Myosin Light Chain Kinase Functions Downstream of Ras/ERK to Promote Migration of Urokinase-type Plasminogen Activator-stimulated Cells in an Integrin-selective Manner

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Abstract. Urokinase-type plasminogen activator (uPA) activates the mitogen activated protein (MAP) kinases, extracellular signal-regulated kinase (ERK) 1 and 2, in diverse cell types. In this study, we demonstrate that uPA stimulates migration of MCF-7 breast cancer cells, HT 1080 fibrosarcoma cells, and uPA R-overexpressing MCF-7 cells by a mechanism that depends on uPA receptor (uPAR)-ligation and ERK activation. Ras and MAP kinase (MEK) were necessary and sufficient for uPA-induced ERK activation and stimulation of cellular migration, as demonstrated in experiments with dominant-negative and constitutively active mutants of these signaling proteins. Myosin light chain kinase (MLCK) was also required for uPA-stimulated cellular migration, as determined in experiments with three separate MLCK inhibitors. When MCF-7 cells were treated with uPA, MLCK was phosphorylated by a MEK-dependent pathway and apparently activated, since serine-phosphorylation of myosin II regulatory light chain (RLC) was also increased. Despite the transient nature of ERK phosphorylation, MLCK remained phosphorylated for at least 6 h. The uPA-induced increase in MCF-7 cell migration was observed selectively on vitronectin-coated surfaces and was mediated by a b2-integrin (probably aVb1) and aVb5. When MCF-7 cells were transfected to express aVb3 and treated with uPA, ERK was still phosphorylated; however, the cells did not demonstrate increased migration. Neutralizing the function of aVb3, with blocking antibody, restored the ability of uPA to promote cellular migration. Thus, we have demonstrated that uPA promotes cellular migration, in an integrin-selective manner, by initiating a uPAR-dependent signaling cascade in which Ras, MEK, ERK, and MLCK serve as essential downstream effectors.

Key words: urokinase-type plasminogen activator • myosin light chain kinase • integrins • vitronectin • cellular migration

Urokinase-type plasminogen activator (uPA)1 is a serine proteinase which is synthesized as a 54-kD single chain (sc) zymogen and converted into the active two chain form (tcuPA) by various proteinases, including plasmin (de Munk and Rijken, 1990). Both scuPA and tcuPA bind with high affinity (Kd of 0.1–1.0 nM) to the cell-surface receptor, uPA R (Ellis and Dano, 1992). Binding to uPA R may cause scuPA to express intrinsic proteinase activity (Manchanda and Schwartz, 1991; Wang et al., 1997), promote the conversion of scuPA to tcuPA (Ellis et al., 1989), and accelerate the activation of cell-associated plasminogen (Cohen et al., 1991; Ellis and Dano, 1992). These reactions increase cell-surface proteinase activity, which may be important for cancer cell invasion and metastasis in vivo (for review see Ndeasean et al., 1997).

uPA R is linked to the cell surface by a glycosyl-phosphatidylinositol (GPI) anchor (Ploug et al., 1991) and thus lacks transmembrane and intracytoplasmic domains. Nevertheless, uPA R expresses multiple activities, unrelated to proteinase localization, which regulate the interaction of cells with the microenvironment. First, uPA R binds di-
rectly to vitronectin and in some cells may augment or substitute for the function of αv-containing integrins (Wei et al., 1994). uPA also associates with integrins (Bohuslav et al., 1995; Simon et al., 1996; Sitrin et al., 1996; Wei et al., 1996; Xu et al., 1997) and may, by direct interaction, modulate the function of specific β1 and β2 subunit-containing integrins (Wei et al., 1994; Sitrin et al., 1996; May et al., 1998). Finally, uPA functions as a signaling receptor, probably with the aid of a transmembrane adaptor protein or multiprotein-complex which includes integrins (Chapman, 1997).

The signaling cascades that are activated when uPA binds to uPAR are partially characterized. Known protein tyrosine kinases in the Src-family, including p60<sup>src</sup>, p53/p56<sup>lyn</sup>, p56/p59<sup>ck</sup>, and p59<sup>fr</sup>, have been immunoprecipitated with uPAR (Dumlur et al., 1993; Bohuslav et al., 1995; Resnati et al., 1996; Konakova et al., 1998) and at least p56/p59<sup>ck</sup> is activated by uPA (Resnati et al., 1996; Konakova et al., 1998). uPA also activates protein kinase Cε (Busso et al., 1994), the J A K1/StA T1 signaling pathway (Koshelsnick et al., 1997; Dumlur et al., 1998), focal adhesion kinase (Tang et al., 1998), and the mitogen-activated protein (MAP) kinase extracellular-signal regulated kinase (ERK) 1 and 2 (Kanse et al., 1997; Konakova et al., 1998; Nguyen et al., 1998; Tang et al., 1998). The proteolytic activity of uPA is not required for these signal transduction responses.

uPA promotes the migration of diverse cell types (Odekon et al., 1992; Busso et al., 1994; Resnati et al., 1996; Stefansson and Lawrence, 1996; Nguyen et al., 1998), however, it is not clear how the various activities of the uPA/uPAR system are integrated to generate this response. In MCF-7 breast cancer cells, activation of ERK is apparently essential for uPA-promoted cellular migration since the M A P kinase kinase (MEK) inhibitor, PD098059, completely blocks the uPA response without affecting the basal level of cellular migration (Nguyen et al., 1998). Once activated, ERK may translocate to the nucleus and regulate gene expression by modifying transcription factors (Gille et al., 1992; Marais et al., 1993; Hipskind et al., 1994; Hill and Treisman, 1995); however, ERK also modulates cellular functions that are independent of growth factor domains of uPA, such as signal transduction from the uPA-specific chromogenic substrate, γ-protoctysin-glycyl-arginine-p-nitroanilide HCl (Bachem). Type I collagen was from Collaborative Biochemical Products. Vitronectin was purified according to the method of Y atoh et al. (1988). The expression vector, pEGFP, which encodes green fluorescent protein (GFP), was from Clontech. Polyclonal anti-human uPA antibody 399R, which recognizes uPA and blocks uPA-binding to uPAR and monoclonal anti-human uPA antibody, which is directed against the growth factor domain of uPA, were from A ericam Diagnostica. In control experiments, we confirmed that each of these antibodies, at 25 μg/ml, inhibits >95% of the specific binding of 125I-DIP-uPA to MCF-7 cells (data not shown). The two antibodies also blocked ERK1/2 phosphorylation in response to exogenously added scuPA in MCF-7 cells. Polyclonal antibody specific for phosphorylated ERK1/2 was from Promega or Calbiochem. Polyclonal antibodies which recognize total ERK antigen and polyclonal antibodies which recognize phosphoserine residues were from Zymed. Monoclonal anti-R-LC antibody was from ICN Biomedicals. Monoclonal anti-hemagglutinin (HA) 12CA5 was from Babco. M LCK-specific monoclonal antibody (clone K 36) was from Sigma Chemical Co. Integrin-specific blocking antibodies directed against human α<sub>5</sub>β<sub>1</sub>, α<sub>1</sub>β<sub>1</sub>, α<sub>1</sub>β<sub>2</sub> (L M 609), and β<sub>1</sub>-containing integrins (656) were from Chemicon International. The MEK inhibitor, PD 098059, the MLCK inhibitors, ML-7, ML-9, and W-7, and actinomycin D were from Calbiochem. Calcein-AM was from Molecular Probes. Leupeptin was from Boehringer Mannheim. Cycloheximide, PM SF, aprotinin, benzamidine, NaF, sodium orthovanadate, and G 418 were from Sigma Chemical Co.

