351).

The relationship between the cytoskeleton and integrins has been well studied. Adhesion to extracellular matrix proteins has been shown to play a role in cytoskeletal organization (495). The α6β1 integrin localizes to the focal adhesion, functioning to link the extracellular matrix to the actin cytoskeleton via the β1 cytoplasmic domain for both signal transduction and mechanical stability of the cell during migration (155,189,378,496-498). This interaction has been shown to be important for integrin signaling and recruitment of secondary molecules such as paxillin and F-actin (499,500) in other integrin models.

This chapter presents an extension of the studies of α6p integrin in order to determine the functional role of the variant. Previous studies suggested production of α6p was a regulated event which differed from that of α6 integrin during differentiation. The α6 integrin participates in adhesion events via hemidemosomes and focal adhesions. It is not known what the role of the α6p variant in these same structures. The following studies demonstrated the α6 and α6p integrins respond differently to the alteration of

actin filaments and the action of serine/threonine phosphatase inhibitors. These data may provide insight into the function of the newly identified α6 integrin variant, α6p.

Results

Disruption of the actin cytoskeleton significantly reduced production of α6p, but not α6 integrin.

In addition to participating within the hemidesmosome, the α6 integrin paired with β1 subunit is a component of the focal adhesion complex. In order to examine the role of the α6p variant in this structure, the human prostate carcinoma DU145H cell line was used due to the abundance of the α6β1 and α6pβ1 integrins (494). The normal cytoskeletal elements of the DU145H cells were examined by immunocytochemistry, using fluorescent probes for actin and tubulin as described in "Materials and Methods". Cells were grown on glass coverslips to approximately 80% confluency then stained for actin (Figure 23A) with Alexa Fluor 568 phalloidin (Molecular Probes, Eugene, OR, USA) (445), or tubulin (Figure 23B) with anti-tubulin antibody YL1/2 (Chemicon International, Inc. CA, USA).

Observation of actin staining in the DU145H cells revealed primarily a cortical actin pattern surrounding the periphery of the cells with few stress fibers noted. Microtubule networks were observed to radiate throughout the cytoplasm, originating from the microtubule organization centers near the nuclei of the DU145H cells. Disruption of the actin cytoskeleton in DU145H cells was accomplished by treatment

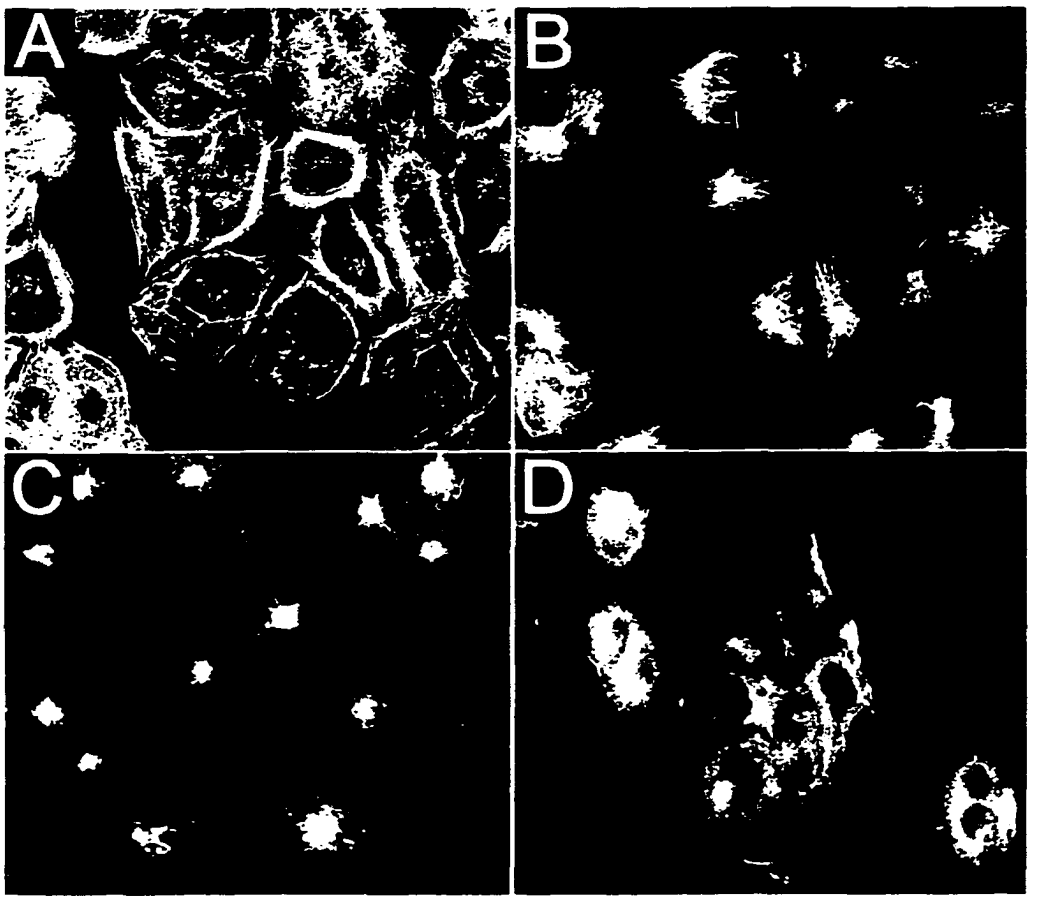

Figure 23: Cytoskeletal elements in DU145H cells were sensitive to cytoskeletal inhibitors; cytochalasin D and nocodazole.

Cytoskeletal actin and tubulin in human prostate carcinoma DU145H cells were visualized by immunocytochemistry using fluorescent probes. Untreated cells were immunostained for actin (Panel A) and tubulin (Panel B). Note actin was expressed primarily as cortical filaments with few stress fibers present, while the tubulin network was fairly well-developed. Disruption of either actin or tubulin was accomplished by 18 hour treatment with either 10 μM cytochalasin D (Panel C) or 8 μM nocodazole (Panel D), respectively.

with 10μM cytochalasin D for 18 hours (Figure 23C). Observation of the cytochalasin D-treated DU145H cells revealed loss of cortical actin organization which was replaced with peri-nuclear distribution of disorganized actin. Disruption of the microtubule network in the DU145H cells was accomplished by 8μM nocodazole for 18 hours (Figure 23D). Upon disruption of the microtubule network with nocodazole, anti-tubulin staining revealed loss of tubulin network organization in the DU145H cells and was redistributed in a peri-nuclear pattern. Both cytochalasin D and nocodazole treatment of DU145H cells resulted in altered cell spreading. Cells were adherent, but rounded and not spread out as was observed in cells treated with DMSO vehicle.

The actin cytoskeleton influences integrin behavior such as integrin clustering, dispersal from focal adhesions and integrin-mediated adhesion to extracellular matrix proteins (377). Alteration of the actin cytoskeleton has previously been shown to inhibit α6β1-mediated cell adhesion to laminin (501). In order to determine whether the α6p integrin variant could be altered in response to disruption of the cytoskeleton as is seen with α6β1 integrin, cytoskeletal inhibitors were used.

Using 10 μM cytochalasin D to disrupt the actin cytoskeleton, the total amount of α6 and α6p integrins was examined at timepoints 0, 1, 3, 6, 12 and 18 hours (Figure 24, Panel A). 10-15μg total protein was examined by SDS-PAGE under non-reducing conditions. The blot was then probed using anti-α6 integrin antibody, AA6A. The protein bands were quantitated and graphed (Figure 24,B). Quantitation of the protein bands demonstrated a time-dependent decrease in total α6p protein levels while total α6 integrin protein levels was relatively unaltered. The differential change in the α6 and α6p

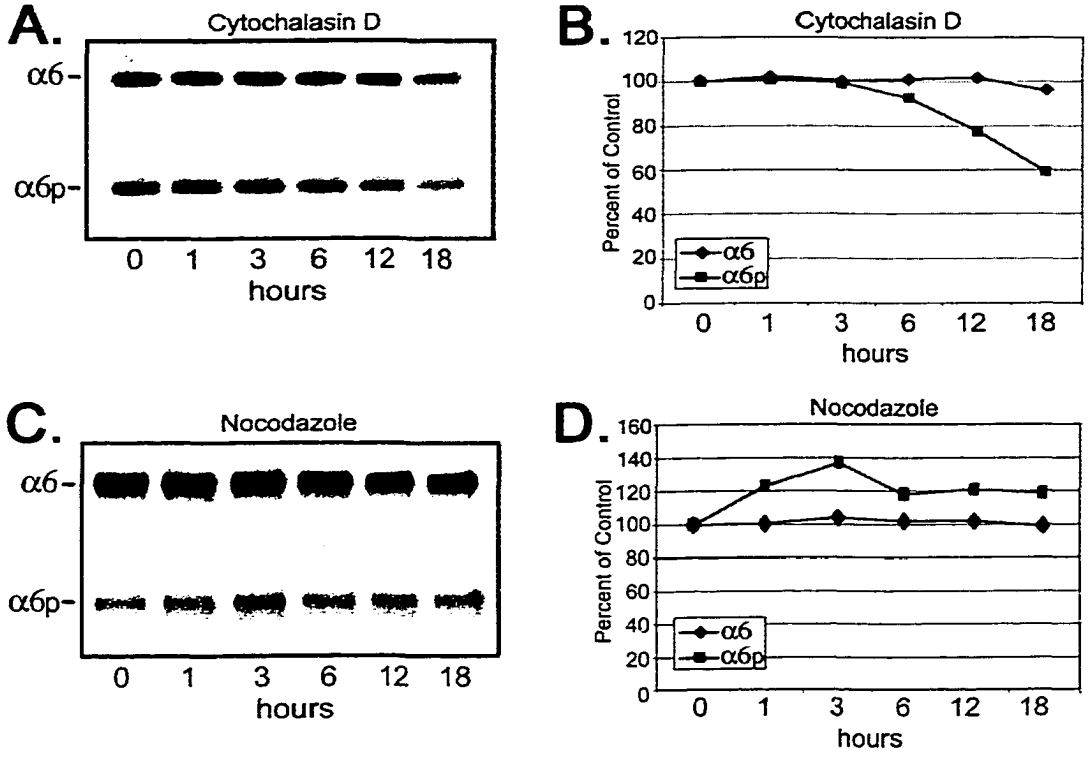

Figure 24: Disruption of the actin cytoskeleton reduced total protein expression of α6p, but not α6 integrin.

