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 uPAR-overexpressing MCF-7 cells by a mechanism that depends on uPA receptor (uPAR)-ligation and ERK activation. Ras and MAP kinase 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 β_1-integrin (probably $\alpha_V\beta_1$) and $\alpha_V\beta_5$. When MCF-7 cells were transfected to express $\alpha_V\beta_3$ and treated with uPA, ERK was still phosphorylated; however, the cells did not demonstrate increased migration. Neutralizing the function of $\alpha_V\beta_3$, 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

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$\bf U$ROKINASE-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 (K_d of 0.1–1.0 nM) to the cell-surface receptor, uPAR (Ellis and Dano, 1992). Binding to uPAR 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 Andreasen et al., 1997).

uPAR is linked to the cell surface by a glycosyl-phosphatidylinositol (GPI) anchor (Ploug et al., 1991) and thus lacks transmembrane and intracytoplasmic domains. Nevertheless, uPAR expresses multiple activities, unrelated to proteinase localization, which regulate the interaction of cells with the microenvironment. First, uPAR binds di-

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1. *Abbreviations used in this paper:* ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; GPI, glycosyl-phosphatidylinositol; HA, hemagglutinin; MAP kinase, mitogen activated protein kinase; MEK, MAP kinase kinase; MLCK, myosin light chain kinase; PAI, plasminogen activator inhibitor; RLC, myosin II regulatory light chain; sc, single chain; tc, two chain; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor.

rectly to vitronectin and in some cells may augment or substitute for the function of α_V-containing integrins (Wei et al., 1994). uPAR also associates with integrins (Bohuslav et al., 1995; Simon et al., 1996; Sitrin et al., 1996; Wei et al., 1996; Xue et al., 1997) and may, by direct interaction, modulate the function of specific β_1 and β_2 subunit–containing integrins (Wei et al., 1994; Sitrin et al., 1996; May et al., 1998). Finally, uPAR 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 only partially characterized. Known protein tyrosine kinases in the *Src*-family, including p60[fyn], p53/p56[lyn], p56/p59[hck], and p59[fgr], have been coimmuno-precipitated with uPAR (Dumler et al., 1993, 1998; Bohuslav et al., 1995; Resnati et al., 1996; Konakova et al., 1998) and at least p56/p59[hck] is activated by uPA (Resnati et al., 1996; Konakova et al., 1998). uPA also activates protein kinase Cϵ (Busso et al., 1994), the JAK1/STAT1 signaling pathway (Koshelnick et al., 1997; Dumler et al., 1998), focal adhesion kinase (Tang et al., 1998), and the mitogen activated protein (MAP) kinases, 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 MAP 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 modifies multiple cytoplasmic substrates (Kanemitsu and Lau, 1993; Lin et al., 1993; Klemke et al., 1997). Klemke et al. (1997) demonstrated that ERK phosphorylates and thereby activates myosin light chain kinase (MLCK), promoting cellular migration. Activated ERK also suppresses the function of specific integrins (Hughes et al., 1997) and may promote rapid disassembly of focal contacts (Xie et al., 1998), allowing more effective cytoskeletal contraction at the rear of the migrating cell (for review see Lauffenberger and Horwitz, 1996).

The goal of this investigation was to elucidate the mechanism by which uPA promotes cellular migration. Our results demonstrate that the uPA response requires uPAR-ligation and activation of the Ras/ERK-signaling pathway. MLCK functions downstream of Ras/ERK and is also an essential mediator of uPA-promoted cellular migration, suggesting that the uPA/uPAR system may regulate cytoskeletal contractility. MCF-7 cell migration on vitronectin was promoted by uPA only when migration was under the control of $\alpha_V\beta_5$ and a β_1 subunit–containing integrin (probably $\alpha_V\beta_1$), but not $\alpha_V\beta_3$, thus defining yet an-

other novel functional relationship between integrins and the uPA/uPAR system. The activity of MLCK in uPA-promoted cellular migration and the integrin-selectivity of the response suggest a model in which uPAR-initiated signal transduction orchestrates integrin function and cytoskeletal reorganization during cellular migration.

Materials and Methods

Antibodies and Reagents

scuPA and tcuPA were provided by Drs. Jack Henkin and Andrew Mazar of Abbott Laboratories. tcuPA was inactivated with 20 mM diisopropyl-fluorophosphate (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, L-pyroglutamyl-glycyl-arginine-*p*-nitroanilide HCl (Bachem). Type I collagen was from Collaborative Biochemical Products. Vitronectin was purified according to the method of Yatohgo et al. (1988). The expression vector, pEGFP, which encodes green fluorescent protein (GFP), was from Clontech. Polyclonal anti–human uPAR antibody 399R, which recognizes uPAR and blocks uPA-binding to uPAR, and monoclonal anti–human uPA antibody, which is directed against the growth factor domain of uPA, were from American Diagnostica. In control experiments, we confirmed that each of these antibodies, at 25 μg/ml, inhibits >95% of the specific binding of ^{125}I-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-RLC antibody was from ICN Biomedicals. Monoclonal anti-hemagglutinin (HA) antiserum 12CA5 was from Babco. MLCK-specific monoclonal antibody (clone K36) was from Sigma Chemical Co. Integrin-specific blocking antibodies directed against human $\alpha_V\beta_5$ (P1F6), $\alpha_V\beta_3$ (LM609), and β_1-containing integrins (6S6) were from Chemicon International. The MEK inhibitor, PD098059, 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, PMSF, aprotinin, benzamidine, NaF, sodium orthovanadate, and G418 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) (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 uPAR, 5×10^5 cells were transfected with 2 μg of the uPAR 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 uPAR overexpression by flow cytometry. Cell-surface uPAR was quantitated by measuring specific binding of ^{125}I-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 determinations. 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 uPAR/cell from B_{max} values.