**Cell Culture and Transfection Methods**

Low-passage MCF-7 cells were kindly provided by Dr. Richard Santen (University of Virginia, Charlottesville, VA) and cultured in RPMI (Life Technologies, Inc.) supplemented with 10% FBS (HyClone), penicillin (100 U/ml), and streptomycin (100 μg/ml). Low-passage MCF-7 cells were kindly provided by Dr. Richard Santen (University of Virginia, Charlottesville, VA) and cultured in RPMI (Life Technologies, Inc.), HT 1080 human fibrosarcoma cells were from the ATCC. These cells were cultured in MEM supplemented with FBS, penicillin, and streptomycin. Cells were passaged with enzyme-free cell dissociation buffer and maintained in culture at 37°C for 48 h before conducting experiments. The full-length human uPAR cDNA was obtained from the ATCC and subcloned into pBK-CMV (Stratagene). To generate stable MCF-7 cell lines which overexpress uPA, 5 × 10<sup>5</sup> cells were transfected with 2 μg of the uPA expression construct, using 10 μl of Superfect (Qiagen) for 2.5 h at 37°C. The cells were then washed with serum-free RPMI, cultured in serum-supplemented medium for 48 h, and selected in G418 (1 mg/ml) for 14 d. Single-cell clones were prepared by serial dilution and screened for uPA overexpression by flow cytometry. Cell-surface uPA was quantified by measuring specific binding of 125I-DIP-uPA, as described previously (Nguyen et al., 1998). To determine the average mass of the MCF-7 cell, suspended cells were counted with a hemocytometer or Coulter counter (yielding equivalent results) and then extracted for protein determination. The mean cellular mass was 0.94 ± 0.07 ng (n = 5). This value was used to calculate the number of copies of cell-surface uPA R/ cell from B<sub>max</sub> values.

Expression constructs which encode constitutively active rat MEK 1 (S218→D/S222→D) and dominant-negative rabbit MEK 1 (S217→A), in pCHA and pBABE, respectively, were described previously (Cowley et al., 1994; Catling et al., 1995). Expression constructs which encode constitutively active H-Ras (G 12→V) and dominant-negative H-Ras

**Materials and Methods**

**Antibodies and Reagents**

scuPA and tcuPA were provided by Drs. J ack Henkin and A ndrew Mazer of A bbott Laboratories. tcuPA was inactivated with 20 mM disopropylfluorophosphate (Sigma Chemical Co.), as described previously (Nguyen et al., 1998). In the resulting preparation (DIP-uPA), >95% of the enzymatic activity of tcuPA was abolished, as determined by the hydrolysis rate of the uPA-specific chromogenic substrate, γ-protoctysin-glycyl-arginine-p-nitroanilide HCl (Bachem). Type I collagen was from Collaborative Biochemical Products. Vitronectin was purified according to the method of Y atoh et al. (1988). The expression vector, pEGFP, which encodes green fluorescent protein (GFP), was from Clontech. Polyclonal anti-human uPA antibody 399R, which recognizes uPA and blocks uPA-binding to uPAR, and monoclonal anti-human uPA antibody, which is directed against the growth factor domain of uPA, were from A ericam Diagnostica. In control experiments, we confirmed that each of these antibodies, at 25 μg/ml, inhibits >95% of the specific binding of 125I-DIP-uPA to MCF-7 cells (data not shown). The two antibodies also blocked ERK1/2 phosphorylation in response to exogenously added scuPA in MCF-7 cells. Polyclonal antibody specific for phosphorylated ERK1/2 was from Promega or Calbiochem. Polyclonal antibodies which recognize total ERK antigen and polyclonal antibodies which recognize phosphoserine residues were from Zymed. Monoclonal anti-R-LC antibody was from ICN Biomedicals. Monoclonal anti-hemagglutinin (HA) 12CA5 was from Babco. M LCK-specific monoclonal antibody (clone K 36) was from Sigma Chemical Co. Integrin-specific blocking antibodies directed against human α<sub>5</sub>β<sub>1</sub>, α<sub>1</sub>β<sub>1</sub>, α<sub>1</sub>β<sub>2</sub> (L M 609), and β<sub>1</sub>-containing integrins (656) were from Chemicon International. The MEK inhibitor, PD 098059, the MLCK inhibitors, ML-7, ML-9, and W-7, and actinomycin D were from Calbiochem. Calcein-AM was from Molecular Probes. Leupeptin was from Boehringer Mannheim. Cycloheximide, PM SF, aprotinin, benzamidine, NaF, sodium orthovanadate, and G 418 were from Sigma Chemical Co.
of these constructs (5.0 μg) and with 1.25 μg of pEGFP, using 30 μl of Super-Perfect. The cells were then maintained in culture for 24-36 h before analysis. Transfection efficiencies were 20-30%, as determined by fluorescence microscopy to detect GFP-positive cells. Cotransfection efficiencies were determined by immunofluorescence microscopy. In these analyses, cells were cotransfected with pEGFP and each of the four constructs, fixed with 1% paraformaldehyde, permeabilized with 1.1% Triton X-100, and incubated with a mouse monoclonal antibody (1:1,000) which recognizes the HA epitope-tag. The cells were then washed and incubated with Texas red-conjugated rabbit anti–mouse IgG (1:2,000, Vector Laboratories). Cotransfection was demonstrated by the presence of Texas red fluorescence and GFP fluorescence in the same cells and was always >90%.

The full-length CDNA for the β3-integrin subunit was kindly provided by Dr. D. avid Cheres (Scripps Research Institute, La Jolla, CA). The CDNA was subcloned into pBK-CMV and transfected into MCF-7 cells (2 μg CDNA/5 × 10⁶ cells) using 10 μl SuperPerfect. The cells were cultured for 36-48 h and selected in G 418 (1 mg/ml) for 14 d. The transfectants were then subjected to flow cytometry, using antibody L M 609 and fluorescein-conju- gated anti–mouse IgG, to detect cell-surface αvβ3 expression. A fitter flow cytometry, the cells were cultured in RPMI supplemented with G 418 (25 μg/ml), FBS, penicillin, and streptomycin. ERK activation experiments and migration assays were performed within 2 wk of obtaining flow cyto-metry results.

ERK Activation in Transfected Cells

To confirm that ERK activity was regulated in cells transfected to express mutant MEK1 or R-Ha, 5 × 10⁵ MCF-7 cells were transfected with 5 μg of each cDNA construct and with 1.25 μg of a construct encoding HA-tagged ERK1, as described by Chu et al. (1996). The cells were main- tained in serum-supplemented medium for 24 h, serum-starved for 4 h, and then treated with 10 nM DIP-uPA or with vehicle for 1 min. Cells were extracted in RIPA buffer, which contains 0.1% SDS, 1% deoxycho- late, 1% NP-40, 10 mM sodium phosphate, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 10 mM sodium phosphate, pH 8.1, for 30 min on ice and centrifuged at 800 × g. The eluates were treated with 10 nM DIP-uPA or with vehicle for 1 min. Cells were then incubated with a mouse monoclonal antibody (1:1,000) which recognizes the αvβ3 integrin. The cells were then cultured in RPMI supplemented with G 418 (25 μg/ml), FBS, penicillin, and streptomycin. ERK activation experiments and migration assays were performed within 2 wk of obtaining flow cyto-metry results.

