

# Proteinase Suppression by E-cadherin-mediated Cell-Cell Attachment in Premalignant Oral Keratinocytes\*

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**The expression and activity of epithelial proteinases is under stringent control to prevent aberrant hydrolysis of structural proteins and disruption of tissue architecture. E-cadherin-dependent cell-cell adhesion is also important for maintenance of epithelial structural integrity, and loss of E-cadherin expression has been correlated with enhanced invasive potential in multiple tumor models. To address the hypothesis that there is a functional link between E-cadherin and proteinase expression, we have examined the role of E-cadherin in proteinase regulation. By using a calcium switch protocol to manipulate junction assembly, our data demonstrate that initiation of *de novo* E-cadherin-mediated adhesive contacts suppresses expression of both relative matrix metalloproteinase-9 levels and net urinary-type plasminogen activator activity. E-cadherin-mediated cell-cell adhesion increases both phosphatidylinositol 3'-kinase (PI3-kinase)-dependent AKT phosphorylation and epidermal growth factor receptor-dependent MAPK/ERK activation. Pharmacologic inhibition of the PI3-kinase pathway, but not the epidermal growth factor receptor/MAPK pathway, prevents E-cadherin-mediated suppression of proteinases and delays junction assembly. Moreover, inhibition of junction assembly with a function-blocking anti-E-cadherin antibody stimulates proteinase-dependent Matrigel invasion. As matrix metalloproteinase-9 and urinary-type plasminogen activator potentiate the invasive activity of oral squamous cell carcinoma, these data suggest E-cadherin-mediated signaling through PI3-kinase can regulate the invasive behavior of cells by modulating proteinase secretion.**

teinases have also been implicated in the invasion and metastasis of malignant cells (4–6). Predominant among these enzymes are the matrix metalloproteinases (MMPs)<sup>1</sup> and the plasminogen activator (PA) urinary-type PA (uPA) (7–10). MMPs are a large family of metalloendopeptidases with activity directed against a variety of extracellular matrix substrates (11). MMP-9 (gelatinase B), a 92-kDa gelatinase that efficiently degrades native type IV collagen, has been implicated in tumor dissemination, as evidenced by enhanced MMP-9 expression in tumor samples exhibiting matrix invasion and distant metastases (12). This is supported by studies using tumor-bearing MMP-9-deficient mice, which exhibit decreased propensity to develop metastatic foci, indicating that MMP-9 plays a critical role in tumor development (5). Post-translational regulation of MMP-9 activity is mediated by interaction with tissue inhibitor of metalloproteinases (TIMP)-1 which forms a 1:1 noncovalent inactive enzyme-inhibitor complex (12). Under many conditions, secretion of MMP-9 and TIMP-1 is coordinately regulated (12).

In addition to MMP-9, up-regulation of uPA expression has also been correlated with malignant progression of a wide variety of neoplasms (9). uPA is a serine proteinase that functions in the conversion of the circulating zymogen plasminogen to the active enzyme plasmin (9). Plasmin is a broad spectrum serine proteinase that can directly cleave a number of protein substrates (9), as well as activate many additional proteinase zymogens including pro-MMP-9 (13). uPA is localized to the cell surface via interaction with a glycosylphosphatidylinositol-anchored receptor, designated uPA receptor or uPAR (9). Proteolytic activity is also regulated by the serpin plasminogen activator inhibitor 1 (PAI-1) which forms a covalent enzyme-inhibitor complex with both free and receptor-localized uPA (9). In a number of tumor models, down-regulation of either uPA or its receptor decreases invasion and reduces metastatic potential (14, 15).

Like proteolytic enzymes, dysregulation of adhesion molecules is often observed in malignant cells. Cadherins are a family of cell surface adhesion molecules that participate in Ca<sup>2+</sup>-dependent cell-cell adhesion (16) and thus are essential for maintenance of tissue integrity. E-cadherin is a widely distributed transmembrane intercellular adhesion molecule

Degradation of the extracellular matrix by proteolytic enzymes is necessary for a number of normal and pathological processes, including embryonic development, tissue resorption and remodeling, angiogenesis, and wound healing (1–3). Pro-

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<sup>1</sup> The abbreviations used are: MMP-9, matrix metalloproteinase 9; TIMP-1, tissue inhibitor of metalloproteinases 1; uPA, urinary-type plasminogen activator; uPAR, uPA receptor; PAI-1, plasminogen activator inhibitor 1; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PI3-kinase, phosphatidylinositol 3'-kinase; ELISA, enzyme-linked immunosorbent assay; EGFR, epidermal growth factor receptor; PBS, phosphate-buffered saline; pp, phosphoprotein.

(17). In addition to functioning in cell-cell adhesion, the cytoplasmic tail of E-cadherin binds to  $\beta$ - or  $\gamma$ -catenin/plakoglobin, thereby providing a mechanism for association with additional proteins, including signaling molecules such as phosphatidylinositol 3'-kinase (PI3-kinase) and epidermal growth factor receptor (18–21). Loss of E-cadherin expression is frequently observed in carcinomas (22, 23), and transfection of ectopic E-cadherin into breast (24), colon (25), and prostate cancer cells (26) decreases cellular invasion.

In multiple tumor models, loss of E-cadherin expression and increased proteinase activity correlate with more invasive and metastatic tumors (9, 12, 14, 15, 22, 23, 27). To address the hypothesis that there is a functional link between E-cadherin and proteinase expression, in the current study we have examined the role of E-cadherin in the regulation of proteinase expression in premalignant oral keratinocytes. Our data demonstrate that initiation of *de novo* E-cadherin-mediated cell-cell adhesion suppresses both relative MMP-9 levels and net uPA activity in premalignant oral keratinocytes. Concomitant with decreased proteinase expression, secretion of TIMP-1 and PAI-1 is also down-regulated. E-cadherin-mediated cell-cell adhesion increases PI3-kinase-dependent AKT activation and epidermal growth factor receptor (EGFR)-dependent mitogen-activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK) activation. Inhibition of the PI3-kinase pathway, but not the EGFR-MAPK pathway, interferes with formation of adherens junctions and prevents E-cadherin-mediated suppression of proteinases. Furthermore, prevention of junction assembly with a function-blocking E-cadherin antibody stimulates proteinase-dependent Matrigel invasion. Together these data support the hypothesis that E-cadherin-mediated signaling via PI3-kinase can regulate the invasive behavior of cells by modulating proteinase expression.

