The Cytoplasmic Extension of the Integrin β6 Subunit Regulates Epithelial–to–Mesenchymal Transition

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Abstract. *Prognosis for oral cancer patients has not improved in over 60 years due to invasion and recurrence. To understand the invasive behavior of this tumor, we evaluated the role of the αvβ6 integrin. Invasive oral SCC cells express the αvβ6 integrin, which contains an 11-amino-acid extension on its β-subunit unique to the integrin family. We determined that this β6 cytoplasmic extension regulates the composition of the intermediate filament network and the organization of signaling structures called focal contacts. The auto-phosphorylation of FAK, which is localized to focal contacts, was also regulated by the β6-cytoplasmic tail, as were the transcription factors Notch and STAT3. Lastly, we also determined that activation of MAPK required the full-length β6 integrin. Together these results indicate that the signaling critical to epithelial-to-mesenchymal transition (EMT) is regulated by the β6 integrin cytoplasmic domain.*

Squamous cell carcinoma (SCC) accounts for approximately 96% of all oral cancers (1). This disease has a 5-year survival rate of approximately 50%, in part, due to its invasive behavior and aggressive rate of recurrence. The extracellular matrix (ECM) surrounding invasive oral SCC is dynamic and regulates many aspects of cell behavior including survival, growth and invasion. The cytoskeleton provides mechanical strength and architectural design that is needed to establish cell shape. The cytoskeleton is made-up of three kinds of protein filaments: actin filaments, intermediate filaments, and microtubules. The composition of intermediate filaments is dynamic and subject to external stresses like the local microenvironment. During epithelial-to-mesenchymal transition (EMT), the intermediate filament composition of the cells changes from cytokeratin to vimentin (2). This sets-up the framework for tumor cell motility. Vimentin is a major cytoskeletal component of poorly-differentiated mesenchymal cells. Vimentin is used as a marker of cells undergoing EMT.

Tumor cells acquire signals from the ECM through cell surface receptors such as those of the integrin family. Integrins are transmembrane receptors that mediate the attachment between a cell and the ECM. Thus, integrin binding to the ECM connects the extracellular environment to the intracellular components such as, paxillin and vinculin through the formation of focal contacts. Focal contacts are rich in signaling kinases including Src family kinases (src, fyn, and yes) and focal adhesion kinase (FAK).

Previously, we determined that the expression of the αvβ6 integrin was one of the initial changes that occur during progression from oral dysplasia to SCC. The αvβ6 integrin is epithelial-specific and acts as a receptor for fibronectin, tenascin-C, and the latency-associated peptide of TGFβ1. αvβ6 integrin is up-regulated in several epithelial cancers and is associated with poor prognosis of the disease. The β6 subunit contains a unique 11-amino acid carboxyl terminal extension reported to induce a cell proliferative response (4). The β6 cytoplasmic domain has also been suggested to be a binding site for ERK and is associated with a variety of post-ligand binding events (4).

During EMT, epithelial tumor cells lose polarization and cell-cell contacts. The expression of the αvβ6 integrin and a variety of co-factors promote EMT (2). For example, Notch is a transcription factor than can act as either a tumor promoter or suppressor depending on the microenvironment. Snail is under the direct influence of Notch and modulates migration under hypoxic conditions (5). The end result of EMT is invasion and in this article, we demonstrate that many of the invasive properties seen in oral SCC are dependent upon the mesenchymal-promoting cytoplasmic tail of β6 integrin.