ERK Phosphorylation Experiments

MCF-7 cells were cultured in 100-mm dishes until 70-80% confluent, washed with phosphate-free RPMI, and metabolically labeled for 2 h at 37°C with 1³⁵Sorthophosphate (250 μCi) in serum-free RPMI supple-mented with 1 mg/ml BSA and 1 mg/ml sodium orthovanadate. The labeled cells were treated with 10 nM DIP-uPA up to 6 h. Control cells were treated with vehicle instead of DIP-uPA. In some cultures, 50 μM PD 098059 was added to inhibit MEK. The PD 098059 was added 15 min before adding the DIP-uPA and remained present throughout the assay. A fitter flow cytometry, the cells were extracted with 1% NP-40, 10 mM Tris-HCl, 140 mM NaCl, 2 mM EDTA, 100 KIU/ml aprotinin, 0.1 mg/ml leupeptin, 2 mM PMSF, 50 mM NaF, 1 mM sodium vanadate, 20 mM sodium pyrophosphate, pH 8.1, for 30 min on ice and centrifuged at 800 g for 10 min. The supernatants were preclared with protein A–agarose for 1 h at 22°C. MLC in the supernatants were then immunoprecipitated by incubation with MLC-specific monoclonal antibody (6 μg) for 12 h at 4°C, rabbit anti-mouse IgG (7.5 μg) for 4 h at 4°C, and finally with protein A–agarose for 1 h at 22°C. The immunoprecipitates were subjected to SDS-PAGE on 8% acrylamide slabs and transferred to nitrocellulose. Phosphorylated MLC was detected by autoradiography.

Serine-phosphorylation of RLC

Suspended MCF-7 cells (10⁶ in 100 μl) were treated with 10 nM DIP-uPA or with vehicle for the indicated times at 37°C. Reactions were terminated by adding SDS sample buffer at 95°C. The whole-cell lysates were then subjected to SDS-PAGE on 3% acrylamide slabs and transferred to nitrocellulose. Immunoblot analysis was performed to detect serine-phosphorylated RLC (primary antibody at 0.5 μg/ml). The same blots were also probed to detect total RLC. In some experiments, the cells were pre-treated for 15 min with drugs that inhibit MEK or MLCK, before adding uPA or vehicle.

Migration Assays

We demonstrated previously that uPA promotes MCF-7 cell migration across serum-coated Transwell membranes irrespective of whether both sides of the membrane were coated with serum or just the underside (Nguyen et al., 1998). The magnitude of the uPA response was greater when both sides of the membrane were serum-coated; however, coating just the underside allows for more rapid cellular migration so that experiments may be completed in 6 h. For this reason, the single-sided coating method was used in this study.

Transwell membranes (6.5 mm, 8.0-μm pores) (Costar) were coated with 20% FBS, purified vitronectin (5 μg/ml), or type I collagen (25 μg/ml) for 2 h at 37°C. Both membrane surfaces were blocked with 10 mg/ml BSA. MCF-7 cells, uPAR-overexpressing MCF-7 cells, and β3-integrin subunit-expressing MCF-7 cells (10⁶ cells in 100 μl) were pretreated with 10 nM DIP-uPA or with vehicle for 15 min in suspension, and then added to the top chamber. Before DIP-uPA exposure, some cells were treated for 15 min with actinomycin D (10 μg/ml), cycloheximide (3 μg/ml), ML-7 (3 μM), ML-9 (30 μM), W7 (51 μM), or with the following antibodies: uPA-specific antibody, uPAR-specific antibody, LM 609, P1F6, or 6S6 (at concentrations up to 32 μg/ml). When cells were pretreated with DIP- uPA, 10 nM DIP-uPA was added to both Transwell chambers. Drugs or antibodies were added to the top chamber. The bottom chamber always contained 10% FBS. A fitter flow cytometry, the cells were removed from the top surface of each membrane using a cotton swab. Cells which penetrated to the underside surfaces of the membranes were stained with Diff-Quik (Dade Diagnostics) and counted. In some experiments, migration of uPAR-overexpressing MCF-7 cells was quantitated by fixing the membranes in methanol and staining the migratory cells with 0.1% crystal violet. The dye was eluted with 10% acetic acid and the absorbance of the eluate was determined at 600 nm. In control experiments, we confirmed that crystal violet absorbance is linearly related to cell number.

HT 1080 cell migration was studied in Transwell chambers containing membranes that were coated on both surfaces with 20% FBS. 5 × 10⁵ cells were added to the top chamber in serum-free medium and allowed to migrate for 6 h in the presence or absence of 10 nM DIP-uPA. FBS was not added to the bottom chamber. Thus, there was no chemotactic or haptotactic stimulus, suggesting that chemokinase was detected. Nonmigrating cells were removed with a cotton swab. Cellular migration was then determined by the crystal violet-staining method.

To study the migration of GFP-expressing cells, translucent Biocoat Cell Culture Inserts (Becton Dickinson) were used instead of Transwell chambers. The insert membranes had 8-μm pores and were coated with serum or purified vitronectin. The response to uPA was not affected in this alternative system as determined by counting Diff-Quik-stained cells. Cells (5 × 10⁵) that were cotransfected with signaling effector mutants and pEGFP, or with pEFP alone, were added to the top chamber and allowed to migrate for 6 h. In some experiments, cells were treated with inhibitors or antibodies and allowed to migrate in the presence or absence of DIP-uPA. Migrating cells were fixed in 4% paraformaldehyde and counted by fluorescence microscopy. To standardize results obtained with transfected and untransfected cells, the pEGFP-transfection efficiency was determined for each experiment. The number of GFP-positive cells which migrated across the membrane was then divided by the transfection efficiency (typically 0.2-0.3).

Mannosamine Treatment Protocol

Mannosamine inhibits a critical enzyme involved in the attachment of GPl-linked proteins to their anchors (Li et al., 1991) and can be used to substantially downregulate the expression of α6β4 integrin (Nguyen et al., 1994; Weaver et al., 1997). MCF-7 cells were treated with 10 mM mannosamine for 6 h at 37°C in glucose-free RPMI. Control cells were incubated in glucose-free RPMI for the equivalent period of time. The cells were then dissociated and added to Transwell chambers in the presence or absence of mannosamine. In control experiments, MCF-7 cells that were mannosamine-treated demonstrated unaltered Trypan blue exclusion.

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Cell Adhesion Assays

Vitronectin-coated cell culture wells were prepared by incubating purified vitronectin (5 μg/ml) in 96-well cell culture plates (Costar) for 2 h at 37°C. The wells were then blocked with BSA. MCF-7 cells that were cotransfected to express GFP and dominant-negative or constitutively active MEK1 or H-Ras (105 cells in 100 μl) were allowed to adhere for the indicated times at 37°C. To assess cellular adhesion, the wells were washed with 10 mM Hepes, 150 mM NaCl, pH 7.4, and adherent cells were quantitated by fluorescence emission. The excitation and emission wavelengths were 488 nm and 507 nm, respectively. Fluorescence emission was also determined for the total number of cells added to each well. Cellular adhesion was quantitated as a percentage of the total number of GFP-expressing cells added. In control experiments, we determined that cell adhesion and spreading do not affect fluorescence emission.

MCF-7 cells that did not express GFP were allowed to adhere to vitronectin-coated cell culture wells in the presence of integrin-specific antibodies (P1F6, LM609, 6S6) or drugs that inhibit MEK or MLCK. After washing, adherent cells were stained with Calcein-AM (Braut-Boucher et al., 1995) and quantitated by fluorescence emission using a Cytofluor 2350.