#### EXPERIMENTAL PROCEDURES

**Materials**—Gelatin, cell culture reagents, D-Val-Leu-Lys-p-nitroanilide, rat anti-E-cadherin (DECMA clone), and peroxidase-conjugated secondary antibodies were purchased from Sigma. Keratinocyte-SFM was obtained from Invitrogen. Plasminogen was purified by affinity chromatography from outdated human plasma as described previously (28). Anti-mouse E-cadherin (HECD-1 clone) and isotype-specific IgG1 antibodies were obtained from Calbiochem; anti-phosphorylated p42/p44 (MAPK/ERK) was obtained from Promega (Madison, WI); anti-ERK1/2 (anti-p42/p44) antibody and anti-AKT antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phosphorylated AKT (Thr-308) was obtained from Upstate Biotechnology, Inc.; anti-uPAR antibody (399R) and function-blocking anti-uPA antibody (394) were obtained from American Diagnostica (Greenwich, CT); and Alexa Fluor 594 goat anti-mouse and anti-rat antibodies were from Molecular Probes (Eugene, OR). TIMP-1 and MMP-9 ELISA kits were from Oncogene Research Products (Boston, MA), and PAI-1 ELISA kit was purchased from American Diagnostica. The mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) inhibitor PD98059, PI3-kinase inhibitor LY294002, and EGFR-specific inhibitor AG1478 were obtained from Calbiochem. The MMP inhibitor GM6001 was purchased from Chemicon (Temecula, CA). Polyvinylidene difluoride membrane was from Amersham Biosciences (Arlington Heights, IL). Supersignal enhanced chemiluminescence (ECL) reagent, EZ-Link Sulfo-NHS-Biotin, and UltraLink Immobilized Streptavidin Gel were obtained from Pierce. Microcon 10 microconcentrators were purchased from Millipore (Bedford, MA).

**Cell Cultures**—Premalignant oral keratinocytes (pp126 cells) were the gift of Dr. D. Oda (University of Washington, Seattle, WA) (29, 30). Early passage (between 3 and 8) pp126 cells were maintained at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> in keratinocyte-SFM containing 0.09 mM calcium and supplemented with 100 units/ml penicillin, 100 ng/ml EGF, and 50  $\mu$ g/ml bovine pituitary extract.

**E-cadherin Activation**—The calcium switch method was utilized to assess the consequences of *de novo* E-cadherin activation (19–21, 31, 32). Briefly, pp126 cells were seeded at a constant density ( $1.5 \times 10^5$  cells/well) in 12-well tissue culture plates. After 24 h, the cells were

starved for 6–8 h, and calcium was removed by incubation with Keratinocyte-SFM containing 4 mM EGTA and 1 mM MgCl<sub>2</sub> at 37 °C. After 30 min the calcium-free medium was removed, and Keratinocyte-SFM containing 0.09 mM calcium was immediately added to induce E-cadherin mediated cell-cell interactions (designated “E-cadherin activation +”). In control experiments, cells received fresh media in the absence of EGTA such that original junctions are maintained, but *de novo* activation of E-cadherin is not induced (designated “E-cadherin activation –”). In selected experiments, either anti-E-cadherin antibody (HECD-1 clone) or isotype-specific control antibody was added to the calcium-containing medium at 10  $\mu$ g/ml. In additional studies, the PI3-kinase inhibitor LY294002 (10  $\mu$ M), the MEK inhibitor PD98059 (5  $\mu$ M), the EGFR inhibitor AG1478 (250 nM), or the appropriate vehicle was added to the medium. Conditioned medium was collected for proteinase analysis after 24–36 h.

**Immunofluorescence Microscopy**—pp126 cells were grown on glass coverslips at 37 °C in Keratinocyte-SFM. Following a 30-min incubation with serum-free medium containing 4 mM EGTA and 1 mM MgCl<sub>2</sub>, cells were incubated with Keratinocyte-SFM containing 0.09 mM Ca<sup>2+</sup> in the presence of either control IgG antibody or anti-E-cadherin antibody (HECD-1, 10  $\mu$ g/ml). In additional studies, the PI3-kinase inhibitor LY294002 (10  $\mu$ M), the MEK inhibitor PD98059 (5  $\mu$ M), the EGFR inhibitor AG1478 (250 nM), or the appropriate vehicle was added to the medium. For immunofluorescence staining, cells were washed with PBS and fixed with ice-cold methanol for 2 min or with 3.7% formaldehyde for 5 min followed by 0.5% Triton X-100 for 7 min. After washing with PBS, cells were blocked with 1% bovine serum albumin in PBS and incubated with mouse anti-E-cadherin antibody (HECD-1) or rat anti-E-cadherin antibody (DECMA) for 1 h at 37 °C. After three washes with PBS, cells were incubated for 1 h at room temperature with Alexa Fluor 594-labeled goat anti-mouse or anti-rat antibody. Glass coverslips were washed with PBS three times, mounted, and examined under a UV microscope (Nikon) using the appropriate filter.

**Analysis of MMP-9 and TIMP-1 Expression**—Gelatinase activities in the conditioned media at 36 h were determined using SDS-PAGE gelatin zymography as described previously (33). Briefly, SDS-PAGE gels (9% acrylamide) were co-polymerized with 0.1% gelatin, and samples were electrophoresed without reduction or boiling using 5 $\times$  Laemmli sample buffer (34). SDS was removed through a 30-min incubation in 2.5% Triton X-100, and gels were incubated in 20 mM glycine, pH 8.3, 10 mM CaCl<sub>2</sub>, 1  $\mu$ M ZnCl<sub>2</sub> at 37 °C for 24–36 h. The gels were stained with Coomassie Blue to visualize zones of gelatinolytic activity. MMP-9 levels in the conditioned media were also quantified by ELISA (Oncogene Research Products) following concentration of the conditioned media 25–30-fold with Microcon 10. Levels of TIMP-1 protein in the conditioned media were quantified by ELISA (Oncogene Research Products) according to the manufacturer's specifications.

**Analysis of uPA Activity and PAI-1 Protein Levels**—Net uPA activity in the conditioned media at 24 h was quantified using a coupled assay to monitor plasminogen activation and resulting hydrolysis of a colorimetric substrate (Val-Leu-Lys-p-nitroanilide) as described previously (28). Levels of PAI-1 protein in the conditioned media were quantified by ELISA (American Diagnostica) according to the manufacturer's specifications.

**Cell Surface Biotinylation**—pp126 cells were grown in a 6-well plate, washed with ice-cold PBS, and incubated at 4 °C with gentle shaking for 30 min with 0.5 mg/ml cell-impermeable Sulfo-NHS-Biotin in ice-cold PBS, followed by washing with 100 mM glycine to quench free biotin. Cells were then detached by scraping, lysed in modified RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 0.1% SDS) with proteinase inhibitors, and clarified by centrifugation. To isolate biotinylated cell-surface proteins, equal amounts of protein from each of the samples were incubated with streptavidin beads at 4 °C for 14 h, followed by centrifugation. After boiling in Laemmli sample dilution buffer (34) to dissociate streptavidin bead-biotin complexes, the samples were analyzed by SDS-PAGE (9% gels) and immunoblotted for uPAR (1:1000, American Diagnostica, clone 399R).

