

Urinary-type Plasminogen Activator (uPA) Expression and uPA Receptor Localization Are Regulated by $\alpha_3\beta_1$ Integrin in Oral Keratinocytes*

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Expression of urinary-type plasminogen activator (uPA) and its receptor (uPAR) is correlated with matrix proteolysis, cell adhesion, motility, and invasion. To evaluate the functional link between adhesion and proteolysis in gingival keratinocytes (pp126), cells were treated with immobilized integrin antibodies to induce integrin clustering. Clustering of α_3 and β_1 integrin subunits, but not α_2 , α_5 , α_6 , or β_4 , enhanced uPA secretion. Bead-immobilized laminin-5 and collagen I, two major $\alpha_3\beta_1$ ligands, also induced uPA expression. Coordinate regulation of the serpin plasminogen activator inhibitor 1 was also apparent; however, a net increase in uPA activity was predominant. $\alpha_3\beta_1$ integrin clustering induced extracellular signal-regulated kinase 1/2 phosphorylation, and both uPA induction and extracellular signal-regulated kinase activation were blocked by the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor PD98059. Integrin aggregation also promoted a dramatic redistribution of uPAR on the cell surface to sites of clustered $\alpha_3\beta_1$ integrins. Co-immunoprecipitation of β_1 integrin with uPAR provided further evidence that protein-protein interactions between uPAR and β_1 integrin control uPAR distribution. As a functional consequence of uPA up-regulation and uPA-mediated plasminogen activation, the globular domain of the laminin-5 α_3 subunit, a major pp126 matrix protein, was proteolytically processed from a 190-kDa form to a 160-kDa species. Laminin-5 containing the 160-kDa α_3 subunit efficiently nucleates hemidesmosome formation and reduces cell motility. Together, these data suggest that multivalent aggregation of the $\alpha_3\beta_1$ integrin regulates proteinase expression, matrix proteolysis, and subsequent cellular behavior.

Urinary-type plasminogen activator (uPA)¹ (urokinase) is a

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¹ The abbreviations used are: uPA, urinary-type plasminogen activator; BPE, bovine pituitary extract; BSA, bovine serum albumin; ECM, extracellular matrix; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-acti-

serine proteinase that functions in conversion of the circulating zymogen plasminogen to the active, broad-spectrum serine proteinase plasmin (reviewed in Ref. 1). uPA is secreted by numerous cell types, and up-regulation of uPA expression has been correlated with malignant progression of a wide variety of neoplasms (1). The biological activity of uPA is regulated post-translationally by a functional interplay between the proteinase, its receptor (uPA receptor (uPAR)), and the serpin plasminogen activator inhibitor 1 (PAI-1). uPAR is tethered in the cell membrane by a glycosylphosphatidylinositol moiety and has been postulated to play a critical role in the initiation of extracellular matrix proteolysis by spatially concentrating uPA at the cell-matrix interface. Receptor-bound uPA (designated uPA/R) catalyzes plasmin formation and is also inhibited by PAI-1, leading to endocytosis of uPAR/uPA/PAI-1 complexes (1).

Recent studies have demonstrated that the glycosylphosphatidylinositol-anchored uPAR can form lateral associations with transmembrane integrins (2–5). Integrins are $\alpha\beta$ heterodimeric proteins that function in cell-matrix adhesion and participate in diverse biological processes, including migration, proliferation, differentiation, and apoptosis (6, 7). Because integrin cytoplasmic domains can couple to both cytoskeletal and signaling proteins, integrin engagement can control a hierarchy of subcellular events based on the physical nature of the specific ligand-receptor interaction. Integrin binding by an ECM ligand can result in receptor occupancy, aggregation, or both, thus functionally coupling the extracellular environment to the actin-based cytoskeleton and to specific signal transduction pathways that modulate distinct cellular responses (8–10). Furthermore, recent studies report that uPAR ligation may result in modulation of integrin signaling, suggesting a potential mechanism whereby uPA/R may participate in regulation of cell cycle progression and cell motility (7, 11, 12).

Integrin engagement has been shown to regulate expression of numerous gene products (6, 7); however, the contribution of integrins to modulation of uPA/R expression is inconclusive. In the current study, we evaluated the functional link between cell-matrix adhesion and proteolysis in premalignant oral keratinocytes. We report that aggregation of $\alpha_3\beta_1$ integrins exerts multifunctional control on the uPA system by inducing expression of uPA and PAI-1 as well as regulating the membrane localization of uPAR. Integrin-induced uPA expression requires extracellular signal-regulated kinase 1/2 (ERK1/2) activation and is blocked by treatment with a MEK inhibitor. As a

vated protein kinase/extracellular signal-regulated kinase kinase; PAI, serpin plasminogen activator inhibitor; PBS, phosphate-buffered saline; uPAR, uPA receptor; uPA/R, receptor-bound uPA.

functional consequence of integrin-induced activation of the uPA/plasmin system, limited proteolytic modification of endogenous matrix-associated laminin-5 was observed, providing support for the hypothesis that integrin-mediated adhesion regulates matrix proteolysis and subsequent cellular behavior.

EXPERIMENTAL PROCEDURES

Materials—Gelatin, BSA, type I collagen, type IV collagen, fibronectin, laminin-1, aprotinin, cycloheximide, herbimycin, actinomycin D, *N*-hydroxy-succinimidobiotin, *D*-Val-Leu-Lys-*p*-nitroanilide, peroxidase conjugates of anti-mouse IgG and anti-goat IgG, and fluorescein isothiocyanate-conjugated anti-mouse IgG were purchased from Sigma. Laminin-5 was generously provided by Desmos (San Diego, CA). Plasminogen and plasmin were purified by affinity chromatography from outdated human plasma as described previously (18). Anti-human integrin β_1 (clone P4C10), α_5 (P1D6), and β_4 (3E1) monoclonal antibodies were products of Life Technologies, Inc. Antibodies against α_2 (MAB1988) and α_3 integrin (MAB2056) were obtained from Chemicon (Temecula, CA), and anti- α_6 (GOH3) was from Coulter. Laminin-5 subunit-specific monoclonal antibodies were derived as described previously (13). Antibody EM11 recognizes an epitope present in both processed (160 kDa) and unprocessed (190 kDa) human laminin-5 α_3 subunit, whereas antibody 12C4 is directed against the α_3 G5 subdomain and thus detects only the intact 190-kDa α subunit. Affinity-purified polyclonal antibody specific for phosphorylated p42/p44 mitogen-activated protein kinase (anti-ACTIVER[®] MAPK p42/p44) was purchased from Promega (Madison, WI). Anti-ERK1/2 (anti-p42/p44), which recognizes both phosphorylated and nonphosphorylated p42/p44, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The MEK1 inhibitor PD98059, which selectively inhibits the MAPK cascade, was purchased from New England Biolabs (Beverly, MA). The tyrosine kinase inhibitors genistein and herbimycin were purchased from Calbiochem (Cambridge, MA). uPA, uPAR, and PAI-1 ELISA kits, as well as anti-human uPAR antibodies (3936, 3937, and 399R), anti-PAI-1 (395G), and anti-catalytic uPA antibodies (394), were obtained from American Diagnostica (Greenwich, CT). Hydrobond-P polyvinylidene difluoride membrane and SuperSignal enhanced chemiluminescence reagents were obtained from Amersham Pharmacia Biotech and Pierce, respectively.