Results

uPAR-stimulated Cellular Migration Is Antagonized by uPAR-specific Antibody and by PD098059 in Parent and uPAR-overexpressing MCF-7 Cells

We demonstrated previously that uPA activates ERK1/2, rapidly but transiently, in MCF-7 cells and stimulates MCF-7 cell migration (Nguyen et al., 1998). The selective MEK inhibitor, PD 098059, blocked the ability of uPA to stimulate MCF-7 cell migration without affecting the basal level of cellular migration, suggesting that ERK activation is essential in the pathway by which uPA increases MCF-7 cell motility. Fig. 1 A confirms and extends our original observations by demonstrating that DIP-uPA (10 nM) increases MCF-7 cell migration, in serum-coated Transwells, and that this activity is blocked by antibodies (25 μg/ml) which bind to uPA or uPAR and prevent uPAR ligation. The uPA- and uPAR-specific antibodies did not affect MCF-7 cell migration in the absence of exogenously added uPA. Furthermore, nonimmune IgG did not affect MCF-7 cell migration, in the presence or absence of uPA. Thus, the motility-stimulating activity of uPA, in MCF-7 cells, requires uPA-binding to uPA R.

MCF-7 cells express 3,300 copies of cell-surface uPA R, as determined by [125I]-DIP-uPA binding and the experimentally determined mean cellular mass (0.94 ng) (Nguyen et al., 1998). To further test the role of ERK activation in uPA-promoted cellular migration, we overexpressed uPAR in MCF-7 cells. Single-cell cloning yielded several clonal cell lines with increased levels of cell-surface uPA R, as determined by flow cytometry. Clone M5 demonstrated increased specific binding of [125I]-DIP-uPA. The Kd (1.2 ± 0.3 nM) was unchanged compared with that determined for untransfected MCF-7 cells; however, the Bmax was increased to 33 ± 5 fmol/mg cell protein (n = 4), corresponding to 19,800 copies of cell-surface uPA R/cell (data not shown). Fig. 1 B shows the results of migration experiments performed with clone M5. In the absence of uPA, the basal rate of M5 cell migration was 536 ± 80 cells/membrane (compared with 98 ± 4 untransfected MCF-7 cells/membrane); however, basal M5 cell migration was not inhibited by uPA- or uPAR-specific antibody or PD 098059. Thus, the increase in basal M5 cell migration did not result from the binding of endogenously produced uPA to uPAR or uPAR-initiated cell signaling as anticipated, since MCF-7 cells produce very low levels of uPA (Nguyen et al., 1998; Webb et al., 1999). When treated with DIP-uPA (10 nM), M5 cell migration increased 2.0 ± 0.2-fold. The response to DIP-uPA was totally inhibited by uPA- and uPAR-specific antibodies and by PD 098059. M5 cell migration was also analyzed by crystal violet staining instead of direct cell counts; DIP-uPA increased cellular migration 2.5 ± 0.2-fold in these experiments. Thus, uPA R-overexpressing MCF-7 cells respond similarly to the parent cells when treated with uPA.

uPAR-stimulated HT 1080 Cell Migration Requires ERK Activation

HT 1080 fibrosarcoma cells express uPA R (Laug et al., 1992) and demonstrate increased levels of activated ERK 2 when treated with uPA (Konakova et al., 1997). In our initial experiments, we demonstrated specific binding of [125I]-
DIP-uPA to HT 1080 cells; the \( K_d \) was 1.2 ± 0.4 nM and the \( B_{max} \) was 49 ± 4 fmol/mg of cell protein \((n = 4)\), corresponding to ~30,000 copies of cell-surface uPAR/cell. In Transwell assays, DIP-uPA (10 nM) increased HT 1080 cell migration 2.6 ± 0.1-fold (Fig. 2 A). The effects of DIP-uPA on HT 1080 cell migration were blocked by uPA- and uPAR-specific antibodies and by PD098059. The same reagents did not affect HT 1080 cell migration in the absence of exogenously added uPA. Thus, uPAR-initiated signal transduction and ERK activation are critical for uPA-stimulated HT 1080 cell migration.

The lack of an effect of uPA- and uPAR-specific antibodies on HT 1080 cell migration, in the absence of exogenously added DIP-uPA, suggested that endogenously produced uPA is insufficient to activate autocrine uPAR-signaling in these cells. However, others have demonstrated that HT 1080 cells express substantial levels of uPA (Tsuboi and Rifkin, 1990; Laug et al., 1992). To address this issue, we measured the concentration of uPA in medium that was conditioned by HT 1080 cells for 24 h, using an activity assay which detects scuPA and tcuPA (Weaver et al., 1997). The uPA concentration was 24 ± 4 pm. The activity assay does not detect uPA-PAI-1 complex; however, immunoblotting experiments demonstrated only trace levels of this complex in the conditioned medium (data not shown). As determined by the equation for fractional receptor saturation \( Y = L/(K_d + L) \), a uPA concentration of 24 pm would be expected to occupy no more than 2% of the uPAR. In separate experiments, HT 1080 cells were incubated with 1 nM DIP-uPA at 4°C. The cells were then extracted and subjected to immunoblot analysis to detect total cellular uPA (Fig. 2 B). Trace levels of endogenously produced uPA were detected in extracts from control cells which had not been treated with DIP-uPA; this uPA may have been surface-associated or contained within intracellular pools. The high molecular mass band in the same lane is probably cell-associated uPA-PAI-1 complex. After incubation with 1 nM DIP-uPA for 4 h at 4°C, the amount of cell-associated uPA was substantially increased. Furthermore, uPAR-specific antibody blocked the increase in uPA recovery. These studies demonstrate that the majority of the uPAR, in HT 1080 cells, is unliganded and available to bind exogenous DIP-uPA under our experimental conditions.

**MEK Is Essential in the uPAR-initiated Signaling Pathway Which Increases MCF-7 Cell Migration**

To further explore the relationship between the uPA/uPAR system and ERK activation in promoting cellular migration, we transfected MCF-7 cells to express dominant-negative or constitutively active MEK1. To demonstrate that the MEK1 mutants were functional as upstream-modulators of ERK activation, MCF-7 cells were cotransfected to express HA-tagged ERK1. The transfectants were treated with DIP-uPA (10 nM) or vehicle for 1 min; cell extracts were immunoprecipitated using an antibody directed against the HA-epitope and phosphorylated ERK1 was detected by immunoblot analysis. As shown in Fig. 3 A, MCF-7 cells that were transfected to express dominant-negative MEK1 did not contain detectable levels of phosphorylated HA-ERK1, irrespective of whether these cells were treated with DIP-uPA or not. By contrast, substantial levels of phosphorylated HA-ERK1 were detected in cells that were transfected to express constitutively active MEK1. However, when these cells were treated with DIP-uPA, no further increase in phosphorylated HA-ERK1 was observed. As a control, we cotransfected MCF-7 cells with the HA-tagged ERK1 construct and with the empty vector (pcHA) which had been used to prepare the constitutively active MEK1 mutant. In the absence of uPA, only trace levels of phosphorylated HA-ERK1 were observed; however, DIP-uPA substantially increased phosphorylated HA-ERK1 in these cells. These results demonstrate that the MEK1 mutants were functional as regulators of ERK activation in the presence and absence of uPA.

To study the migration of MCF-7 cells, which were tran-
siently transfected to express mutant forms of MEK1, we cotransfected the cells with pEGFP. Cotransfection efficiencies were always >90%, allowing us to selectively detect the migration of transfected cells by GFP fluorescence. As shown in Fig. 3 B, cells that were transfected only with pEGFP demonstrated increased migration when treated with 10 nM DIP-uPA. When MCF-7 cells were transfected to express dominant-negative MEK1, basal migration was not significantly altered (24% decrease, \( P > 0.1 \)); however, the ability of uPA to stimulate cellular migration was entirely blocked. MCF-7 cells that were transfected to express constitutively active MEK1 demonstrated increased migration in the absence of uPA; however, these cells demonstrated no change in migration when treated with DIP-uPA. Since uPAR synthesis may be regulated by a MAP kinase-dependent pathway (Tkachuk et al., 1996), control experiments were performed to rule out the possibility that increased uPAR expression was responsible for the increase in the migration of constitutively active MEK1-expressing cells. First, GFP-expressing cells were isolated by flow cytometry and studied in \(^{125}\)I-DIP-uPA-binding experiments; no change in cell-surface uPAR was detected (data not shown). Furthermore, migration of constitutively active MEK1-expressing MCF-7 cells was not inhibited by uPA - or uPAR-specific antibodies (data not shown). Thus, active MEK operates downstream of uPAR, as a necessary and sufficient effector in the signal transduction pathway by which uPA stimulates MCF-7 cell migration.