Materials and Methods

Cell culture. The SCC9 cell line was derived from a base of tongue lesion and was a generous gift from Dr. James Rheinwald (Brigham and Woman's Hospital, Harvard School of Medicine). The SCC9β6

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and SCC9CAFyn cell lines were established by stable transfection of the SCC9 cells with cDNAs for the full-length β6 and a constitutively active Fyn (CAFyn), respectively (6, 12). The SCC9β6KDFyn and SCC9β6D1 lines, were established by transducing the SCC9β6 cells with the cDNA for a kinase-dead Fyn or a truncated β6 cDNA lacking the C-terminal 11 AA, respectively (6, 13). The HSC4 cell line was a generous gift from Dr. Randall H. Kramer (University of California, San Francisco). The HSC4 cell line was retrovirally-transduced with the full-length β6 or the truncated β6 to derive the HSC4β6 and HSC4β6D1 cell lines, respectively. The Fyn cDNAs were a generous gift of Dr H. Kawakatsu (University of California, San Francisco, USA). The cDNA for the full-length β6 and the 11-amino-acid cytoplasmic deletion were generously provided by Dr. Dean Sheppard (University of California, San Francisco, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

Western Blotting. Cells were plated onto fibronectin (FN) (10 μg/ml) for pre-determined time intervals (60 min, 24 h). The cells were then lysed in Nonidet P-40 lysis buffer (1.4% Nonidet P-40, 150 mM NaCl, 0.2% SDS, 1 mM EDTA, 20 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na_3VO_4, 50 mM NaF). BCA Protein Assay Kit (Pierce, Rockford, Ill.) was used to determine protein concentration. The proteins were separated using SDS-PAGE and transferred to PVDF membrane (EMD Millipore Corp., Billerica, MA, USA) on semi-dry blotting apparatus (Bio Rad, Hercules, CA, USA). The immunoblots were visualized using the Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA) and UltraCruz Autoradiography Film (Santa Cruz Biotechnology Inc., Dallas TX, USA). Blots were quantified and assigned relative value units (RVUs) using an image analysis program (NIH image).

Immunofluorescence microscopy. A total of 2×10^5 cells/ml were plated onto fibronectin-coated glass coverslips (10 μg/ml), cultured serum–free for 24 h and then fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton® X-100. The cells were incubated with monoclonal antibody for 1 h, rinsed with phosphate-buffered saline (PBS) and then incubated with goat anti-mouse or anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) for 30 min at room temperature. Coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Cell reaction with the antibody was determined using a Nikon 80i immunofluorescence microscope.

Reporter assay. To evaluate Notch and MAPK, we used the Cancer 10 Pathway Reporter Assay kit. (Qiagen Inc., Valencia, CA, USA.). Each reporter consisted of a mixture of a specific targeted transcription factor coupled to firefly luciferase plus a constitutively-expressing Renilla luciferase construct. The targeted transcription factor for the Notch pathway was RBP-Jk. The target for phosphorylated MAPK/ERK was ELK1/SRF. Assay medium consisted of DMEM plus 0.5% FBS. The negative control is a noninducible GFP reporter and the positive control is a constitutively expressing GFP reporter. Dual-luciferase results are calculated for each transfectant. The change in the activity of each signaling pathway is determined by comparing the normalized luciferase activities of the reporter in treated versus untreated transfectants. Assay results are read in a luminometer.

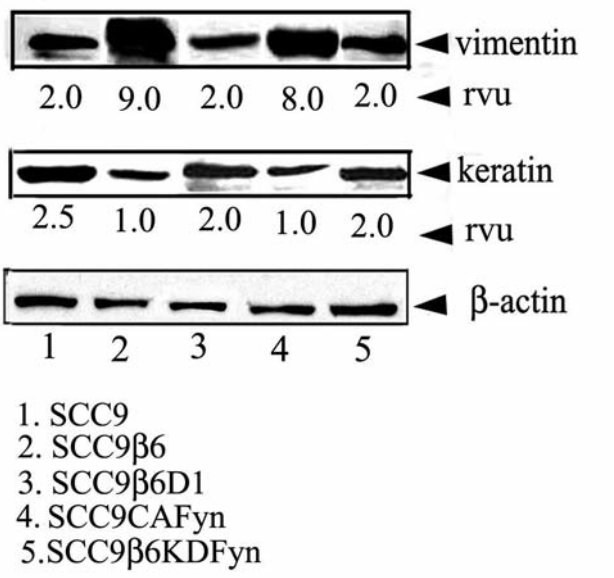

Figure 1. *Regulation of intermediate filament composition. SCC9, SCC9β6, SCCβ6D1, SCC9CAFyn, and SCC9β6KDFyn cells were grown, serum-free, on FN for 24 h, lysed and processed for western blotting using monoclonal antibodies against vimentin (upper panel) or keratin (middle panel). β-actin was used as a loading control (bottom panel).*