Human prostate carcinoma DU145H cells were treated with either 10 μM Cytochalasin D (Panel A,B) or 8 μM nocodazole (Panel C,D) over a 24 hour period of time. Whole cell lysates (10-15μg) were loaded and electrophoresed on a 7.5% polyacrylamide gel under non-reducing conditions. Proteins were transferred to a PVDF membrane followed by Western analysis for α6 integrin (Panel A, C). The α6 and α6p protein bands were scanned and quantitated using Scion Image Analysis software and graphed in Excel (Panel B, D). Data shown are representative of three experiments.

integrin proteins was first apparent by 12 hours post-addition of cytochalasin D and by 18 hours, the α6p integrin form had decreased to 60% of the vehicle control (DMSO). Data shown were representative of three independent experiments. This differential regulation of the α6 and α6p integrins was interesting, suggesting the production of the two forms was regulated by different mechanisms.

Previously the actin cytoskeleton, but not the microtubule network, has been implicated in integrin-mediated events (501). Because there was a dramatic alteration in the levels of α6 and α6p integrins upon disruption of the actin cytoskeleton in the DU145H cells, it was next determined whether this phenomenon was a general effect, or specific for actin microfilament bundles. Using 8 μM nocodazole to disrupt the microtubules, the total amount of α6 and α6p integrins was examined at timepoints 0, 1, 3, 6, 12 and 18 hours. Equal amounts of protein (10-15μg whole cell lysate) were loaded in each well and separated by 7.5% SDS-PAGE under non-reducing conditions. To examine α6 and α6p integrin forms, the blot was then probed using anti-α6 integrin antibody, AA6A (Figure 24, Panel C). The protein bands were quantitated and graphed (Panel D). Data shown were representative of three independent experiments. No significant difference was observed in the total amount of the α6 and α6p integrin forms upon depolymerization of the microtubules.

Disruption of the actin cytoskeleton significantly reduced cell surface expression of α6 and β1, but not α6p integrins.

The integrin family plays an active role at the cell surface by interacting with the extracellular matrix (183,189,372). Because integrins cannot be activated unless they are on the surface of the cell, the effect of disruption of the actin cytoskeleton on the surface levels of the α6, β1 and α6p integrins was examined. In order to distinguish between surface and cytoplasmic integrin subunits, cells surface proteins were labeled using biotin before adding cytochalasin D to the cells. After an 18 hour incubation, cells were lysed and prepared for immunoprecipitations with anti-α6 integrin antibody, J1B5. The resulting samples were examined by 7.5% SDS-PAGE analysis under non-reducing conditions and the membrane blot was probed with horseradish peroxidase conjugated to streptavidin (Figure 25A). The protein bands corresponding to α6, β1 and α6p integrins from three independent experiments were quantitated and graphed in Panel B. Results indicated α6 and β1 integrin cell surface levels were significantly decreased to 36% and 30%, respectively, as was expected. The surface levels of the α6p integrin were decreased to 67% of control, indicating that surface α6p protein was not as responsive to changes in the actin cytoskeleton as α6 and β1 integrins.

In order to determine whether the change in surface integrin levels was a general phenomenon due to disruption of the cytoskeleton, the effect of disruption of the microtubule network was examined using nocodazole. The DU145H cells were first treated with 8 μM nocodazole or vehicle for 18 hours in serum free media plus 0.1% BSA conditions. The cells were then surface labeled with biotin for 30 minutes and then

lysed. Equal amounts of total whole cell lysate (200 μg) were used for immunoprecipitations using anti-α6 integrin antibody, J1B5. Captured proteins were separated by 7.5% SDS-PAGE under non-reducing conditions. The protein blot was incubated with streptavidin conjugated to horseradish peroxidase (Figure 25,A). Protein bands corresponding to α6, β1 and α6p from three independent experiments were quantitated and graphed (Figure 25,B). No significant difference was observed between treated versus control samples for α6, β1 or α6p integrins which suggested that alteration of surface integrin levels was specific for the actin cytoskeleton, but not the microtubule network.

The α6, but not α6p integrin was altered by Serine/Threonine phosphatase inhibitors

Inhibition of serine/threonine phosphatases has previously been shown to decrease cell-cell adhesion (502,503) and integrin-dependent adhesion and motility (504-507). For example, inhibition of serine/threonine phosphatases using okadaic acid resulted in phosphorylation of β1 integrin and selective loss from the focal adhesion (504). Phosphorylation of the β1 integrin decreased binding and cell movement on fibronectin but did not alter integrin surface or total levels (505,506). Okadaic acid has also been shown to inhibit α3β1 integrin-mediated adhesion in PC3 cells to collagen IV via decreased interaction of calreticulin with the α3 subunit (507).

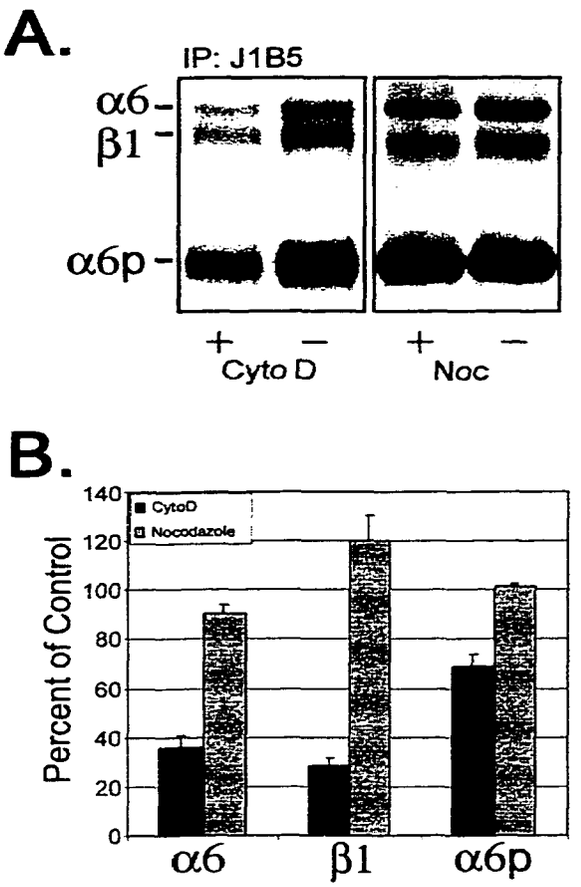

Figure 25: Disruption of the actin cytoskeleton significantly reduced cell surface expression of α6 and β1, but not α6p integrins.

Surface changes of α6, β1 and α6p were determined by surface of DU145H cells with biotin prior to treatment with either 10 μM cytochalasin D or 8 μM nocodazole for 18 hours. Labeled cells were lysed and 200μg total protein was used for immunoprecipitations with anti-α6 integrin antibody, J1B5. Samples were separated on a 7.5% polyacrylamide gel under non-reducing conditions. Proteins were transferred to PVDF membrane followed by incubation with horseradish peroxidase conjugated to streptavidin (Panel A). Resulting α6, β1 and α6p integrin protein bands were quantified and graphed (Panel B).