In Transwell migration assays, increased cellular migration may reflect increased cellular penetration of the Transwell membranes or a change in the kinetics of cellular adhesion to the membranes. To rule out the latter possibility, GFP-expressing MCF-7 cells were allowed to adhere to vitronectin-coated cell culture wells for 10, 20, 30, 40, or 60 min. Adhesion was detected by measuring fluorescence emission in a Cytofluor 2350. The kinetics of MCF-7 cell adhesion were not affected by DIP-uPA or by either MEK mutant (data not shown). PD 098059 also did not affect MCF-7 cell adhesion, in the presence or absence of uPA. Thus, the differences observed in the Transwell assays reflect changes in the migration of cells which have already adhered to the Transwell membranes.

**Ras Is Essential in the uPAR-initiated Signaling Pathway Which Increases MCF-7 Cell Migration**

Ras activation is frequently but not always necessary as an upstream activator of ERK in growth factor-stimulated cells (Frost et al., 1997). To test whether Ras is involved in the uPAR-initiated signaling pathway which leads to ERK activation, MCF-7 cells were cotransfected to express HA-tagged ERK1 and the specified MEK mutants or the empty vector, pCHA. HA-tagged ERK1 was immunoprecipitated from cell extracts 1 min after treating the cells with 10 nM DIP-uPA (+) or with vehicle (-). Phosphorylated and total levels of ERK1 were determined by immunoblot analysis. As shown in Fig. 4 A, phosphorylated HA-ERK1 was not detected in cells that were transfected to express dominant-negative H-Ras or constitutively active H-Ras. A s shown in Fig. 4 A, phosphorylated HA-ERK1 was not detected in cells that were transfected to express dominant-negative H-Ras, irrespective of whether the cells were treated with uPA or not. By contrast, cells that were transfected to express constitutively active H-Ras demonstrated substantial levels of phosphorylated HA-ERK1; however, the level of phosphorylated HA-ERK1 was not further increased by uPA. A s a control, we transfected cells with the empty vector, pDCR. Trace levels of phosphorylated HA-ERK1 were detected in the absence of uPA; however, when these cells were treated with DIP-uPA, a substantial increase in phosphorylated HA-ERK1 was observed. In other control experiments, we demonstrated unchanged binding of \(^{125}\)I-DIP-uPA to constitutively active H-Ras-expressing MCF-7 cells, indicating that cell-surface uPAR expression is not altered in these cells (data not shown).

To determine whether the H-Ras mutants affect the ability of uPA to stimulate cellular migration, MCF-7 cells were cotransfected to express GFP. As shown in Fig. 4 B, MCF-7 cells that expressed dominant-negative H-Ras demonstrated only a slight decrease in migration in the absence of uPA; however, these cells failed to demonstrate increased migration...
when treated with DIP-uPA. MCF-7 cells that expressed constitutively active H-Ras demonstrated 2.3 ± 0.3-fold increased migration in the absence of uPA, but no further increase in motility when DIP-uPA was added. Antibodies against uPA and uPAR had no effect on the migration of constitutively active H-Ras-expressing MCF-7 cells (data not shown). Thus, constitutively active H-Ras did not increase migration by regulating uPA or uPAR expression. Instead, Ras functioned as an essential mediator of the uPAR-initiated signal transduction response which stimulates MCF-7 cell migration.

**uPA-stimulated MCF-7 Cell Migration Does Not Require New Gene Transcription or Protein Synthesis**

When activated, ERK may translocate to the nucleus and regulate gene expression by modifying transcription factors, such as Elk-1 (Gille et al., 1992; Marais et al., 1993; Hill and Treisman, 1995). To determine whether new gene transcription and/or protein synthesis are required for uPA-stimulated MCF-7 cell migration, Transwell assays were performed in the presence of actinomycin D or cycloheximide (Fig. 5). Neither agent had any effect on the migration of MCF-7 cells, in 6 h, in the absence of uPA. Furthermore, cycloheximide and actinomycin D did not affect the response of cells to DIP-uPA. In control experiments, we demonstrated that [35S]methionine incorporation into total cellular protein was inhibited by >90% when MCF-7 cells were cultured in the presence of 3 μg/ml cycloheximide for 1–6 h (data not shown). We also demonstrated that cycloheximide and actinomycin D do not significantly affect the migration of MCF-7 cells that are transfected to express constitutively active MEK1 (115 ± 18% and 112 ± 20% of control, respectively) or H-Ras (107 ± 5% and 89 ± 17% of control, respectively). These results demonstrate that ERK promotes MCF-7 cell migration in uPA-stimulated cells by a transcription-independent pathway.

**MLCK Is Phosphorylated and Activated by a MEK-dependent Pathway in uPA-treated Cells**

MLCK is a Ca2+/calmodulin-dependent kinase that phosphorylates RLC, promoting contraction of the actin-based cytoskeleton (Lamb et al., 1988; Goeckeler and Wysolmerski, 1995). Several enzymes, including cyclic AMP-dependent protein kinase, protein kinase C, and Ca2+/calmodulin-dependent protein kinase II, phosphorylate MLCK near the calmodulin-binding domain, increasing the concentration of Ca2+ required for MLCK activation and thereby effectively decreasing MLCK activity (for reviews see A delstein et al., 1982; Stull et al., 1993). p21-activated kinase also decreases MLCK activity by directly affecting the enzyme Vmax (Sanders et al., 1999). However, Klemke et al. (1997) demonstrated that ERK phosphorylates and thereby activates MLCK and that this reaction may be critical in growth factor or integrin-initiated pathways which lead to accelerated cellular migration.

To determine whether uPAR-initiated signal transduction results in MLCK phosphorylation, MLCK was immu-
noprecipitated from MCF-7 cells that had been metabolically labeled with \(^{32}\text{P}\)orthophosphate and treated with DIP-uPA. Fig. 6 A shows that MLCK was phosphorylated within 1 h of exposure to uPA; however, unlike ERK1/2, MLCK phosphorylation was sustained for at least 6 h. PhosphorImager analysis was used to quantitate phosphorylated MLCK. When the results were standardized for total MLCK recovery, we did not detect significant variation in the level of phosphorylated MLCK between 1 and 6 h (Fig. 6 B). Pretreatment of MCF-7 cells with PD098059, before DIP-uPA exposure, blocked MLCK phosphorylation. Thus, uPA-induced MLCK phosphorylation is MEK-dependent. We have demonstrated that purified ERK1 directly phosphorylates MLCK in vitro (data not shown), confirming the work of others (Klemke et al., 1997; Wu et al., 1998); however, in intact uPA-treated cells, MLCK may be phosphorylated directly by ERK or by another kinase which is downstream of MEK.