Results

The β6 cytoplasmic tail modulates the composition of the intermediate filaments. Normal epithelial cells are highly differentiated and rich in keratin filaments; whereas, poorly-differentiated mesenchymal cells are rich in vimentin filaments (2). We have previously shown that full-length β6 integrin is required to promote EMT. We wished to determine if the β6 integrin cytoplasmic tail modulated intermediate filament composition.

The SCC9, SCC9β6, and SCC9β6D1 cells were grown on FN for 24 h and then analyzed by western blotting (Figure 1). We also grew the SCC9CAFyn and the SCC9β6KDFyn cells on fibronectin (FN) to evaluate the role of Fyn. Western blotting showed that the SCC9β6 and the SCC9CAFyn cells expressed roughly 4-times the level of vimentin compared to the SCC9, SCC9β6D1 and SCC9β6KDFyn cell lines (Figure 1). The SCC9, SCC9β6D1 and SCC9β6KDFyn cells expressed twice the level of keratin compared to the SCC9β6 and the SCC9CAFyn cells (Figure 1). Immunofluorescence confirmed these results (data not shown). These results indicate that the cytoplasmic domain of β6, which activates Fyn, modulates the composition of the intermediate filaments thus regulating EMT.

Truncation of the β6 cytoplasmic domain promotes focal contact formation. Focal contacts are specialized structures

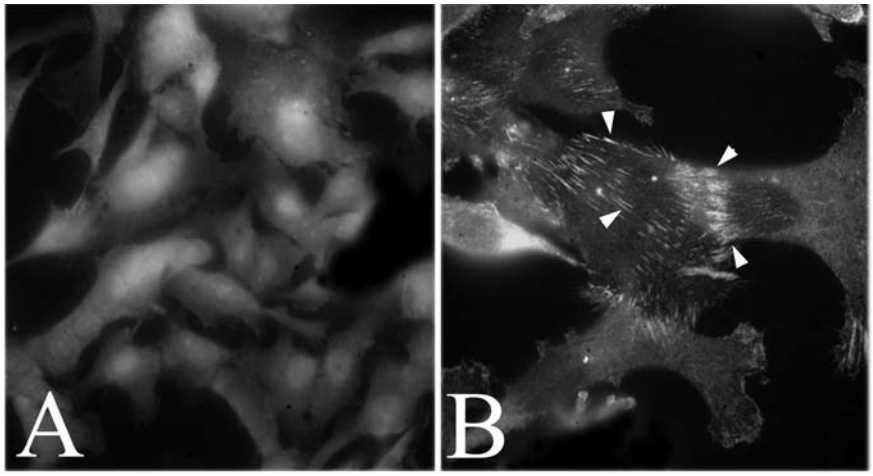

Figure 2. *Deletion of the β6 cytoplasmic tail promotes focal contact formation. SCC9β6 (panel A) and SCC9β6D1 cells (panel B) were grown on FN for 24 h serum-free and then processed for immunofluorescence using monoclonal antibodies against paxillin (arrows denote focal contact formation).*

in which the ECM, the integrin, and the cytoskeleton come together. Specific structural and signaling proteins, such as FAK, Src and the β6 integrin are concentrated at focal contacts (6). To determine whether the β6 cytoplasmic domain contributes to the formation of focal contacts, the SCC9β6 and the SCC9β6D1 cells were grown on FN for 24 h. The cells were processed for immunofluorescence and paxillin, a marker of focal contacts, was immuno-localized using monoclonal antibodies. Focal contact expression was lightly detected in the SCC9β6 cell line (Figure 2A). In contrast, the SCC9β6D1 cells (Figure 2B) formed large, prominent, paxillin-positive focal contacts. These results indicate that the cytoplasmic tail of the β6 integrin suppresses the formation of focal contacts thus altering intracellular signaling.