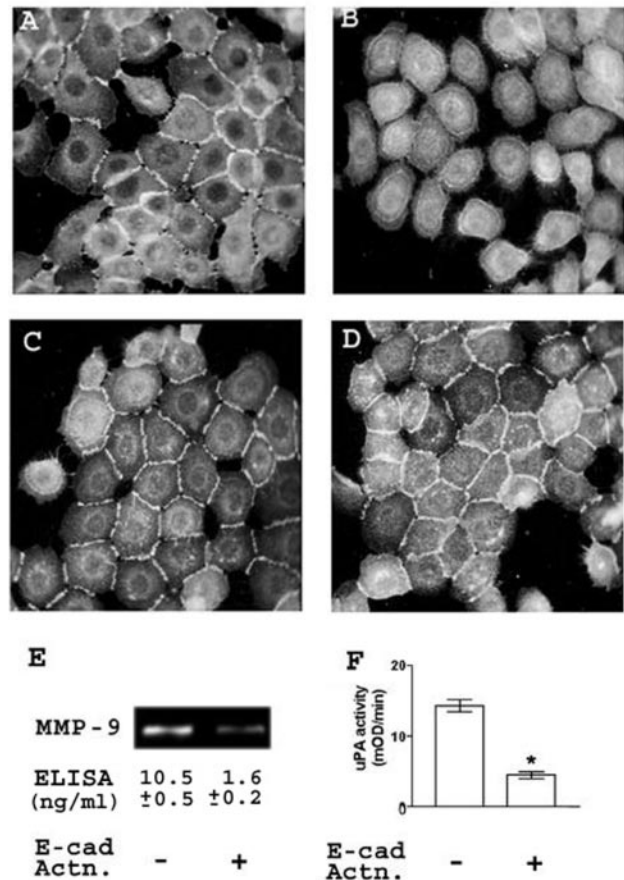
**MAPK and AKT Activation**—E-cadherin was activated by the calcium switch method as described above, and at the indicated time points cells were lysed in modified RIPA buffer containing 20 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1  $\mu$ g/ml aprotinin, 1  $\mu$ M pepstatin, and 10  $\mu$ M leupeptin. The samples were analyzed by SDS-PAGE (9% gels), and the blots were probed with anti-ERK1/2 antibody (1:1000) or with anti-AKT antibody (1:1000) to detect total ERK1/2 or AKT expression or with anti-phosphorylated ERK1/2 antibody (1:1000) or anti-phosphorylated AKT antibody (1:1000) to detect active (phosphorylated) forms of ERK or AKT.

**Analysis of Invasion**—Invasive activity was quantified using a Boyden Chamber (8- $\mu$ m pore size) coated with Matrigel (10  $\mu$ g). Cells ( $2 \times 10^5$ ) were added to the chamber in 500  $\mu$ l of serum-free medium with 25  $\mu$ g/ml of E-cadherin function-blocking antibody (HECD-1 clone) or isotype-specific control antibody, followed by incubation for 40 h. Nonmigrating cells were removed from the upper chamber with a cotton swab; filters were fixed and stained with Diff-Quik Stain, and migrating cells adherent to the underside of the filter were enumerated using an ocular micrometer and counting a minimum of 10 high-powered fields. Data are expressed as relative migration (number of cells/field). In selected experiments, the proteinase dependence of invasion was determined by quantifying invasion in the presence of the MMP inhibitor GM6001 (2.5  $\mu$ M) (Chemicon) or the function-blocking anti-uPA antibody (15  $\mu$ g/ml) (American Diagnostica, clone 394).

## RESULTS

**Calcium-dependent Cell-Cell Adhesion Modulates Proteinase and Inhibitor Expression**—E-cadherin dependent cell-cell adhesion is important for the maintenance of epithelial structural integrity, and the loss of E-cadherin expression has been shown to correlate with increased invasive potential of both carcinoma cell lines and human tumor samples (21, 22, 27, 35). Recent data demonstrate that specific proteinases, including tumor-associated MMPs, can modulate cell-cell adhesion by cleaving E-cadherin (36–38). As E-cadherin itself couples to signal transduction pathways (18–21), the hypothesis that E-cadherin may participate in proteinase regulation was tested in premalignant gingival keratinocytes (pp126 cells). The calcium switch method, in which E-cadherin-mediated cell-cell adhesion was disrupted with EGTA treatment and restored by replacing  $Ca^{2+}$ , was utilized to initiate *de novo* adherens junction assembly (19–21, 31, 32). Control cells present a typical pattern of E-cadherin staining at the level of cell-cell contacts (Fig. 1A); however, in cells treated with EGTA, E-cadherin is absent from cell junctions (Fig. 1B). After addition of calcium, adherens junctions are again formed (Fig. 1C), with complete restoration by 1 h of treatment (Fig. 1D). To evaluate the effect of E-cadherin activation on proteinase expression, after 24–36 h conditioned media were collected and analyzed. Gelatin zymography demonstrated that calcium-mediated cell-cell adhesion (designated *E-cad Actn* +) decreased relative MMP-9 levels (Fig. 1E). This was confirmed using an ELISA kit that recognizes both free and TIMP-1-complexed MMP-9, demonstrating a 6.5-fold decrease in MMP-9 levels in concentrated conditioned media following calcium-mediated cell-cell adhesion (Fig. 1E). Concomitant with MMP-9 down-regulation, *net* uPA activity was also decreased by 2–3-fold (Fig. 1F). Evaluation of proteinase inhibitor expression by ELISA indicated a coordinate decrease in both TIMP-1 and PAI-1 levels (Table I). Disruption of cell-cell junctions using the calcium switch method did not affect cell proliferation (data not shown).

**Proteinase Suppression Following Cell-Cell Adhesion Requires Engagement of E-cadherin**—To confirm that proteinase suppression is due to E-cadherin engagement and is not an unrelated consequence of calcium modulation, a function-blocking anti-E-cadherin antibody (HECD-1 clone) was utilized. To verify that the HECD-1 antibody blocked cell-cell attachment following the calcium switch protocol, cells were treated with EGTA to dissociate cell-cell junctions and then incubated for 40 min in calcium-containing medium in the presence of either function-blocking E-cadherin antibody (HECD-1, 10  $\mu$ g/ml) or isotype-matched control IgG. Cells were then processed for immunofluorescence microscopy using rat anti-E-cadherin antibody (DECMA clone). Similar to the control experiment shown in Fig. 1B, no E-cadherin staining is visible at cell-cell junctions following EGTA treatment (Fig. 2A). Following calcium restoration, junctional E-cadherin is prevalent in IgG-treated cells (Fig. 2B), but not in anti-E-cadherin-treated cells (Fig. 2C), demonstrating that the antibody indeed blocks cell-



**FIG. 1. Calcium-mediated cell-cell adhesion decreases proteinase expression.** A–D, pp126 cells were left untreated (A) or treated with 4 mM EGTA for 30 min (B–D). To induce E-cadherin activation, the EGTA-containing medium was then replaced with calcium-containing Keratinocyte-SFM (0.09 mM) for 30 min (C) or 1 h (D). Cells were fixed, incubated with anti-E-cadherin antibody (HECD-1), and detected with Alexa Fluor 594-conjugated anti-mouse antibody. E, E-cadherin was activated (designated *E-cad Actn* +) in pp126 cells using the calcium switch method as described under “Experimental Procedures.” Control cells (designated *E-cad Actn* -) were left untreated. Conditioned media were collected at 36 h and analyzed for MMP-9 expression by gelatin zymography and by ELISA as described under “Experimental Procedures.” F, uPA activity was analyzed in the conditioned media at 24 h using a coupled colorimetric plasminogen activation assay as described under “Experimental Procedures.” The results represent the mean  $\pm$  S.E. of five different experiments. \*, significantly different from control with  $p < 0.001$ .

TABLE I

*Calcium-mediated cell-cell attachment suppresses TIMP-1 and PAI-1*

E-cadherin (E-cad)-mediated cell-cell adhesion was disrupted by incubating pp 126 cells with 4 mM EGTA for 30 min and then re-initiated by replacing the medium with calcium-containing keratinocyte-SFM (0.09 mM, designated E-cad activation +). Conditioned media were collected after 24–36 h, and TIMP-1 and PAI-1 levels were analyzed by ELISA according to the manufacturer’s specifications. The results represent the mean  $\pm$  S.E. of three individual experiments.