To determine whether MLCK, which is phosphorylated in uPA-treated cells, is also activated, we examined RLC phosphorylation in whole-cell extracts using an antibody which is specific for phosphoserine. As shown in Fig. 6 C, the major serine-phosphorylated species demonstrated an apparent mass of 23 kD. This species was identical in mobility to RLC, as determined by probing the same blots with RLC-specific antibody. DIP-uPA significantly increased RLC phosphorylation. In five separate experiments, the increase in RLC phosphorylation was 2.4 ± 0.2-fold and 3.0 ± 0.4-fold after treatment with 10 nM DIP-uPA for 45 and 60 min, respectively. A number of enzymes may phosphorylate RLC on serine, in addition to MLCK (Bresnick, 1999); however, the increase in RLC phosphorylation was blocked by the specific MLCK inhibitors, ML-7 (3 μM) and ML-9 (30 μM), and by the general antagonist of Ca\(^{2+}\)/calmodulin-dependent kinases, W-7 (51 μM) (Table I). Each of these inhibitors was present at a concentration that was 10-fold higher than the reported \(K_i\) value for MLCK inhibition (Saitoh et al., 1987). PD098059 also blocked the increase in RLC phosphorylation, suggesting a dependency on MEK activity. In experiments that are not shown, increased RLC phosphorylation was also demonstrated in uPA-treated cells by isoelectric focusing, using the method described by Garcia et al. (1995). These results support the conclusion that MLCK is activated when phosphorylated by ERK, as proposed by Klemke et al. (1997). Interestingly, none of the MLCK inhibitors significantly decreased RLC phosphorylation, in the absence of uPA, when analyzed by phosphoserine immunoblotting 75 min after adding the drugs.

**MLCK Activity Is Necessary for uPA-promoted Cellular Migration**

To determine whether MLCK activity is necessary for uPA-promoted MCF-7 cell migration, Transwell assays were performed in the presence of the MLCK inhibitors. In the absence of uPA, M L-7 and M L-9, at concentrations up to 10-fold the reported \(K_i\) values for MLCK inhibition, had little or no effect on MCF-7 cell migration (Fig. 7 A). W-7 was also inactive at concentrations up to 51 μM, which is 10-fold the reported \(K_i\) for MLCK inhibition. These results suggest that MLCK activity is not essential.

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**Figure 6.** MLCK and RLC are phosphorylated in uPA-treated MCF-7 cells. (A) MCF-7 cells were metabolically labeled with \(^{32}\text{P}\)orthophosphate and then treated with vehicle (control) or with 10 nM DIP-uPA for the indicated times. Some MCF-7 cells were treated with 50 μM PD098059 (+), beginning 15 min before adding uPA and continuing for the entire assay. MLCK was immunoprecipitated from detergent extracts, subjected to SDS-PAGE, and analyzed by autoradiography. (B) Densitometry was performed using low-exposure autoradiography films from the specified number of experiments \((n=2-4)\). In each study, phosphorylated MLCK was not readily detected in the absence of uPA. Thus, band intensity was standardized against that observed with cells that had been treated with DIP-uPA for 1 h. (C) Suspended MCF-7 cells were treated with 10 nM DIP-uPA or with vehicle (control) for the indicated times. Cells lysates were subjected to SDS-PAGE and immunoblot analysis to detect RLC serine-phosphorylation.
for basal MCF-7 cell migration. W-7, at concentrations exceeding 0.1 mM, blocked MCF-7 cell migration, probably reflecting the ability of W-7 to inhibit Ca$^{2+}$/calmodulin-dependent kinases other than MLCK (Hidaka et al., 1981, 1988).

When MCF-7 cells were treated with DIP-uPA, in the presence of the MLCK inhibitors, the uPA-induced increase in cellular migration was blocked (Fig. 7B). Equivalent results were obtained in experiments with uPAR-overexpressing MCF-7 cells and HT 1080 cells. None of the three drugs affected the basal level of migration of these two cell lines; however, uPA-stimulated cellular migration was inhibited. In control experiments, ML-7, M L-9, and W-7 had no effect on MCF-7 cell adhesion to vitronectin (data not shown). These studies demonstrate a critical role for MLCK activity in uPA-stimulated cellular migration in three distinct model systems.

### Table I. RLC Serine-phosphorylation in uPA-treated MCF-7 Cells (% of Controls*)

| Inhibitor | -uPA | +uPA |
|-----------|------|------|
| None      | 100 ± 20 | 300 ± 44 |
| ML-7      | 117 ± 7 | 115 ± 12 |
| ML-9      | 97 ± 9 | 84 ± 5 |
| W-7       | 97 ± 8 | 79 ± 12 |
| PD098059  | 80 ± 17 | 95 ± 11 |

*a uPA treatments were for 60 min. Inhibitors were added 15 min before uPA or vehicle. Serine phosphorylation is expressed as a percentage of that observed in the absence of uPA or drugs.

uPA-promoted MCF-7 Cell Migration Is Matrix Protein–selective

When surfaces are coated with serum, vitronectin serves as the major attachment and spreading factor (H aym an et al., 1985). To determine whether uPA-promoted MCF-7 cell migration is matrix protein–selective, Transwell membranes were coated with purified vitronectin or type I collagen, instead of serum. DIP-uPA increased MCF-7 cell migration on vitronectin 3.1 ± 0.2-fold (n = 5), as anticipated (Fig. 8). MCF-7 cells migrated more rapidly on type I collagen-coated surfaces, in the absence of uPA; however, DIP-uPA failed to stimulate cellular migration further. In control experiments, we demonstrated that MCF-7 cell migration across collagen-coated membranes occurs as a linear function of time (1–6 h), precluding the possibility that we missed a uPA response due to the assay time (data not shown).

Mannosamine inhibits the membrane-anchoring of all GPI-linked proteins, including uPAR (Lisanti et al., 1991; Wei et al., 1994; Weaver et al., 1997). When MCF-7 cells were treated with mannosamine, cellular migration across type I collagen-coated membranes was not significantly affected. By contrast, mannosamine substantially inhibited the migration of MCF-7 cells on vitronectin, in the presence and absence of uPA. These studies suggest that uPAR or another GPI-anchored protein is critical for MCF-7 cell migration on vitronectin. In control experiments, we demonstrated that MCF-7 cell adhesion and migration on vitronectin are totally blocked by the integrin antagonists, EDTA (1 mM) and GRGDSP (0.5 mM) (data
not shown). Thus, whereas uPAR apparently plays an important role in MCF-7 cell migration on vitronectin, this process is still dependent on integrins.

**αvβ3 Mediates MCF-7 Cell Adhesion to Vitronectin**

Meyer et al. (1998) demonstrated that MCF-7 cells express αvβ3 and αvβ1, but not αvβ5. Our flow cytometry experiments, with antibodies directed against αvβ5 and αvβ3, confirmed their results (data not shown). We also demonstrated substantial levels of cell-surface β1 subunit; however, our antibody was not specific for αvβ1. To compare the function of various integrins in MCF-7 cell adhesion to vitronectin, in the presence and absence of DIP-uPA (10 nM), integrin-neutralizing antibodies were used. In the absence of uPA, antibody P1F6, which blocks αvβ3, substantially inhibited MCF-7 cell adhesion whereas LM609, which blocks αvβ5, was ineffective as anticipated (Fig. 9). Interestingly, when the β1-integrin–blocking antibody, 6S6, was added in combination with P1F6, the extent of inhibition was significantly increased (P < 0.05). These results suggest that a β1 subunit–containing integrin (probably αvβ3) plays a significant supporting role in MCF-7 cell adhesion to vitronectin. DIP-uPA neither promoted nor inhibited MCF-7 cell adhesion in the presence or absence of any of the antibodies, suggesting that uPA does not affect the function of the major vitronectin-binding integrins as mediators of MCF-7 cell adhesion.