Phosphorylation of FAK 397 is suppressed by the truncation of the β6 cytoplasmic tail. Integrin-based cell signaling occurs through the activation of FAK, which is localized to focal contacts. FAK is a non-receptor protein tyrosine kinase that becomes tyrosine phosphorylated and activated by integrin adhesion to various matrix proteins.

To expand our study we used the poorly-invasive oral SCC cell line HSC4. The HSC4 and SCC9 cells were transfected with either full-length β6 cDNA or the truncated β6D1 subunit to generate the HSC4β6, SCC9β6 cells or the HSC4β6D1 and SCC9β6D1 cell lines, respectively. All cell lines were serum-starved for 24 h prior to use. The cells were allowed to attach to FN for 60 minutes then lysed, and evaluated for phosphorylation of Tyr^{397} by western blotting (Figure 3). Tyr^{397} auto-phosphorylation was suppressed by 50% when SCC9β6D1 and HSC4β6D1 cells were compared to SCC9β6 and HSC4β6 cell lines. Our results demonstrate

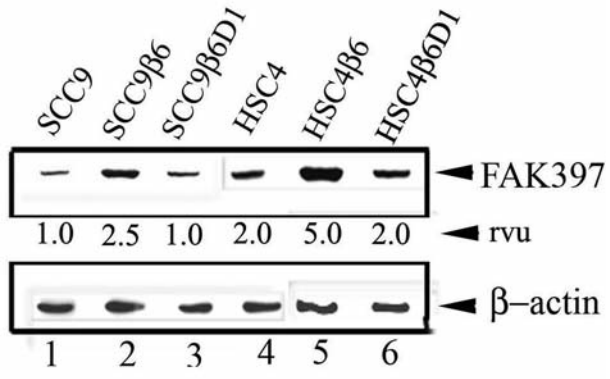

Figure 3. *Autophosphorylation of FAK^{397} is modulated by the β6 integrin. SCC9, SCC9β6, SCC9β6D1, HSC4, HSC4β6 and HSC4β6D1 cells were plated onto FN serum-free for 24 h. Cells were then processed for western blotting using monoclonal phosphor-antibodies against FAK^{397} (upper panel); β-actin was used as a loading control (lower panel).*

that the full-length β6 cytoplasmic domain is required for the full auto-phosphorylation of Tyr^{397} to occur.

The cytoplasmic aspect of the β6 integrin regulates the Notch pathway. Notch signaling is an important contributor to EMT (7). EMT is orchestrated by interactions between extracellular molecules, intracellular proteins and the cell membrane receptors. The cells were serum starved for 24 h prior to use. To evaluate Notch expression, we examined the expression of the target transcription factor RBP-Jk using the Cancer 10-pathway Reporter Array. All three cell lines were transfected with either the RBP-Jκ reporter, negative control

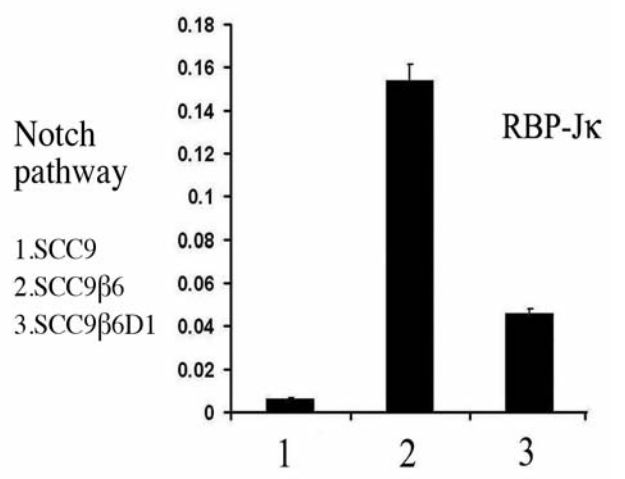

Figure 4. *Expression of Notch is regulated by the β6 integrin cytoplasmic domain. To evaluate Notch expression, we used the Cancer 10-pathway Reporter Array (SABiosciences). The cells were transfected with RBP-Jκ reporter, negative control and positive control.*