Expression constructs which encode constitutively active rat MEK1 (S218→D/S222→D) and dominant-negative rabbit MEK1 (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 (G12→V) and dominant-negative H-Ras

(S17→N), in pDCR, were described by White et al. (1995) and Cai et al. (1990), respectively. MCF-7 cells (2×10^6) were cotransfected with each of these constructs (5.0 μg) and with 1.25 μg of pEGFP, using 30 μl of Superfect. 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 in 4% 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 β_3-integrin subunit was kindly provided by Dr. David Cheresh (Scripps Research Institute, La Jolla, CA). The cDNA was subcloned into pBK-CMV and transfected into MCF-7 cells (2 μg cDNA/5×10^5 cells) using 10 μl Superfect. The cells were cultured for 36–48 h and selected in G418 (1 mg/ml) for 14 d. The transfectants were then subjected to flow cytometry, using antibody LM609 and fluorescein-conjugated anti–mouse IgG, to detect cell-surface $\alpha_V\beta_3$ expression. After flow cytometry, the cells were cultured in RPMI supplemented with G418 (25 μg/ml), FBS, penicillin, and streptomycin. ERK activation experiments and migration assays were performed within 2 wk of obtaining flow cytometry results.

ERK Activation in Transfected Cells

To confirm that ERK activity was regulated in cells transfected to express mutant MEK1 or H-Ras, 5×10^5 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 maintained 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% deoxycholate, 1% NP-40, 10 mM sodium phosphate, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate, 0.1 mM sodium vanadate, 2 mM PMSF, 0.1 μg/ml leupeptin, and 100 KIU/ml aprotinin. HA-ERK1 was immunoprecipitated using anti-serum 12CA5, as described previously (Catling et al., 1995). Samples were then subjected to SDS-PAGE and transferred to nitrocellulose. Phosphorylated HA-ERK1 and total HA-ERK1 were detected by immunoblot analysis (Nguyen et al., 1998).

ERK phosphorylation, in β_3-integrin subunit-expressing MCF-7 cells, was also detected by immunoblot analysis. In brief, MCF-7 cells, that had been transfected and selected, were transferred to serum-free medium for 6 h and then treated with 10 nM DIP-uPA, 25 ng/ml EGF, or vehicle. Cell extracts were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies for phosphorylated ERK1/2 and total ERK1/2, as described previously (Nguyen et al., 1998).

MLCK 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 [^{32}P]orthophosphate (250 μCi) in serum-free RPMI supplemented with 1 mg/ml BSA and 1 mg/ml sodium orthovanadate. The labeled cells were treated with 10 nM DIP-uPA for up to 6 h. Control cells were treated with vehicle instead of DIP-uPA. In some cultures, 50 μM PD098059 was added to inhibit MEK. The PD098059 was added 15 min before adding the DIP-uPA and remained present throughout the assay. After washing the cultures, 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 precleared with protein A–agarose for 1 h at 22°C. MLCK in the supernatants was then immunoprecipitated by incubation with MLCK-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 MLCK was detected by autoradiography.

Serine-phosphorylation of RLC

Suspended MCF-7 cells (10^5 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 15% 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 pretreated 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 are 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 β_3-integrin subunit-expressing MCF-7 cells (10^5 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), W-7 (51 μM), or with the following antibodies: uPA-specific antibody, uPAR-specific antibody, LM609, 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. After terminating a study, 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. 5×10^5 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 chemokinesis 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 (5×10^5) that were cotransfected with signaling effector mutants and pEGFP, or with pEGFP 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 GPI-linked proteins to their anchors (Lisanti et al., 1991) and can be used to substantially downregulate the level of cell-surface uPAR (Wei 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.

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 (10^5 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

uPA-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, PD098059, 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 uPAR.

MCF-7 cells express 3,300 copies of cell-surface uPAR, as determined by ^{125}I-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 cell lines with increased levels of cell-surface uPAR, as determined by flow cytometry. Clone M5 demonstrated increased specific binding of ^{125}I-DIP-uPA. The K_d (1.2 ± 0.3 nM) was unchanged compared with that determined for untransfected MCF-7 cells; however, the B_{max} was increased to 33 ± 5 fmol/mg cell protein (n = 4), corresponding to 19,800 copies of cell-surface uPAR/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 PD098059. Thus, the increase in basal M5 cell migration
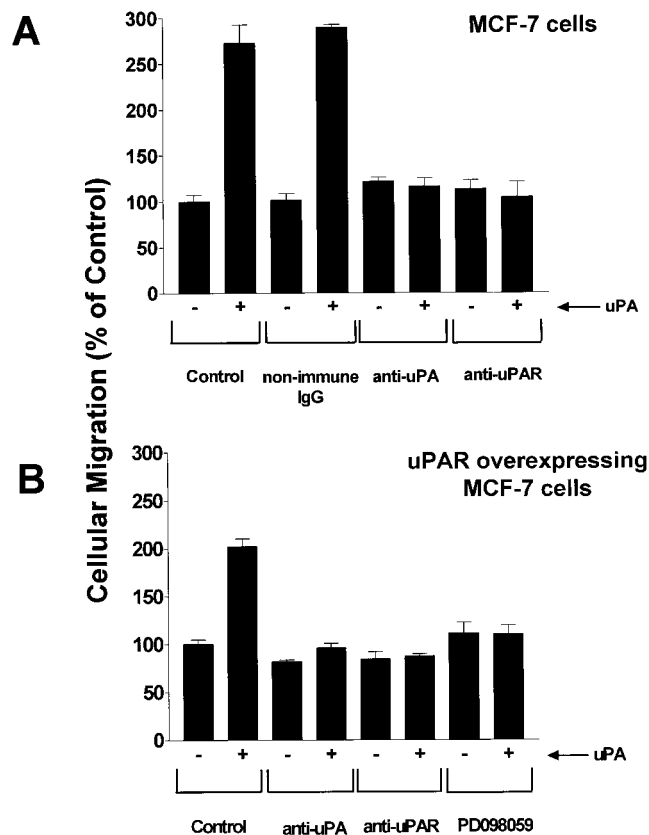
Figure 1. uPA promotes cellular migration by a uPAR-dependent mechanism which is inhibited by PD098059 in parent and uPAR-overexpressing MCF-7 cells. Each of the designated cell types was treated with 10 nM DIP-uPA (+) or with vehicle (−) and allowed to migrate for 6 h on serum-coated Transwell membranes. Some cells were treated with uPA-specific antibody, uPAR-specific antibody, nonimmune IgG (each at 25 μg/ml), or with PD098059 (50 μM). Cellular migration is expressed as a percentage of that observed with control cells, which were not treated with DIP-uPA, antibodies, or PD098059 (mean ± SEM, n = 4).

