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

Supurna Ghosh‡, Renee Brown‡, Jonathan C. R. Jones§¶, Shawn M. Ellerbroek∥∥, and M. Sharon Stack‡§§

From the Departments of ‡Obstetrics & Gynecology and ¶Cell & Molecular Biology and the §Robert H. Lurie Comprehensive Cancer Center, Northwestern University Medical School, Chicago, Illinois 60611

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 $\alpha_3$ and $\beta_1$ integrin subunits, but not $\alpha_m$, $\alpha_6$, $\beta_4$, or $\beta_5$, 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 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 $\beta_1$ integrin with uPAR provided further evidence that protein-protein interactions between uPAR and $\beta_1$ integrin control uPAR distribution. As a functional consequence of uPA up-regulation and uPA-mediated plasminogen activation, the globular domain of the laminin-5 $\alpha_5$ 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 $\alpha_5$ 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)† (urokinase) is a 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 posttranslationally 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 uPAR) 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 uPAR 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 uPAR 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

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†To whom correspondence should be addressed: Northwestern University Medical School, 303 E. Chicago Ave., Tarry 4-751, Chicago, IL 60611. Tel.: 312-908-8216; Fax: 312-908-8773; E-mail: ms130@nwu.edu.

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-activated 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 endog-
ogenous matrix-associated laminin-5 was observed, providing sup-
port for the hypothesis that integrin-mediated adhesion regu-
lates matrix proteolysis and subsequent cellular behavior.

EXPERIMENTAL PROCEDURES

Materials—Gelatin, BSA, type I collagen, type IV collagen, fibronec-
tin, laminin-1, aprotinin, cycloheximide, herbimycin, actinomycin D, N-
hydroxy-succinimidobiotin, n-Val-Leu-Lys-p-nitroanilide, peroxidase 
conjugates of anti-mouse IgG and anti-goat IgG, and fluorescein-
conjugated anti-rabbit IgG were acquired from Sigma. Laminin-5 was 
generously provided by Desmos (San Diego, CA). Plas-
minogen and plasmin were purified by affinity chromatography from 
outdated human plasma as described previously (18). Anti-human in-
tegrin β1 (clone P4C10), α5 (P1D6), and β3 (3E1) monoclonal antibodies 
are products of Life Technologies, Inc. Antibodies against α5 
(MAB1988) and α3 integrin (MAB2056) were obtained from Chemicon 
(Temecula, CA), and anti-α6 (G0H3) 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 α5 subunit, whereas antibody 12C4 is directed against the α5 subdo-
mument in only the intact (250 kDa) α3 or α6 laminin-5 subunit. Affinity-
purified polyclonal antibody specific for phosphorylated p42/p44 mito-
gen-activated protein kinase (anti-activateR MAPK p42/p44) was 
purchased from Promega (Madison, WI). Anti-ERK2/1 (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 casc-
cade, was purchased from New England Biols (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). Hydroboud-P polyvinylidi-
dene difluoride membrane and SuperSignal enhanced chemilumines-
cence reagents were obtained from Amersham Pharmacia Biotech and 
Fierce, respectively.