**A β1-Integrin and αvβ3 Function in uPA-promoted MCF-7 Cell Migration**

In the absence of uPA, αvβ3-blocking antibody had no effect on MCF-7 cell migration on vitronectin, as anticipated (Fig. 10 A). However, αvβ3-blocking antibody was also inactive and β1 subunit–blocking antibody inhibited migration by <25%. When added in combination, αvβ3-blocking antibody and the β1-blocking antibody inhibited migration by up to 79 ± 5%. These results suggest that αvβ3 and a β1-containing integrin (probably αvβ3) function interchangeably in MCF-7 cell migration on vitronectin, in the absence of uPA.
mote pancreatic carcinoma cell migration on vitronectin by a mechanism that is dependent on endogenously synthesized uPA when the cells express \( \alpha_v \beta_3 \) but not when they express \( \alpha_v \beta_2 \). To determine whether the pattern of integrin expression influences the ability of MCF-7 cells to respond to exogenously added uPA, MCF-7 cells were transfected to express the \( \beta_3 \)-integrin subunit. \( \alpha_v \beta_3 \) expression was demonstrated in cells that had been selected in G418 for 14 d, by flow cytometry (Fig. 11 A). DIP-uPA stimulated ERK 1/2 phosphorylation in the \( \beta_3 \)-expressing cells (Fig. 11 B). Interestingly, ERK phosphorylation was sustained for an increased period of time (at least 40 min), compared with the parent cell line (<5 min) (Nguyen et al., 1998). Previous studies have shown that \( \alpha_v \beta_3 \) may affect the duration of ERK activation in other cell systems as well (Elicieri et al., 1998).

\( \alpha_v \beta_3 \)-expressing MCF-7 cells demonstrated increased migration on serum-coated membranes, in the absence of uPA, compared with the parent cell line (control) and MCF-7 cells which had been transfected with empty vector (Fig. 11 C). However, DIP-uPA (10 nM) did not stimulate migration of the \( \alpha_v \beta_3 \)-expressing cells. When LM609 was added to block the activity of \( \alpha_v \beta_3 \), cellular migration returned to the pretransfection level; however, the cells also regained responsiveness to DIP-uPA. Identical results were obtained in transient transfection experiments; cells that were cotransfected to express GFP and \( \beta_3 \)-integrin subunit demonstrated 2.6-fold increased migration compared with cells that were transfected with pEGFP alone. However, these cells did not respond to DIP-uPA (data not shown). A nitrobody LM609 decreased the migration of the transiently transfected cells but restored responsiveness to uPA. These results suggest that \( \alpha_v \beta_3 \) serves as the dominant integrin responsible for the migration of \( \beta_3 \)-transfected cells and that uPA does not promote \( \alpha_v \beta_3 \)-mediated MCF-7 cell migration. When \( \alpha_v \beta_3 \) is blocked, the naturally occurring vitronectin-binding integrins remain available and the function of these integrins is enhanced by uPA.

\( \alpha_v \beta_3 \)-Mediated MCF-7 Cell Migration

Is MLCK-independent

Since the ability of uPA to promote MCF-7 cell migration depends on MLCK activity, we performed experiments to determine whether \( \alpha_v \beta_3 \)-expressing cells, which were refractory to uPA stimulation, are also refractory to MLCK inhibitors. Migration of \( \beta_3 \)-expressing cells was studied in the presence of ML-7, ML-9, and W-7, at concentrations that abolished the uPA response in untransfected cells. As shown in Table II, none of the inhibitors significantly affected the migration of \( \beta_3 \)-expressing MCF-7 cells on vitronectin. Thus, MLCK does not play an essential role in \( \alpha_v \beta_3 \)-mediated MCF-7 cell migration.

Discussion

Cellular migration is an integrated, multistep process which is regulated by growth factors and extracellular matrix proteins that bind integrins (Lauffenburger and Horwitz, 1996). The uPA/uPAR system also regulates cellular migration; this activity may reflect the ability of uPAR to
bind directly to vitronectin, associate with and modulate the function of integrins, initiate cell-signaling responses, or the function of uPA as a proteinase (Andreasen et al., 1997; Chapman, 1997). Although uPA-deficient (−/−) mice are viable and demonstrate only modest phenotypic abnormalities in the absence of exogenous challenge (Carmeliet et al., 1994), major deficiencies in cellular migration may be observed under specific circumstances. For example, uPA−/− mice demonstrate inadequate inflammatory cell recruitment and significantly increased mortality when challenged by pulmonary infection with Cryptococcus neoformans (Gyetko et al., 1996) and may also be predisposed to other infections (Shapiro et al., 1997). Similarly, mice that are uPAR-deficient demonstrate deficient recruitment of inflammatory cells to inflamed peritoneum (May et al., 1998).

Table II. The Effects of MLCK Inhibitors on αvβ3-mediated Cellular Migration

| Transfectants | Inhibitors | Cell migration (% of control) |
|---------------|------------|-----------------------------|
| Control       | None       | 100 ± 6                     |
| β3            | None       | 304 ± 29                    |
|               | ML-9 (30 μM) | 291 ± 8                   |
|               | ML-7 (3 μM)  | 310 ± 26                   |
|               | W-7 (50 μM)  | 249 ± 41                   |

* MCF-7 cell migration was quantitated as a percentage of that observed with untransfected cells (control) that were not treated with inhibitors.
uPA R-specific antibodies, which block association of uPA with uPA R, prevented uPA-stimulated cellular migration. PD 098059 also blocked uPA-stimulated cellular migration in all three cell lines. Thus, the ability of uPA to bind to uPA R and activate ERK may be pivotal in promoting cellular migration in different cell types with varying levels of uPA R expression.

Although recent studies have elucidated diverse signaling pathways that may be activated by uPA (Chapman, 1997), little information is available regarding the relationship of these pathways to the biological activities of uPA such as the ability to stimulate cellular migration. Mirashashi et al. (1997) demonstrated that uPA-stimulated ovarian cancer cell migration depends on tyrosine kinase activation. Using a transfection strategy that included dominant-negative and constitutively active Ras and MEK mutants, we have now demonstrated that these proteins are essential for uPA-stimulated MCF-7 cell migration. Dominant-negative H-Ras and MEK1 completely blocked the response of MCF-7 cells to uPA. Constitutively active H-Ras and MEK1 independently promoted MCF-7 cell migration but also blocked the uPA response. We interpret these results to mean that Ras and MEK are necessary and sufficient in the pathway by which uPA R ligation is linked to ERK activation and increased cellular migration. Since the level of cellular migration observed in cells expressing constitutively active H-Ras or MEK1 was no greater than that observed when control cells were treated with DIP-uPA, the uPA R-initiated signaling cascade appears to be sufficiently potent to optimally activate the operational downstream effectors of motility stimulation. Furthermore, since the dominant-negative H-Ras and MEK1 mutants entirely blocked uPA-stimulated cellular migration, other uPA-signaling pathways, which do not include Ras and MEK, apparently do not affect MCF-7 cell migration or, less likely, have offsetting activities.