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 PD098059. 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 (results not shown). Thus, uPAR-overexpressing MCF-7 cells respond similarly to the parent cells when treated with uPA.

uPA-stimulated HT 1080 Cell Migration Requires ERK Activation

HT 1080 fibrosarcoma cells express uPAR (Laug et al., 1992) and demonstrate increased levels of activated ERK2 when treated with uPA (Konakova et al., 1997). In our initial experiments, we demonstrated specific binding of ^{125}I-
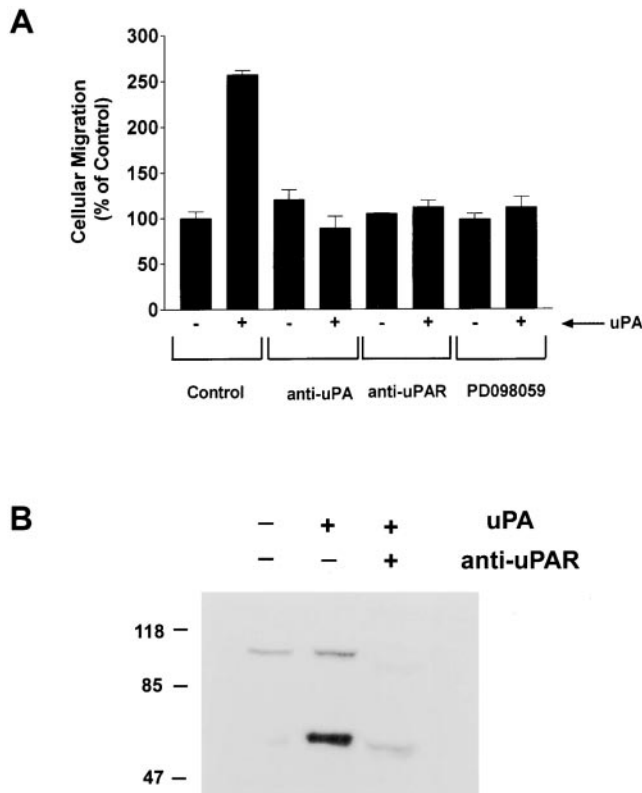
A

B

Figure 2. uPA-stimulated HT 1080 cell migration requires ERK activation and uPAR ligation. (A) HT 1080 cells were treated with 10 nM DIP-uPA (+) or with vehicle (−) and allowed to migrate for 6 h on serum-coated Transwell membranes. Some cells were treated with uPA-specific antibody, uPAR-specific antibody (each at 25 μg/ml), or PD098059 (50 μM). Cellular migration is expressed as a percentage of that observed with control cells that were not treated with DIP-uPA, antibodies, or inhibitors. (B) HT 1080 cells (2.5×10^5 cells) were incubated with 1 nM DIP-uPA or with vehicle for 4 h at 4°C as indicated. Some cells were pretreated with uPAR-specific antibody (25 μg/ml) for 20 min and then with DIP-uPA in the presence of antibody. HT 1080 cell detergent-extracts were subjected to SDS-PAGE on 8% slabs and transferred to nitrocellulose. Total cell-associated uPA (endogenously produced and exogenously added) was detected by immunoblot analysis, using uPA specific antibody (1:10,000), and HRP-conjugated anti–mouse IgG (Sigma, 1:10,000).

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 antibody 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 demon-

strated 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.
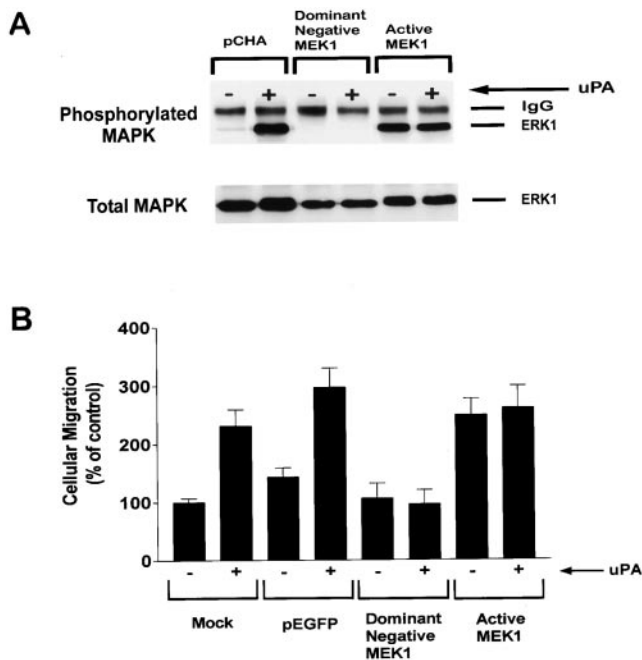
To study the migration of MCF-7 cells, which were tran-

Figure 3. MEK is required for uPA-induced MAP kinase activation and motility stimulation. (A) 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. (B) MCF-7 cells were transfected to express the specified MEK mutants and GFP, or GFP alone (pEGFP). Mock-transfected cells were treated with Superfect in the absence of cDNAs. Cells were allowed to migrate in the presence (+) or absence (−) of 10 nM DIP-uPA for 6 h on serum-coated membranes. Cellular migration of transfected cells was determined using fluorescence microscopy to detect GFP-expressing cells. Migration of mock-transfected cells was determined by Diff-Quik staining. Mock-transfected cells migrated identically to MCF-7 cells that were not Superfect-treated, in the presence and absence of uPA (data not shown). To compare results obtained with GFP-expressing and mock-transfected cells, the number of GFP-expressing cells that penetrated the membrane was divided by the transfection efficiency. For each bar, cellular migration is expressed as a percentage of that observed with mock-transfected cells that were not treated with DIP-uPA.