Cell Culture—Premalignant oral keratinocytes (pp126 cells) were a gift from Dr. D. Oda (University of Washington, Seattle, WA) (14). pp126 cells were obtained by immortalization of normal human gingival keratinocytes with HPV16 DNA. These cells do not stratify in liquid-air interphase organotypic culture models; however, they display normal keratin synthesis and some degree of differentiation and are representative of a premalignant transformation to oral squamous cell carcinoma (15). Cells were maintained in Keratinocyte-SFM (Life Technologies, Inc.) supplemented with 20 mM *L*-glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin, 5 ng/ml EGF, and 50 μ g/ml bovine pituitary extract (BPE) (14). Prior to treatments, cell monolayers were released from culture flasks by the addition of trypsin/EDTA, seeded at a constant density of 0.7×10^5 cells/well into 24-well tissue culture plates, and allowed to attach overnight in the medium described above. Cells were then washed twice with PBS, incubated for 1 h in medium lacking BPE and EGF, and supplemented with fresh BPE/EGF-free medium prior to treatment with soluble antibodies (5–10 μ g/ml), antibody-conjugated latex beads (8–10 μ g/ml), or protein-conjugated latex beads as indicated below. Following incubation, conditioned media were collected for uPA activity determination, ELISA analyses, and Western blotting as described below. Cell lysates were prepared using 50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS. Total protein concentration of lysates was analyzed using a bicinchoninic acid protein detection kit (Sigma). In some experiments, actinomycin D, herbimycin, genistein (in Me_2SO), or cycloheximide (in culture medium) was added to culture wells 30 min prior to the introduction of integrin antibodies. Cells were found to be >95% viable by exclusion of trypan blue at the highest concentrations of all the inhibitors.

Adhesion Assays—Adhesive preferences of pp126 cells were analyzed by evaluating cellular adhesion to ECM-coated 24-well culture plates as described previously (16). Briefly, plates were coated by passive adsorption with the ECM proteins collagen I, collagen IV, fibronectin, laminin-1, laminin-5, or bovine serum albumin (as control), washed with PBS, and blocked by incubating the plates at 37 °C with minimum Eagle's medium containing 3% BSA immediately before use. pp126 cells (10^5) were seeded in BPE/EGF-free medium and allowed to adhere for 45 min at 37 °C. After washing, bound cells were fixed with 50% methanol/50% acetone (–20 °C) and enumerated with an ocular mi-

croscoper by counting a minimum of 10 high powered fields. Adhesion assays were performed in duplicate and repeated three times.

Preparation of Latex Bead-immobilized Antibodies and Proteins—Anti-integrin subunit-specific monoclonal antibodies or control isotype-matched IgGs were passively adsorbed onto 2.97- μ m latex beads (Sigma) as described (9) with the following modifications. A 1% (final concentration) suspension of latex beads was incubated in 50 mM 4-morpholineethanesulfonic acid buffer (pH 6.1) with 75 μ g/ml of the appropriate antibody overnight at 4 °C with gentle agitation (17). Antibody-conjugated beads were blocked with 10 mg/ml BSA for 90 min at room temperature, centrifuged for 3 min at 3000 rpm, and washed twice by resuspension in 2 volumes of culture medium. Bead-immobilized antibodies were resuspended in BPE/EGF-free medium at a final concentration of 1% by volume. Determination of total protein concentration in unblocked bead suspensions using a bicinchoninic acid detection kit (Sigma) indicated that 60–70% of immunoglobulins were adsorbed, resulting in a final concentration of 8–10 μ g/ml antibody beads in culture wells. The same protocol was used to generate collagen I-, laminin-1-, or laminin-5-conjugated beads.

Analysis of uPA, uPAR, and PAI-1—Net plasminogen activator activity in conditioned media was quantified using a coupled assay to monitor plasminogen activation and the resulting plasmin hydrolysis of a colorimetric substrate (*D*-Val-Leu-Lys-*p*-nitroanilide) as described previously (18). Control reactions contained 10 μ g/ml of the anti-catalytic uPA antibody 398 (American Diagnostica). Levels of uPA, uPAR, and PAI-1 in conditioned media and cell lysates were quantified by ELISA (American Diagnostica) according to the manufacturer's specifications. Statistical analyses were performed using Graph Pad Prism. Qualitative analysis of PAI-1 antigen levels was provided by Western blotting. Briefly, samples were electrophoresed on 9% SDS-polyacrylamide gels (19), electroblotted to polyvinylidene difluoride membranes (20), and blocked overnight at 4 °C with Tris-buffered saline containing 3% BSA and 0.1% Tween 20 (TBST). Blots were probed with anti-human PAI-1 antibody (1:2000, 2 h), washed (six times, 10 min each) with TBST, and incubated with peroxidase-conjugated secondary IgG (1:10,000) for 1 h at room temperature. After washing, immunoreactive bands were visualized using enhanced chemiluminescence detection.

Immunocytochemical Staining—Untreated cells were plated on 22-mm² glass coverslips placed into six-well tissue culture plates (1.5×10^5 cells/well), cultured overnight, washed with PBS, and treated with antibody-conjugated latex beads (α_3 , β_1 , or IgG beads) in BPE/EGF-free medium for 4 h. Coverslips were then gently washed with PBS, and cells were fixed for 15 min at room temperature with 3.7% formaldehyde. Coverslips were blocked for 45 min with PBS containing 1% BSA, followed by the addition of biotinylated anti-human uPAR monoclonal antibody 3936 (1:200, 1 h, room temperature). After washing (three times, 10 min each with PBS containing 1% BSA), streptavidin-fluorescein isothiocyanate solution was added, and coverslips were gently agitated for 1 h at room temperature in the dark and washed as described above. Control coverslips included cells treated with α_3 beads followed by streptavidin-fluorescein isothiocyanate in the absence of biotinylated anti-uPAR. Mounted coverslips were viewed using a Zeiss Confocal LSM510 microscope.