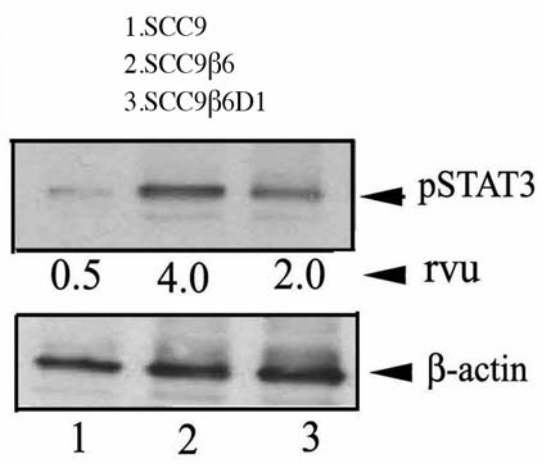

Figure 5. *Phosphorylation of STAT3 is modulated by the cytoplasmic domain of β6 integrin. The Cancer 10-pathway Reporter Array (SABiosciences) was used to evaluate phosphorylation of STAT3. Cells were transfected with Elk-1/SRF reporter, negative control and positive control.*

or the positive control. The SCC9, SCC9β6 and SCC9β6D1 cell lines were grown on FN for 60 min in 0.5% FBS, and processed for immunoassay/ELISA.

The expression of RBP-Jk was 14 fold greater in the SCC9β6 cells when compared to the SCC9 cells (Figure 4). The truncation of the β6 cytoplasmic tail decreased RBP-Jk expression by 75% thereby also manipulating the Notch pathway. These results demonstrated that the Notch pathway is under the control of signals provided by the β6 cytoplasmic domain.

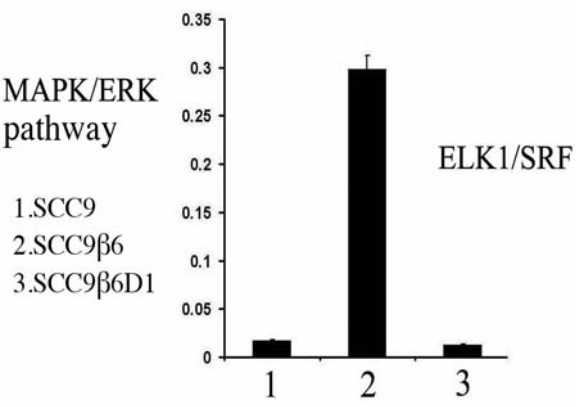

Figure 6. *Phosphorylation of MAPK is regulated by β6 integrin. The Cancer 10-pathway Reporter Array (SABiosciences) was used to evaluate for MAPK activation. Cells were transfected with Elk-1/SRF reporter, negative control and positive control.*

pSTAT expression regulated by the β6 integrin. Signal transducers and activators of transcription (STAT3) are a family of transcription factors that regulate cell growth and differentiation. These transcription factors promote the oncogenesis of some epithelial cancers such as breast cancer (8).

The αvβ6 integrin regulates expression of EGFR thus activating the Ras/MAPK and STAT3 pathways (9). The cells were serum-starved for 24 h and then plated on FN for 60 min. The cells were harvested, lysed, and evaluated by western blotting. Phosphorylated STAT3 was identified in the SCC9β6 cells and was decreased by 50% when the truncated β6 was expressed (Figure 5). Phosphorylated STAT was not detected in the SCC9 cell line, suggesting that the full-length β6 integrin is required for activation of STAT.