E-cad activation	TIMP-1	PAI-1
	<i>ng/ml</i>	
-	72 $\pm$ 3	40 $\pm$ 2
+	45 $\pm$ 3 <sup>a</sup>	16 $\pm$ 1 <sup>b</sup>

<sup>a</sup> Value significantly different from control with  $p < 0.005$ .

<sup>b</sup> Value significantly different from control with  $p < 0.001$ .

cell attachment. To assess the effect of E-cadherin function blocking antibodies on proteinase suppression, cell-cell junctions were disrupted by calcium chelation, and junction assembly was initiated by the addition of calcium in the presence of

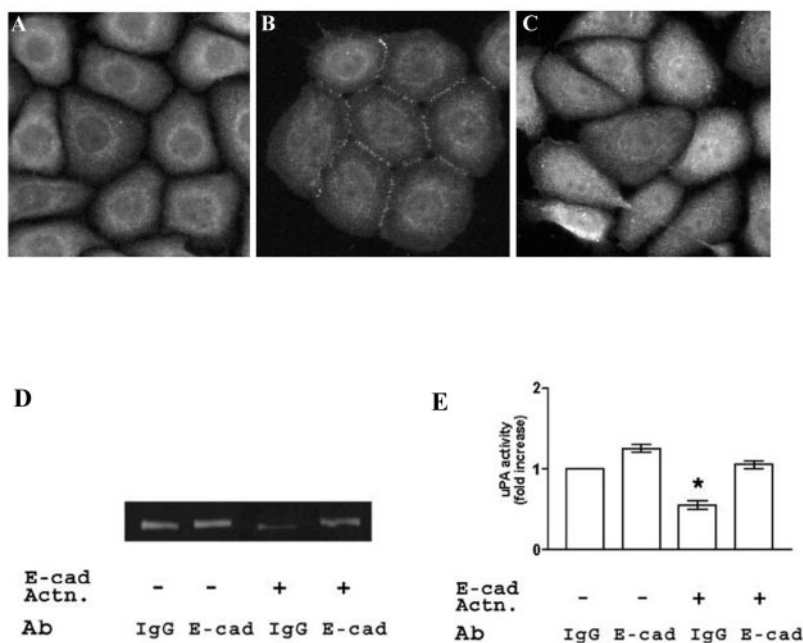


FIG. 2. **Proteinase suppression following cell adhesion requires engagement of E-cadherin.** A–C, pp126 cells were treated with 4 mM EGTA for 30 min (A–C). The EGTA-containing medium was replaced with Keratinocyte-SFM containing 0.09 mM calcium for 45 min in the presence of control IgG (10  $\mu$ g/ml) (B) or function-blocking anti-E-cadherin antibody (HECD-1, 10  $\mu$ g/ml) (C). Cells were fixed, incubated with anti-E-cadherin antibody (DECMA), and detected with Alexa Fluor 594-conjugated anti-rat antibody. D and E, cells were either left untreated or underwent activation of E-cadherin using the calcium switch method in the presence of function-blocking anti-E-cadherin antibody (Ab) (HECD-1, 10  $\mu$ g/ml) or isotype-matched IgG (10  $\mu$ g/ml) as described under “Experimental Procedures.” D, conditioned media were collected at 36 h and analyzed for MMP-9 activity by gelatin zymography. E, uPA activity was analyzed in the conditioned media at 24 h using a coupled colorimetric plasminogen activation assay as described under “Experimental Procedures.” The results represent the mean  $\pm$  S.E. of three different experiments. \*, significantly different from control with  $p < 0.01$ .

blocking antibody (10  $\mu$ g/ml) to prevent formation of adherens junctions. Control samples contained an equal concentration of isotype-specific IgG. In additional controls, antibodies were added without prior disruption of cell-cell junctions by calcium chelation (designated *E-cad Actn* –). Blocking E-cadherin engagement prevented the adhesion-mediated suppression of both the relative MMP-9 levels (Fig. 2D, 4th lane) and the net uPA activity (Fig. 2E). Addition of the function-blocking HECD-1 antibody at 10  $\mu$ g/ml without prior junction disruption (designated *E-cad Actn* –) was ineffective (Fig. 2, D, 2nd lane, and E). Moreover, in IgG-treated control samples, in which junction reformation was not prevented, proteinase expression was suppressed (Fig. 2, D, 3rd lane, and E). Similar results were obtained for TIMP-1 and PAI-1, in which suppression of expression by E-cadherin engagement was also blocked by anti-E-cadherin antibody (Table II). In control experiments, treatment of cells with EGTA in the presence or absence of blocking antibody did not alter proliferation (data not shown). Together these data demonstrate that *de novo* E-cadherin engagement suppresses proteinase expression.

Because uPA activity can also be modulated by cell surface association (9), the effect of E-cadherin-mediated cell-cell adhesion on uPAR expression was evaluated. Cell-cell junctions were disrupted by calcium chelation, and the samples were treated with either HECD-1 blocking antibody or control IgG as described above. Cells were then incubated with cell-impermeable NHS-biotin to label surface proteins and lysed in modified RIPA buffer. Following precipitation of surface-labeled proteins with streptavidin beads, samples were electrophoresed and probed for uPAR by immunoblotting. There was no change in uPAR surface expression induced by E-cadherin-mediated cell-cell adhesion (Fig. 3). In addition, there was no change in total cellular uPAR protein levels (surface and cytoplasmic) as measured by Western blot (data not shown). These data sug-

TABLE II  
Suppression of TIMP-1 and PAI-1 following cell adhesion requires E-cadherin

E-cadherin (E-cad)-mediated cell-cell adhesion was disrupted by incubating pp 126 cells with 4 mM EGTA for 30 min and then re-initiated by replacing the medium with calcium-containing keratinocyte-SFM (0.09 mM, designated E-cad activation +) in the presence of anti-E-cadherin antibody (HECD-1, 10  $\mu$ g/ml) or isotype-matched IgG (10  $\mu$ g/ml). Conditioned media were collected after 24–36 h, and TIMP-1 and PAI-1 levels in the conditioned media were analyzed by ELISA according to the manufacturer’s specifications.

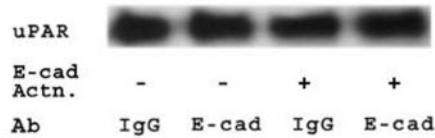
Antibody	E-cad activation	TIMP-1	PAI-1
		ng/ml	
IgG	–	94 $\pm$ 6	25 $\pm$ 2
E-cad	–	83 $\pm$ 5	23 $\pm$ 2
IgG	+	46 $\pm$ 3 <sup>a</sup>	13 $\pm$ 1 <sup>b</sup>
E-cad	+	70 $\pm$ 5	21 $\pm$ 1

<sup>a</sup> Values significantly different from control with  $p < 0.001$ .