Immunoprecipitation—pp126 cells were cultured in 24-well plates coated by passive adsorption with collagen I, fibronectin, or BSA (16) for 24 h prior to lysis in (25 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM MgCl_2 , 1% Brij, 200 kallikrein inhibitory units/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride) as described (21). Lysates (500 μ g) were incubated with anti-uPAR antibody (5 μ g, antibody 3937, American Diagnostica) in a total volume of 1 ml at 4 °C overnight. Immune complexes were precipitated by the addition of 30 μ l of a 50% slurry of protein G beads (Sigma) for 2 h at 4 °C followed by centrifugation at 2500 rpm. Pelleted protein G complexes were washed five times in cold lysis buffer, resuspended in 35 μ l of Laemmli sample dilution buffer containing β -mercaptoethanol, and boiled. Samples were electrophoresed on duplicate 9% SDS-polyacrylamide gels and electroblotted to polyvinylidene difluoride membranes as described above. Blots were probed with either biotinylated anti-uPAR (1:200 dilution, antibody 399R, American Diagnostica) followed by streptavidin-conjugated peroxidase or with anti- β_1 integrin monoclonal antibodies (1:1000 dilution, P4C10) followed by peroxidase-conjugated goat-(anti-mouse) secondary antibodies (1:5000). Blots were developed using enhanced chemiluminescence detection.

ERK (Mitogen-activated Protein Kinase) Activation Assays—To evaluate ERK (mitogen-activated protein kinase) activation, cells (0.7×10^5) were cultured overnight in serum-free medium followed by treatment with IgG, α_3 , or β_1 beads as described above. At varying time points, cells were lysed with RIPA buffer including 1 mM sodium or-

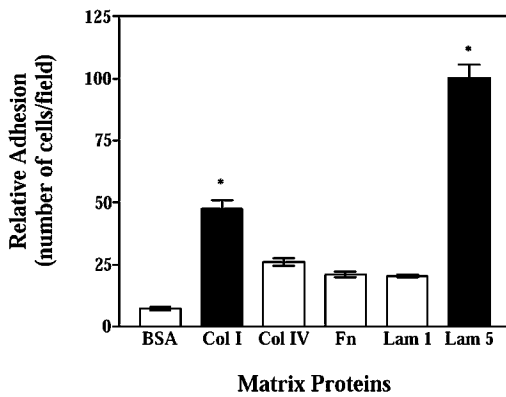


FIG. 1. **Analysis of pp126 adhesive profiles.** Wells were coated by passive adsorption with BSA, collagen I, collagen IV, fibronectin, laminin-1, or laminin-5. Cells (1×10^5) were added to wells for 45 min at 37 °C. After washing to remove nonadherent cells, bound cells were fixed and enumerated using an ocular micrometer by counting 10 high power fields. Data represent the mean and S.D. from triplicate experiments (*, $p < 0.05$ relative to BSA).

thovanadate (21) and extracted on ice for 20 min. The lysates were centrifuged, protein concentration determined using the Bio-Rad protein assay kit, and equal amounts of cellular protein (20 μ g) were electrophoresed on SDS-polyacrylamide gels and electroblotted to Immobilon. Blots were probed with anti-ERK1/2 antibody (1:1000) to detect total ERK1/2 expression or with anti-ACTIVE-MAPK p42/p44 (1:5000) to detect the phosphorylated, active forms of ERK. In control experiments, ERK activation was blocked using the MEK inhibitor PD98059 (2 μ M), and both ERK activation and β_1 bead-stimulated uPA expression were evaluated as described above.

Evaluation of pp126 Laminin-5 Matrix—The major protein component of the pp126 matrix is the heterotrimeric protein laminin-5 (13). The integrity of the laminin-5 α_3 subunit in pp126 matrix following treatment of cells with α_3 or β_1 integrin-conjugated latex beads was evaluated by Western blotting of extracted matrix proteins as described previously (13). Briefly, cells were cultured for 48 h to permit matrix accumulation and then treated with anti- α_3 or β_1 integrin beads (to induce uPA expression) or control IgG beads for 20 h. Plasminogen (0.02 μ M) was added to the culture medium for 30 min, and matrices were solubilized in 10 mM Tris-HCl, pH 6.8, containing 8 M urea, 1% SDS, and 15% β -mercaptoethanol (22). Samples were electrophoresed on 6% SDS-polyacrylamide gels, electroblotted to polyvinylidene difluoride membranes as described above, and probed with laminin-5 α_3 subunit-specific monoclonal antibodies EM11 (1:500) or 12C4 (1:1000). Blots were then incubated with peroxidase-conjugated goat anti-mouse IgG and developed using enhanced chemiluminescence detection.

RESULTS

Cellular Adhesion and β_1 Integrin Expression—To identify the integrins that mediate binding of pp126 cells to ECM proteins, adhesive profiles were evaluated. Preferential adhesion to laminin-5 and interstitial type I collagen was observed (Fig. 1), implicating β_1 integrins in cell-matrix binding. Adhesion to laminin-5 and collagen I was efficiently blocked by an inhibitory β_1 integrin antibody (10 μ g/ml), and both immunocytochemical staining and Western blotting of cell membrane extracts confirmed the expression of β_1 integrins (data not shown). Integrin binding by an ECM ligand can induce distinct cellular events based on the physical nature of the specific ligand-receptor interaction. To mimic cellular interaction with a three-dimensional matrix and promote multivalent integrin aggregation, cells were treated with β_1 integrin subunit-specific monoclonal antibodies immobilized on latex beads (designated β_1 beads). β_1 beads bound avidly to pp126 cells (Fig. 2, B and D) relative to the loosely adherent IgG-coated control beads (Fig. 2, A and C). To confirm that β_1 beads bound to β_1 integrins, bead-treated cells were lysed with modified RIPA buffer, and beads were precipitated by centrifugation. Western blot analysis of proteins co-precipitating with β_1 beads demonstrated the presence of β_1 integrins (data not shown).