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). PD098059 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 either dominant-negative H-Ras or constitutively active H-Ras. As 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. As 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, which was not statistically significant. However, these cells failed to demonstrate increased migration
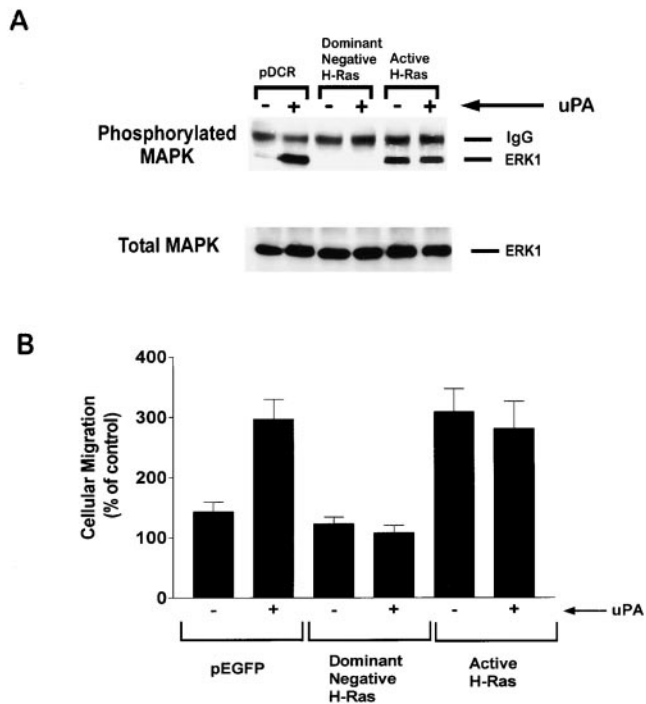
Figure 4. Ras is required for uPA-induced MAP kinase activation and MCF-7 cell migration. (A) MCF-7 cells were cotransfected to express HA-tagged ERK1 and dominant-negative H-Ras, constitutively active H-Ras, or the empty vector, pDCR. The cells were then treated with 10 nM DIP-uPA (+) or vehicle (−) for 1 min. HA-tagged ERK1 was recovered by immunoprecipitation. Phosphorylated and total levels of ERK1 were determined by immunoblot analysis. (B) MCF-7 cells were transfected to express the specified H-Ras mutants and GFP or GFP alone. The cells were then treated with 10 nM DIP-uPA (+) or vehicle (−) and allowed to migrate for 6 h on serum-coated membranes. Cellular migration was standardized to that observed with mock-transfected cells that were not uPA-treated.

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 [^{35}S]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 Ca^{2+}/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 Ca^{2+}/calmodulin-dependent protein kinase II, phosphorylate MLCK near the calmodulin-binding domain, increasing the concentration of Ca^{2+} required for MLCK activation and thereby effectively decreasing MLCK activity (for reviews see Adelstein et al., 1982; Stull et al., 1993). p21-activated kinase also decreases MLCK activity by directly affecting the enzyme V_{max} (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-
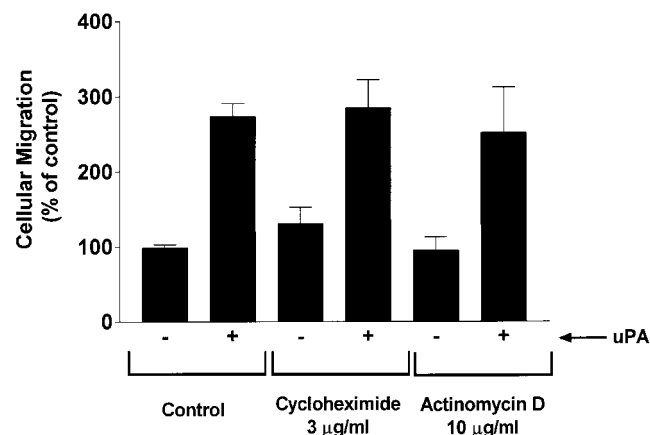

