

Phosphatidylinositol 3-Kinase Activity in Epidermal Growth Factor-stimulated Matrix Metalloproteinase-9 Production and Cell Surface Association¹

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ABSTRACT

Activation of the epidermal growth factor (EGF) receptor regulates many processes associated with metastasis, including modulation of cell:cell and cell:substrate interactions, production of matrix-degrading proteases, and cellular migration. We have demonstrated previously that EGF stimulates migration and matrix metalloproteinase (MMP)-9-dependent invasion of ovarian cancer cells. In this study, we compare the roles of EGF-induced phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) activities in regulation of cellular responses associated with ovarian tumor cell metastasis. Inhibition of PI3K and MAPK activity impairs EGF-stimulated cell migration, *in vitro* invasion, and MMP-9 production. PI3K activity is not required for growth factor disruption of cell:cell junctions, whereas inhibitors of extracellular signal-regulated kinase (ERK)1/ERK2 activation and p38 MAPK activity block EGF-dependent junction dissolution. EGF promotes pro-MMP-9 binding to the cell surface through a mechanism that is independent of extracellular enzyme concentration. Interestingly, inhibition of PI3K activity abolishes EGF-induced cell surface association of pro-MMP-9, whereas inhibitors of MAPKs only partially block the response. These data suggest that EGF receptor activation promotes a PI3K-dependent induction of a cell surface pro-MMP-9 binding component that may facilitate gelatinase-mediated cellular invasion and supports an expanded role for elevated PI3K activity in cellular responses associated with ovarian tumor metastasis. In addition, our findings support the hypothesis that divergent kinase activities regulate distinct cellular events associated with growth factor-induced invasion of ovarian cancer cells.

INTRODUCTION

Overexpression of EGF³ receptor by ovarian tumors is associated with a poor patient prognosis (1–3). Elevated levels of EGF receptor ligands have been detected in tumors, ascites fluid, and urine of ovarian cancer patients (2–6), suggesting that receptor overexpression studies may underestimate the contribution of EGF receptor signaling to ovarian cancer pathologies. EGF stimulates proliferation of both normal ovarian epithelial cells and ovarian cancer cells (7–9), and reduction of EGF receptor expression via antisense inhibition decreases cellular proliferation (10). These findings suggest that aberrant EGF receptor expression or activity may support clonal expansion of cells within a primary ovarian cancer lesion.

The mechanisms by which EGF receptor-dependent signaling con-

tributes to ovarian cancer metastatic potential are poorly understood. Ovarian epithelial tumor cell metastasis often involves dissociation of tumor cells from the primary lesions, dissemination and attachment to the peritoneal mesothelial cell wall, and modification of the underlying connective tissue barrier through proteolysis to promote invasion and growth (11–13). Serine protease and MMP activity generated or used by ovarian cancer cells appears to contribute to many, if not all, of these metastatic steps (13). Several lines of evidence suggest that MMP-9 is involved in tumor metastasis. Experimental findings indicate a relationship between elevated MMP-9 expression and metastatic potential *in vivo* for melanoma (14) and prostate tumors (15), and experimental metastasis is suppressed in MMP-9-deficient mice (16). In ovarian tumors, the ratio of MMP-9:MMP-2 is elevated in tumor tissue compared with normal ovaries (17), and elevated levels of MMP-9 protein have been detected in invasive epithelial ovarian carcinoma patient specimens (18). Moreover, primary ovarian tumor cells in short-term culture produce MMP-9; however, expression is lost with time in culture, indicating that extracellular factors, such as EGF, regulate ovarian cancer cell production of proteolytic activity in a paracrine fashion (19).

In support of this hypothesis, we demonstrated previously that EGF stimulates production of both urokinase-type plasminogen activator and MMP-9 in several ovarian tumor cell lines (12). Cell lines that display EGF-stimulated MMP-9 protein expression are more invasive than non-MMP-9-producing cell lines, and anticatalytic antibodies to MMP-9 strongly inhibit EGF-stimulated invasion. In this work, we investigate the contributions of several EGF receptor-mediated signaling pathways to the regulation of MMP-9 production and localization, junctional dissolution, cell migration, and *in vitro* invasion of OVCA 429 ovarian tumor cells. Our results demonstrate that MMP-9 binding to the cell surface is regulated by intracellular PI3K activity and provide mechanistic insight into the downstream effectors of EGF-stimulated ovarian tumor cell migration and invasion.

MATERIALS AND METHODS

Materials. Epidermal growth factor was purchased from Biomedical Technologies, Inc. (Stoughton, MA). BSA, protein G-agarose, and cell culture reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum and protein A-agarose were acquired from Life Technologies, Inc. (Gaithersburg, MD). Matrigel was purchased from Becton Dickinson (Bedford, PA). PD98059, SB202190, LY294002, and wortmannin were purchased from CalBiochem (La Jolla, CA) and were dissolved in DMSO. Aprotinin, pepstatin, leupeptin, and E64 were obtained from Alexis Biochemicals (San Diego, CA). Human pro-MMP-9 containing TIMP-1 and anti-MMP-9 AB805 rabbit polyclonal were acquired from Chemicon (Temecula, CA). Phospho-specific phospho p44/42 MAPK kinase (Thr-202/Tyr-204), phospho AKT (Ser-473), and phospho p38 MAP kinase (Thr-180/Tyr-82) antibodies were purchased from New England Biolabs (Beverly, MA). To control for total protein, a pan-ERK antibody was acquired from Transduction Labs (Lexington, KY), and an AKT antibody was acquired from New England Biolabs. SuperSignal chemiluminescent detection system was purchased from Pierce Corp. (Rockford, IL). Peroxidase-conjugated anti-(rabbit-IgG) was pur-

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³ The abbreviations used are: EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; JNK, c-jun NH₂-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MMP, matrix metalloproteinase; PI3K, phosphatidylinositol 3-kinase; TIMP, tissue inhibitor of metalloproteinase; CM, conditioned medium.

chased from Promega Corp. (Madison, WI). Anti-JNK and anti-p38 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [γ - 32 P]ATP was acquired from New England Nuclear (Boston, MA). The construct pGEX-2T-c-Jun (1–79 amino acids) was the kind gift of Dr. Daniel Mueller (Department of Medicine, University of Minnesota). PGEX-3X-ATF-2 was the generous gift of Dr. Benoit Dérjard (Center de Biochimie, Nice, France). GST fusion proteins were expressed and purified as described previously (20).