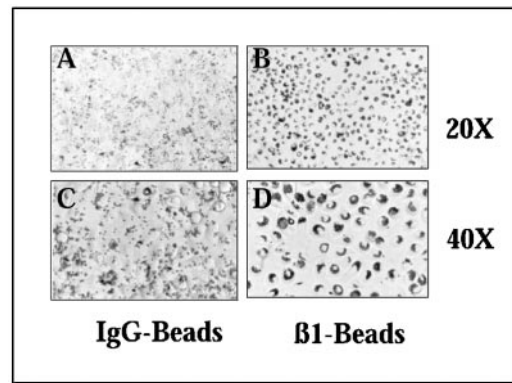


FIG. 2. **Binding of β_1 integrin antibody-conjugated beads to pp126 cells.** Latex beads were coated with control IgG (*IgG beads*) (A and C) or β_1 integrin antibody clone P4C10 (*β_1 beads*) (B and D). Beads (8–10 μ g/ml) were added to pp126 cells (10^5 /well) in 25-well culture plates and allowed to attach for 2 h prior to examination by phase contrast microscopy at the indicated magnifications.

Effect of Integrin Aggregation on uPA and PAI-1 Expression—To determine whether β_1 integrin aggregation regulates proteinase expression, cells were incubated with β_1 or control IgG beads for 12–48 h. Conditioned medium was evaluated for uPA expression by ELISA and uPA activity using a coupled colorimetric plasminogen activation assay. A significant increase in both uPA protein and activity was observed in the conditioned medium of β_1 bead-treated pp126 cells (Fig. 3, *solid bars*) relative to IgG beads (Fig. 3, *hatched bars*) or untreated cells (Fig. 3, *open bars*). In control experiments, activity was completely blocked by preincubating with a uPA-specific anti-catalytic antibody (Fig. 3), confirming that the induced protease was uPA. Induction of uPA was detectable as early as 6 h after treatment with β_1 beads and was maximal at 24–36 h. To determine whether multivalent aggregation of β_1 integrins is required to induce uPA expression, pp126 cells were treated with soluble antibodies to promote divalent integrin engagement. No significant change in uPA activity was observed (Table I), suggesting that β_1 integrin aggregation, rather than simple integrin occupation, is necessary for uPA induction.

β_1 integrins can pair with numerous α subunits in oral keratinocytes to facilitate binding to diverse ECM proteins (23–25). To assess the effect of α subunit aggregation on uPA activity, latex beads were coated with antibodies directed against the α_2 , α_3 , α_5 , or α_6 integrin subunits and incubated with pp126 cells as described above, and the conditioned medium was analyzed for uPA activity. In control experiments, cells were incubated with either β_1 or β_4 beads. Although all α subunit beads bound to pp126 cells, only α_3 and β_1 beads enhanced uPA activity, whereas the remaining samples were indistinct from IgG controls (Table II), suggesting that the $\alpha_3\beta_1$ integrin regulates uPA expression in pp126 cells.

As modulation of PAI-1 levels may alter uPA function (1), the effect of integrin clustering on PAI-1 expression was evaluated by Western blotting (Fig. 4A) and ELISA (Fig. 4B). Similar to results observed with uPA, aggregation of either α_3 or β_1 integrin resulted in increased PAI-1 expression. However, as shown in Fig. 3, a net increase in uPA activity was apparent, indicative of an overall imbalance in the uPA:PAI-1 ratio in favor of uPA.

The Effect of β_1 Integrin Aggregation on uPAR Expression and Distribution—Because uPAR binding is a primary mechanism for posttranslational control of uPA activity (1), the effect of β_1 integrin aggregation on uPAR expression was also evaluated. ELISA of cell membrane extracts indicated that uPAR expression was unaffected by β_1 bead treatment, suggesting a lack of coordinate regulation between uPA and uPAR

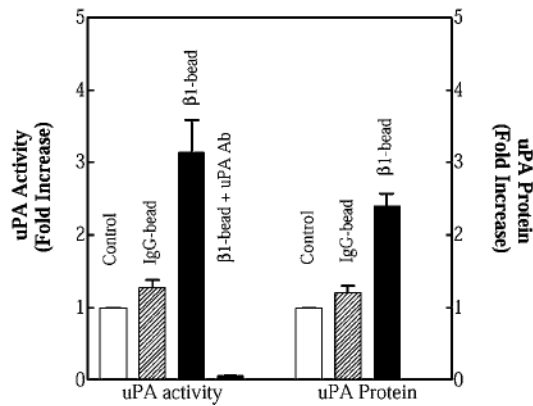


FIG. 3. β_1 integrin regulation of uPA expression. pp126 cells (0.7×10^5) were cultured in 24-well tissue culture plates and left untreated as a control (open bars) or incubated with latex bead-immobilized IgG (hatched bars) or β_1 integrin antibodies (solid bars) for 18 h at 37 °C. Conditioned media were collected and analyzed for uPA activity using a coupled colorimetric plasminogen activation assay or for uPA antigen by ELISA. uPA activity was also evaluated in the presence of an anti-catalytic uPA antibody (10 μ g/ml) as indicated. Data represent the mean and S.D. of three experiments repeated in triplicate, and are expressed as fold increase relative to untreated controls (designated as 1).

TABLE I

Effect of soluble β_1 integrin antibodies on uPA expression

Cells (0.7×10^5) were cultured with soluble β_1 integrin antibodies or isotype-matched control IgG for 24 or 48 h. Conditioned media were removed after 24 or 48 h and evaluated for uPA activity using a coupled colorimetric plasminogen activation assay as described under "Experimental Procedures." Results are expressed as fold increase in uPA activity relative to untreated controls (designated as 1) and shown as the mean and S.D. of three experiments repeated in triplicate.

Treatment	uPA Activity (fold increase)	
	24 h	48 h
Control	1.000	1.000
IgG (5 μ g/ml)	0.861 \pm 0.037	0.723 \pm 0.136
IgG (10 μ g/ml)	0.773 \pm 0.084	0.903 \pm 0.095
β_1 (5 μ g/ml)	0.986 \pm 0.102	0.856 \pm 0.227
β_1 (10 μ g/ml)	0.993 \pm 0.090	0.847 \pm 0.069

TABLE II

Effect of integrin α subunit aggregation on uPA induction

Cells (0.7×10^5) were cultured for 18 h with integrin α subunit-specific monoclonal antibodies conjugated to latex beads as described under "Experimental Procedures." Control experiments included beads conjugated with β subunits or isotype-matched IgG. Conditioned media were evaluated for uPA activity as described under "Experimental Procedures." Results are expressed as fold increase in uPA activity relative to untreated controls (designated as 1) and show the mean and S.D. of five experiments.