Figure 5. Gene transcription and protein synthesis are not required for uPA-promoted MCF-7 cell migration. MCF-7 cells were allowed to migrate for 6 h on serum-coated Transwell membranes in the presence (+) or absence (−) of 10 nM DIP-uPA. Some MCF-7 cells were treated with cycloheximide (3 μg/ml) or actinomycin D (10 μg/ml). Cellular migration is expressed as a percentage of that observed with control cells that were not treated with DIP-uPA or with inhibitors.
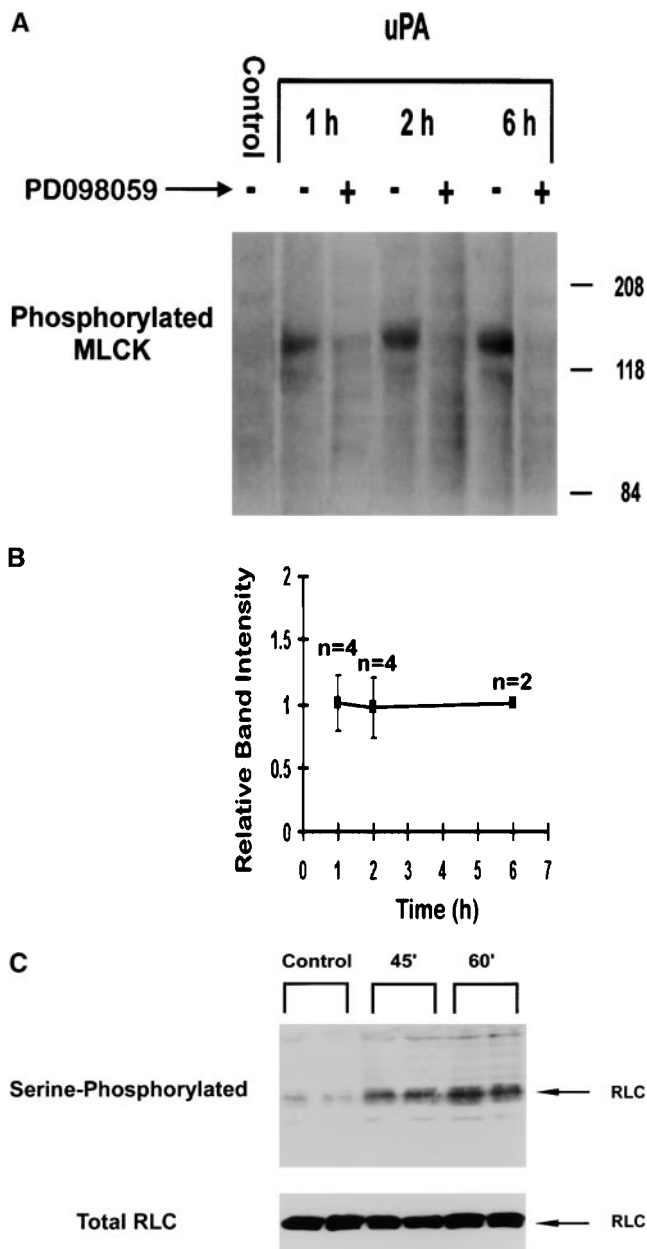
A

B

C

Figure 6. MLCK and RLC are phosphorylated in uPA-treated MCF-7 cells. (A) MCF-7 cells were metabolically labeled with [^{32}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.

noprecipitated from MCF-7 cells that had been metabolically labeled with [^{32}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, ML-7 and ML-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

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

*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.

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. 7 B). Equivalent results were obtained in experiments with uPAR-overexpressing MCF-7 cells and HT 1080 cells. None of 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, ML-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.

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 (Hayman 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
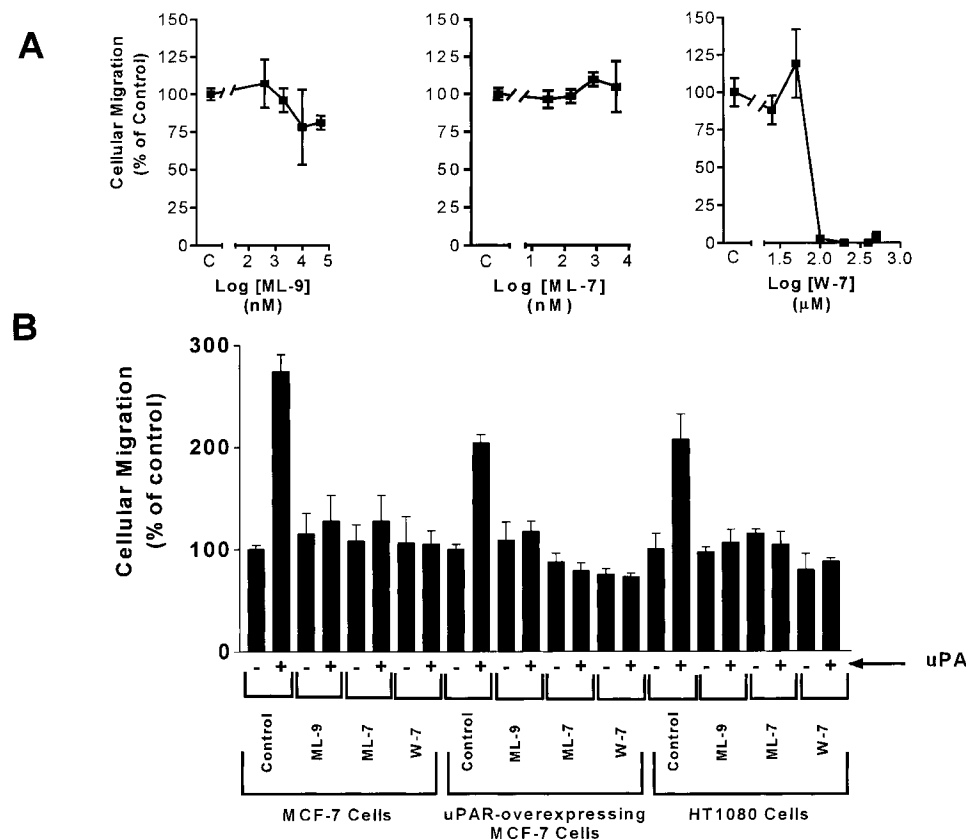