Cell Culture and Treatment. Ovarian carcinoma cell line OVCA 429 was generously provided by Dr. Robert Bast, Jr. (M. D. Anderson Cancer Center, Houston, TX) and grown as described previously (12). For experiments using pharmacological inhibitors, cells were pretreated for the indicated times, and every treatment condition received an equal concentration of DMSO (not in excess of 0.25% for any experiment). Incubation with inhibitors did not compromise cell viability, as determined by trypan blue exclusion. For experiments involving growth factor addition, OVCA 429 cells were placed into MEM containing 0.1% (w/v) BSA for 24 h prior to growth factor addition (12).

Western Blot Analysis and Kinase Assays. Control and treated cells were washed with ice-cold PBS and harvested in lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% w/v bromophenol blue]. Cell lysates were clarified at (10,000 \times g at 4°C for 10 min), and 10 μ g of total cell lysate were resolved by electrophoresis through 10% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA), and probed with the indicated antibodies according to the vendor's instructions and as described previously (20, 21). JNK and p38 activity was measured by the immunocomplex assay described previously (20). Substrate phosphorylation was detected by autoradiography and quantified using a Bio-Rad Model GS-700 imaging densitometer.

In Vitro Invasion and Cell Motility. Cells were plated onto Matrigel-coated well inserts (11 μ g/filter; 8 μ m pore size; Becton Dickinson, Bedford, MA) in serum-free medium and incubated at 37°C for 4 h to allow for cellular adherence and spreading on the filter. The inhibitors LY294002 (10 μ M), SB202190 (10 μ M), PD98059 (50 μ M), or DMSO vehicle control (0.25%) were then added directly to wells and incubated for an additional hour before administration of EGF (100 nM). Cells were allowed to invade through the Matrigel over a period of 72 h at 37°C under standard culture conditions. Noninvading cells were removed from the top of the wells with a cotton swab tip, followed by fixation and staining with the Diff-Quick staining kit (Fisher Scientific). All experiments were completed in triplicate, and at least 20 fields/filter were counted. Evaluation of colony dispersion (cell scattering) was performed as described previously (20). Results shown are representative of at least three independent experiments.

Zymogram Analysis. OVCA 429 cells were serum deprived for 24 h prior to growth factor treatment in fresh serum-free medium. Samples of conditioned medium collected from control and growth factor-treated cell cultures were analyzed for proteinase activity by substrate-gel zymography as described previously (12). Briefly, equal total protein from experimental samples was resolved on 8 or 10% SDS-polyacrylamide gels containing 0.1% gelatin. After electrophoresis, gels were washed with 2.5% Triton X-100 for 30 min at room temperature and then incubated with substrate buffer (50 mM Tris, 0.2 M NaCl, 5 mM CaCl₂, and 0.02% Brij, pH 7.6) 24–48 h at 37°C. Proteinase activity is visualized as zones of clearance in a Coomassie blue-stained gel. Results shown are representative of a minimum of three independent experiments.

TIMP-1 ELISA. TIMP-1 ELISA kits were obtained from Amersham (Arlington Heights, IL) and performed according to the manufacturer's protocol. Fifty μ g of protein were tested to estimate the concentration of TIMP-1 in cell lysates.

Analysis of MMP Cell Surface Association. Cells were grown to 80–90% confluency on 10-cm tissue culture dishes before switching to fresh serum-free medium. Cells were washed with PBS and given 5 ml of fresh serum-free medium containing LY294002 (10 μ M), SB202190 (10 μ M), PD98059 (50 μ M), or DMSO vehicle control (0.25% v/v). After a 1-h incubation, EGF (100 nM) was added directly to cells. In some experiments, purified human MMP-9 (80 ng/ml) was added. After a 36-h incubation, cells were washed two times with 5 ml of PBS, and then surface proteins were labeled with 2.5 ml of 500 μ g/ml sulfo-NHS Biotin (Pierce) in PBS under gentle shaking at 4°C for 30 min. Cells were washed two times in 5 ml of PBS and then incubated with 5 ml of 100 mM glycine/PBS (pH 7.2) for an additional 20 min under gentle shaking at 4°C. Cells were washed three times in 5 ml of PBS and then lysed

with 1 ml of buffered detergent [50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 1% NP40, 1 μ g/ml aprotinin, 1 μ M pepstatin, 10 μ M leupeptin, and 10 μ M E64]. Lysates were collected with a cell scraper and clarified (10,000 \times g at 4°C for 10 min). Protein concentrations were calculated using the Bio-Rad D_C protein assay kit and diluted to a concentration of 750 μ g/ml. One ml of diluted lysate was added to 40 μ l of ImmunoPure immobilized monomeric avidin gel (Pierce) in a 1.5-ml screw cap tube and incubated overnight at 4°C on a rotator. Gels were washed by centrifugation (10,000 \times g at 4°C for 30 s), followed by aspiration of supernatant and addition of 1 ml of fresh lysis buffer. Washes were repeated for a total of five times. Forty μ l of 10 mM free D-Biotin/PBS (Pierce) were added to compete bound protein overnight at 4°C. Thirty- μ l aliquots were analyzed by gelatin substrate zymography as described above.