Cell Culture—Premalignant oral keratinocytes (pp126 cells) were a 
distillate in liquid-adherent oral keratinocyte culture models; however, they display normal keratin synthesis and some degree of differentiation and are representa-
tive 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 
μg/ml streptomycin, 5 ng/ml EGF, and 50 μg/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 con-
stant density of 0.7 × 105 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 μg/ml), antibody-con-
gjugated latex beads (8–10 μg/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 μl 
Tris, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS. Total protein concen-
tration of lysates was analyzed using a bichinonic acid protein de-
tection kit (Sigma). In some experiments, actinomycin D, herbimycin, 
genistein (in Me2SO), 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—Adhesion preferences of pp126 cells were analyzed by 
evaluating cellular adhesion to ECM-coated 24-well culture plates as 
described previously (16). Briefly, plates were covered by passive ados-
orption of ECM components (30 μg/ml) to culture wells, coated by 
collagen-I, 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 
(105) 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-
crometer 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— 
Antigen-integrin subunit-specific monoclonal antibodies or control isotype-
matched IgGs were passively adsorbed onto 2.97-μm latex beads (Sig-
avent) as described (9) with the following modifications. A 1% (final 
concentration) suspension of latex beads was incubated in 50 mM 4-
methoxyphenylthanesulfonic acid buffer (pH 6.1) with 75 μM 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 anti-
body was resuspended in BPE/EGF-free medium at a final concen-
tration of 1% by volume. Determination of total protein concentration in 
unblocked bead suspensions using a bichinonic acid detection kit 
(Sigma) indicated that 60–70% of immunoglobulins were adsorbed, 
resulting in a final concentration of 8–10 μg/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 (n-Val-Leu-Lys-p-nitroanilide) as described 
previously (18). Control reactions contained 10 μM of the anti-
catalytic uPA antibody (398), an activating uPA antibody (398), and 
anti-human PAI antibody (1:2000, 2 h), washed (six times, 10 min each) 
with TBST, and incubated with peroxidase-conjugated secondary IgG 
(1:10000) for 1 h at room temperature. After washing, immunoreactive 
bands were visualized using enhanced chemiluminescence detection.

Immunocytochemical Staining—Untreated cells were plated on 22-
mM glass coverslips placed into six-well tissue culture plates (1.5–105 
cells/well), cultured overnight, washed with PBS, and treated with 
antibody-conjugated latex beads (α5, β1, 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% formalde-
hyde. Coverslips were blocked for 45 min with PBS containing 1% BSA, 
and nonimmune (or 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-fluores-
cine 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 
α6 beads followed by streptavidin-fluorescein isothiocyanate in the absence 
of biotinylated anti-uPA or anti-uPAR. Mounted coverslips were viewed using a Zeiss 
Immunofluorescence Kit LS510 microscope.

Immunoprecipitation—pp126 cells were cultured in 24-well plates 
coated by passive adsorption with collagen I, fibronecin, or BSA (16) for 
24 h prior to lysis in (25 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 
1% Brij, 200 millikrein inhibitory units/ml aprotinin, 1 μg/ml leupeptin, 
1 μg/ml pepstatin, 1 mm phenylmethylsulfonyl fluoride) as described 
(21). Lysates (500 μg) were incubated with anti-uPAR antibody (5 μg, 
antibody 3937, American Diagnostica) in a total volume of 1 ml at 4 °C 
ownight. Immune complexes were precipitated by the addition of 30 μl 
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 μl of Laemmli sample 
dilution buffer containing β-mercaptoethanol, and boiled. Samples were 
electrophoresed on duplicate 9% SDS-polyacrylamide gels and electro-
blotted 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-conju-
gated peroxidase or with anti-β1 integrin monoclonal antibodies (1:1000 
dilution, P1C10) followed by peroxidase-conjugated goat-(anti-mouse) 
secondary antibody. Immune complexes were visualized using enhanced 
chemiluminescence detection. ERK (Mitogen-activated Protein Kinase) Activation Assays—To 
evaluate ERK (mitogen-activated protein kinase) activation, cells (0.7 × 105) 
were cultured overnight in serum-free medium followed by treat-
ment with IgG, α6, or β1 beads as described above. At varying time 
points, cells were lysed with RIPPA buffer including 1 mM sodium or-

Regulation of uPA by α3β1 Integrin

Matrix Proteins

![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).](Image)

![Fig. 2. Binding of β1 integrin antibody-conjugated beads to pp126 cells. Latex beads were coated with control IgG (IgG beads) (A and C) or β1 integrin antibody clone P4C10 (β1 beads) (B and D). Beads (8–10 μg/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.](Image)