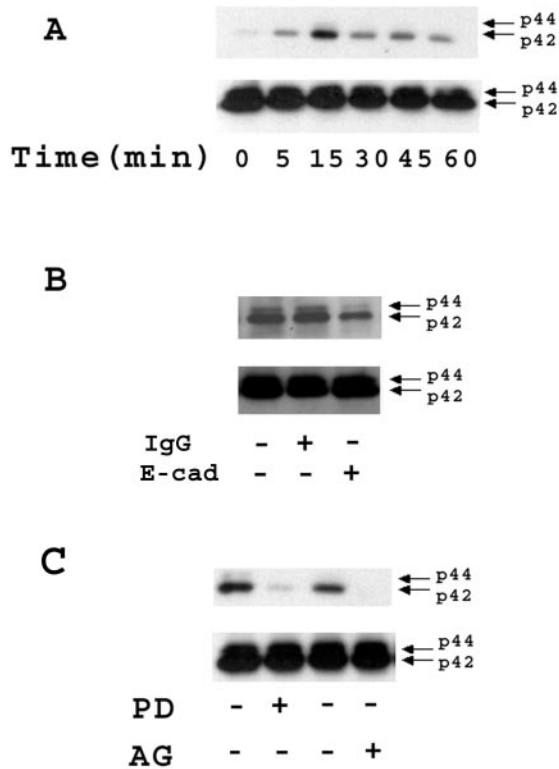
<sup>b</sup> Values significantly different from control with  $p < 0.01$ .

gest that, although the net uPA activity is suppressed, the receptor remains available.

**Inhibition of MAPK Does Not Block E-cadherin-mediated Suppression of Proteinases**—Because we have reported previously (30) that MAPK activation regulates proteinase expression in pp126 cells, levels of phosphorylated (active) ERK1/2 were assessed in pp126 cells following the calcium switch. Cells were lysed at various time points after calcium-induced initiation of junction assembly, and samples were analyzed by Western blotting using antibodies directed against total ERK1/2 or the phosphorylated (active) species of ERK 1/2. A time-dependent phosphorylation of ERK1/2 following *de novo* engagement of E-cadherin was observed in pp126 cells (Fig. 4A), with maximal MAPK activation at  $\sim$ 15 min. There was no change in the total amount of ERK1/2 protein (Fig. 4A). To confirm that

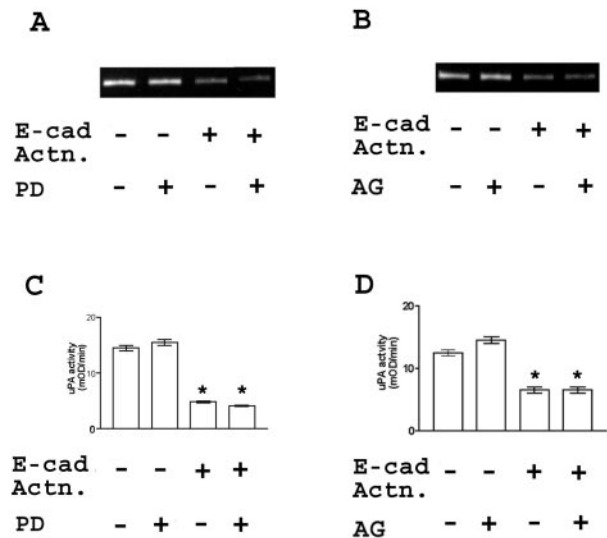


**FIG. 3. E-cadherin-mediated cell-cell adhesion does not affect surface uPAR expression.** Cells were either left untreated (designated *E-cad Actn.* -) or underwent activation of E-cadherin (designated *E-cad Actn.* +) using the calcium switch method in the presence of function-blocking anti-E-cadherin antibody (*Ab*) (HECD-1, 10  $\mu$ g/ml) or isotype-matched IgG (10  $\mu$ g/ml) as described under "Experimental Procedures." After 24 h the cells were surface-biotinylated and lysed. Samples were immunoprecipitated with streptavidin beads to isolate cell-surface proteins and electrophoresed on a 9% SDS-polyacrylamide gel. The membranes were immunoblotted with anti-uPAR antibody followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection.



**FIG. 4. E-cadherin-mediated adhesion enhances EGFR-mediated MAPK activity.** *A*, using the calcium switch method, E-cadherin was activated in pp126 cells. The cells were lysed at the indicated times following E-cadherin activation. The lysates were separated by SDS-PAGE (9% gels), transferred to polyvinylidene difluoride membrane, and probed with anti-phospho-ERK1/2 antibody to detect the phosphorylated, active form of ERK (*upper panel*) or with anti-ERK1/2 antibody to detect total ERK1/2 expression (*lower panel*). *B*, pp126 cells underwent activation of E-cadherin using the calcium switch method in the presence of function-blocking anti-E-cadherin antibody (HECD-1, 10  $\mu$ g/ml) or isotype-matched IgG (10  $\mu$ g/ml) as described under "Experimental Procedures." The cells were lysed at 15 min after calcium restoration. The lysates were analyzed for phospho-ERK (*upper panel*) or for total ERK expression (*lower panel*). *C*, pp126 cells were treated with the MEK inhibitor, PD98059 (PD, 5  $\mu$ M), EGFR inhibitor, AG1478 (AG, 250 nM), or with an equal amount of Me<sub>2</sub>SO vehicle as control at the time of disruption of cell-cell junctions as described above. The cells were lysed at 15 min after calcium restoration. The lysates were analyzed for phospho-ERK (*upper panel*) or for total ERK expression (*lower panel*). The results are representative of three independent experiments.

MAPK phosphorylation was a specific consequence of E-cadherin engagement, junction assembly was initiated in the presence of the function-blocking anti-E-cadherin antibody or con-

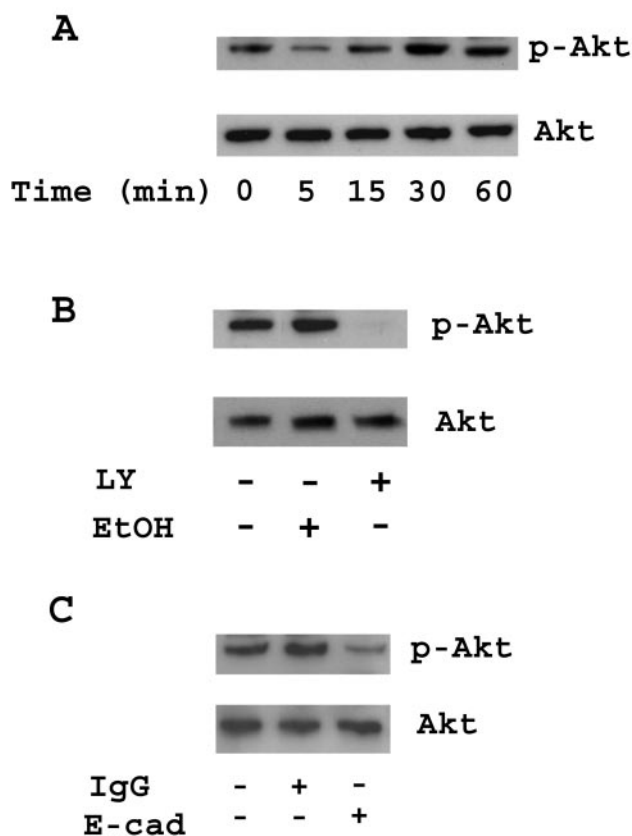


**FIG. 5. EGFR-MAPK pathway is not involved in E-cadherin-mediated suppression of proteinases.** Serum-starved pp126 cells were either left untreated (designated *E-cad Actn.* -) or underwent activation of E-cadherin (designated *E-cad Actn.* +) using the calcium switch method in the presence of 5  $\mu$ M PD98059 (PD), 250 nM AG1478 (AG), or an equal volume of Me<sub>2</sub>SO as described under "Experimental Procedures." *A* and *B*, conditioned media were collected at 36 h and analyzed for MMP-9 activity by gelatin zymography. *C* and *D*, uPA activity was analyzed in the conditioned media at 24 h using a coupled colorimetric plasminogen activation assay. The results represent the mean  $\pm$  S.E. of three different experiments. \*, significantly different from control with  $p < 0.005$ .