In order to examine the role for protein phosphatase inhibitors on α6 and α6p integrin forms, calyculin A and okadaic acid were tested. Calyculin A is a potent inhibitor of protein phosphatase type 1 and 2A, while okadaic acid inhibits both, it preferentially inhibits type 2A (508,509). Thus, usually higher concentrations and longer incubation periods are necessary to achieve the same effect with okadaic acid as is seen with calyculin A (510,511). Using 15 nM calyculin A to inhibit serine/threonine phosphatases, the total amount of α6 and α6p integrins was examined at timepoints 0, 1, 3, 6, 12 and 18 hours. Equal amounts of protein (10-15μg whole cell lysate) were loaded in each well and separated by 7.5% SDS-PAGE under non-reducing conditions. To examine α6 and α6p integrin forms, the blot was then probed using anti-α6 integrin antibody, AA6A (Figure 26,A). The protein bands were quantitated and graphed (Figure 26,B). Data shown in Panel A and B were representative of three independent experiments. After treatment with 15 nM calyculin A, we observed a 50% decrease in total protein level of α6 integrin,

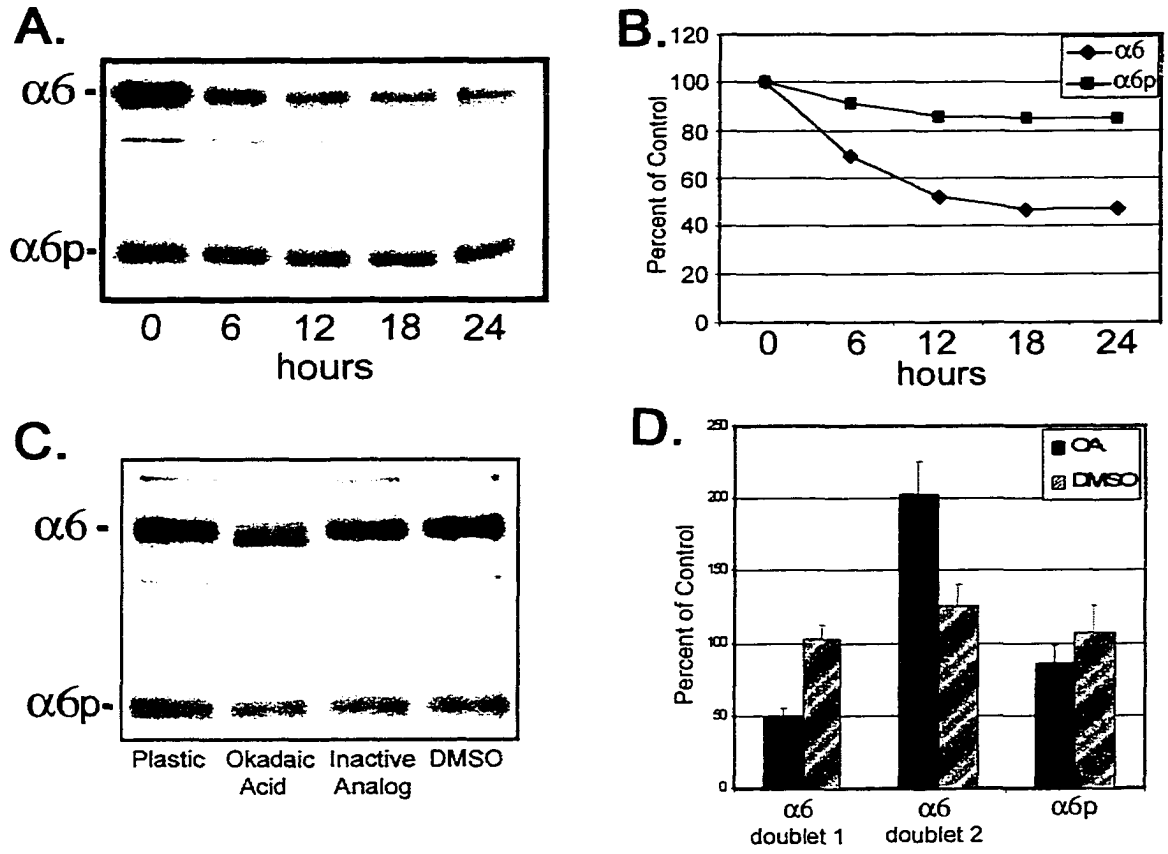

Figure 26: Okadaic Acid and Calyculin A treatment of DU145H cells decreased α6 integrin protein levels but not α6p.

Human prostate carcinoma DU145H cells were treated with 15nM Calyculin A over a 24 hour period of time. Whole cell lysate (10-15μg) was loaded and electrophoresed on a 7.5% polyacrylamide gel under non-reducing conditions. Proteins were transferred to PVDF membrane followed by Western analysis for α6 integrin (Panel A). Protein bands in panel A were scanned and quantitated using Scion Image Analysis software and graphed in Excel (Panel B). Data shown were representative of three independent experiments. DU145H cells were treated with 50μM okadaic acid, the inactive analog 1-nor-okadaone or vehicle (DMSO) for 18 hours. Whole cell lysates were examined for α6 integrin protein expression as above (Panel C). Resulting α6 and α6p bands from three independent experiments were quantitated and graphed (Panel D).

but only a 10% decrease in the variant α6p form, both of which were observed to begin at 6 hours post-addition of drug. Similar results were obtained with 50 μM okadaic acid. These data suggested that production of α6 and α6p integrin variants was differentially regulated by serine-threonine phosphatases.

Protein samples treated with 50 μM okadaic acid for 18 hours resulted in an α6 integrin doublet, the lower protein band migrated more quickly than that observed in the vehicle control (Figure 26,C). The molecular weight shift observed in the lower doublet was consistent with a dephosphorylated α6 integrin protein. The α6 integrin doublets from three separate experiments similar to Panel C were quantitated and graphed using Excel software (Figure 26,D). In cells treated with okadaic acid, there was a 200% increase of the α6 integrin doublet 2 (lower doublet), with a corresponding 50% decrease in the upper α6 doublet protein band. Although okadaic acid is a phosphatase inhibitor, the observed shift from the upper to lower doublet was consistent with accumulation of a dephosphorylated protein. This was suggestive that okadaic acid altered an intermediate protein which secondarily resulted in dephosphorylation the α6 integrin. No doublets or alteration in electrophoretic mobility of α6p integrin was observed. Due to profound changes in the adhesion status of the DU145H cells when treated with these compounds, we were unable to assay cell surface alteration of α6, β1 or α6p integrins.

Alteration of α6 and α6p levels was not transcriptionally regulated

Long incubation times were needed to observe changes in the α6 and α6p integrin forms, which was not suggestive of proteolytic processing to explain the alterations in α6 and α6p integrin protein levels after drug treatment with either cytochalasin D, calyculin A or okadaic acid. In order to determine whether or not the alterations in protein levels observed in both α6 and α6p were due to transcriptional regulation, the α6 mRNA levels were examined in treated cells. Previously, only one mRNA transcript was identified in the DU145H cells which corresponded to α6 (494). The DU145H cells were treated with either: 15nM Calyculin A; 50μM Okadaic Acid; 50μM 1-Nor-Okadaone; 10μM Cytochalasin D or vehicle (DMSO) for 18 hours in the dark. Total mRNA was isolated and examined for α6 message levels as indicated in "Materials and Methods". GAPDH was used as a loading control. The α6 mRNA levels were normalized to the GAPDH mRNA levels and the calculated ratio of α6 mRNA to GAPDH mRNA was indicated below the sample lanes (Figure 27). The α6/GAPDH ratio for cells treated with calyculin A and okadaic acid were increased 130% and 180%, respectively, over both untreated and vehicle control samples.

Although alteration of the α6 mRNA levels was apparent, there was no correlation with the protein alterations observed by Western blot analysis. The mRNA levels for both calyculin A- and okadaic acid-treated DU145H cells, which previously showed a decrease in total α6 protein levels, indicated an increase in α6 integrin mRNA

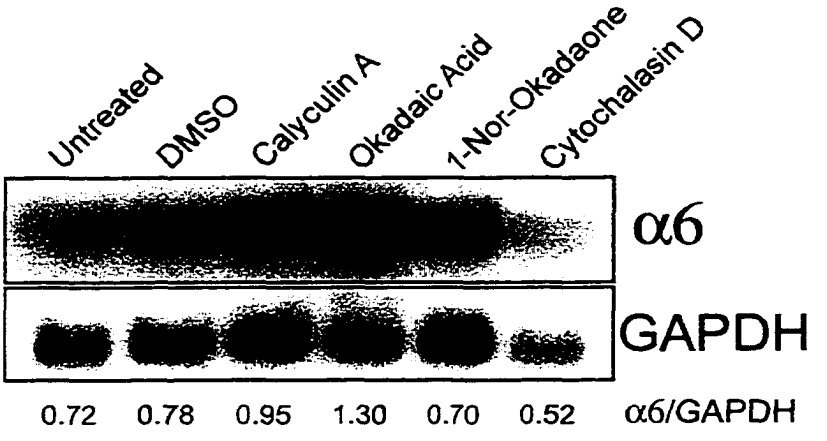

Figure 27: Northern analysis indicated alteration of α6 and α6p levels was not transcriptionally regulated

Human prostate carcinoma DU145H cells were treated for 18 hours with drug (Calyculin A, 15nM; Okadaic Acid, 50μM; 1-nor-okadaone, 50μM; or Cytochalasin D, 10μM) or vehicle. Total RNA was extracted from cultured cells by acid guanidinium thiocyanate-pheno-chloroform extraction. Total RNA (15μg) was separated on a 1% agarose gel containing 1.85% formaldehyde, transferred to nylon membranes and cross-linked by ultraviolet light. Blots were probed with a random primed ^{32}P-labeled probe of a 1958-bp *Bgl-I* fragment of human alpha 6 integrin, then exposed to X-OMAT AR film. A 1.2-kb PstI fragment of human GAPDH was used for loading control. The ratio of α6 mRNA/GAPDH is indicated below each lane.

levels. These results suggested the protein regulation observed in the α6 and α6p integrins was not transcriptionally regulated.

Pharmacological inhibitors were not toxic at 18 hours in DU145H cells.