Immunofluorescence. OVCA 429 cells were seeded in a Lab Tek II chamber slide system (Nalge Nunc International, Naperville, IL). After 1 day of growth, cultures were switched to serum-free medium. Cell chambers received 350 μ l of 10 μ M LY294002, 50 μ M PD98059, or 10 μ M SB202190 in fresh serum-free medium or DMSO load control. After 2 h, EGF was administered to chamber wells at the concentrations and times indicated in the figure legends. For detection of desmosomal junctions, cells were fixed for 2 min in ice-cold, dehydrated methanol and then blocked in 1.5% BSA in PBS containing calcium and magnesium overnight at 4°C. A chicken anti-plakoglobin antibody (ab1407; generous gift of Dr. K. Green, Northwestern University) was added at a 1:200 dilution for 1 h at 37°C. Cells were washed four times in PBS containing calcium and magnesium and then incubated with FITC-labeled goat antichick antibody (AVES Labs, Inc., Tigard, OR) at 1:500 dilution for 1 h at 37°C. For detection of adherens junctions, cells were fixed for 2 min in ice-cold methanol, incubated at 37°C for 2 h with a 1:5 dilution (PBS) of hybridoma supernatant containing anti- β catenin primary antibody clone 5H10 (generous gift of Dr. M. Wheelock, University of Toledo, Toledo, OH), washed three times with PBS, and then incubated with a 1:500 dilution of sheep antimouse secondary antibody tagged with fluorescein (Boehringer Mannheim, Indianapolis, IN) for 1 h at 37°C. For detection of cell surface-associated MMP-9, cells were fixed with 3.7% formaldehyde/PBS for 7 min and then washed two times for 5 min in PBS in a Coplin jar. Cell chambers were incubated with 100 μ l of rabbit IgG or anti-MMP-9 AB805 rabbit polyclonal (1:150) for ~4 h at 37°C and then washed three times for 5 min with PBS. Chambers were incubated with 100 μ l of a 1:250 dilution of donkey antirabbit fluorescein conjugate secondary antibody (Chemicon) for 1 h at 37°C before washing three times for 5 min with PBS. Slides were set under coverslip using fresh gelvatol (Sigma) and analyzed using a Zeiss Photomicroscope (Thornwood, NY). Images were digitized using Zeiss imaging software and compiled using Adobe Photoshop Software.

RESULTS

Stimulation of Signal Transduction Pathways by EGF. EGF stimulates the ERK, JNK, and p38 MAPK in OVCA 429 cells (Fig. 1). EGF-stimulated ERK activation in OVCA 429 cells is sustained for an extended period of time (>4 h), as has been reported in other epithelial models, including human keratinocytes (20, 22) and mammary epithelial tumor cells (23). In contrast, EGF-dependent stimulation of JNK and p38 in OVCA 429 cells is transient and maximal at 15 min after EGF addition (Fig. 1, B and C).

EGF stimulation of OVCA 429 cells promotes activation of AKT/PKB, a downstream target of PI3K (Fig. 2A). Both basal and EGF-dependent AKT/PKB activation was ablated by two mechanistically distinct pharmacological inhibitors of PI3K catalytic activity, LY294002 and wortmannin (Fig. 2A; data not shown). PI3K has been reported to be an upstream activator of both JNK and ERK in fibroblasts (24–26), thereby providing a potential mechanism for EGF-dependent JNK activation in OVCA 429 cells. To determine whether PI3K activity is involved in EGF-stimulated MAPK activation, cells were treated with LY294002 or wortmannin prior to EGF treatment. Exposure to LY294002 or wortmannin did not interfere with EGF stimulation of the ERK, JNK, or p38 MAPK cascades (Fig. 2, B–D; data not shown). This demonstrates that LY294002 is an

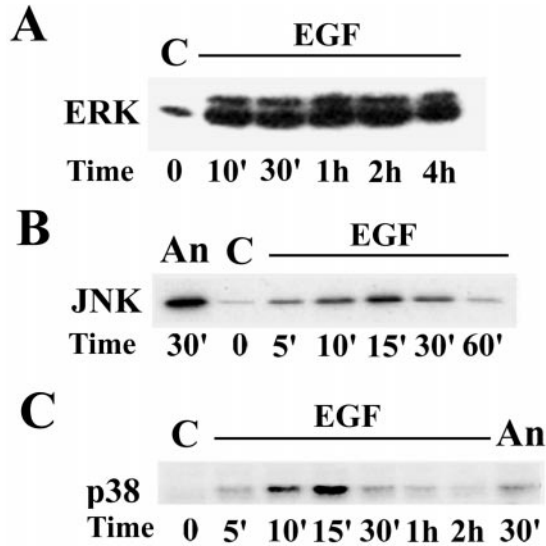


Fig. 1. Time course of MAPK activation by EGF in OVCA 429 cells. OVCA 429 cells were incubated for the indicated times with 20 nM EGF, and cell lysates were collected for analysis of MAPK activation. A, the active, dually phosphorylated forms of ERK1/2 were detected by immunoblot analysis using a phospho-specific (Thr-202/Tyr-204) monoclonal antibody (New England Biolabs) as described in "Materials and Methods." A pan-ERK antibody confirmed equal loading of cell lysates (data not shown). JNK (B) and p38 (C) activities were measured by substrate phosphorylation using GST-c-Jun (*JNK*) or GST-ATF-2 (*p38*) fusion proteins as substrates (see "Materials and Methods"). Anisomycin (*An*) was used as a positive control for activation of JNK and p38 MAPKs. 10', 10 min; 30', 30 min.

effective and selective inhibitor of PI3K activity, as reported previously (27), and that the following observations of PI3K-dependent responses in OVCA 429 cells are not attributable to indirect disruption of the MAPK signaling cascades.

Signal Transduction Requirements of EGF-dependent *in Vitro* Invasion. EGF stimulation of OVCA 429 cells promotes *in vitro* invasion through an artificial basement membrane (12). To address the signaling requirements of EGF-induced invasion, cells were pre-treated with various kinase inhibitors, and invasion through Matrigel was assessed (Fig. 3). PD98059 prevents activation of MEK1, thereby selectively inhibiting the ERK pathway (28), and SB202190 is a direct inhibitor of p38 catalytic activity (29). Inhibition of PI3K by LY294002 or wortmannin, p38 by SB202190, or ERK activation by PD98059 was sufficient to reduce invasion to near basal levels, indicating that multiple EGF-stimulated signal transduction pathways contribute to an invasive response in ovarian cancer cells.