trol IgG as described above. Inhibition of E-cadherin engagement decreased ERK1/2 phosphorylation, indicating that engagement of E-cadherin leads to MAPK activation (Fig. 4B). Moreover, E-cadherin-mediated MAPK activation was blocked with the MEK inhibitor PD98059, demonstrating the involvement of the MEK-ERK pathway (Fig. 4C). Interestingly, the epidermal growth factor receptor (EGFR)-specific tyrosinase inhibitor AG1478 also completely abrogated E-cadherin-mediated MAPK activation (Fig. 4C), supporting the observation that E-cadherin-mediated activation of MAPK is dependent on EGFR (20).

By using the MEK inhibitor PD98059, the role of MAPK in the E-cadherin mediated suppression of proteinases was evaluated. Cells were incubated with EGTA and 5  $\mu$ M PD98059 or an equal amount of Me<sub>2</sub>SO vehicle for 30 min before the addition of calcium-replete medium containing either 5  $\mu$ M PD98059 or Me<sub>2</sub>SO. Under basal conditions (designated *E-cad Actn.* -), PD98059 did not affect the relative MMP-9 levels or the net uPA activity (Fig. 5, *A*, 2nd lane, and *C*). However following E-cadherin activation by EGTA treatment and calcium restoration, inhibition of MEK activity did not prevent the suppression of proteinase expression (Fig. 5, *A*, 4th lane, and *C*). Similar results were obtained with TIMP-1 and PAI-1 (data not shown).

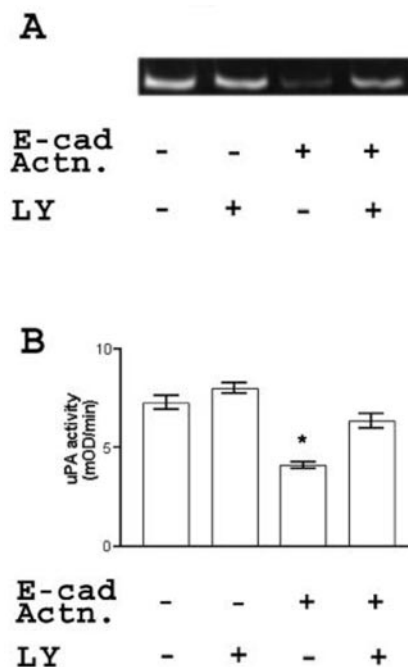
To evaluate further the role of MAPK in the control of E-cadherin-regulated proteinase expression, the EGFR kinase-specific inhibitor tyrosinase AG1478 was employed. Cells were treated with EGTA followed by calcium restoration in the presence of AG1478 (250 nM). Under basal conditions (designated *E-cad Actn.* -) AG1478 (250 nM) did not affect MMP-9 or uPA expression (Fig. 5, *B*, 2nd lane, and *D*). Similar to the results obtained with PD98059, specific inhibition of the EGFR kinase also failed to abrogate the suppressive effect of E-cadherin engagement on the relative MMP-9 levels or the net uPA activity (Fig. 5, *B*, 4th lane, and *D*). Together these data demonstrate that, although E-cadherin engagement can activate



**FIG. 6. E-cadherin-mediated adhesion induces PI3-kinase mediated AKT activity.** *A*, using the calcium switch method, E-cadherin was activated in pp126 cells. The cells were lysed at the indicated times following E-cadherin activation. The lysates were separated by SDS-PAGE (9% gels), transferred to polyvinylidene difluoride membrane, and probed with an antibody that specifically recognizes phosphorylation on Thr-308 (phospho-AKT). The membrane was then reprobed with an antibody against total AKT. *B*, pp126 cells were treated with the PI3-kinase inhibitor, LY294002 (LY, 10  $\mu$ M), or an equal volume of ethanol for 30 min at the time of disruption of cell-cell junctions as described above. The cells were lysed at 30 min after calcium restoration and analyzed for phospho-AKT (upper panel) and total AKT (lower panel) as described above. *C*, pp126 cells were either left untreated or underwent activation of E-cadherin using the calcium switch method in the presence of function-blocking anti-E-cadherin antibody (HECD-1, 10  $\mu$ g/ml) or isotype-matched IgG (10  $\mu$ g/ml) as described under "Experimental Procedures." The cells were lysed at 30 min after calcium restoration and analyzed for phospho-AKT or for total AKT expression as described above. The results are representative of three independent experiments.

MAPK in pp126 cells via a mechanism involving the EGFR kinase, this signaling pathway does not modulate E-cadherin regulation of proteinase expression.

**Inhibition of PI3-kinase Blocks E-cadherin-mediated Suppression of Proteinases**—Formation of *de novo* E-cadherin-mediated cell-cell contact activates PI3-kinase (19–21, 31, 32, 39–41) and induces physical association of PI3-kinase with E-cadherin (see Refs. 19 and 21 and data not shown). To determine whether PI3-kinase activation may play a role in the E-cadherin regulation of proteinase expression, activation of PI3-kinase was assessed in pp126 cells following calcium-induced adherens junction assembly by evaluating activation (phosphorylation) of the downstream substrate AKT. Cells were lysed at various time points and samples analyzed by Western blotting using antibodies directed against total AKT or the phosphorylated (active) species. A time-dependent phosphorylation of AKT following E-cadherin activation was observed in pp126 cells (Fig. 6A, upper panel) with no change in

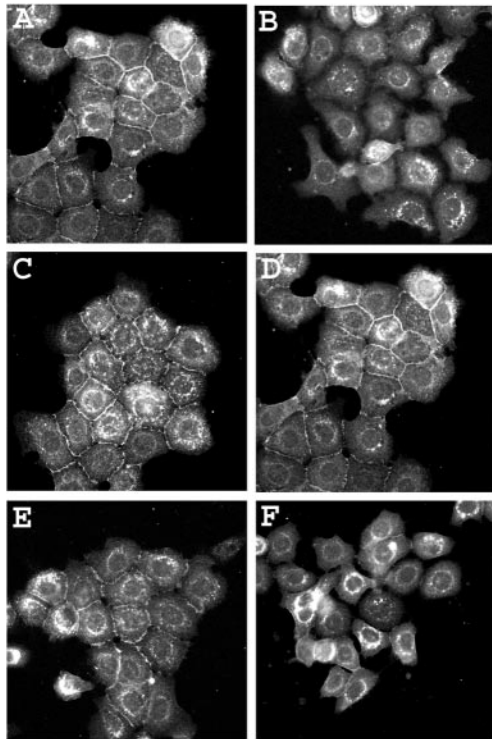


**FIG. 7. Inhibition of PI3-kinase blocks E-cadherin-mediated suppression of proteinases.** Serum-starved pp126 cells were either left untreated (designated *E-cad Actn.* -) or underwent activation of E-cadherin (designated *E-cad Actn.* +) using the calcium switch method in the presence of 10  $\mu$ M LY294002 (LY) or an equal volume of ethanol for 30 min as described under "Experimental Procedures." *A*, conditioned media were collected at 36 h and analyzed for MMP-9 activity by gelatin zymography. *B*, uPA activity was analyzed in the conditioned media at 24 h using a coupled colorimetric plasminogen activation assay. The results represent the mean  $\pm$  S.E. of three different experiments. \* significantly different from control with  $p < 0.05$ .