Treatment	uPA activity
	Fold increase
Control	1.0
IgG	1.167 \pm 0.058
α_2	0.933 \pm 0.153
α_3	2.067 \pm 0.208 ^a
α_5	1.067 \pm 0.153
α_6	0.987 \pm 0.103
β_1	2.160 \pm 0.250 ^a
β_4	1.000 \pm 0.089

^a $P < 0.001$ relative to untreated control.

(Table III). Although uPAR expression levels were unaltered, immunocytochemical staining demonstrated that integrin aggregation induced a significant redistribution of uPAR on the cell surface. Whereas uPAR staining was diffuse in pp126 cells treated with IgG-coated control beads (Fig. 5C), uPAR immunoreactivity was redistributed to the periphery of β_1 or α_3 beads bound to pp126 cells (Fig. 5, A and B). These data suggest

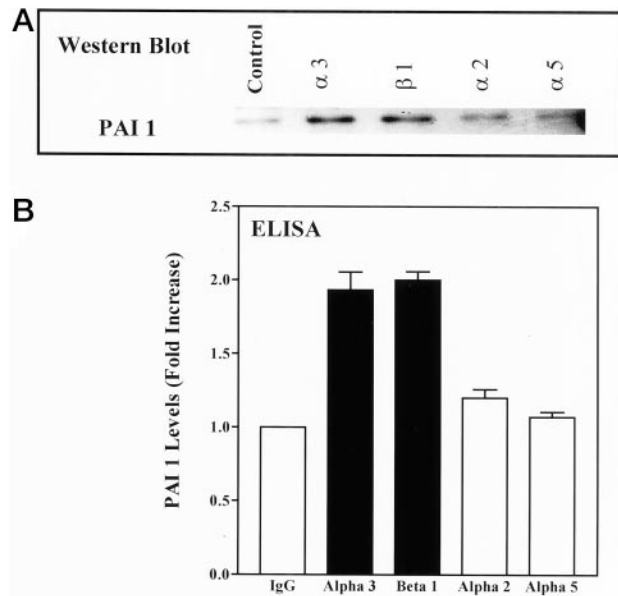


FIG. 4. Effect of integrin clustering on PAI-1 expression. pp126 cells (0.7×10^5) were incubated for 18 h at 37 °C with the bead immobilized anti-integrin subunit-specific antibodies or control IgG as indicated. Conditioned media were collected and analyzed for PAI-1. A, Western blot. Samples were electrophoresed on 9% SDS-polyacrylamide gels and immunoblotted for PAI-1 using monoclonal antibody 395G (1:2000) followed by peroxidase-conjugated secondary antibody (1:10,000) and developed using enhanced chemiluminescence detection. B, ELISA. Samples were analyzed by ELISA according to the manufacturer's specifications.

TABLE III

Effect of β_1 integrin aggregation on uPAR expression

Cells (0.7×10^5) were cultured for 18 h with β_1 integrin antibody-conjugated latex beads or isotype-matched control IgG beads. Quantitation of uPAR expression in cell lysates was performed by ELISA. Results are expressed as fold increase in uPAR expression relative to untreated controls (designated as 1) and show the mean and S.D. of six experiments.

Treatment	uPAR expression
Fold increase	
Control	1.000
IgG bead	1.020 \pm 0.028
β_1 integrin bead	1.000 \pm 0.010

that protein-protein interactions between uPAR and $\alpha_3\beta_1$ integrin may alter the cell surface distribution of uPAR and thereby regulate its function. To confirm this observation, cells were cultured on collagen I or fibronectin-coated surfaces, to engage $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrins, respectively, and the association of β_1 integrin with uPAR was evaluated by co-immunoprecipitation and Western blotting. A significant increase in β_1 integrin co-precipitating with an anti-uPAR antibody was observed in cells cultured on collagen I (Fig. 6, left lane) relative to those cultured on fibronectin (Fig. 6, middle lane) or plastic (Fig. 6, right lane), providing further evidence that $\alpha_3\beta_1$ integrin aggregation promotes physical interaction between uPAR and β_1 integrin.

ERK Activation and uPA Expression—In keratinocytes plated on fibronectin, association of uPAR with β_1 integrins increases both the magnitude and duration of ERK activation (3). Furthermore, inhibition of ERK activity using a dominant negative mutant blocks transcription of a reporter gene from the uPA promoter in oral carcinoma cells (26). To determine whether $\alpha_3\beta_1$ integrin aggregation induced ERK activation, cells were incubated with α_3 or IgG beads, and both total ERK1/2 expression and ERK activation were evaluated by