Figure 7. MLCK activity is critical for uPA-promoted cell migration. (A) MCF-7 cells were treated with the specified MLCK inhibitors and allowed to migrate for 6 h on serum-coated Transwell membranes. (B) MCF-7 cells, uPAR overexpressing MCF-7 cells, and HT 1080 cells were treated with ML-9 (30 μM), ML-7 (3 μM), or W-7 (51 μM), in the presence (+) or absence (−) of DIP-uPA, and allowed to migrate for 6 h. Control cells were not treated with any of the MLCK inhibitors. Cellular migration was expressed as a percentage of that observed with control cells, of the same cell line, in the absence of DIP-uPA and inhibitors.
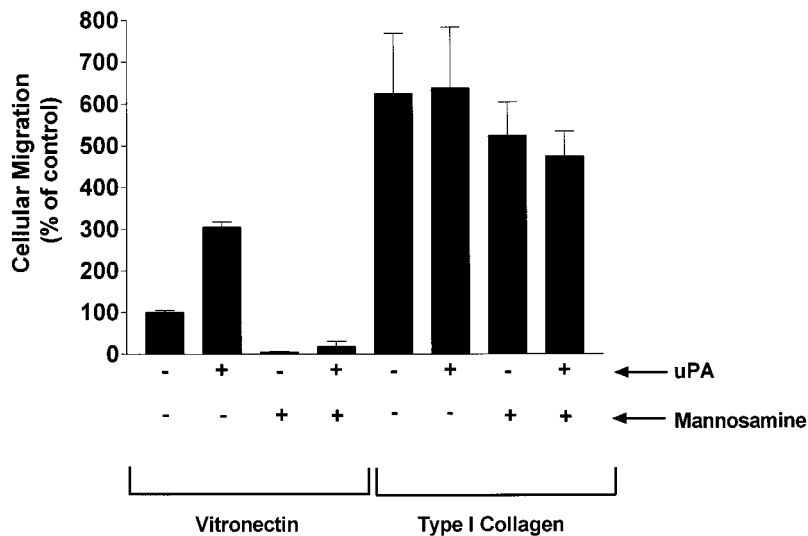
Figure 8. uPA-promoted cellular migration is matrix protein–selective. Transwell membranes were coated with purified vitronectin or with type I collagen. MCF-7 cells that were treated with mannosamine (+) and untreated cells (−) were allowed to migrate for 6 h in the presence of 10 nM DIP-uPA (+) or vehicle (−). Migration was expressed as a percentage of that observed with control cells (no mannosamine or DIP-uPA treatment) on vitronectin-coated membranes.

not shown). Thus, whereas uPAR apparently plays an important role in MCF-7 cell migration on vitronectin, this process is still dependent on integrins.

$\alpha_V\beta_5$ Mediates MCF-7 Cell Adhesion to Vitronectin

Meyer et al. (1998) demonstrated that MCF-7 cells express $\alpha_V\beta_5$ and $\alpha_V\beta_1$, but not $\alpha_V\beta_3$. Our flow cytometry experiments, with antibodies directed against $\alpha_V\beta_5$ and $\alpha_V\beta_3$, confirmed their results (data not shown). We also demonstrated substantial levels of cell-surface β_1 subunit; however, our antibody was not specific for $\alpha_V\beta_1$. 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 $\alpha_V\beta_5$, substantially inhibited MCF-7 cell adhesion whereas LM609, which blocks $\alpha_V\beta_3$, was ineffective as anticipated (Fig. 9). Interestingly, when the β_1-integrin–blocking antibody, 6S6, was added in combination with P1F6, the extent of inhibition was significantly increased ($P < 0.05$). These re-

sults suggest that a β_1 subunit–containing integrin (probably $\alpha_V\beta_1$) 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 β_1-Integrin and $\alpha_V\beta_5$ Function in uPA-promoted MCF-7 Cell Migration

In the absence of uPA, $\alpha_V\beta_3$-blocking antibody had no effect on MCF-7 cell migration on vitronectin, as anticipated (Fig. 10 A). However, $\alpha_V\beta_5$-blocking antibody was also inactive and β_1 subunit–blocking antibody inhibited migration by <25%. When added in combination, $\alpha_V\beta_5$-blocking antibody and the β_1-blocking antibody inhibited migration by up to 79 ± 5%. These results suggest that $\alpha_V\beta_5$ and a β_1-containing integrin (probably $\alpha_V\beta_1$) function interchangeably in MCF-7 cell migration on vitronectin, in the absence of uPA.
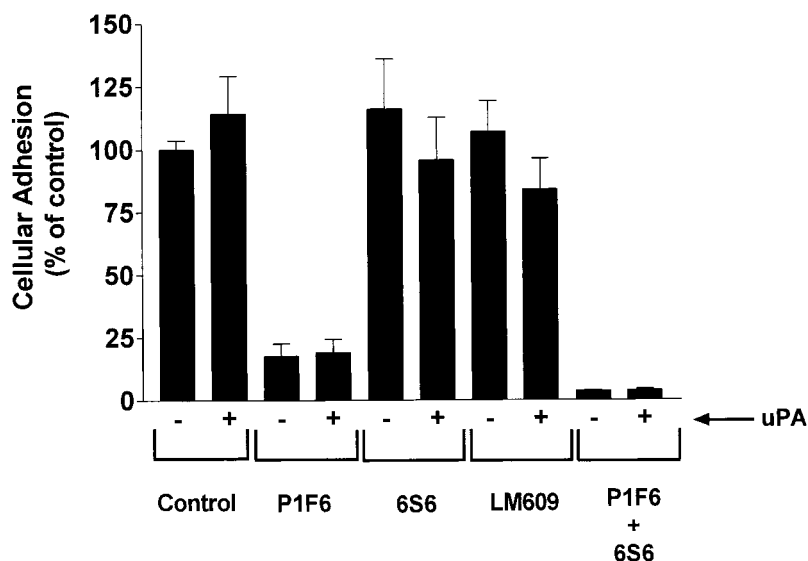