MAPK, but not PI3K, Activity Is Required for EGF-dependent Disruption of Cell:Cell Junctions. We reported previously increased cell migration and elevated MMP-9 production as components of EGF-induced ovarian tumor cell invasion (12). EGF treatment of OVCA 429 cells promotes colony dispersion (Fig. 4E), a response that represents both increased cell migration and disruption of cell:cell adhesive junctions. Consistent with the *in vitro* invasion assay, administration of the PI3K inhibitor LY294002 or the MAPK inhibitors SB202190 or PD98059 effectively impeded cell scattering (Fig. 4, F–H). In contrast, divergence in EGF-dependent signaling requirements was observed for disruption of desmosomal and adherens junctions (Fig. 5). EGF treatment promotes redistribution of plakoglobin (γ -catenin; Fig. 5B) and β -catenin (Fig. 5G) from the cell periphery to the cytoplasm. Inhibition of PI3K activity by LY294002 prior to ligand stimulation did not alter EGF-dependent disruption of either desmosomal junctions (Fig. 5C) or adherens junctions (Fig. 5H). In contrast, inhibition of EGF-stimulated ERK and p38 activation by PD98059 or SB202190, respectively, eliminated EGF-dependent

disruption of desmosomal (Fig. 5, D and E) and adherens (Fig. 5, I and J) junctions. These data strongly implicate MAPK activity in EGF-induced junctional dissolution, whereas PI3K activity appears to play a role in other aspects of migration and invasion.

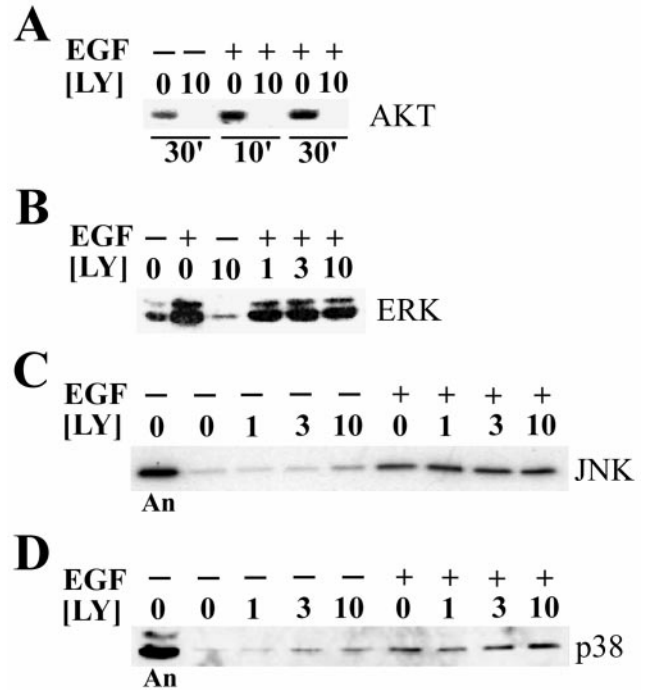


Fig. 2. Inhibition of PI3K does not interfere with MAPK activation. A, OVCA 429 cells were treated for the indicated time [10 min (10') or 30 min (30')] with (+) or without (-) EGF and in the absence or presence of the PI3K inhibitor LY294002 (10 μ M). Cell lysates were collected, and phospho-AKT/PKB was detected by immunoblot analysis using a phospho-specific monoclonal antibody (New England Biolabs). B, OVCA 429 cells were treated without (-) or with (+) EGF (20 nM) for 30 min, and ERK activation was detected as described in the legend to Fig. 1. C and D, cells were treated without (-) or with (+) EGF (20 nM) and the indicated concentrations of LY294002 [*LY*] for 15 min prior to collection. JNK (C) activity was measured as described in the legend to Fig. 1. The active, dually phosphorylated form of p38 was detected by immunoblot analysis using a phospho-specific (Thr-180/Tyr-82) polyclonal antibody (New England Biolabs) as described in "Materials and Methods." The same results were obtained using a p38 immunocomplex kinase assay (data not shown). For each experiment (A–D), inhibitor was added for 30 min before EGF addition.

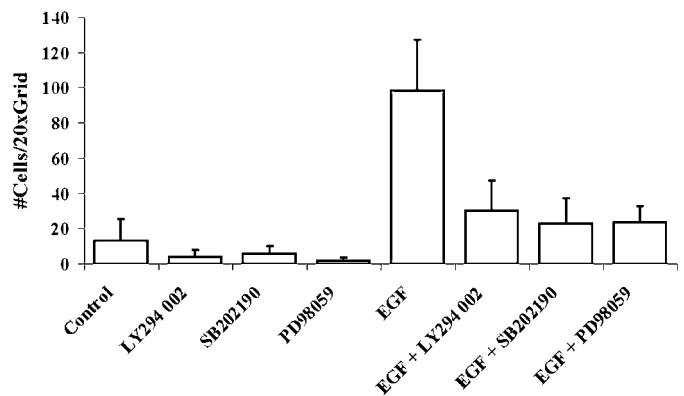


Fig. 3. Signaling requirements for EGF-stimulated OVCA 429 invasion. *In vitro* invasion of OVCA 429 cells through a reconstituted basement membrane was performed as described in "Materials and Methods." To assess signaling requirements of EGF-induced invasion, adherent cells were treated with vehicle control (0.25% v/v DMSO), the PI3K inhibitor LY294002 (10 μ M), the MEK1 inhibitor PD98059 (50 μ M), and the p38 inhibitor SB202190 (10 μ M) for 1 h prior to treatment with EGF (100 nM). The concentrations of PD98059 or SB202190 used are consistent with those reported for complete inhibition of MEK1 (28) or p38 (29) *in vitro*. Cells were allowed to invade through the Matrigel for 72 h under standard culture conditions. All invasion assays were conducted in triplicate with at least 20 fields counted/filter. Values are expressed as number of invasive cells detected with a set grid using a 20 \times objective. Bars, SD.