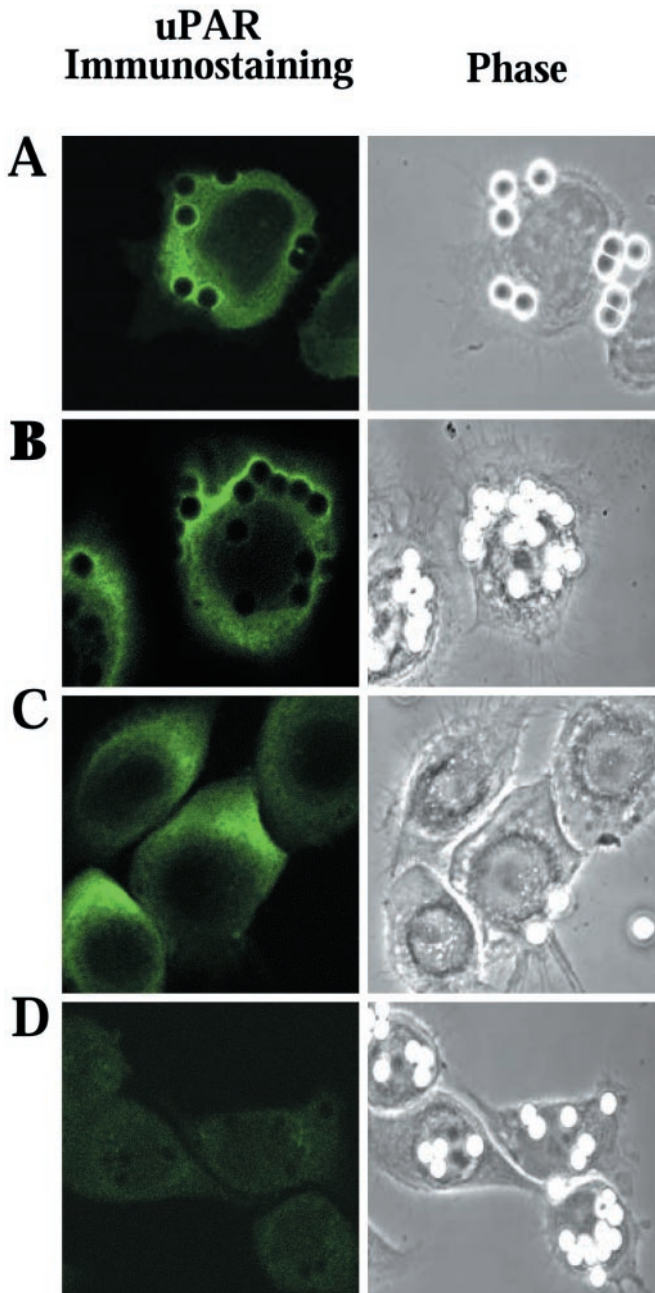


FIG. 5. Clustering of $\alpha_3\beta_1$ integrins induces uPAR redistribution. pp126 cells were plated at 50% confluence on 22-mm glass coverslips and cultured for 18 h at 37 °C prior to treatment with β_1 beads (A), α_3 beads (B and D), or IgG beads (C) for 4 h. Cells were fixed in 3.7% formaldehyde, blocked with BSA and incubated with biotinylated anti-uPAR antibody (A–C) followed by streptavidin-conjugated fluorescein isothiocyanate (A–D). In control experiments, biotinylated anti-uPAR was omitted from cells treated with α_3 beads (D). Cells were visualized by confocal (left column) or phase contrast (right column) microscopy, as indicated.

Western blotting. Although total ERK1/2 expression levels were unchanged (Fig. 7B), α_3 integrin aggregation resulted in a significant increase in ERK phosphorylation (Fig. 7A, second lane from right) relative to cells cultured with IgG beads (Fig. 7A, second lane from left) or untreated controls (Fig. 7A, left lane). Treatment of pp126 cells with α_3 beads in the presence of the MEK inhibitor PD98059 blocked both ERK phosphorylation (Fig. 7A, right lane) and uPA induction (Fig. 7C), indicating that $\alpha_3\beta_1$ integrin aggregation induces a signal propagated through the MEK-ERK pathway resulting in up-regulation of uPA expression. The requirement for tyrosine kinase signaling

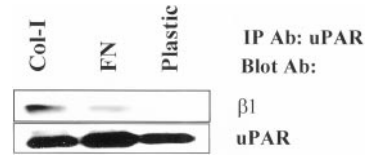


FIG. 6. Co-immunoprecipitation of uPAR and β_1 integrin. pp126 cells were cultured on plastic, collagen I (Col-I), or fibronectin (FN) for 24 h. Cells were lysed and subjected to immunoprecipitation with an anti-uPAR antibody (antibody 3937). Immunoprecipitates were electrophoresed on 9% SDS-polyacrylamide gels and immunoblotted with anti β_1 integrin (clone P4C10, 1:1000) followed by peroxidase-conjugated secondary antibody (top panel) or biotinylated anti-uPAR (antibody 399R, 1:200) (bottom panel) followed by streptavidin-peroxidase and enhanced chemiluminescence detection.

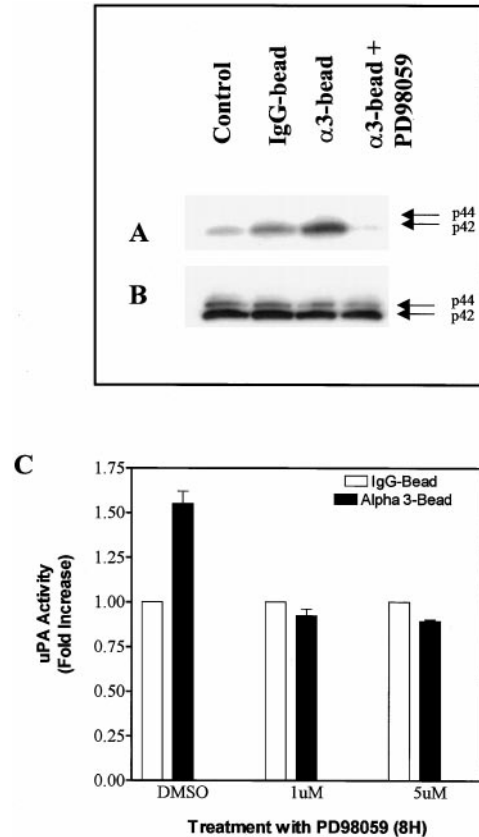


FIG. 7. Analysis of ERK activation and uPA expression. Cells (10^5) were cultured overnight in serum-free medium followed by treatment with buffer, IgG, or α_3 beads. After 3 h, cells were lysed with RIPA buffer and lysates (20 μ g) evaluated by Western blotting for ERK activation (A) or ERK expression (B). Blots were probed with anti-ACTIVE-MAPK p42/p44 (1:5000) to detect the phosphorylated, active form of ERK (A) or with anti-ERK1/2 antibody (1:1000) to detect total ERK1/2 expression (B). The arrows designate the migration positions of p42 and p44. Left lane, untreated control; second lane from left, IgG bead-treated cells; second lane from right, α_3 bead-treated cells; right lane, α_3 bead-treated cells incubated with the MEK inhibitor PD98059 (2 μ M). C, inhibition of ERK activation blocks α_3 bead-mediated uPA induction. Cells (10^5) were treated with IgG beads (open columns) or α_3 beads (solid columns) for 8 h in the presence of the MEK inhibitor PD98059 at the indicated concentrations or with vehicle control. Conditioned media were evaluated for uPA activity as described above.

with subsequent activation of transcription and *de novo* protein synthesis is supported by data showing that β_1 integrin-stimulated uPA expression is blocked by the general tyrosine kinase inhibitors genistein and herbimycin, as well as inhibitors of transcription (actinomycin D) and translation (cycloheximide) (Fig. 8).