Cellular Adhesion and β1 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 β1 integrin subunit aggregation on uPA expression, rather than simple integrin occupation, is necessary for uPA induction. β1 integrins can pair with numerous α subunits in oral keratinocytes to facilitate binding to diverse ECM proteins (23–25). To assess the effect of a subunit bound to pp126 cells, only α3 and β1 beads enhanced uPA activity, whereas the remaining samples were indistinct from IgG controls (Table II), suggesting that the αβ1 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 α3 or β1 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 β1 Integrin Aggregation on uPAR Expression and Distribution—Because uPAR binding is a primary mechanism for posttranslational control of uPA activity (1), the effect of β1 integrin aggregation on uPAR expression was also evaluated. ELISA of cell membrane extracts indicated that uPAR expression was unaffected by β1 bead treatment, suggesting a lack of coordinate regulation between uPA and uPAR.
that protein-protein interactions between uPAR and α₃β₁ 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 α₃β₁ and α₅β₁ integrins, respectively, and the association of β₁ integrin with uPAR was evaluated by co-immunoprecipitation and Western blotting. A significant increase in β₁ 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 α₃β₁ integrin aggregation promotes physical interaction between uPAR and β₁ integrin.

**ERK Activation and uPA Expression**—In keratinocytes plated on fibronectin, association of uPAR with β₁ 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 α₅β₁ integrin aggregation induced ERK activation, cells were incubated with α₅G or IgG beads, and both total ERK1/2 expression and ERK activation were evaluated by

**TABLE I**

Effect of soluble β₁ integrin antibodies on uPA expression

Cells (0.7 × 10⁵) were cultured with soluble β₁, 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 experiments repeated in triplicate.

| Treatment     | uPA Activity (fold increase) |
|---------------|------------------------------|
|               | 24 h | 48 h |
| Control       | 1.000 | 1.000 |
| IgG (5 μg/ml) | 0.861 ± 0.037 | 0.723 ± 0.136 |
| IgG (10 μg/ml)| 0.773 ± 0.084 | 0.903 ± 0.095 |
| β₁ (5 μg/ml)  | 0.986 ± 0.102 | 0.856 ± 0.227 |
| β₁ (10 μg/ml)| 0.993 ± 0.090 | 0.847 ± 0.069 |

**TABLE II**

Effect of integrin α subunit aggregation on uPA induction

Cells (0.7 × 10⁵) 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 shown as the mean and S.D. of five experiments.

| Treatment     | uPA activity |
|---------------|--------------|
|               | Fold increase |
| Control       | 1.0          |
| IgG           | 1.167 ± 0.058 |
| α₂           | 0.933 ± 0.153 |
| α₂          | 2.067 ± 0.208* |
| α₂          | 1.067 ± 0.153 |
| α₅          | 0.987 ± 0.103 |
| β₁          | 2.160 ± 0.250* |
| β₄          | 1.000 ± 0.089 |

* P < 0.001 relative to untreated control.
Western blotting. Although total ERK1/2 expression levels were unchanged (Fig. 7B), a3 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 a3 beads in the presence of the MEK inhibitor PD98059 blocked both ERK phosphorylation (Fig. 7A, right lane) and uPA induction (Fig. 7C), indicating that a3β1 integrin aggregation induces a signal propagated through the MEK-ERK pathway resulting in up-regulation of uPA expression. The requirement for tyrosine kinase signaling with subsequent activation of transcription and de novo protein synthesis is supported by data showing that β1 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 a3β1 integrin for adhesion to both collagen I and laminin. Clustering of a3β1 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 β1 beads (A), a3 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 a3 beads (D). Cells were visualized by confocal (left column) or phase contrast (right column) microscopy, as indicated.

Fig. 5. Clustering of a3β1 integrins induces uPAR redistribution. 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 3997). Immunoprecipitates were electrophoresed on 9% SDS-polyacrylamide gels and immunoblotted with anti β1 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. 6. Co-immunoprecipitation of uPAR and β1 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 3997). Immunoprecipitates were electrophoresed on 9% SDS-polyacrylamide gels and immunoblotted with anti β1 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 (10⁵) were cultured overnight in serum-free medium followed by treatment with buffer, IgG, or a3 beads. After 3 h, cells were lysed with RIPA buffer and lysates (20 μg) 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, a3 bead-treated cells; right lane, a3 bead-treated cells incubated with the MEK inhibitor PD98059 (2 μM). C, inhibition of ERK activation blocks a3 bead-mediated uPA induction. Cells (10⁵) were treated with IgG beads (open columns) or a3 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.
Regulation of uPA by αβ integrin