In MCF-7 cells, uPA-induced ERK activation is highly transient; however, the effects of uPA on cellular migration are sustained (Nguyen et al., 1998). Increased MCF-7 cell migration is demonstrable for at least 24 h, even when the cells are pulse-exposed to uPA for only 30 min and then washed. The transient nature of ERK activation may be explained by the activity of MAP kinase phosphatases (Clarke, 1994; K eyse, 1995) or by negative feedback loops which limit Ras activation (Corbalan-Garcia et al., 1996). It is also possible that activated ERK, which becomes associated with the cytoskeleton, is not completely extracted by the detergents used in the immunoblotting experiments. We demonstrated that uPA-promoted MCF-7 cell migration does not require new gene transcription or protein synthesis. A c tinomycin D and cycloheximide had no effect on the rate of MCF-7 cell migration in 6 h, in the presence or absence of DIP-uPA. Thus, we undertook studies to identify ERK substrates, other than transcription factors, that may be responsible for uPA-promoted MCF-7 cell migration. Onc activated, ERK localizes not only to the nucleus, but also to the plasma membrane (Gonzalez et al., 1993) and to the cytoskeleton (Reszka et al., 1995). MLCK has been identified previously as an ERK substrate which may be involved in stimulating cellular migration (Klemke et al., 1997). When MLCK is phosphorylated by ERK, the MLCK is apparently activated and thus phosphorylates RLC in the presence of decreased concentrations of Ca$^{2+}$/calmodulin (Klemke et al., 1997). Phosphorylation of RLC on serine-19 stimulates the actin-activated ATPase activity of myosin-II and promotes myosin-binding to filamentous actin, which drives contraction of the actin-cytoskeleton (Lamb et al., 1988; Goekeler and Wysolmerski, 1995). In stationary cells, MLCK may be involved in the formation of strong focal adhesions and stress fibers (chrzanowska-Wodnicka and Burridge, 1996). However, in migrating cells, MLCK probably functions to enhance contraction of the cytoskeleton at the rear of the cell and within leading lamellipodia (Cande and Ezell, 1986; K olega and Taylor, 1993; J ay et al., 1995; M oores et al., 1996; M atsumura et al., 1998).

In uPA-treated MCF-7 cells, MLCK was phosphorylated by a MEK-dependent pathway and remained phosphorylated for at least 6 h, which was the duration of our standard Transwell migration assay. The phosphorylated MLCK was also apparently activated since levels of serine-phosphorylated RLC increased and this increase was prevented by ML-7 and ML-9. The same MLCK inhibitors blocked the motility-stimulating activity of uPA in three separate model systems. Thus, MLCK provides a link between uPAR ligand and increased cytoskeletal contractility which drives cellular migration. Although the increase in RLC phosphorylation was only about two- to threefold, our analysis was not sensitive to possible compartmentalization of the enzyme. Thus, it is possible that RLC phosphorylation was increased to a much greater level within specific regions of the cell. Since MLCK phosphorylation and activation are sustained in uPA-treated cells, continuous ERK activity may not be necessary for motility stimulation. We propose that the uPA R-activated signaling pathway, which promotes cellular motility, includes Ras→ Raf→ MEK→ ERK→ MLCK.

In the absence of uPA, the level of phosphorylated MLCK in MCF-7 cells was near or below the detection limit of our assay. Furthermore, MLCK inhibitors, which entirely blocked uPA-stimulated MCF-7 cell migration, did not affect the level of phosphorylated RLC or cellular migration, in the absence of uPA. Similar results were obtained in migration assays with uPA R-overexpressing MCF-7 cells and HT 1080 cells. These results may be explained if RLC phosphorylation is stable in the time course of our experiments, so that only new RLC phosphorylation is blocked by the inhibitors. However, it is also possible that MLCK does not play a major role in regulating the basal level of RLC phosphorylation, in the absence of uPA R, in MCF-7 cells and H T 1080 cells. Evidence supporting the latter hypothesis has been reported in other systems (M ita and W alsh, 1997). Enzymes which may phosphorylate RLC, other than MLCK, include: Ca$^{2+}$/calmodulin-dependent protein kinase II, Rho-associated kinase, MAP kinase-activated protein kinases, and p21-activated protein kinase (Tan et al., 1992; A mano et al., 1996; Komatsu et al., 1997; E yk et al., 1998; B resnick, 1999).

Yebra et al. (1996) demonstrated that endogenously synthesized uPA is required for phorbol ester- or transforming growth factor-α-promoted FG cell migration. Although the mechanism of uPA action was not fully characterized, it is tempting to speculate that in the FG cells,
endogenously synthesized uPA functioned comparably to exogenously added uPA, as defined in the present study. Plasminogen activator inhibitor (PAI)-1 and PAI-2 inhibited FG cell migration, suggesting a role for uPA proteinase activity in this process (Yebra et al., 1996) and a major difference in the mechanism of uPA activity in the FG cells compared with MCF-7 and HT 1080 cells. Alternatively, the activity of PAI-1 and PAI-2, in the FG cell system, may reflect the ability of these proteinase inhibitors to influence cellular catabolism of endogenously produced uPA and thereby alter the concentration of uPA which is available to ligate uPAR (Conese et al., 1995; Webb et al., 1999).

$\alpha_\text{v}\beta_3$ mediates cellular migration in the absence of exogenous stimulants whereas $\alpha_\text{v}\beta_3$ may mediate cellular migration only in cells that are treated with growth factors such as EGF or insulin-like growth factor-1 (Klemke et al., 1994; Filardo et al., 1996; Brooks et al., 1997). Since the uPA-signaling pathway defined here is similar to that triggered by many growth factors, we undertook studies to determine whether the ability of uPA to promote cellular migration depends on the integrins which are functional. Initially, we identified a substratum selectivity; uPA-promoted migration on vitronectin but not type I collagen. We then demonstrated that uPA functions to promote cellular migration on vitronectin only when certain integrins are functional. In the parent MCF-7 cells, $\alpha_\text{v}\beta_3$ and a $\beta_2$ subunit containing integrin (probably $\alpha_\text{v}\beta_2$) mediated cellular migration. When these cells were transfected to express $\alpha_\text{v}\beta_3$, the cells still responded to uPA, as determined in E R K phosphorylation experiments; however, uPA no longer stimulated cellular migration. Furthermore, M L C K antagonists did not inhibit the migration of $\alpha_\text{v}\beta_3$-expressing cells. Thus, the R as/E R K-dependent uPA-R-signaling pathway may not promote motility in uPA-treated cells that utilize $\alpha_\text{v}\beta_3$ to migrate on vitronectin. Interestingly, despite expression of $\alpha_\text{v}\beta_3$ in the transfected cells, alternative vitronectin-binding integrins were still available and competent to mediate cellular migration when $\alpha_\text{v}\beta_3$ was blocked with antibody. Under these conditions, the ability of uPA to promote cellular migration was restored.

HT 1080 cells express substantial levels of $\alpha_\text{v}\beta_3$ and utilize this integrin, as opposed to $\alpha_\text{v}\beta_3$, to adhere and migrate on vitronectin (Conforti et al., 1994, and unpublished results). Thus, the ability of uPA to promote HT 1080 cell migration on vitronectin by a R A S/E R K-dependent pathway is consistent with the pattern of vitronectin-receptor expression in this cell line. In each of our model systems, we focused our studies on the response to exogenously added uPA and consistently demonstrated a dominant role of uPA R-initiated signaling in mediating changes in cellular motility. However, we also obtained evidence for uPA-independent uPA R activities which may affect cellular migration. First, uPA R-overexpressing M CF-7 cells demonstrated increased migration on vitronectin in the absence of exogenously added uPA. Since M CF-7 cells express very low levels of uPA (Nguyen et al., 1998; Webb et al., 1999) and the increase in migration was not blocked by uPA-specific antibody or PD 098059, uPA R overexpression apparently increased basal M CF-7 cell migration by a uPA-independent mechanism. Sec-ondly, mannosamine treatment inhibited MCF-7 cell migration on vitronectin, in the presence and absence of exogenously added uPA. These uPA-independent uPA R activities may be related to the ability of uPA R to bind vitronectin or modulate integrin function (Wei et al., 1994, 1996).

The reason why uPA-promoted activation of E R K and M L C K stimulates cellular migration in an integrin-selective manner remains to be determined. Various integrin properties, including their subcellular localization, avidity for ligand, and strength of focal adhesions may be involved. The ability of uPA to regulate R L C phosphorylation, as well as integrin function (results presented here and by Wei et al., 1996), suggests a model in which uPA and uPA R orchestrate diverse aspects of cellular migration.

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