In order to determine the viability of human prostate carcinoma DU145H cells treated with various cytoskeletal inhibitors, a colorimetric assay (XTT based) was used for the quantitation of cell proliferation and viability as described in Materials and Methods. Each drug treatment (Calyculin A, 15nM; Okadaic Acid, 50μM; Cytochalasin D, 10μM; Nocodazole, 8μM) was performed in 6 well replicates for an 18 hour timepoint. Data shown were representative of three independent experiments (Figure 28). The absorbance at 490 nm was recorded. Cell survival (percent of control, either vehicle for Calyculin A, Okadaic Acid and Cytochalasin D, or untreated for Nocodazole) was calculated by first averaging the 6 replicate wells, then dividing the treatment group by the appropriate control group and converting to a percentage. The data did not indicate significant toxicity to the DU145H cells at any of the drug concentrations used for the 18 hour timepoint.

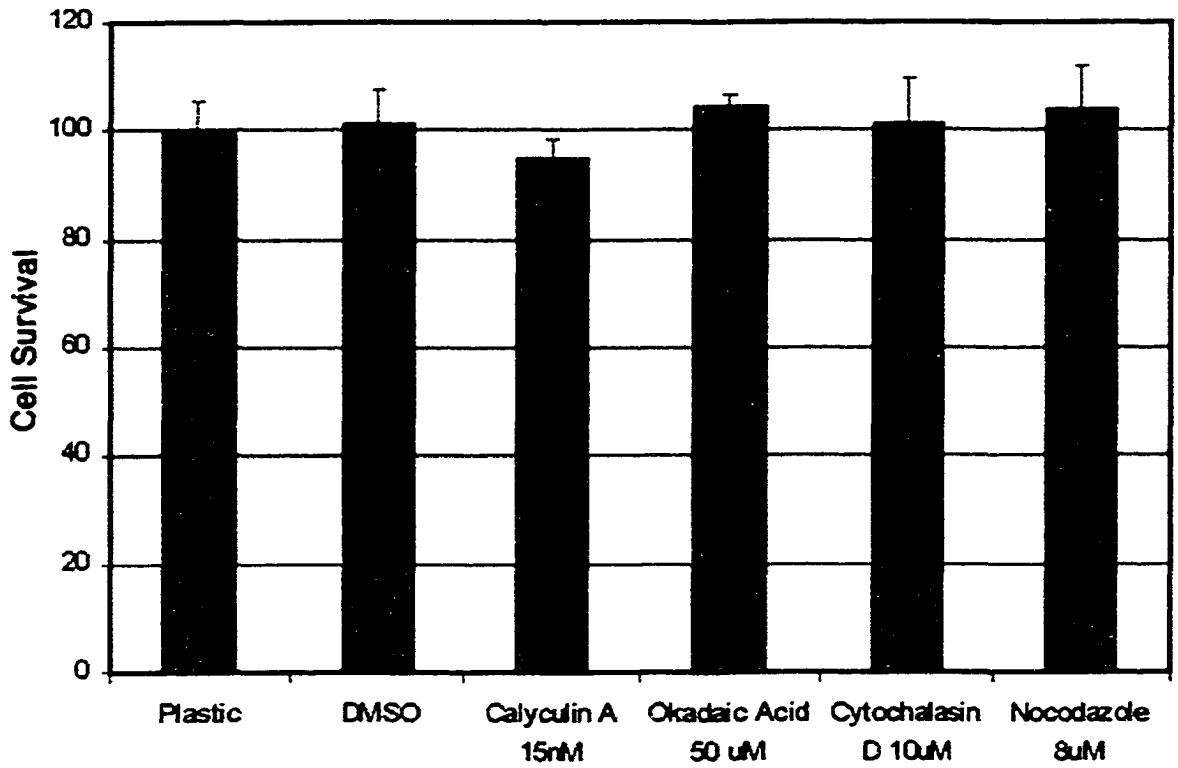

Figure 28: Drug concentrations used on DU145H cells were not cytotoxic after 18 hours of treatment.

The viability of human prostate carcinoma DU145H cells treated with various cytoskeletal inhibitors was determined using an XTT metabolic survival assay. Cells were seeded at a density of 10,000 cells per well in a 96 well plate and treated for 18 hours with drug (Calyculin A, 15nM; Okadaic Acid, 50μM; Cytochalasin D, 10μM; and Nocodazole, 8μM) or vehicle. Absorbance was read at 490 nm and 6 replicate wells were averaged for each treatment group. Cell survival was calculated as a percentage by dividing the absorbance of the drug-treated group by the absorbance of the corresponding vehicle-treated group.

The α6 but not α6p integrin was associated with the hemidesmosome

The α6 integrin is found in both hemidesmosomes and focal adhesions (313,372). The α6β4 integrin serves as a 'seed' site for hemidesmosome formation (77,340). In order to determine whether the α6p variant was associated with functional components of the hemidesmosome, co-immunoprecipitation experiments. For these studies, the normal immortalized keratinocyte cell line, HaCaT, was used due to the abundance of α6β4 and α6pβ4 integrin heterodimer expression (494). Whole cell lysates were immunoprecipitated with anti-α6 integrin antibody, J1B5; anti-β4 integrin antibody, ASC3; anti-laminin-5 antibody, BM165; and anti-CD151 antibody, 14A2.H1. The samples were then analysed by SDS-PAGE under non-reducing conditions in a 7.5% polyacrylamide gel. The membrane blot was then probed with anti-α6 integrin antibody, AA6A. For a positive control, the α6 and α6p integrins were immunoprecipitated with anti-α6 integrin antibody, J1B5 (Figure 29). The resulting protein bands migrated at 160 and 70 kD, respectively, as previously shown (494). The α6 integrin was retrieved by immunoprecipitation with anti-β4 integrin, anti-laminin-5 or anti-CD151 antibodies. However, the α6p variant was found only to co-immunoprecipitate with the β4 integrin subunit and was not found to co-immunoprecipitate with either laminin-5 or CD151. These results demonstrated that although α6p paired with the β4 subunit, it was not strongly associated with two components of the hemidesmosome.

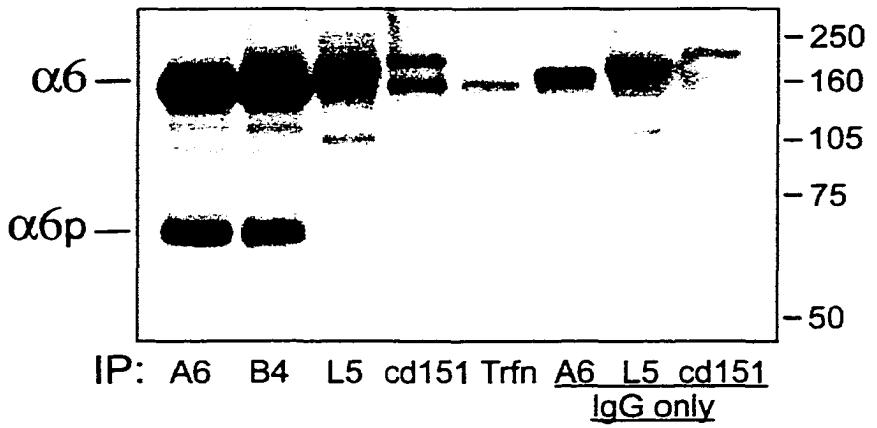

Figure 29: The α6, but not α6p integrin was co-immunoprecipitated with normal hemidesmosomal components.

Normal immortalized, human keratinocytes (HaCaT) were lysed and α6 and α6p integrins were co-immunoprecipitated using antibodies against components of the hemidesmosome. Immunoprecipitation with anti-α6 integrin antibody, J1B5 was used for a positive control. Antibodies against β4 integrin (ASC3), laminin-5 (BM165) and CD151 (14A2.H1) were used to assess association of α6 and α6p integrins with the hemidesmosome. The transferrin receptor is not a component of the hemidesmsome and was used for a negative control. Migration of broad range molecular weight markers are indicated.

Discussion:

Previous studies have indicated the α6 integrin-containing heterodimer is altered in prostate carcinoma progression, shifting from the α6β4 to α6β1 integrin. The previous chapter presented studies which identified a novel variant of the α6 integrin, called α6p (494). This variant was observed on epithelial cell surfaces as α6pβ1 and α6pβ4 heterodimers. Two-dimensional gel analysis indicated the primary alteration in the α6p variant was in the extracellular β-propeller domain and that the intracellular light chain was immunologically identical. In the present study, the role of the α6p integrin in the hemidesmosome and the focal adhesion was further investigated. In order to determine whether α6p and α6 were regulated in a similar or distinct manner, three different experimental strategies were employed. Cytoskeletal inhibitors were used to investigate the known dependence of integrins on the intact cytoskeleton. Secondly, serine/threonine phosphatase inhibitors were used due to their known effects on integrin phosphorylation and disruption of adhesion. Lastly, co-immunoprecipitations were used to determine α6 and α6p variant association in the hemidesmosome. Together, the data demonstrated that the α6p variant was differentially regulated from the full length α6 integrin and suggested α6p functioned as an inactive subunit.