total AKT protein (Fig. 6A, lower panel). AKT activation was blocked with the PI3-kinase inhibitor LY294002, demonstrating the involvement of the PI3-kinase-AKT pathway (Fig. 6B). To confirm that AKT activation resulted from E-cadherin engagement, junction assembly was initiated in the presence of the function-blocking anti-E-cadherin antibody or control IgG as described above. Inhibition of E-cadherin engagement decreased AKT activation (Fig. 6C), indicating that engagement of E-cadherin leads to PI3-kinase activation.

By using the inhibitor LY294002, the role of PI3-kinase in E-cadherin-mediated suppression of MMP-9 and uPA expression was evaluated. Cells were preincubated with EGTA and LY294002 (10  $\mu$ M) or an equal amount of ethanol vehicle for 30 min before the addition of calcium-replete medium containing LY294002 or ethanol. Under control conditions when the adherens junctions were not disrupted with EGTA treatment (designated *E-cad Actn.* -), LY294002 did not alter the relative MMP-9 levels or the net uPA activity (Fig. 7, A, 2nd lane, and B). In EGTA-treated cells, calcium-induced engagement of E-cadherin decreased the relative MMP-9 levels and the net uPA activity, as demonstrated previously (Fig. 7, A, 3rd lane, and B). However, concomitant treatment with LY294002 abrogated the E-cadherin-mediated proteinase suppression and restored the relative MMP-9 levels and the net uPA activity (Fig. 7, A, 4th lane, and B), implicating PI3-kinase in E-cadherin-mediated proteinase regulation. Similar results were obtained with TIMP-1 and PAI-1 (data not shown).

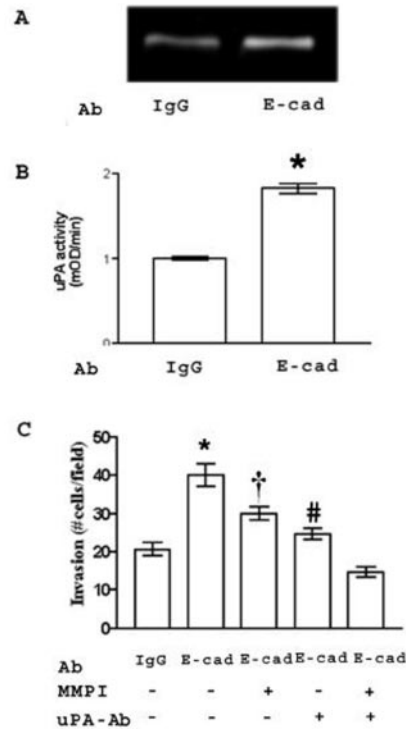
**PI3-Kinase Controls Integrity of Adherens Junctions in pp126 Cells**—Recent data indicate that PI3-kinase can regulate either the assembly or maintenance of adherens junctions (21, 32). As both PI3-kinase inhibition (using LY294002) and prevention of E-cadherin junction formation (with blocking an-



**FIG. 8. PI3-kinase controls the integrity of adherens junctions in pp126 cells.** Serum-starved pp126 cells were either left untreated (A) or treated with 4 mM EGTA for 30 min (B–F) containing either  $\text{Me}_2\text{SO}$  as control (A–C), 5  $\mu\text{M}$  PD98059 (D), 250 nM AG1478 (E), or 10  $\mu\text{M}$  LY294008 (F). The EGTA containing medium was then replaced with Keratinocyte-SFM (0.09 mM  $\text{Ca}^{2+}$ ) containing  $\text{Me}_2\text{SO}$  or the corresponding inhibitor for 45 min. Cells were fixed, incubated with anti-E-cadherin antibody (HECD-1), and detected with Alexa Fluor 594-conjugated anti-mouse antibody.

tibodies) restored proteinase expression, these data suggest that PI3-kinase may participate in regulation of E-cadherin junctions in pp126 cells. To test this hypothesis, the effect of PI3-kinase inhibition (10  $\mu\text{M}$  LY294002) on junction formation was evaluated. Control experiments included inhibitors of EGFR tyrosine kinase (250 nM AG1478) or MEK (5  $\mu\text{M}$  PD98059). Cells were pre-incubated with EGTA in the presence of inhibitor or  $\text{Me}_2\text{SO}$  vehicle for 30 min before the addition of calcium-replete medium containing the specific inhibitor or  $\text{Me}_2\text{SO}$ . After 45 min, cells were processed for immunofluorescence microscopy using anti-E-cadherin antibody. Control cells present a typical pattern of E-cadherin staining at the level of cell-cell contacts (Fig. 8A). After 30 min of treatment with EGTA, E-cadherin was absent from sites of cell-cell contact (Fig. 8B). Following calcium addition, E-cadherin-mediated adherens junctions were again formed at 45 min (Fig. 8C). Similar results were observed in cells treated with PD98059 or AG1478 (Fig. 8, D and E). In contrast, cells treated with LY294002 showed significantly reduced E-cadherin staining at sites of cell-cell contact (Fig. 8F), indicating that PI3-kinase participates in the formation of *de novo* E-cadherin-mediated adherens junctions in pp126 cells.

**Prevention of Cell-Cell Adhesion Enhances pp126 Cell Invasion**—To assess the functional consequences of E-cadherin-regulated proteinase expression, the impact of preventing cell-cell adhesion on cellular invasive activity was evaluated. pp126 cells were seeded into Boyden chambers overlaid with Matrigel to provide a three-dimensional, protein-rich barrier to invasion in the presence of E-cadherin blocking antibody (HECD-1) or control IgG. Prevention of *de novo* E-cadherin cell-cell contacts resulted in an increase in the relative MMP-9 levels and the net



**FIG. 9. Disruption of cell-cell adhesion enhances pp126 cell invasion.** pp126 cells were plated with either control antibody (Ab) (IgG) or anti-E-cadherin antibody (HECD-1, designated *E-cad*). A, conditioned media were collected at 36 h and analyzed for MMP-9 activity by gelatin zymography. B, uPA activity was analyzed in the conditioned media at 24 h using a coupled colorimetric plasminogen activation assay. \*, significantly different from control with  $p < 0.001$ . C, cells ( $2 \times 10^5/500 \mu\text{l}$ ) were added to porous polycarbonate filters (8- $\mu\text{m}$  pore) coated with Matrigel (10  $\mu\text{g}$ ) in the presence 25  $\mu\text{g}/\text{ml}$  of control antibody (IgG) or anti-E-cadherin antibody (HECD-1, designated *E-cad*) for 40 h. In wells containing E-cadherin antibody, either MMP inhibitor, GM6001 (2.5  $\mu\text{M}$ ), function-blocking uPA antibody (American Diagnostica number 394, 15  $\mu\text{g}/\text{ml}$ ), or both were added. Nonmigrating cells were removed from the upper chamber, and filters were fixed and stained, and invading cells were enumerated using an ocular micrometer. The results represent the mean  $\pm$  S.E. of three different experiments. \*, significantly different from IgG treated cells with  $p < 0.001$ . †, significantly different from E-cadherin antibody treated cells with  $p < 0.05$ . #, significantly different from E-cadherin antibody treated cells with  $p < 0.01$ .