To evaluate the potential functional link between adhesion and proteolysis in premalignant oral keratinocytes, in the current study we have analyzed the role of cell-matrix interactions in modulation of uPA expression. Although association between uPA/R 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 αβ 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 αβ integrin, as clustering of αβ, αα, or ββ subunits does not influence uPA expression. In addition to antibody-induced integrin aggregation, integrin clustering with the intact subepithelial matrix αβ 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 αβ integrin aggregation, integrin clustering induces a dramatic redistribution of uPAR on the cell surface. Furthermore, co-

In pp126 cells were incubated with actinomycin D (ACT-D) (1 ng/ml), cycloheximide (CHX) (1 ng/ml), genistein (GEN) (1 μg/ml), or herbimycin (HERB) (160 nM) prior to the addition of IgG beads (open columns) or β3 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.

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), αβ 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 αβ integrin aggregation in the regulation of uPA activity in pp126 cells. This is supported by data showing that clustering of αβ subunits, 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 α3 subunit and thus alters epithelial cell behavior (13). Intact laminin-5 contains a 190-kDa α3 subunit and promotes cell motility, whereas plasmin cleavage of laminin-5 produces a 160-kDa α3 subunit, leading to diminished motility and increased hemidesmosome assembly. As uPA efficiently catalyzes pericellular plasmin formation, the effect of αβ integrin-induced uPA expression on the laminin-5 α3 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 α3 subunit to the 160-kDa α3 subunit form was observed in the matrix of β3 bead-treated cells (Fig. 9A, right lane) or α3 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 α3 subunit (EM11 (13)). This result was confirmed using an antibody specific for the laminin-5 α3 G5 subdomain (12C4 (13)), showing diminished immunoreactivity of intact (190 kDa) laminin-5 in matrices extracted from β3 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 current study we have analyzed the role of cell-matrix interactions in modulation of uPA expression. Although association between uPA/R 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 αβ 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 αβ integrin, as clustering of αα, αβ, or ββ subunits does not influence uPA expression. In addition to antibody-induced integrin aggregation, integrin clustering with the intact subepithelial matrix αβ 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 αβ integrin aggregation, integrin clustering induces a dramatic redistribution of uPAR on the cell surface. Furthermore, co-

### Table IV

Effect of αβ integrin ligands on uPA expression

| Treatment | uPA activity | Fold increase |
|-----------|--------------|---------------|
| Control   | 1.000        |               |
| BSA       | 1.03 ± 0.026 |               |
| Laminin-1 | 1.06 ± 0.02  |               |
| Laminin-5 | 1.00 ± 0.03  |               |
| Collagen I| 1.06 ± 0.035 |               |
| BSA beads | 1.100 ± 0.100|               |
| Laminin-1 beads | 1.200 ± 0.100 |               |
| Laminin-5 beads | 2.227 ± 0.261 |               |
| Collagen 1 beads | 1.947 ± 0.150 |               |

αP < 0.001 relative to untreated controls.
Regulation of uPA by α₃β₁ Integrin

immunoprecipitation of uPAR and β₁ integrin is observed when cells are cultured under conditions of α₃β₁ integrin ligation, providing additional support for a physical interaction between uPAR and β₁ integrin. These data suggest that protein-protein interactions between the glycosylphosphatidylinositol-anchored uPAR and the transmembrane β₁ integrin may control uPAR cell surface distribution. This hypothesis is supported by previous studies that show uPAR 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 β₁ and β₂ 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 substrate (5). In addition to regulation of uPAR localization, uPAR-integrin associations may also modify integrin function. For example, ligation of uPAR with uPA promoted uPAR/β₁ integrin association in kidney 293 cells and resulted in altered adhesion profiles (2). A more recent study demonstrated that uPAR association with fibronectin-ligated α₃β₁ integrin increased both the magnitude and duration of ERK activation (3), indicating that uPAR 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 uPAR/integrin association may modulate cell behavior.