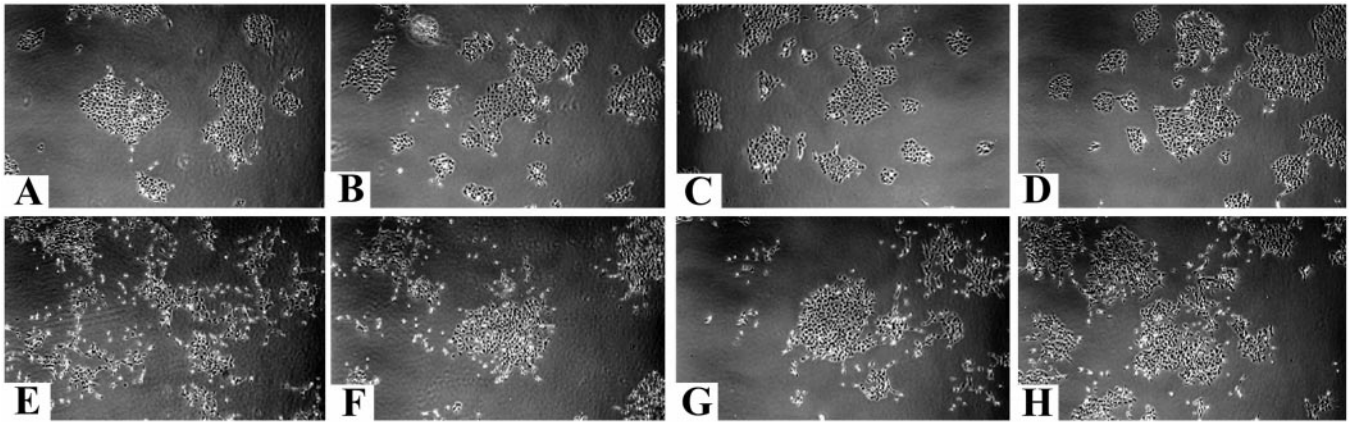


Fig. 4. Signaling requirements for EGF-stimulated colony dispersion. OVCA 429 cells were maintained in serum-free medium containing 0.1% BSA (w/v) for 24 h prior to treatment without (A–D) EGF or with (E–H) EGF (20 nM). Cells also received DMSO (0.1% v/v) as vehicle control (A and E); 10 μ M LY204002 (B and F); 50 μ M PD98059 (C and G) or 10 μ M SB202190 (D and H). All cultures were incubated for 24 h before documentation by photography. Each of the three inhibitors impaired EGF-dependent colony dispersion (compare F, G, and H with E). The results are representative of at least three independent experiments.

Multiple Signaling Cascades Regulate MMP-9 Production in OVCA 429 Cells. EGF-stimulated OVCA 429 cell invasion through modified basement membrane matrix (Matrigel) requires MMP-9 activity (12). To identify the signaling requirements for the proteolytic component of EGF-induced invasion, MMP-9 protein expression in CM was detected by gelatin zymography. Both basal and EGF-stimulated pro-MMP-9 production were inhibited by LY294002, PD98059, and SB202190 in a concentration-dependent manner (Fig. 6). These data indicate that multiple kinase pathways converge to regulate MMP-9 expression. In contrast, MMP-2 expression was refractory to EGF stimulation of OVCA 429 cells (12); however, inhibition of p38 activity in control or stimulated cells consistently depressed MMP-2 levels in CM, suggesting a role for this MAPK in basal MMP-2 production.

Requirement of ERK Activation for EGF Induction of TIMP-1 Expression. MMP expression is often coordinately regulated with production of their inhibitors, TIMPs. To evaluate signaling requirements for EGF-stimulated expression of TIMP-1, a well-characterized inhibitor of MMP-9, both CM and Triton-soluble cell lysates were analyzed by ELISA assays. EGF stimulates a 2-fold induction of TIMP-1 protein expression through an ERK-dependent signaling pathway, as determined by loss of induction in the presence of PD98059 (Table 1). In contrast, inhibition of PI3K and p38 activation

minimally disrupted EGF-stimulated TIMP-1 expression. These results illustrate differences in the EGF-dependent signaling requirements for MMP-9 and TIMP-1 induction.

Inhibition of PI3K Activity Results in Loss of Cell Surface Association of MMP-9. The discovery of *trans*-membrane-anchored MMPs has fueled the hypothesis that localization of MMP activity to the cell surface contributes significantly to matrix remodeling events that occur during cellular migration and invasion. Recent findings indicate that MMP-9 can associate with the cell surface (30–32) and that cell surface association is stimulated by exposure to phorbol ester tumor promoter (33). To determine whether EGF induces MMP-9 association with the cell membrane, surface proteins were labeled with a cell membrane-impermeable biotin analogue (s-NHS-Biotin), isolated on monomeric avidin gels, eluted by competition with free D-biotin, and analyzed by gelatin zymography for the presence of surface-associated MMP activity. EGF promotes a dramatic association of pro-MMP-9 with the cell surface (Fig. 7A, Lanes 2 and 4). Although inhibition of ERK activation by PD98059 and p38 activity by SB202190 reduces EGF-stimulated MMP-9 expression to near control levels in CM (Fig. 7B, upper panel, Lanes 1, 4, and 5), considerable pro-MMP-9 binding to the cell surface was retained under these conditions (Fig. 7B, lower panel, Lanes 1, 4, and 5). In contrast, inhibition of EGF-induced PI3K signaling abolished pro-