Because the integrins are known to be regulated by the cytoskeleton (345,404,475,495,500), the effect of cytoskeletal inhibitors on the total and surface protein levels of the integrins was first investigated for α6 and α6p integrins. It was observed that actin and microtubule networks were sensitive to pharmacological

inhibition in human prostate carcinoma DU145H cells by addition of either cytochalasin D or nocodazole (Figure 23). Total production of the α6p integrin, but not α6, was dramatically reduced 60% in a time-dependent manner until 18 hours post-incubation with cytochalasin D (Figure 24). In contrast, no alteration in either the α6 or α6p total protein levels was observed upon disruption of the microtubule network by nocodazole, demonstrating that altered α6p integrin production was specifically regulated by the actin cytoskeleton.

Conversely, upon disruption of the actin cytoskeleton the cell surface expression of α6 and β1 integrins was significantly reduced to 36% and 30% of vehicle control, respectively. The α6p variant was less significantly effected and decreased 67% of control upon actin depolymerization (Figure 25). This alteration was not reproduced upon depolymerization of the microtubule network, again suggesting the specificity of actin in integrin regulation.

While the production of the α6p variant was dependent upon an intact actin cytoskeleton, the cell surface distribution of the variant was less responsive to disruption of actin than α6 or β1 integrins, suggesting that α6p was not associated with the focal adhesion. There is much evidence in eukaryotic cells that polyribosomes, mRNAs and components of protein synthesis machinery are associated with and modulated by the actin cytoskeleton (512,513). Also, it is known that cytoskeletal attachment to integrins is important for modulation of integrin clustering and dispersal from focal contacts and 'inside-out' signaling (377). Recently, Haier and others demonstrated inhibition of α6β1 integrin-mediated cell adhesion to laminin after depolymerization of the actin

cytoskeleton in colon cells (501) and depolymerization of tubulin did not alter α6β1 integrin-mediated cell adhesion to laminin. Together, the data suggest that expression of α6 and α6p integrins are differentially regulated by the actin cytoskeleton. Furthermore, the failure of actin depolymerization to significantly alter cell surface distribution of the α6p variant suggested it did not associate with the actin-containing focal contact, despite association with the β1 subunit.

The second experimental approach used to examine α6 and α6p function was the use of serine/threonine phosphatase inhibitors, which have previously been shown to alter integrin phosphorylation and decrease adhesion (504-506). The α6 integrin may function as a signaling receptor in the focal adhesion. Phosphorylation of the α6 cytoplasmic tail on serine residue 1041 has been described (390,391), however physiological relevance of this event is still unclear. Previous studies have indicated that serine/threonine phosphatase inhibitors, such as okadaic acid and calyculin A, disrupted integrin-mediated cell adhesion and migration (505-507,514,515). Specifically, phosphorylation changes have been described including dephosphorylation of α4 (516) and phosphorylation of β1 integrins (504) upon treatment with okadaic acid. Additionally, serine/threonine phosphatase inhibitors have been used to induce hyperphosphorylation of vimentin and reorganization of intermediate filaments (517).

Treatment with calyculin A and okadaic acid, demonstrated differential alteration of the electrophoretic properties of α6 and α6p integrins (Figure 26). It is difficult to ascribe a specific interaction with the α6 integrin due to these drugs because significant alterations in the biology of the cell occur with long exposure to these inhibitors (518-

520). The observed alteration in α6 integrin resulted in an increased electrophoretic mobility, and a protein doublet emerged which was consistent with phosphorylated and dephosphorylated products. The α6p variant was not altered by treatment with serine/threonine phosphatase inhibitors. Although okadaic acid is a phosphatase inhibitor, the observed shift from the upper to lower doublet was consistent with accumulation of a dephosphorylated protein. This was suggestive that okadaic acid altered an intermediate protein which secondarily resulted in dephosphorylation the α6 integrin. While significance of phosphorylation of the α6 integrin cytoplasmic domain is incompletely understood, it may function in signal transduction. The α6 integrin has been shown to induce tyrosine phosphorylation of paxillin and several other unknown proteins upon ligand binding (389,521). These studies were suggestive that the cytoplasmic domain of the α6 integrin was responsive to a signaling event, while the α6p variant was not, despite having identical cytoplasmic domains (494). This again is consistent with the emerging role of α6p as an inactive receptor for signal production.

Lastly, co-immunoprecipitation experiments demonstrated α6β4, but not α6pβ4 integrin was associated with laminin-5 and CD151; two components of the hemidesmosome (320,351,356). The current hypothesis of integrin ligand binding is based on studies performed on the α5 and α4 integrin subunits. These studies demonstrate loops contained within the N-terminal β-propeller domain are necessary for confirming ligand specificity (196,197,202,203). The current data suggest that the α6p variant contains the cytoplasmic domain and a portion of the stalk region; exons 1

through 12 of the extracellular domain are missing, suggesting the β-propeller is not present (494). Current data suggest the α6p integrin variant contained the same cytoplasmic light chain, therefore it is not suprising that the variant paired to the β4 subunit, but did not recognize the laminin-5 ligand extracellularly. Additionally, it is presently hypothesized that tetraspanin association with the integrin is dependent upon an extracellular epitope within the α subunit (487). For recruitment of CD151 into the hemidesmosome, the α6 subunit must be associated with β4 integrin (351). Significant alteration of the extracellular domain of the α6p variant may obliterate an epitope which is important for CD151 recruitment. The literature suggests laminin-5 and the α6 integrin are not necessary for hemidesmosome formation (341) and probably would not influence recruitment of secondary molecules such as HD1/plectin, BP180 and BP230. It would be interesting to determine whether α6pβ4 integrin is still capable of recruiting these proteins. Functions which are dependent on interaction with the extracellular domain of the α6 integrin would be effected by the α6p variant.

Together, the data suggest the α6p integrin variant serves as an inactive receptor which functionally differs from that of the full length α6 integrin. Integrins are conformationally dependent molecules with dynamic ligand interactions which can transduce signals bidirectionally across the cell membrane (482,522). Extracellular signals can be transduced through the molecule to the intracellular domain (483). Due to the nature of shortened extracellular domain of α6p, it is logical that the variant functions differently from α6 integrin. Data presented in this chapter suggest the α6p integrin

variant may function as an inactive receptor despite its ability to pair with both β1 and β4 integrin subunits. Recently, Sastry and others dissected the function of the α6A cytoplasmic domain from that of the full length molecule. The α6A cytoplasmic domain was ectopically expressed in myoblasts and the resulting phenotype was suppression of proliferation, induction of differentiation and suppression of focal adhesion signaling via focal adhesion kinase and mitogen-activated protein kinase with a lesser effect on paxillin phosphorylation (209,210). These data further support the role of the α6p integrin variant in inactivation of the growth stimulatory role of the α6 integrin. It remains to be determined if ectopic expression of α6p can elicit the same response.

VI. PRELIMINARY DATA REGARDING DIFFERENTIAL EFFECTS OF SYNTHETIC PEPTIDES ON α6 AND α6P INTEGRINS

Introduction:

The progression of cancer is contingent upon the ability of a given carcinoma for cellular invasion and metastatic spread. These processes are both highly dependent upon the function of integrins (78,116,155,158,283,292,523). Modulation of integrin-ligand interaction has been a subject of many scientific articles (524,525). Previously, a synthetic antagonist to laminin was shown to inhibit the formation of osteolytic metastases by human melanoma cells in nude mice (526). There has been much interest in the possibility of identifying new potential therapuetic targets using small molecules for the management and treatment of cancer (527-530).

This chapter will briefly examine the preliminary effects of two ligand antagonists identified in our laboratory. Using the "one-bead one-compound" combinatorial library method (446) the peptides which supported cell adhesion were sequenced. From those sequences, a hybrid sequence was formulated called HYD1 (single-letter amino acid code, KIKMVISWKG). Another sequence called C1 (AKRNRIIIYAWKG) was postulated based on a homology model of the α6 integrin structure and consideration of the binding loops. Both peptides were found to be active in inhibiting integrin-mediated cell adhesion and bind to the cell surface, but do not enter

cells. Two inactive, scrambled equivalents were generated using the same amino acids, but placed in random order HYDS (WIKSMKIVKG) and CS (KAIKNAWYRIIRG).

Our preliminary data demonstrated adherence of human prostate cancer DU145H cells to either HYD1 or C1 peptides differentially altered the α6 and α6p integrins as detected by Western blot analysis.

Results:

Adhesion to immobilized peptides differentially altered α6 and α6p integrins

Human prostate carcinoma cells were allowed to adhere for 18 hours to peptides HYD1, C1, HYDS or CS, in serum free IMDM containing 0.1% BSA, as described in "Materials and Methods". Cells and media were collected with a cell scraper, centrifuged and washed 2 times in HEPES buffer before being lysed in RIPA buffer containing protease inhibitors. Whole cell lysates (15 μg) were separated on a 7.5% polyacrylamide gel under non-reducing conditions, transferred to PVDF membrane followed by Western blot analysis with anti-α6 integrin antibody, AA6A (Figure 30).