In addition to promoting uPAR redistribution, our current data demonstrate that β₁ integrin aggregation induced expression of uPAR via signal transduction through a MEK/ERK-dependent pathway. Pharmacologic inhibition of MEK blocked both ERK activation and uPAR 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 Shc adaptor protein Shc, leading to Ras signaling and subsequent activation of MAPK (34). Although the α₃β₁ integrin was not shown to activate the Shc pathway, a recent study demonstrated that α₃β₁ signaling via 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 uPAR gene expression via activation of ERK-2 (36). Together, these data suggest that α₃β₁ 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 α₃β₁ 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 uPAR/R ligation, thus potentiating ERK activation and subsequent uPA transcription. Studies are currently under way to determine whether uPAR 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 α₃β₁ integrin signaling. Whether physical association of uPAR with α₃β₁ 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 (α₁β₁γ₂) 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 α₃ 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 α₃β₁ integrin clustering by either antibodies or matrix ligands (including laminin-5). Under these conditions, the plasmin-modified (160 kDa) laminin-5 α₃ subunit was the predominant form present in pp126 matrix, indicative of a transition from a pro-migratory to an adhesive substrate. Interestingly, this modified form of laminin-5 is susceptible to additional cleavage within the γ₂ 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.

REFERENCES

1. Andreassen, P. A., Kjoller, L., Christensen, L., and Duffy, M. J. (1997) Int. J. Cancer 72, 1–22
2. Wei, Y., Lukashev, M. Simon, D. L., Birdsey, S. C., Rosenberg, S., Doyle, M. V., and Chapman, H. A. (1996) Science 274, 1551–1555
3. Aguirre Ghiso, J. A., Kovalski, K., and Ossowski, L. (1999) J. Cell Biol. 147, 89–103
4. Chapman, H. A. (1997) Curr. Opin. Cell Biol. 9, 714–725
5. Xue, W., Mizukami, I., Todd, R. F., and Petty, H. R. (1997) Cancer Res. 57, 1682–1689
6. Humphries, M. J. (1996) Curr. Opin. Cell Biol. 8, 632–640
7. Boudreau, N. J., and Jones, P. L. (1999) Biochem. J. 339, 481–488
8. Miyamoto, S., Teramoto, H., Coso, O. A., Silvio-Gutkind, J., Burboela, P. D., Akiyama, S. K., and Yamada, K. M. (1995) J. Biol. Chem. 270, 719–725
9. Miyamoto, S., Akiyama, S. K., and Yamada, K. M. (1995) Science 267, 883–885
10. Laffrej, R. M., and Yamada, K. M. (1996) J. Cell. Biochem. 61, 543–553
11. Wei, Y., Yang, X., Liu, Q., Wilkins, J. A., and Chapman, H. A. (1999) J. Cell Biol. 144, 1285–1294
12. Yebra, M., Gorlatz, L., Pfeifer, M., and Mueller, B. H. (1999) Exp. Cell Res. 250, 231–240