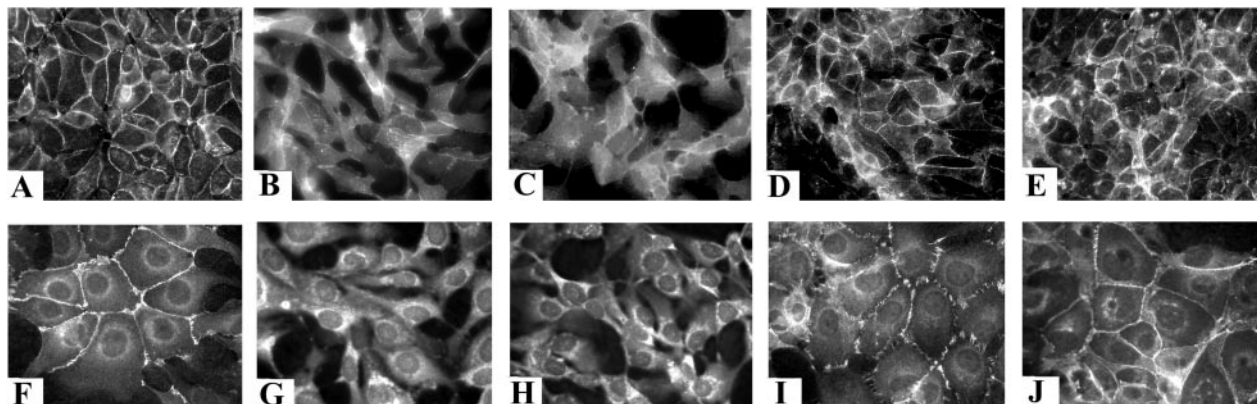


Fig. 5. Signaling requirements for EGF-stimulated disruption of desmosomal and adherens junctions. OVCA 429 cells were seeded in Lab Tek II chamber slides and treated without (A and F) EGF or with (B–E and G–J) EGF (20 nM) in the absence (A, B, F, and G) or presence of inhibitors: 10 μ M LY294002 (C and H), 50 μ M PD98059 (D and I), and 10 μ M SB202190 (E and J). Plakoglobin (A–E) and β -catenin (F–J) were detected by immunofluorescence as described in “Materials and Methods.” Disruption of junctions is evident by redistribution of plakoglobin (B and C) or β -catenin (G and H) from the cell periphery to more diffuse cytoplasmic staining. Note that inhibition of the ERK or p38 MAPKs by PD98059 or SB202190, respectively, interferes with EGF-dependent junction dissolution. In contrast, EGF-stimulated junction disruption occurs in the presence of the PI3K inhibitor LY294002 (C and H).

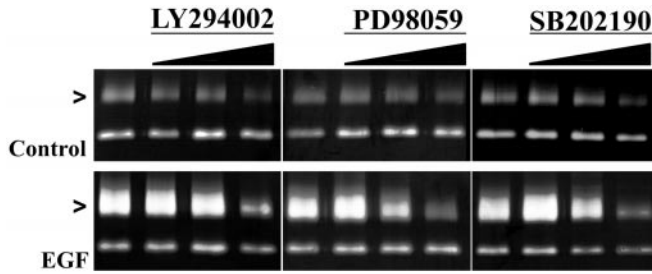


Fig. 6. Signaling requirements for EGF-stimulated MMP-9 production. Cells were treated without (*top panel*) or with (*bottom panel*) EGF (100 nM) subsequent to administration of vehicle control (0.1% v/v DMSO); 1, 3, or 10 μ M LY294002; 1, 10, or 50 μ M PD98059; 1, 3, or 10 μ M SB202190. After 36 h, gelatinase secretion into CM was measured by 8% gelatin substrate zymography (see "Materials and Methods"). PI3K and MAPK signaling regulates both basal and EGF-stimulated expression of pro-MMP-9 expression (*top band, >*). pro-MMP-2 expression (*bottom band*) is not induced by EGF or controlled by these kinase pathways, with the noted exception of p38 MAPK, which consistently inhibited enzyme expression.

Table 1 ERK1/2 signaling is required for EGF induction of TIMP-1 expression

Cells were treated without (control) or with (100 nM) EGF in the presence or absence of the indicated kinase inhibitors (10 μ M LY294002, 50 μ M PD98059, and 10 μ M SB202190). After 36 h, CM and Triton-soluble lysate (mRIPA) was collected, and 100- μ l aliquots (CM) or 50 μ g of protein (lysate) were added to anti-TIMP-1 ELISA wells. The concentration of free and pro-MMP-9-complexed TIMP-1 was measured according to the manufacturer's protocol and expressed as ng/mg (lysate) or ng/ml (CM).

Condition	mRIPA lysate (ng/mg lysate)	CM (ng/ml)
Control	8.98 \pm 0.22	67.73 \pm 8.42
EGF	15.57 \pm 4.65	106.75 \pm 11.97
EGF/LY294002	11.65 \pm 2.21	93.67 \pm 5.65
EGF/PD98059	9.93 \pm 0.22	51.35 \pm 2.55
EGF/SB202190	22.62 \pm 4.87	98.52 \pm 4.54

MMP-9 association with the cell surface (Fig. 7B, Lane 3), indicating that PI3K activity is an important regulator of gelatinase association with the cell surface. The doublet of cell surface gelatinase activity (Fig. 7B, bracket) was not inhibited by the zinc⁽²⁺⁾ chelating agent 1,10-phenanthroline, suggesting that it is likely a cell surface serine protease (*i.e.*, plasmin; data not shown). EGF stimulation of MMP-9 cell surface association was confirmed by immunofluorescence (Fig. 8B), and MMP-9 binding was not detected on the surface of cells treated with PI3K inhibitors (Fig. 8C).

To evaluate temporal requirements of PI3K activity for EGF-induced pro-MMP-9 membrane binding, cells were treated with 10 μ M LY294002 at various times before or after EGF stimulation, and MMP-9 surface association was assessed. Inhibition of PI3K activity by LY294002 at either 0.5 or 1.5 h after EGF stimulation (Fig. 9A, Lanes 5 and 6) was as effective as exposure to inhibitors before EGF addition (Fig. 9A, Lane 4) with regard to disruption of cell surface gelatinase association. However, addition of LY294002 2 h prior to collection of surface proteins had no effect on cell surface MMP-9 levels (Fig. 9A, Lane 7). To address whether pro-MMP-9 binding is regulated by extracellular concentrations of the enzyme, an excess of exogenous purified human MMP-9 was added to cells under different treatment conditions (Fig. 9B). Addition of pro-MMP-9 to control cells did not promote enzyme binding to the cell surface (Fig. 9B, Lane 2) or recover binding to cells stimulated with EGF and concurrently treated with LY294002 (Fig. 9B, Lane 4). Together, these data indicate that EGF stimulated pro-MMP-9 membrane association involves a PI3K-sensitive pathway.

DISCUSSION

The association of EGF receptor and erbB-2 overexpression with a poor prognosis for ovarian cancer patients (1-3) suggests that a

relationship exists between these proteins and cellular responses involved with metastasis, including migration, protease production, and cell adhesion. EGF receptor activation stimulates multiple signal transduction mechanisms in target cells, including the PI3K and MAPK cascades. Interestingly, aberrant activity of the PI3K signaling pathway is detected in many human tumors, and the catalytic subunit of PI3K has been implicated as a putative oncogene in ovarian cancer (34). Although the impact of PI3K activity on cell survival is well documented, our findings suggest that PI3K may be involved in other aspects of tumor progression, including invasion and metastasis.