Figure 9. $\alpha_V\beta_5$ and β_1-containing integrins mediate MCF-7 cell adhesion to vitronectin. MCF-7 cells were allowed to adhere to vitronectin-coated surfaces for 1 h in the presence (+) or absence (−) of 10 nM DIP-uPA. Some MCF-7 cells were treated with blocking antibodies (each at 32 μg/ml) against $\alpha_V\beta_3$ (LM609), $\alpha_V\beta_5$ (P1F6), and/or β_1 subunit (6S6). Adherent cells were stained with Calcein AM and measured by fluorescence emission using a Cytofluor 2350. Cellular adhesion was quantitated as a percentage of that observed with control cells that were not exposed to DIP-uPA or antibodies.
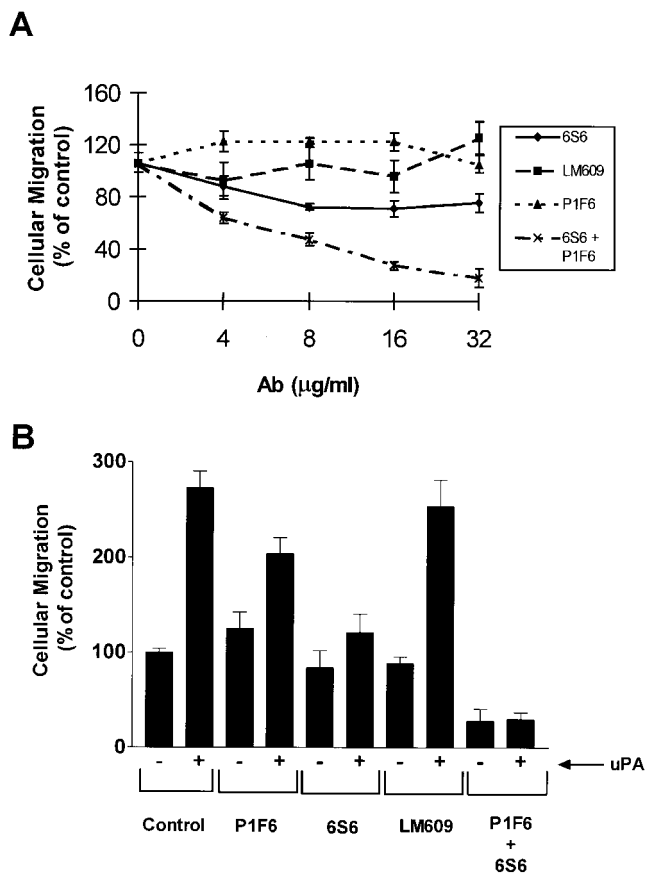
A

B

Figure 10. $\alpha_V\beta_5$ and a β_1-containing integrin mediate MCF-7 cell migration in the presence and absence of uPA. (A) MCF-7 cells were treated with increasing concentrations of blocking antibodies against $\alpha_V\beta_3$ (LM609), $\alpha_V\beta_5$ (P1F6), and/or β_1-integrin subunit (6S6) and allowed to migrate for 6 h in Transwell chambers with vitronectin-coated membranes. (B) MCF-7 cells were treated with 10 nM DIP-uPA (+) or with vehicle (−) in the presence of the specified antibodies (32 μg/ml) and allowed to migrate for 6 h on vitronectin-coated membranes. Control cells were not treated with antibodies. Migration was expressed as a percentage of that observed with control cells in the absence of DIP-uPA.

Fig. 10 B compares the activities of the vitronectin-binding integrins in mediating uPA-stimulated MCF-7 cell migration. The $\alpha_V\beta_3$-blocking antibody, LM609, had no effect on the uPA response. Antibody P1F6, which neutralizes $\alpha_V\beta_5$, reduced the magnitude of the uPA response; however, a statistically significant increase in cellular migration was still observed ($P < 0.005$). The uPA response was reduced still further when migration was allowed to proceed in the presence of antibody 6S6. Importantly, when antibodies P1F6 and 6S6 were added in combination, the response of MCF-7 cells to uPA was entirely abrogated. These results demonstrate that uPA-stimulated MCF-7 cell migration on vitronectin is mediated by the same integrins which function in the absence of uPA. A β_1-containing integrin (probably $\alpha_V\beta_1$) and $\alpha_V\beta_5$ are both involved.

uPA Does Not Promote the Migration of MCF-7 Cells that Express $\alpha_V\beta_3$

Yebra et al. (1996) demonstrated that growth factors pro-

mote pancreatic carcinoma cell migration on vitronectin by a mechanism that is dependent on endogenously synthesized uPA when the cells express $\alpha_V\beta_5$ but not when they express $\alpha_V\beta_3$. 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 β_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 ERK1/2 phosphorylation in the β_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 β_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). Antibody 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 β_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 β_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 β_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
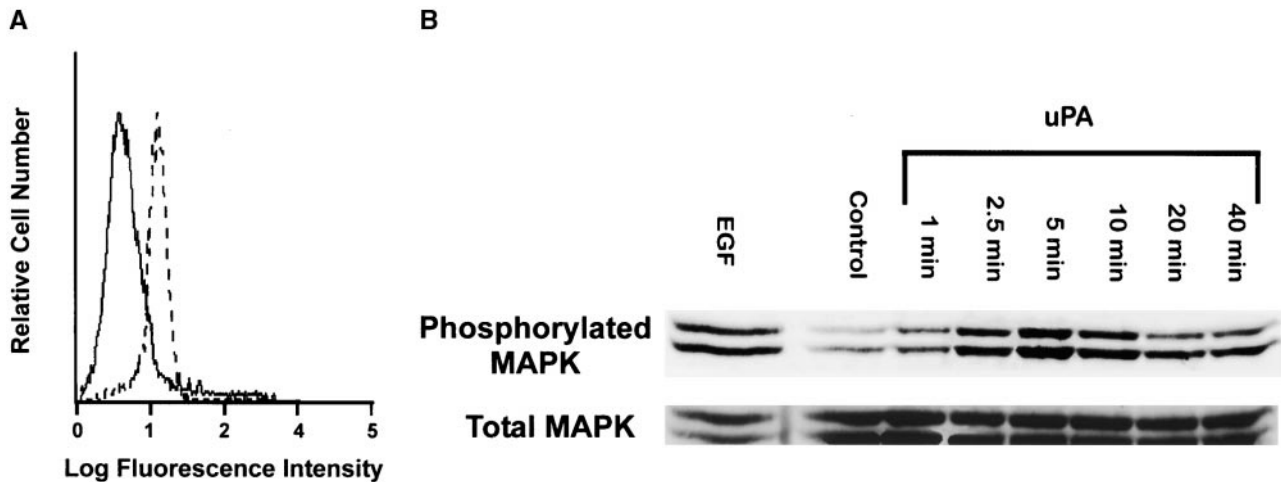
Figure 11. MCF-7 cells which express $\alpha_V\beta_3$ are refractory to uPA-stimulation in migration assays. (A) MCF-7 cells were transfected to express β_3-integrin subunit. After selection for 14 d, cell-surface $\alpha_V\beta_3$ expression was detected by flow cytometry using antibody LM609. Transfected cells are shown with the broken line. The solid line shows untransfected MCF-7 cells. (B) $\alpha_V\beta_3$-expressing MCF-7 cells were treated with DIP-uPA for the specified times, with EGF (25 ng/ml) for 5 min, or with vehicle (control). Phosphorylated and total levels of ERK1/2 were determined by immunoblot analysis. (C) $\alpha_V\beta_3$-expressing MCF-7 cells (β_3), untransfected cells (control), and cells that were transfected with the empty vector that was used to prepare the β_3-expression construct (pBK-CMV) were treated with 10 nM DIP-uPA (+) or with vehicle (−) and allowed to migrate for 6 h in Transwell chambers with vitronectin-coated membranes. Migration of $\alpha_V\beta_3$-expressing cells was also studied in the presence of antibody LM609 (32 μg/ml). Cells which penetrated the membranes were detected by Diff-Quik staining. Migration was expressed as a percentage of that observed with untransfected cells in the absence of DIP-uPA.