In the serum control sample, the α6 and α6p integrins were detected at 160 kD and 70 kD, respectively as described previously (494). The α6 and α6p integrins were differentially altered when the DU145H cells were allowed to adhere to the active peptides, HYD1 or C1. The α6p integrin in the HYD1 and C1 samples, but not α6, was both decreased in abundance and had an altered electrophoretic mobility of 65 kD, compared to the 70 kD α6p observed in control samples. This alteration of the α6p integrin variant was not observed in the cell lysates from cells allowed to adhere to

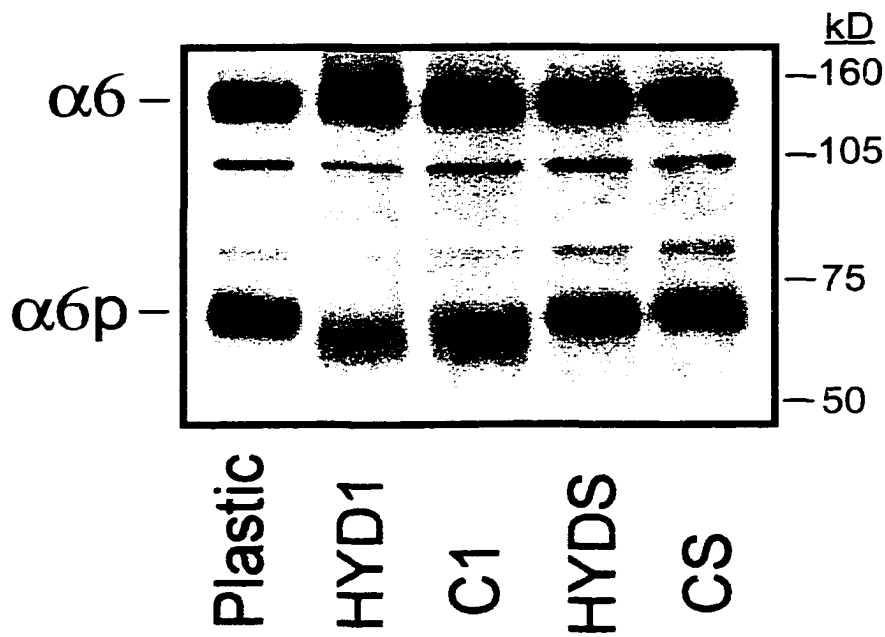

Figure 30: Adhesion to immobilized peptides differentially alter α6 and α6p integrin.

Human prostate carcinoma DU145H cells were allowed to attach for 18 hours to either active peptides (HYD1 or C1), scrambled peptides (HYDS or CS) in serum free media conditions. Cell lysates from DU145H cells grown in media containing 10% serum were used as a standard for α6 and α6p integrin migration. Total protein (15 μg) was separated in a 7.5% polyacrylamide gel under non-reducing conditions, followed by Western blot analysis using anti-α6 integrin antibody, AA6A.

scrambled peptides HYDS or CS, indicating peptide sequence was important for conferring this alteration. Comparison of the scrambled peptide samples with the untreated control sample show that there was no effect due to serum free conditions on the α6 or α6p integrins.

Alterations of the α6p integrin due to adhesion on HYD1 or C1 was reversible.

Human prostate carcinoma cells were allowed to adhere for 24 hours to HYD1 or C1 peptides in serum free media containing 0.1% BSA, as described in "Materials and Methods". To determine whether the α6p variant could recover phenotypically as judged by Western blot analysis, fetal bovine serum was added back cells which were adhered to peptide for 24 hours for a final concentration of 10%. Cells were allowed to recover for 24 hours post addition of serum. It is significant to note that addition of serum did not restore the normal cell spread phenotype. Cells and media were collected with a cell scraper, centrifuged and washed 2 times in HEPES buffer before being lysed in RIPA buffer containing protease inhibitors. Whole cell lysates (15 μg) were separated on a 7.5% polyacrylamide gel under non-reducing conditions, transferred to PVDF membrane followed by Western blot analysis with anti-α6 integrin antibody, AA6A (Figure 31).

The α6 and α6p integrins contained in lysates from DU145H cells grown in serum were detected at 160 kD and 70 kD, respectively. In total protein lysates derived from cells allowed to adhere to either HYD1 or C1 peptides, the α6p integrin variant was decreased in abundance and had an apparent molecular weight of 65 kD, compared to the 70 kD control samples. The α6 integrin was not altered. Twenty-four hours following the

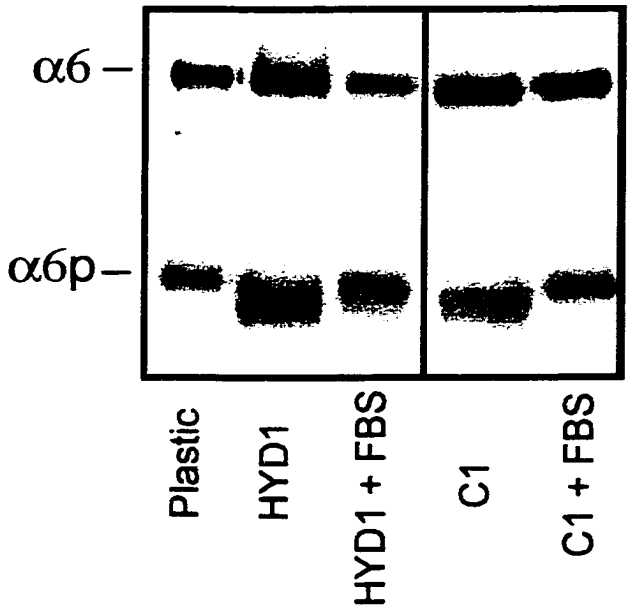

Figure 31: Alterations of the α6p integrin due to adhesion on HYD1 or C1 was reversible.

Human prostate carcinoma DU145H cells were allowed to attach for 24 hours to active peptides HYD1 or C1 in serum free media conditions. For recovery, 10% serum was added to cells on peptides in tissue culture dishes and cells were grown for another 24 hours. Cell lysates from DU145H cells grown in media containing 10% serum were used as a standard for α6 and α6p integrin migration. Total protein (15 μg) was separated in a 7.5% polyacrylamide gel under non-reducing conditions, followed by Western blot analysis using anti-α6 integrin antibody, AA6A.

addition of serum to cells grown on either HYD1 or C1, the α6p integrin was phenotypically restored to the 70 kD molecular weight as judged by Western blot analysis.

External α6 and β1 integrin epitopes were altered by cell adhesion to C1, but not HYD1.

In order to assay the external conformational epitopes, immunoprecipitations with anti-α6 and anti-β1 integrin antibodies were performed. Human prostate carcinoma cells were allowed to adhere to peptides for 18 hours. Cells and media were collected with a cell scraper, centrifuged and washed 2 times in HEPES buffer before being lysed in RIPA buffer containing protease inhibitors. Whole cell lysates (200 μg) were used for immunoprecipitation overnight with either anti-α6 integrin antibody, GoH3 (429) or anti-β1 integrin antibody, P4C10 (421). Retrieved proteins were separated on a 7.5% polyacrylamide gel under non-reducing conditions, transferred to PVDF membrane followed by Western blot analysis with anti-α6 integrin antibody, AA6A (Figure 32).

The GoH3 antibody retrieved both α6 and α6p integrins from cells allowed to adhere to HYD1, HYDS, C1, CS or biotin (Figure 32,A). In HYD1 samples, the α6p variant was observed at an altered electrophoretic mobility of 65 kD, compared to 70 kD in scrambled peptide HYDS. Interestingly in the cells which were grown on C1, the α6p integrin was observed at 70 kD, despite its apparent molecular weight of 65 kD when whole cell lysates were examined. None of the altered α6p variant (65 kD) was retrieved

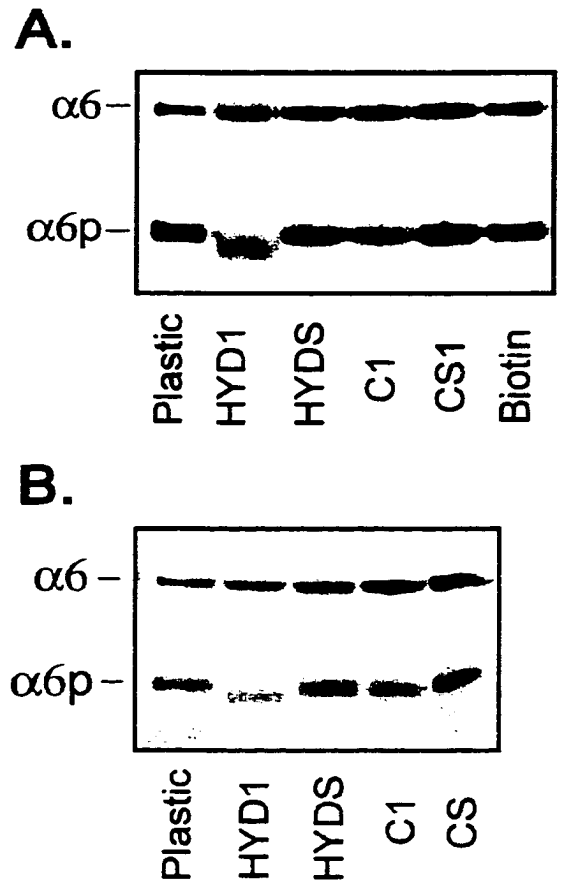

Figure 32: External α6 and β1 integrin epitopes were altered by cell adhesion to C1, but not HYD1.