Functional Consequences of uPA Induction—Epithelial cells utilize $\alpha_3\beta_1$ integrin for adhesion to both collagen I and lami-

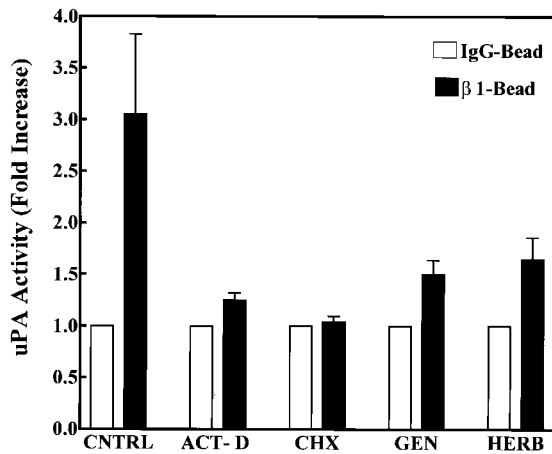


FIG. 8. **Effect of inhibitors on β_1 integrin-mediated uPA induction.** pp126 cells were incubated with actinomycin D (*ACT-D*) (1 ng/ml), cycloheximide (*CHX*) (1 ng/ml), genistein (*GEN*) (1 μ g/ml), or herbimycin (*HERB*) (160 nM) prior to the addition of IgG beads (*open columns*) or β_1 beads (*solid columns*). After 18 h at 37 °C, conditioned media were analyzed for uPA activity as described above. Data represent the mean and S.D. of six experiments.

nin-5 (24, 27). To evaluate the effect of these natural ligands on uPA expression, cells were cultured on collagen I-, laminin-5-, or BSA-coated surfaces. As observed in experiments using soluble antibodies (Table I), $\alpha_3\beta_1$ engagement was not sufficient for uPA induction (Table IV). However, treatment of pp126 cells with bead-immobilized collagen I or laminin-5 resulted in up-regulation of uPA expression (Table IV), providing additional evidence of the requirement for $\alpha_3\beta_1$ integrin aggregation in the regulation of uPA activity in pp126 cells. This is supported by data showing that clustering of $\alpha_6\beta_4$, a predominant laminin-5-binding integrin on epithelial cells (24, 27), did not alter proteinase production (Table II). In control experiments using laminin-1 or BSA-coated beads, no modulation of uPA expression was observed (Table IV).

Although collagen I is not susceptible to cleavage by uPA or plasmin, we have previously demonstrated that limited plasmin proteolysis removes the G5 subdomain of the laminin-5 α_3 subunit and thus alters epithelial cell behavior (13). Intact laminin-5 contains a 190-kDa α_3 subunit and promotes cell motility, whereas plasmin cleavage of laminin-5 produces a 160-kDa α_3 subunit, leading to diminished motility and increased hemidesmosome assembly. As uPA efficiently catalyzes pericellular plasmin formation, the effect of $\alpha_3\beta_1$ integrin-induced uPA expression on the laminin-5 α_3 subunit structure was evaluated by Western blot analysis of endogenous laminin-5 present in the subcellular matrix deposited by pp126 cells. Increased proteolysis of the 190-kDa α_3 subunit to the 160-kDa form was observed in the matrix of β_1 bead-treated cells (Fig. 9A, right lane) or α_3 bead-treated cells (not shown) relative to IgG bead-treated controls (Fig. 9A, left lane) when probed with an antibody that recognizes both intact and plasmin-modified laminin-5 α_3 subunit (EM11 (13)). This result was confirmed using an antibody specific for the laminin-5 α_3 G5 subdomain (12C4 (13)), showing diminished immunoreactivity of intact (190 kDa) laminin-5 in matrices extracted from β_1 bead-treated cells (Fig. 9B, right lane) relative to IgG bead controls (Fig. 9B, left lane). Together, these data suggest that integrin-mediated proteinase induction may modulate cellular behavior by modifying matrix proteins to support specific physiological functions.

DISCUSSION

To evaluate the potential functional link between adhesion and proteolysis in premalignant oral keratinocytes, in the cur-

TABLE IV

Effect of $\alpha_3\beta_1$ integrin ligands on uPA expression

Cells (0.7×10^5) were cultured for 18 h in tissue culture wells passively adsorbed with a thin layer of laminin-1, laminin-5, or collagen I or in the presence of latex bead-conjugated proteins. Conditioned media were evaluated for uPA activity using a coupled colorimetric assay as described under "Experimental Procedures." Results are expressed as fold increase in uPA activity relative to untreated controls (designated as 1) and represent the mean and S.D. of triplicate experiments.

Treatment	uPA activity
	<i>Fold increase</i>
Control	1.000
BSA	1.03 \pm 0.026
Laminin-1	1.06 \pm 0.02
Laminin-5	1.00 \pm 0.03
Collagen I	1.06 \pm 0.035
BSA beads	1.100 \pm 0.100
Laminin-1 beads	1.200 \pm 0.100
Laminin-5 beads	2.227 \pm 0.261 ^a
Collagen I beads	1.947 \pm 0.150 ^a

^a $P < 0.001$ relative to untreated controls.

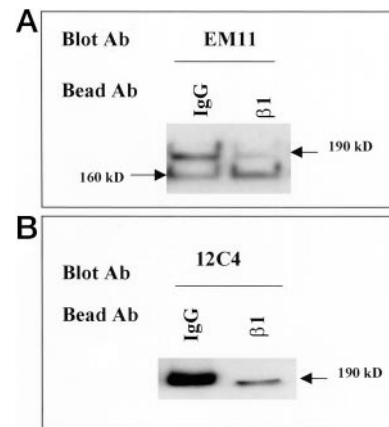


FIG. 9. **Processing of laminin-5 α_3 subunit.** pp126 cells were treated with IgG beads (*left lanes*) or β_1 beads (*right lanes*) as indicated. After incubation for 20 h, plasminogen (0.02 μ M) was added for 30 min at 37 °C prior to removal of cells and harvest of the subcellular matrix using 10 mM Tris-HCl, pH 6.8, containing 8 M urea, 1% SDS and 15% β -mercaptoethanol. Samples were electrophoresed on 6% gels and immunoblotted using the following anti-human laminin-5 α_3 subunit-specific monoclonal antibodies: EM11, which recognizes both intact (190 kDa) and processed (160 kDa) laminin-5 α_3 chain (A), and 12C4, which recognizes only the 190-kDa α_3 subunit (B).

rent study we have analyzed the role of cell-matrix interactions in modulation of uPA expression. Although association between uPAR and matrix proteins has been shown to regulate cell adhesion (2, 4, 11), matrix proteolysis (28–30), and cellular invasion (1), the influence of integrin-mediated matrix binding on uPA expression is not well characterized. Our results demonstrate that multivalent aggregation of the $\alpha_3\beta_1$ integrin in pp126 oral keratinocytes induces expression of both uPA and its primary inhibitor PAI-1; however, a net increase in proteolytic activity is predominant. Proteinase induction is specific to the $\alpha_3\beta_1$ integrin, as clustering of α_2 , α_5 , α_6 , or β_4 subunits does not influence uPA expression. In addition to antibody-induced integrin aggregation, integrin clustering with the intact subepithelial matrix $\alpha_3\beta_1$ ligands laminin-5 and collagen I also induce uPA expression. Furthermore, multivalent integrin engagement is necessary for uPA induction, as ligation with soluble antibodies or thin layer matrix proteins is not sufficient to alter expression.