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

Table II. The Effects of MLCK Inhibitors on $\alpha_V\beta_3$-mediated Cellular Migration

Transfectants	Inhibitors	Cell migration (% of control*)
Control	None	100 ± 6
β_3	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.

that are uPAR-deficient demonstrate deficient recruitment of inflammatory cells to inflamed peritoneum (May et al., 1998).

We demonstrated previously that uPA promotes MCF-7 cell migration, in vitro, in serum-coated Transwell chambers (Nguyen et al., 1998). This activity did not require uPA proteolytic activity since the amino-terminal fragment of uPA, which contains the receptor-binding domain but lacks the serine proteinase domains, was also active. Furthermore, uPA-promoted MCF-7 cell migration was blocked by PD098059, leading us to hypothesize that ERK activation is critical in the pathway by which uPA stimulates cellular migration. MCF-7 cells express <4,000 copies of cell-surface uPAR (Nguyen et al., 1998; Webb et al., 1999), yet demonstrate increased migration when stimulated by uPA at concentrations as low as 50 pM (Webb et al., 1999). This may suggest that the uPAR-initiated signaling response is exquisitely sensitive to low levels of uPAR ligation. However, other investigators have presented evidence for the possible existence of a second cell-surface uPA-binding site which may be involved in uPA signaling (Koopman et al., 1998). Thus, in the present investigation, we tested the hypothesis that uPAR ligation and ERK activation are responsible for promoting cellular migration in three separate model systems. In MCF-7 cells, uPAR-overexpressing MCF-7 cells, and HT 1080 cells, uPA- and

uPAR-specific antibodies, which block association of uPA with uPAR, prevented uPA-stimulated cellular migration. PD098059 also blocked uPA-stimulated cellular migration in all three cell lines. Thus, the ability of uPA to bind to uPAR and activate ERK may be pivotal in promoting cellular migration in different cell types with varying levels of uPAR 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. Mirashahi 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 uPAR 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 uPAR-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; Keyse, 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. Actinomycin 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. Once 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 in vitro (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; Goeckeler 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 Ezzell, 1986; Kolega and Taylor, 1993; Jay et al., 1995; Moores et al., 1996; Matsumura 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 ligation 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 uPAR-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 uPAR-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 stimulants such as uPA, in MCF-7 and HT 1080 cells. Evidence supporting the latter hypothesis has been reported in other systems (Mita and Walsh, 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; Amano et al., 1996; Komatsu et al., 1997; Eyk et al., 1998; Bresnick, 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 the 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).

$\alpha_V\beta_3$ mediates cellular migration in the absence of exogenous stimulants whereas $\alpha_V\beta_5$ 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 cellular 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, $\alpha_V\beta_5$ and a β_1 subunit containing integrin (probably $\alpha_V\beta_1$) mediated cellular migration. When these cells were transfected to express $\alpha_V\beta_3$, the cells still responded to uPA, as determined in ERK phosphorylation experiments; however, uPA no longer stimulated cellular migration. Furthermore, MLCK antagonists did not inhibit the migration of $\alpha_V\beta_3$-expressing cells. Thus, the Ras/ERK-dependent uPAR-signaling pathway may not promote motility in uPA-treated cells that utilize $\alpha_V\beta_3$ to migrate on vitronectin. Interestingly, despite expression of $\alpha_V\beta_3$ in the transfected cells, alternative vitronectin-binding integrins were still available and competent to mediate cellular migration when $\alpha_V\beta_3$ was blocked with antibody. Under these conditions, the ability of uPA to promote cellular migration was restored.

HT 1080 cells express substantial levels of $\alpha_V\beta_5$ and utilize this integrin, as opposed to $\alpha_V\beta_3$, to adhere and migrate on vitronectin (Conforti et al., 1994, and our unpublished results). Thus, the ability of uPA to promote HT 1080 cell migration on vitronectin by a RAS/ERK-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 uPAR-initiated signaling in mediating changes in cellular motility. However, we also obtained evidence for uPA-independent uPAR activities which may affect cellular migration. First, uPAR-overexpressing MCF-7 cells demonstrated increased migration on vitronectin in the absence of exogenously added uPA. Since MCF-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 PD098059, uPAR overexpression apparently increased basal MCF-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 uPAR activities may be related to the ability of uPAR to bind vitronectin or modulate integrin function (Wei et al., 1994, 1996).

The reason why uPA-promoted activation of ERK and MLCK 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 RLC phosphorylation, as well as integrin function (results presented here and by Wei et al., 1996), suggests a model in which uPA and uPAR orchestrate diverse aspects of cellular migration.

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