The cytoplasmic domain of β6 regulates MAPK/ERK. Mitogen-activated protein (MAP) kinases are a family of ubiquitous proline-directed, protein-serine/threonine kinases, which participate in signal transduction pathways that control intracellular events including acute hormone responses and changes occurring in development. To evaluate the MAPK/ERK pathway, we examined the expression of the specific target transcription factor ELK1-SRF using the Cancer 10-pathway Reporter Array. ELK1 complexes with SRF over SRE and activates gene expression. ELK1 is phosphorylated by MAPK resulting in activation of target genes. All three cell lines were transfected with either the ELK1/SRF reporter, negative control or the positive control. The SCC9, SCC9β6 and SCC9β6D1 cell lines were grown on FN for 60 min in 0.5% FBS, and processed for immunoassay/ELISA. Truncation of the β6 integrin completely suppressed expression of the ELK-1/SRF reporter indicating that the complete β6 integrin is required for expression of MAPK (Figure 6).

Discussion

Recurrence and metastasis are the major factors contributing to oral cancer's poor prognosis. Epithelial tumor cells must acquire a flexible and migratory phenotype in order to disseminate from the primary tumor and invade neighboring tissues or vessels. For example, in colorectal cancer the expression of αvβ6 in the tumor worsens the prognosis (10). We previously demonstrated that the expression of αvβ6 integrin promotes EMT. We suggest that the β6 cytoplasmic extension stabilizes the poorly-differentiated state.

The integrin β6 subunit cytoplasmic domain is necessary for initiation of cell spreading and motility and the suppression of focal contacts. The cytoplasmic domain contains binding sites for paxillin, FAK, and c-Src suggesting that cell signaling can be orchestrated *via* the 11 amino acid extension (11).

Integrin-based cell signaling occurs through the activation of FAK, which is localized to focal contacts. FAK is a non-receptor protein tyrosine kinase (NRPTK) that becomes tyrosine phosphorylated and activated by integrin adhesion to various matrix proteins, including FN. Activation of FAK signaling by integrin clustering leads to auto-phosphorylation at Tyr^{397}, which is a binding site for Src family kinases PI3K and PLCγ (12-15). This a major signaling site in the FAK molecule. Recruitment of Src family kinases results in the further phosphorylation of FAK at tyrosine residues located in the catalytic ($Tyr^{407, 576, 577}$) and carboxyl-terminal region of FAK ($Tyr^{871, 925}$) (16, 17). FAK plays an important role in the control of several biological processes, including cell spreading, migration, and survival. We now show that truncation of the β6 cytoplasmic domain promotes focal contact formation which suppresses the Src/FAK^{397} pathway.

The Notch signaling pathway has been shown to be an important contributor to EMT (7). Although Notch can serve as a tumor suppressor in most cases, its activation is primarily oncogenic (18). In oral SCC, we suggest that Notch acts as a tumor promoter. Using the RBP-Jκ reporter system we determined that the Notch pathway is inhibited by 75% when the β6 subunit is truncated.

EMT occurs through a multiplicity of actions and some of them are under the influence of transcription factors such as Signal Transducers and Activators of Transcription (STATs). This family appears to be important for the continued progression and oncogenesis of epithelial cancers in head and neck carcinogenesis through its role in blocking apoptosis.

Phosphorylation of MAPK (42/44 kDa) is requisite for successful EMT. The activation of MAPK is significantly reduced by the truncation of the β6 cytoplasmic unit. This identifies the β6 integrin as a regulator of EMT. αvβ6 ligand binding is essential for generating the initial signaling events surrounding EMT. The conformational changes associated with the 11-amino-acid extension recruits distinct signaling molecules that physically result in activation of multiple signaling pathways.

Our results indicate that the β6 integrin cytoplasmic tail regulates the mesenchymal phenotype associated with EMT. The β6 C- terminal 11 amino acids may prove to be a future therapeutic target for oral cancer and other epithelial cancers expressing αvβ6.

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