Human prostate carcinoma DU145H cells were allowed to attach for 18 hours to either HYD1, C1, HYDS, CS or biotin in serum free media conditions. The α6 integrins were retrieved from cell lysates by immunoprecipitation with either anti-α6 integrin GoH3 (Panel A) or anti-β1 integrin P4C10 (Panel B) antibodies. Immunoprecipitated proteins were separated in a 7.5% polyacrylamide gel under non-reducing conditions, followed by Western blot analysis using anti-α6 integrin antibody, AA6A. For α6 and α6p standard, DU145H cells were grown in media containing 10% serum and used for immunoprecipitation with either anti-α6 or anti-β1 integrin antibodies, to monitor any altered migration of α6 and α6p integrins due to serum free conditions.

by GoH3 from cell lysates from C1 peptide samples, suggesting the ability of GoH3 to retrieve the 65 kD variant was decreased.

In chapter IV, the α6p integrin was shown to modified by N-linked glycosylation. The altered electrophoretic mobility of α6p integrin observed in cells grown on either HYD1 or C1 peptides was consistent with deglycosylation of the integrin. Because deglycosylation of integrin subunits has previously been shown to result in disruption of subunit heterodimerization (531,532), the association of α6p with β1 integrin was investigated.

Immunoprecipitation with anti-β1 integrin antibody P4C10, retrieved both α6 and α6p integrins from cells allowed to adhere to HYD1, HYDS, C1 and CS or serum (Figure 32,B). In HYD1 samples, the α6p variant was observed at the altered electrophoretic mobility of 65 kD, compared to 70 kD in scrambled peptide HYDS, suggesting that alteration of α6p by HYD1 did not disrupt the α6β1 integrin heterodimer. Interestingly, β1 integrin immunoprecipitations from the cells which were grown on C1 demonstrated a 70 kD α6p integrin, despite its apparent molecular weight of 65 kD when examined via whole cell lysates. These data suggested that the alteration induced in α6p by the C1 peptide resulted in its inability to pair with the β1 subunit and thus explaining why only the normal 70 kD α6p protein was retrieved.

Discussion:

Previously, we demonstrated that increased expression of the α6 integrin in prostate cancer was associated with an increased migratory and invasive phenotype (155,158) and that a novel, smaller variant of the α6 integrin exists was present in several prostate carcinoma cell lines (494). We have now identified synthetic peptides which differentially alter α6 and α6p integrins. Both peptides resulted in a decrease of the appparent molecular weight of the α6p integrin variant to 65 kD, but did not alter the α6 integrin, as judged by Western blot analysis (Figure 30). This phenotypic alteration of α6p was reversible after 24 hours post-addition of serum (Figure 31).

The α6 integrin has been reported to be modified post-translationally by glycosylation and phosphorylation (249,390,391). Previously, we demonstrated both the α6 and α6p integrins were modified by N-linked glycosylation (494). The previous experiments using endoglycosidase H and F demonstrated the integrins were glycosylated in the Golgi apparatus and contained mature oligosaccharide complexes. It may be suprising that small peptides which are extracellular could alter integrin post-translational modification, yet integrin biology demonstrates three amino acid peptides (RGD) are the key recognition site in many extracellular matrix proteins (533,534). The interaction between ligand and integrin plays an important role in numerous cell processes including cell adhesion, migration, proliferation, signaling, differentiation and cell survival (182-189). Thus is it not unreasonable that a small peptide signal changes in intracellular processes, such as protein processing.

Alteration of the α6p variant by adhesion to HYD1 or C1 peptides resulted in an increased electrophoretic mobility which was consistent with a deglycosylated product. Studies examining the effect of glycosylation on integrins have demonstrated deglycosylation resulted in decreased adhesion to fibronectin (531,535,536) and laminin-1 (537,538). Additionally, deglycosylation of α5β1 integrin resulted in dissociation of the heterodimer (532). Because both HYD1 and C1 have been found to inhibit integrin-mediated adhesion, if the peptides induced deglycosylation, it would be predicted that the the α6 subunit binding to β1 would be altered or disrupted.

In order to examine whether DU145H cell adhesion to either HYD1 or C1 peptides resulted in dissociation of α6β1 or α6pβ1, immunoprecipitations using anti-α6 and anti-β1 integrin antibodies were performed (Figure 32). The results indicated that alteration of α6pβ1 due to HYD1 did not affect the ability of either anti-α6 or anti-β1 integrin antibodies to retrieve α6 and α6p proteins. Interestingly, the C1 peptide-induced 65 kD α6p integrin which was observed by Western blot analysis of whole cell lysates was not observed in either the anti-α6 or anti-β1 integrin immunoprecipitations. Yet both antibodies retrieved the 70 kD α6p integrin similar to that seen in the control samples, suggesting immunoprecipitation increased the retrieval of uneffected α6p from the whole cell lysate. These data were suggestive that the C1 peptide-induced alteration of α6p, reduced its ability to be retrieved by either α6 or β1 immunoprecipitations. This may occur if the α6p 65 kD alteration induced by C1 does not go to completion in the DU145H cells.

Many questions remain as to the nature of the alteration induced by HYD1 and C1 synthetic peptides and the biological effect of such change. In summary, our data indicated significant modification of the α6p integrin variant occurred (referred to as 65 kD α6p) with both peptides which was consistent with deglycosylation. Immunoprecipitation experiments suggested that the 65 kD α6p alteration induced with both peptides differ from one another, based on the ability of GoH3 and P4C10 to retrieve the 65 kD protein from cells grown on HYD1 or C1. Furthermore, 65 kD α6p protein observed in C1 samples was not retrieved by either anti-α6 or anti-β1 integrin immunoprecipitations, suggesting that the C1 peptide resulted in altered α6pβ1 association. It would be of interest to examine whether this 65 kD α6p integrin is located on the cell surface when cells are adhered to the peptides. More research would need to be done to clarify the mechanism which results in altered electrophoretic mobility of α6p integrin.

VII. CONCLUDING STATEMENTS

Prostate cancer is a national health problem for men in the United States. The etiology of the disease is still elusive and unfortunately, our understanding of the factors which regulate clinical progression is relatively naïve. Observations of early molecular events, such as changes in the tumor microenvironment may be key to improving diagnosis and therapy of prostate carcinoma. Specifically, noted changes in integrin expression are of interest because they occur early in the progression of the disease and preceed alterations of known tumor suppressors and oncogenes which are commonly observed in other epithelial carcinomas.

Expression of the basally polarized α6β4 integrin is lost in prostate carcinoma, and there is a subsequent increase in α6 integrin pairing with the β1 subunit. Increased expression of α6β1 integrin has been correlated with increased cellular adhesion, invasion and metastatic phenotypes in prostate carcinoma models. The extracellular ligand for α6β4 integrin, laminin-5, is also lost in prostate cancer. Previously, it was not known whether this combined defect was progressive, and whether or not other epithelial carcinomas demonstrated the same defect. The data presented in this dissertation demonstrated that loss of laminin-5 and β4 integrin could be traced through progressive lesions in prostate cancer, observable in the PIN lesions. The combined defect appears to be unique to prostate when other epithelial carcinomas (e.g. breast and colon) were examined. Although the mechanism of loss of these proteins and other components of the hemidesmosome in prostate cancer is still unknown, data presented here were suggestive

that the interaction between laminin-5 and α6β4 integrin contributed to stabilization of β4 on the surface of prostate cancer cells. More research is warranted to substantiate this hypothesis.

Analysis of genetic blistering skin diseases which also show loss of hemidesmosomes, may provide insight into the mechanism of α6β4 integrin loss in prostate cancer. Numerous mutations which have been identified in α6β4 integrin and laminin-5 subunits, result in functional loss of these proteins in patients presenting with blistering skin phenotypes. Because genetic blistering diseases are rare and due to recessive alleles, it is unlikely that the same mechanism exists for prostate cancer, given the high prevalence of the disease. It remains to be determined whether novel mutations exist which could functionally inactivate the protein product from one allele, leading to loss of hemidesmosome structures in prostate cancer.

Identification of a novel variant of the α6 integrin in prostate and other normal and cancerous epithelial cells is important because the α6 integrin has been implicated in cell adhesion and metastasis. Loss of a large portion of the extracellular domain, including the putative ligand-binding domain in the N-terminal region has profound effects on the function of the receptor in its ability to associate with proteins which require extracellular epitopes on the α6 integrin. Our data indicated the α6p variant functioned as an inactive receptor and was regulated differently than the α6 integrin. Figure 33 illustrates the hypothesized role of α6p acting as an inactive receptor in the hemidesmosome.