uPA activity (Fig. 9, A and B). Concomitant with enhanced proteinase expression, inhibition of junction formation significantly increased Matrigel invasion (Fig. 9C). The enhanced invasive activity was partially blocked with either a broad spectrum MMP inhibitor (2.5  $\mu\text{M}$  GM6001) or by a function-blocking anti-uPA antibody (15  $\mu\text{g}/\text{ml}$ ) and was completely abrogated by a mixture of the two inhibitors (Fig. 9C), implicating both MMP-9 and uPA in Matrigel invasion by pp126 cells. These data demonstrate that the proteinase up-regulation resulting from inhibition of E-cadherin engagement can promote cellular invasive behavior.

#### DISCUSSION

Studies using multiple cancer models have demonstrated that loss of E-cadherin-mediated adherens junctions leads to increased invasion and metastases (22, 23, 27, 35, 42). Additional data suggest a correlation between E-cadherin status and proteinase levels. For example, down-regulation of E-cadherin increased MMP-9 secretion in murine skin carcinoma cell lines (43), whereas overexpression of E-cadherin decreased MMP-2 activity in prostate cancer cells (26) and MT1-MMP in squamous cancer cells (44). However a mechanistic examina-

tion of the potential functional link between cell-cell adhesion and proteolysis has not been reported. Our current data demonstrate that E-cadherin plays a direct role in proteinase regulation in premalignant oral keratinocytes. Initiation of *de novo* E-cadherin-mediated cell-cell junctions resulted in suppression of MMP-9 and uPA expression. Conversely, prevention of junction formation enhanced proteinase expression and consequent cellular invasive behavior, suggesting a biochemical mechanism by which down-regulation of E-cadherin may promote metastasis.

The detailed signal transduction pathway through which E-cadherin regulates proteinase gene expression is unknown. However, our data support a role for PI3-kinase activity in the E-cadherin-mediated suppression of MMP-9 and uPA expression. Formation of *de novo* E-cadherin junctions activates PI3-kinase (Fig. 6) (19–21, 31, 32, 39–41) and leads to the physical association of PI3-kinase with E-cadherin (see Refs. 19 and 21 and data not shown). Activation of PI3-kinase recruits the GTP exchange factor Tiam-1 to the adherens junctions, resulting in activation of Rac-GTPase (45). In keratinocytes, Rac-GTPases play an important role in forming and stabilizing adherens junctions by recruiting F-actin to these junctions (46, 47). Supporting this model, inhibition of PI3-kinase blocks the recruitment of F-actin to sites of cell-cell contact in intestinal epithelial cells (21) and destabilizes adherens junctions in mammary epithelial cells (48). Moreover, additional data indicate that PI3-kinase may participate in maturation and maintenance of cadherin-based adhesions (32, 40), in part via regulation of productive adhesive contact formation following initial homophilic ligation (32). Our current data support the hypothesis that PI3-kinase activity also functions to regulate formation of adherens junctions in pp126 cells. Thus, inhibition of PI3-kinase activity with LY294002 destabilizes junctions, thereby abrogating the suppressive effect of E-cadherin engagement on proteinase expression.

In addition to activation of PI3-kinase, E-cadherin engagement has also been shown to enhance MAPK activity through the recruitment and activation of EGFR (20). A similar effect was observed in the current study, wherein junction formation induced maximal MAPK activation in pp126 cells at 15 min. MAPK activation was completely abrogated with the EGFR kinase inhibitor AG1478, implicating EGFR signaling in MAPK activation. However, inhibition of MEK (with PD98059) or EGFR kinase (with AG1478) was insufficient to restore proteinase expression, suggesting that E-cadherin-mediated proteinase regulation does not involve EGFR-initiated MAPK signaling. Furthermore, inhibition of MEK and EGFR kinase activity at the time of calcium switch did not prevent adherens junction re-formation in pp126 cells. It should be noted, however, that the MAPK pathway is important in both growth factor-induced secretion of MMP-9 in carcinoma cells (49) and in integrin-mediated up-regulation of uPA in pp126 cells (30). These data support the hypothesis that the signaling pathways that regulate formation of adherens junctions (PI3-kinase) may also regulate cadherin-mediated suppression of proteinases. This effect may be, in part, due to sequestration of  $\beta$ -catenin at cell-cell junctions as part of E-cadherin-catenin complex (50, 51).  $\beta$ -catenin can translocate to the nucleus and form a complex with proteins of the T cell factor/lymphoid-enhancer factor family (52). T cell factor/lymphoid-enhancer factor proteins act as transcription factors and have been shown to activate genes that are important in cancer progression including MMP-7, MMP-26, and uPAR (50, 51, 53–58). A number of other MMP promoters, including MMP-9, have T cell factor 4-binding sites and, consequently, may be regulated by  $\beta$ -catenin (54, 57). Although we have not formally addressed the potential contri-

bution of  $\beta$ -catenin signaling, no change in uPAR expression was observed following E-cadherin activation in our system, suggesting that additional mechanisms of proteinase regulation are engaged. Thus, it is interesting to speculate that net extracellular proteinase activity may result from a balance between signaling pathways differentially activated by engagement of cell-cell *versus* cell-matrix adhesion molecules.

In addition to enhanced proteinase expression (uPA, MMP-9) induced by E-cadherin disruption, a coordinate increase in the corresponding inhibitors (PAI-1, TIMP-1) was also observed. Although a coordinate regulation of uPA and PAI-1 is apparent, a *net* increase in uPA activity is obtained following E-cadherin disruption, and this functionally contributes to the increased Matrigel invasion. Similarly, the Matrigel invasion data also suggest that a net increase in MMP-9 activity is also likely. It has been shown previously (24) in T47D and MCF-7 breast cancer cells that disruption of cell junctions with anti-E-cadherin antibodies increases uPA expression and collagen invasion. The enhanced collagen invasion was partially blocked using anti-catalytic uPA antibodies; however, the effect of MMP inhibitors was not evaluated (24). The current data demonstrate that Matrigel invasion by pp126 cells is dependent on both uPA and MMP-9, as inhibiting both proteinases simultaneously completely abrogated the increase in Matrigel invasion by pp126 cells.

Recent studies (36–38) have shown that proteinase expression may regulate cell-cell junction integrity by cleaving E-cadherin. Conversely, our data demonstrate that E-cadherin participates in proteinase regulation via a PI3-kinase-dependent mechanism, providing novel evidence for a bi-directional communication between proteinases and cadherins. As proteinases play a central role in a number of important cellular processes, these findings may provide a framework for a more detailed understanding of the mechanism by which E-cadherin-mediated cell-cell contacts regulate both normal epithelial cell behavior and the invasiveness of carcinoma cells.

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