Although uPAR expression levels are unaffected by $\alpha_3\beta_1$ integrin aggregation, integrin clustering induces a dramatic redistribution of uPAR on the cell surface. Furthermore, co-

immunoprecipitation of uPAR and β_1 integrin is observed when cells are cultured under conditions of $\alpha_3\beta_1$ integrin ligation, providing additional support for a physical interaction between uPAR and β_1 integrin. These data suggest that protein-protein interactions between the glycosylphosphatidylinositol-anchored uPAR and the transmembrane β_1 integrin may control uPAR cell surface distribution. This hypothesis is supported by previous studies that show that uPA/R is localized to focal adhesions, integrin-rich sites of cell-matrix contact (31, 32). Similar results were obtained using fluorescence resonance energy transfer to evaluate uPAR interaction with β_1 and β_3 integrins in HT1080 cells cultured on various matrix protein surfaces, in which lateral association of uPAR with distinct integrin α and β subunits was found to be specified in part by the adhesive substratum (5). In addition to regulation of uPAR localization, uPA/R-integrin associations may also modify integrin function. For example, ligation of uPAR with uPA promoted uPAR/ β_1 integrin association in kidney 293 cells and resulted in altered adhesion profiles (2). A more recent study demonstrated that uPA/R association with fibronectin-ligated $\alpha_5\beta_1$ integrin increased both the magnitude and duration of ERK activation (3), indicating that uPA/R can function as an accessory molecule to promote integrin-mediated signal transduction. Integrin signaling and cell motility were also enhanced in prostate and breast carcinoma cultures following ligation of uPAR with uPA (12, 47), providing further evidence that uPA/R-integrin association may modulate cell behavior.

In addition to promoting uPAR redistribution, our current data demonstrate that β_1 integrin aggregation induced expression of uPA via signal transduction through a MEK/ERK-dependent pathway. Pharmacologic inhibition of MEK blocked both ERK activation and uPA expression. A large body of evidence supports the involvement of ERK in integrin-mediated signaling pathways (reviewed in Ref. 33), and a subset of integrins has been shown to couple via the α subunit to the adaptor protein Shc, leading to Ras signaling and subsequent activation of MAPK (34). Although the $\alpha_3\beta_1$ integrin was not shown to activate the Shc pathway, a recent study demonstrated that $\alpha_3\beta_1$ signaling through MAPK regulates epithelial cell proliferation (35). Furthermore, it has previously been demonstrated that ERK phosphorylation leads to uPA promoter activation (26). In addition, cytoskeletal reorganization caused by pharmacologic disruption of the actin-based microfilament network also induces uPA gene expression via activation of ERK-2 (36). Together, these data suggest that $\alpha_3\beta_1$ integrin aggregation results in ERK phosphorylation and the subsequent activation of transcription factors that control uPA expression. As mono- and divalent integrin ligation and multivalent integrin clustering induce varying degrees of cytoskeletal reorganization (8, 9), it is interesting to speculate that proteinase expression may be controlled by both the specific matrix microenvironment and the cellular integrin repertoire. Thus, in pp126 cells, multivalent aggregation of $\alpha_3\beta_1$ may prompt subsequent matrix remodeling via induction of uPA. Furthermore, these data suggest that the relative structural integrity of specific matrix protein components may dictate subsequent matrix proteolysis via integrin-mediated control of proteinase expression. It should be noted, however, that "inside-out" signaling via Rho-driven actin reorganization also induces integrin clustering and is dependent on structural remodeling of the extracellular matrix (37). Cells may be unable to remodel a thin matrix deposit immobilized to a tissue culture well, thereby limiting the extent of integrin clustering to that dictated solely by the valence of the matrix at immediate sites of cell-matrix contact.

uPA ligation of uPAR has been shown to induce both FAK

phosphorylation and ERK activation in endothelial cells, although the potential requirement for concomitant integrin engagement was not evaluated (38). However, these data suggest a hypothetical regulatory loop wherein integrin-induced uPA expression results in sustained uPA/R ligation, thus potentiating ERK activation and subsequent uPA transcription. Studies are currently under way to determine whether uPA/R ligation is sufficient for ERK activation in pp126 cells. However, preliminary experiments in which uPAR was ligated using bead-immobilized anti-uPAR antibodies showed no effect on uPA expression levels,² supporting an additional requirement for $\alpha_3\beta_1$ integrin signaling. Whether physical association of uPA/R with $\alpha_3\beta_1$ integrin is necessary to induce or sustain uPA expression is currently under investigation.

To assess the potential functional consequences of enhanced uPA activity at sites of cell matrix contact, the structural integrity of the epithelial basement membrane protein laminin-5 was evaluated. This heterotrimeric protein ($\alpha_3\beta_3\gamma_2$) participates in the formation of hemidesmosomes, which promote stable cell-matrix adhesion (39–41). However, laminin-5 has also been identified at the invasive edge of both tumors and healing wounds, where cells are actively migrating (42–45). These apparently contradictory functions of laminin-5 can be explained by the presence of distinct laminin-5 structural isoforms in the subepithelial matrix. We have previously demonstrated that limited plasmin proteolysis of unprocessed laminin-5 specifically modifies the globular domain of the α_3 subunit, resulting in cleavage from 190 to 160 kDa (13). As a functional consequence of plasmin cleavage, epithelial cells exhibit a 3-fold decrease in motility and an 11-fold increase in hemidesmosome number. Results from the current study demonstrate increased uPA and pericellular plasmin activity following $\alpha_3\beta_1$ integrin clustering by either antibodies or matrix ligands (including laminin-5). Under these conditions, the plasmin-modified (160 kDa) laminin-5 α_3 subunit was the predominant form present in pp126 matrix, indicative of a transition from a pro-migratory to an adhesive substratum. Interestingly, this modified form of laminin-5 is susceptible to additional cleavage within the γ_2 subunit by matrix metalloproteinase-2, exposing a cryptic epitope that promotes motility (46). Together, these data suggest that stepwise limited proteolytic modification of matrix proteins may function as a fine regulatory mechanism for control of cellular adhesion and migration. Furthermore, it is interesting to speculate that integrins may play a dual role in proteinase targeting to extracellular matrix substrates by participating in both ligand-induced control of proteinase gene expression and in protein-protein interactions with proteinase receptors to bring about localized changes in enzyme concentration at sites of cell matrix contact.

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