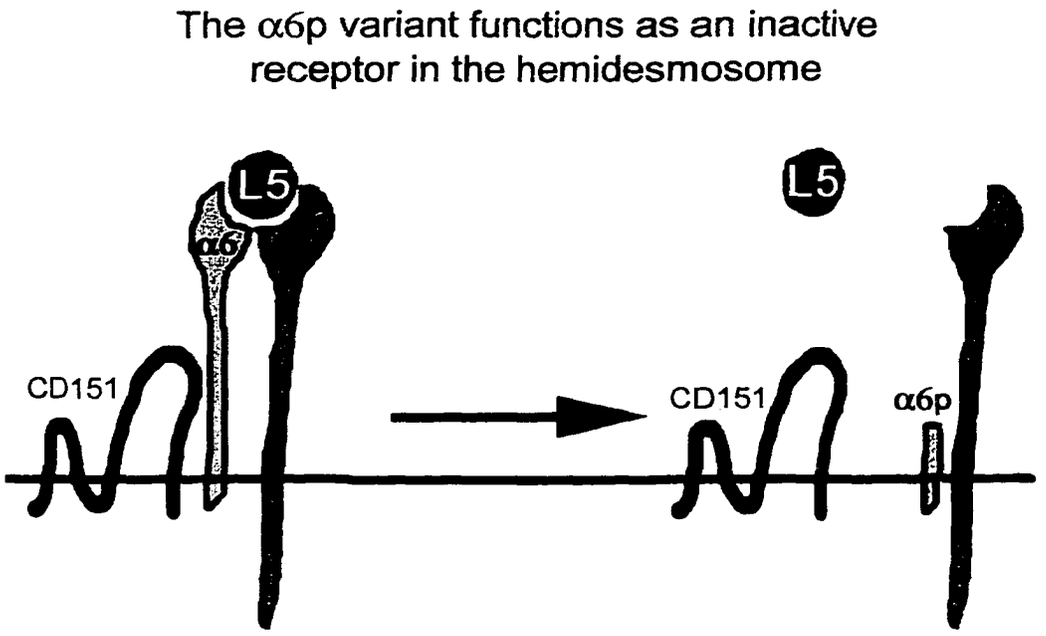

Figure 33: Schematic illustrating the inactive role of the α6p variant in the hemidesmosome.

The schematic illustrates several proteins contained within the hemidesmosome in epithelial cells; α6β4 integrin, laminin-5 and CD151. When the α6p variant is paired with the β4 subunit, co-association hemidesmosome proteins laminin-5 and CD151 with the integrin pair is absent.

Given that the α6p integrin was observed in both normal and cancer cells, the question arises as to the function of the variant in these two settings. Observations that the α6p variant protein expression increased with calcium-induced terminal differentiation - a normal, regulated event in keratinocytes, combined with the observation of the variant in cancer cells may initially be paradoxical. The simplest explanation for these seemingly contradictory observations is that the α6p integrin may provide the cell with a mechanism to disengage cell adhesion from cell signaling. In a normal differentiating keratinocyte model, cells must lose their adhesive interactions with the matrix in order to differentiate. Yet when a normal cell is deprived of its integrin-ligand interactions, it dies by anoikis. The role of the α6p variant in the differentiating keratinocyte model may be to allow differentiation to occur in the absence of adhesion. Additionally, tumor cells are known to exhibit altered adhesion and migratory status. Thus, inappropriate expression or regulation of the α6p variant in a tumor setting may facilitate survival in the absence of adhesion to the extracellular matrix, by bypassing the need for cell adhesion.

Integrin cell surface studies with cytochalasin D and immunoprecipitation studies for CD151 and laminin-5 presented in this dissertation suggest that the α6p subunit functions as an inactive receptor. Further confirmation of the role of α6p as an inactive receptor would be substantiated by determining association of secondary signaling molecules such as paxillin. Because the α6p variant would not be activated by extracellular ligand, it is unlikely that the subunit would be able to transmit signals via 'outside-in' mechanisms. It is unknown whether the α6p-containing heterodimers are

also inactive. The decreased sensitivity of cell surface α6p integrin to alteration of the actin cytoskeleton observed was suggestive that α6p was not a major component of the focal contact, despite association with β1 subunit. Access to a cell culture model system which primarily expresses α6p coupled to β1 integrin and which makes focal contacts would be ideal to further confirm the role of α6p as an inactive subunit.

Data obtained using the serine/threonine phosphatase inhibitors suggested that the α6 integrin was responsive in a signaling event, while the α6p variant was not. Changes in the phosphorylation status of integrins due to use of these inhibitors has been demonstrated for α4 and β1 integrins, which are desphosphorylated and phosphorylated, respectively. Further studies using endoglycosidase digestions would be needed to confirm the suspected phosphorylation changes observed in the α6 integrin. The suggestion that α6p was an inactive receptor would be strengthened if the phosphorylation status of the cytoplasmic tail were found to be different from that of α6 integrin, although physiological significance of α6 phosphorylation is not well understood.

As for the effect of α6p pairing with β4 integrin in the hemidesmosome, current literature suggests that the α6 integrin functions mainly to help the β4 integrin bind extracellular laminin-5. Although recently, it was demonstrated that even laminin-5 was not essential for hemidesmosome formation. Thus it would be of interest to determine whether α6pβ4 integrin heterodimers are capable of recruiting hemidesmosomal proteins to the β4 cytoplasmic tail, such as HD1/plectin, BP180 and BP230. Given the minimal

role of the α6 integrin in hemidesmosome formation, the α6p variant would not be expected to alter recruitment of these proteins by β4 integrin to the hemidesmosome. If this were the case, it would support the idea that α6p can disengage adhesion from cell signaling via α6β4 integrin.

Preliminary studies indicate synthetic peptides can specifically alter the α6p integrin, which may later provide a mechanism for studying the roles of α6p. However questions still remain as to the mechanism of α6p alteration induced by adhesion to peptides and whether or not this alteration was due to deglycosylation. Eventually, model systems such as transgenic mice may help further define the roles of the α6p integrin. One question to answer would be whether the α6p variant could rescue the α6-null mouse phenotype.

One significant question which still remains is how the α6p variant was generated. In general, α6 and α6p do not appear to be subject to transcriptional regulation. The data presented support the hypothesis that the variant arose by a post-transcriptional mechanism such as ribosome skipping. Four pieces of data suggested this. First, sequence analysis by mass spectrometry demonstrated exons 13-25 were present in the α6p variant. Computational analysis of molecular weight was reasonable for that observed by SDS-PAGE analysis. Secondly, RT/PCR only demonstrated one transcript in a cell line which produced both α6 and α6p integrin forms. Thirdly, sequence analysis demonstrated there is an alternative start site which preceeds exon 13, in addition to numerous other sites throughout the molecule. Fourthly, N-glycosylation data

demonstrated the α6p variant contained mature oligosaccharide complexes, suggesting it was post-translationally modified in the Golgi, and thus formed before reaching the cell surface. Further studies are warranted to investigate the mechanism of ribosome skipping for production of the α6p variant.

Together, the work presented in this dissertation examines changes in the α6β4 and α6β1 integrins which occur in prostate carcinoma (summarized in Figure 34). Improved understanding of the α6- and α6p-containing integrin heterodimers may help to identify new targets for improved prostate cancer diagnosis and therapy.

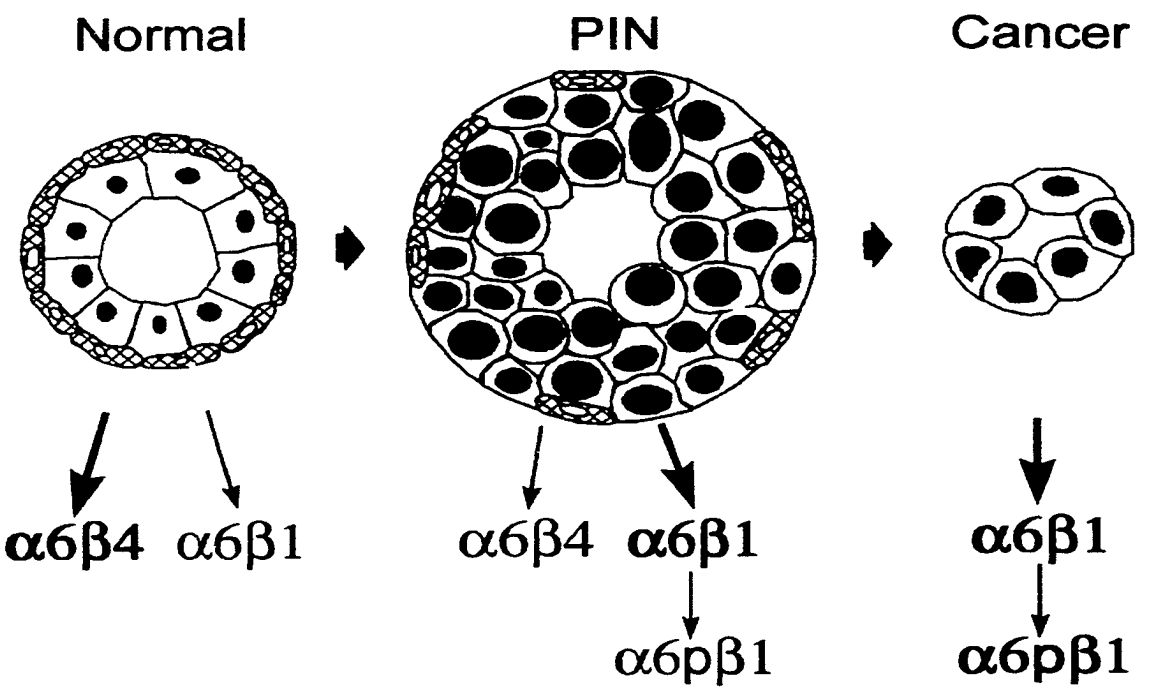

Figure 34: Summary of alterations in α6β4 and α6β1 integrins in prostate carcinoma progression.

The diagram summarizes the changes in the α6-integrin-containing heterodimers thought to occur in the progression of prostate carcinoma. Integrin pairs shown in bold are representative of the primary heterodimer observed in Normal, PIN or Cancer of the prostate.

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