² S. Ghosh and S. Stack, unpublished observations.
13. Goldfinger, L. E., Stack, M. S., and Jones, J. C. R. (1998) J. Cell Biol. 141, 255–265
14. Oda, D., Bigler, L., Lee, P., and Blanton, R. (1996) Exp. Cell Res. 226, 164–169
15. Rheinwald, J. G., and Beckett, M. A. (1981) Cancer Res. 41, 1657–1663
16. Moser, T. L., Pizzo, S. V., Bafetti, L. M., Fishman, D. A., and Stack, M. S. (1996) Int. J. Cancer 67, 695–701
17. Eplerbroek, S. M., Fishman, D. A., Kearns, A. S., Bafetti, L. M., and Stack, M. S. (1999) Cancer Res. 59, 1635–1641
18. Stack, S., Gonzalez-Gronow, M., and Pizzo, S. V. (1990) Biochemistry 29, 4966–4970
19. Laemmli, U. K. (1970) Nature 227, 680–685
20. Matsudaira, P. (1987) J. Biol. Chem. 262, 10035–10038
21. Berditchevski, F. Chang, S., Bodorova, J., and Hemler, M. E. (1997) J. Biol. Chem. 272, 29174–29180
22. Gospodarowicz, D., Delgado, D., Vlodavsky, I. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 4094–4098
23. Sugiyama, M., Speight, P. M., Prime, S. S., and Watt, F. M. (1993) Carcinogenesis 14, 171–176
24. Jones, J., Sugiyama, M., Watt, F. M., and Speight, P. M. (1993) J. Pathol. 168, 235–243
25. VanWaes, C., Suri, D. M., Chen, Z., Kirby, M., Rhim, J. S., Brager, R., Sessions, R. B., Poore, J., Wolf, G. T., and Carey, T. M. (1995) Cancer Res. 55, 5434–5444
26. Lengyel, R., Gum, R., Stepp, E., Juarez, J., Wang, H., and Boyd, D. (1996) J. Cell. Biochem. 61, 430–433
27. Carter, W. G., Ryan, M. C., and Gahr, P. J. (1991) Cell 65, 599–610
28. Yu, W., Kim, J., and Osowski, L. (1997) Cell 137, 767–777
29. Testa, J. E., and Quigley, J. P. (1999) Cancer Metastasis Rev. 18, 353–367
30. Stahl, A., and Mueller, B. M. (1994) Cancer Res. 54, 3066–3073
31. Pollienuz, J., Hedman, K., Nielsen, L. S., Danes, K., and Vaheri, A. (1988) J. Cell Biol. 106, 87–95
32. Ciambrone, G. J., and McKeown-Longo, P. J. (1992) J. Biol. Chem. 267, 13617–13622
33. Schwartz, M. A. (1997) J. Cell Biol. 139, 575–578
34. Wary, K. K., Mainiero, P., Isakoff, S. J., Marcantonio, E. E., and Gianotti, F. G. (1996) Cell 87, 733–743
35. Gonzalez, M., Hann, K., Baker, S. E., Fitchmun, M., Todorow, I., Weitzman, S., and Jones, J. C. R. (1999) Mol. Biol. Cell 10, 259–270
36. Irigoyen, J. P., Besser, D., and Nagamine, Y. (1997) J. Biol. Chem. 272, 1904–1909
37. Schoenfelder, M. B., and Burridge, K. (1999) Curr. Opin. Cell Biol. 11, 274–286
38. Tang, H., Kerins, D. M., Hao, Q., Inagami, T., and Vaughan, D. E. (1998) J. Biol. Chem. 273, 18268–18272
39. Green, K. J., and Jones, J. C. R. (1996) FASEB J. 10, 871–880
40. Langhauer, M., Hopkinson, S. B., and Jones, J. C. R. (1996) J. Cell Sci. 105, 753–764
41. Baker, S. E., Hopkinson, S. B., Fitchmun, M., Andreason, G. L., Frasier, F., Plopper, G., Quaranta, V., and Jones, J. C. R. (1996) J. Cell Sci. 109, 2563–2570
42. Pyke, C., Romer, J., Kallunki, P., Lund, L. R., Ralfkiaer, E., Dano, K., and Tryggvason, K. (1994) Am. J. Pathol. 145, 92–91
43. Ryan, M. C., Tizard, R., VanDevanter, D. R., and Carter, W. G. (1994) J. Biol. Chem. 269, 22779–22787
44. Plopper, G., Falk-Martziller, J., Glaser, S., Fitchmun, M., Gianelli, G., Romano, T., Jones, J. C. R., and Quaranta, V. (1996) J. Cell Sci. 109, 1965–1973
45. Goldfinger, L. E., Hopkinson, S. B., deHart, G. W., Collawn, S., Couchman, J. R., and Jones, J. C. J. Cell Sci. 112, 2615–2622
46. Gianelli, G., Falk-Martziller, J., Schiraldi, O., Stelter-Stevenson, W. G., and Quaranta, V. (1997) Science 277, 225–228
47. Nguyen, D. H. D., Catling, A. D., Webb, D. J., Sankovic, M., Walker, L. A., Somlyo, A. V., Weber, M. J., and Genias, S. L. (1999) J. Cell Biol. 146, 149–164