In this study, we identified divergence in the signaling requirements for specific elements of a migratory or invasive response. EGF-induced motility as assayed by colony dispersion and *in vitro* invasion through a reconstituted basement membrane was suppressed by selective inhibitors of PI3K, p38, and the ERK activating kinase MEK1 (Figs. 3 and 4), confirming the reported involvement of MAPKs and PI3K in cell migration. In contrast, PI3K activity was not required for disruption of cell:cell junctions (Fig. 5) or EGF-stimulated TIMP-1 induction (Table 1). The signaling requirements for junctional disso-

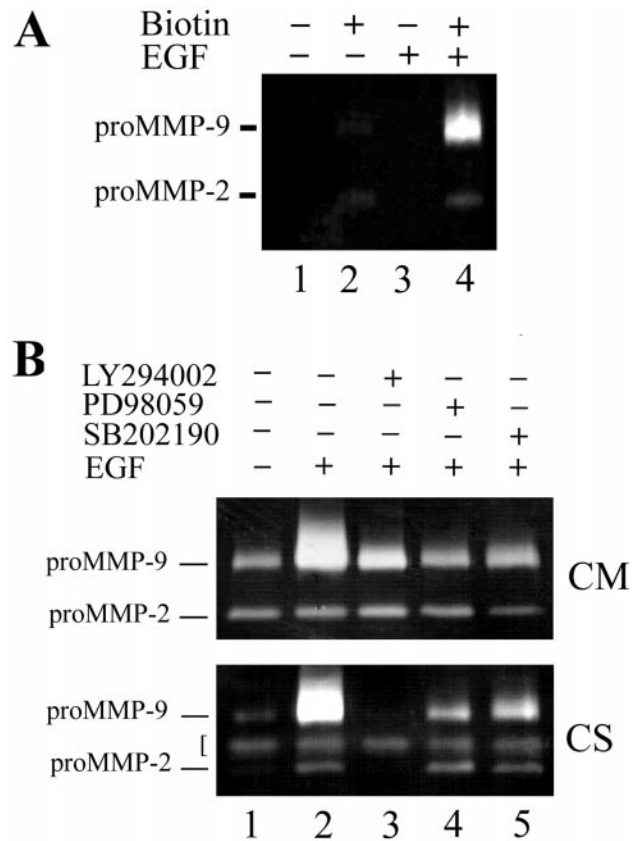


Fig. 7. EGF stimulates cell surface association of pro-MMP-9 through PI3K activity. Surface proteins were labeled with a cell-impermeable biotin analogue, biotin-labeled proteins isolated using monomeric avidin agarose gels, and bound proteins eluted via competition with free D-biotin as described in "Materials and Methods." A, EGF promotes binding of pro-MMP-9 to the cell surface. Specificity for labeled cell surface proteins is demonstrated by the absence of gelatinase activity in assays executed with nonbiotinylated cell lysate preparations (Lanes 1 and 3). In addition, a small amount of pro-MMP-2 also is associated with cell membranes as indicated (Lanes 2 and 4). B, to address the contribution of PI3K or MAPKs to EGF-induced pro-MMP-9 binding, cells were incubated in the presence of 10 μ M LY294002, 50 μ M PD98059, or 10 μ M SB202190 as indicated for 1 h, followed by EGF stimulation (Lanes 2-5). CM and cell surface (CS) samples were analyzed by 8% gelatin substrate zymography. Cell surface binding of pro-MMP-9 was completely abolished in the presence of the PI3K inhibitor (Lane 3), whereas MAPK-inhibited cells bound a significant amount of enzyme compared with nonstimulated cells (Lanes 4 and 5). pro-MMP-2 binding was also eliminated under conditions of PI3K inhibition. Brackets, a doublet of cell surface gelatinase activity that was not inhibited by the zinc⁽²⁺⁾ chelating agent 1,10-phenanthroline, suggesting that it is likely a cell surface serine protease (*i.e.*, plasmin; data not shown).

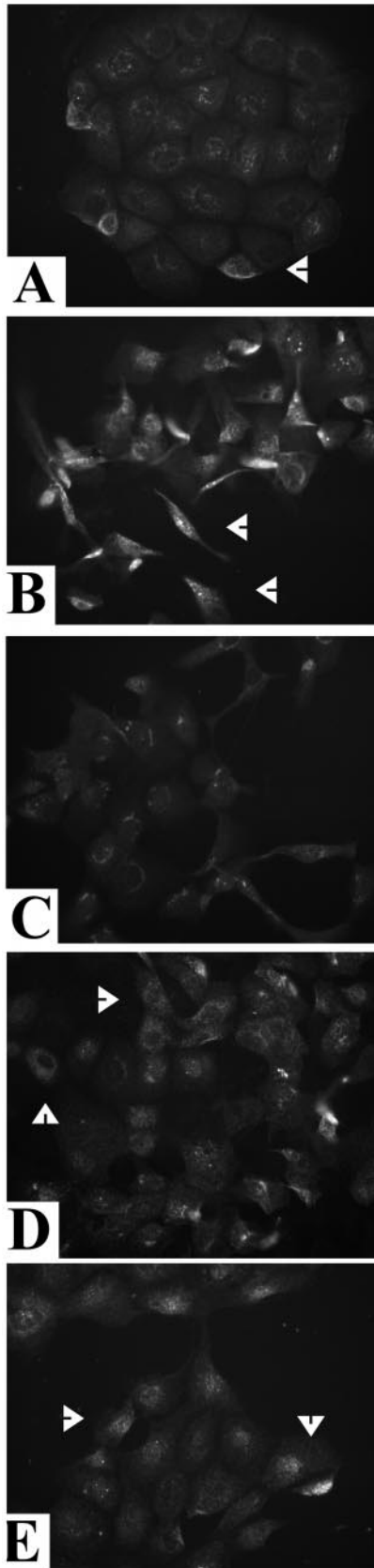


Fig. 8. MMP-9 localization on the cell surface. To confirm the biochemical evidence that EGF stimulates cell surface binding of pro-MMP-9 (Fig. 7), cells were incubated without (A) or with (B–E) EGF (100 nM) subsequent to administration of 0.1% v/v DMSO vehicle control (A and B), 10 μ M LY294002 (C), 50 μ M PD98059 (D), and 10 μ M SB202190 (E). After 36 h, cells were fixed in 3.7% paraformaldehyde without detergent permeabilization and immunostained for MMP-9. Images were captured using a $\times 20$ objective with a $\times 1.4$ photoamplifier. Arrows, examples of positive (A, B, D, and E) MMP-9 immunoreactivity.

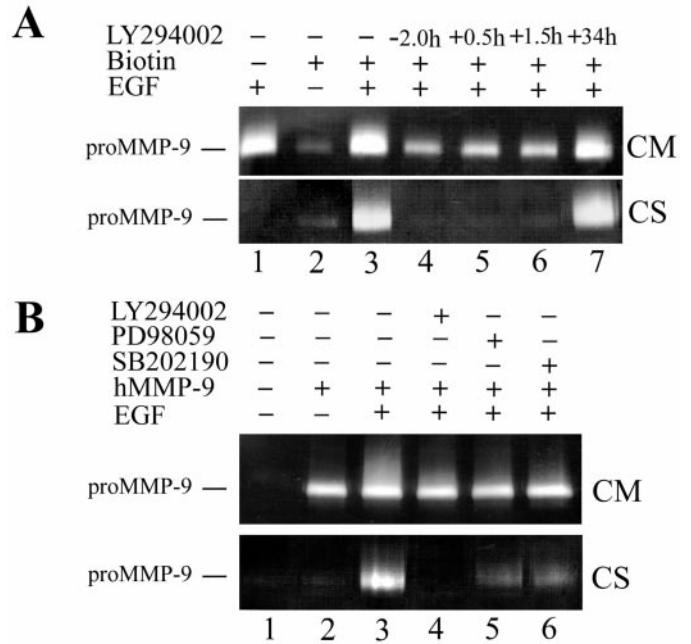


Fig. 9. Requirements of MMP-9 cell surface binding. A, to address the temporal requirements of PI3K signaling for MMP-9 cell surface binding, cells were treated with 10 μ M LY294002 at the following times: 2 h prior to EGF (–2 h), 30 min after EGF (+0.5 h), 90 min after EGF (+1.5 h), and 2 h before collection of lysates (+34 h). CM and cell surface preparations (CS) were analyzed by 8% gelatin zymography. Inhibition of PI3K activity 1.5 h after EGF stimulation was still sufficient to decrease EGF-stimulated pro-MMP-9 expression in CM and block pro-MMP-9 membrane binding to the cell surface (CS). In contrast, inhibition of PI3K activity 2 h before collection of cell surface proteins failed to remove pro-MMP-9 from the cell surface, indicating the involvement of long-term PI3K signaling for this response (Lane 7). B, to discern if pro-MMP-9 membrane association involves an active mechanism that is independent of extracellular pro-MMP-9 concentration, cells were incubated with 10 μ M LY294002, 50 μ M PD98059, and 10 μ M SB02190 for 2 h and then treated with (+) or without (–) EGF (100 nM) or purified human pro-MMP-9 (80 ng/ml). CM and cell surface preparations were analyzed by 8% gelatin zymography. Although levels of pro-MMP-9 were in excess in CM, binding to the cell surface was not promoted in control cells (Lane 2) or recovered in EGF-treated, but PI3K-stimulated, cells (Lane 4).

lution may be cell type and growth factor specific. For example, Potempa and Ridley (35) reported that Ras-dependent activation of MAPK and PI3K was required for hepatocyte growth factor-stimulated disruption of adherens junctions in Madin-Darby canine kidney cells, whereas dissolution of desmosomes and tight junctions occurred through a Ras-independent signaling pathway. In findings similar to our own, breakdown of VE-cadherin-mediated adhesion by vascular endothelial growth factor in endothelial cell monolayers requires MAPK, but not PI3K, activity (36), and EGF-induced morphological changes involving disruption of adherens junctions was reported to be independent of PI3K activity in colon carcinoma cells (37). The findings illustrated in Fig. 5 indicate that PI3K activity is not required for EGF-dependent dissolution of either adherens or desmosomal junctions in OVCA 429 cells but identifies a requirement for MAPK activity.

Although the role of ERK and p38 MAPKs in MMP-9 gene expression has been well documented (20–22, 38–40), involvement of PI3K in EGF-stimulated MMP-9 production has not been studied extensively. Reddy *et al.* (40) reported recently that PI3K inhibition only partially blocks EGF induction of MMP-9 expression in breast epithelial cells, whereas both invasion and MMP-9 production are significantly dependent on MEK signaling. Once expressed, recent evidence suggests that (pro)MMP-9 can bind to the cell surface through interactions the $\alpha 2$ chain of type IV collagen, the putative tumor suppressor RECK, or the transmembrane matrix receptor CD44 (30, 32, 41). However, little is known of mechanisms that regulate

MMP-9 surface binding, including cell surface association in response to growth factor stimulation.

Our work provides insight into growth factor regulation of MMP-9 expression and cell surface association. We found that basal and EGF-induced MMP-9 protein expression involves both PI3K and MAPK signaling (Fig. 6), whereas EGF-induced expression of the inhibitor, TIMP-1, is regulated predominantly by MEK/ERK signaling (Table 1). In contrast, EGF-dependent cell surface association of MMP-9 is highly PI3K dependent (Figs. 7–9). pro-MMP-9 membrane association appears to be a directed event; addition of exogenous pro-MMP-9 does not induce pro-MMP-9 binding to membranes from control or PI3K-inhibited cells (Fig. 9B, *Lanes 2 and 4*). These data suggest that EGF stimulation promotes the cell surface expression of an unidentified pro-MMP-9 binding component(s). As PI3K has been implicated in intracellular membrane and protein trafficking events (42), PI3K-mediated trafficking of pro-MMP-9 or its binding component to the cell surface may be a potential mechanism of gelatinase association. Investigations are currently under way to address the identity of the EGF-regulated pro-MMP-9 binding component(s) and the potential role of PI3K regulation of posttranslational trafficking events.

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